2017

Pharmacokinetics of Micronized Progesterone Administration in Female Dogs

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Recommended Citation

Malbrue, Raphael Anthony, "Pharmacokinetics of Micronized Progesterone Administration in Female Dogs" (2017). *LSU Master's Theses*. 4439.
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PHARMACOKINETICS OF MICRONIZED PROGESTERONE ADMINISTRATION IN FEMALE DOGS

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

Biomedical and Veterinary Medical Sciences
through
The Department of Pathobiological Sciences

by
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B.S., Tuskegee University, 2011
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August 2017

"We stand on the broad shoulders of those pioneers whom came before us". I dedicate my thesis work to the first veterinarian I ever met, my mentor Dr. C.T. Raby. It was through his passion, commitment, and faith that I've been able to make my dreams a reality, and for that I am forever grateful.

Acknowledgements

I extend my sincere gratitude to God, my wife, and parents for their unconditional love, support, and unwavering loyalty towards all my dreams and aspirations. I would also like to deeply thank my residency mentors Dr. Rhett Stout and Dr. David Baker for the opportunity to participate and complete a laboratory animal training program. Additional thanks to my resident mate Dr. Carmen Arsuaga and all the members of my thesis committee for their guidance, honesty, and compassion.

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Abstract

Hypoluteoidism in the bitch is described as a reproductive condition in which insufficient levels of endogenous progesterone are present resulting in failure to maintain a functional secretory endometrium. This condition can prevent normal embryo implantation, development, and ultimately end in pregnancy loss. Hypoluteoidism in the bitch is a rising concern in small animal theriogenology and current medical therapies available to veterinarians are limited. The aim of this study was to determine the pharmacokinetics (PK) of intravaginally (Crinone®, Serono Laboratories, Norwell, MA) and orally delivered micronized progesterone (Prometrium®, Solvay Pharmaceuticals, Inc., Marietta, GA) in the bitch. We hypothesized that both vaginal and oral treatments would result in a dose-dependent increase in concentrations of plasma progesterone. We further hypothesized that oral dosing of micronized progesterone would result in greater, sustained plasma progesterone than those recorded in bitches treated with intravaginal (IVa) micronized progesterone gel. Eight adult sexually intact bitches in anestrus were arranged in a 4x4 Latin square cross over experimental design. Each subject rotated through four different progesterone treatment groups with a minimum seven day-wash out period between treatments: 100 mg oral micronized progesterone, 200 mg oral micronized progesterone, 45 mg intravaginal micronized progesterone and 90 mg intravaginal micronized progesterone. Blood samples from each subject were obtained at time points 0, 0.5, 2, 1.5, 2, 4, 6, 8, 12, 24, 36, 48 and 72 hours following one initial dosing of each treatment. Concentrations of plasma progesterone were determined by RIA (ImmuChem Double Antibody, 125I RIA Kit, MP Biomedicals, Costa Mesa, CA). Pharmacokinetic analysis was carried out using commercially available software (Phoenix WinNonlin 6.4, Certara Inc., Princeton, NJ). One-compartmental (intravaginal) and non-compartmental (oral administration) modeling were performed to analyze data using the mean

concentrations for each dosing to calculate the area under the curve (AUC), maximum plasma concentration (C_{max}), time elapsed to reach C_{max} (T_{max}), and elimination half-life ($t_{1/2}$).

Results for the 100 mg and 200 mg oral doses and 45 mg and 90 mg IVA doses were as follows: AUC, 30.86, 187.96, 90.64, and 226.68 ng.h.mL^{-1}, respectively; C_{max}, 13.47, 169, 8.68, and 13.24 ng.mL^{-1}, respectively; T_{max}, 0.5, 0.5, 0.84, and 1.67 hr, respectively, and half-life, 5.87, 6.76, 6.6, and 10.65 hr, respectively.

Micronized progesterone was readily absorbed in bitches when administered either orally or intravaginally. Contrary to our initial hypothesis, micronized progesterone exposure over time, as indicated by the area under the curve, was greater when intravaginal micronized progesterone was used. The ability of intravaginal preparations of micronized progesterone to induce sustained progesterone exposure may provide an alternative strategy for treating pregnant dogs whenever hypoluteoidism is being suspected.

Chapter 1. Introduction and Literature Review

1.1 Canine Reproductive Cycle and Breeding

In veterinary medicine, it is important that the general practitioner develops a sound understanding of reproductive physiology and endocrinology. The reproductive system not only plays a major role in animal breeding, but its dysfunction can also contribute to the development of several endocrinopathies. The domestic canine (*Canis familiaris*) reproductive cycle has been well described in scientific literature (Heape, 1900). It involves four stages: proestrus, estrus, diestrus, and anestrus. Most dogs will typically have two reproductive cycles per year; however, there have been reports of some breed variation, such as the African basenji dog, displaying only one reproductive cycle annually (Fuller, 1956).

Determining at what specific age to breed a bitch can be difficult for both the practicing veterinarian and the owner. It is recommended to perform a bitch's first breeding after at least one complete normal reproductive cycle (Feldman and Nelson, 2004). Factors that influence the start of a dog's first reproductive cycle include breed, size, and seasonal patterns (Feldman and Nelson, 2004). It is also not uncommon for owners to overlook the bitch's first cycle, as result of a reproductive condition called *silent heat*. This term is often used to describe the loss or decrease of clinical signs usually associated with normal cycling in the bitch, such as swelling of the vulva, vaginal bleeding, male attraction, and changes in behavior (Concannon, 1980).

Determining what phase of the reproductive cycle a bitch is in can also be challenging. It is advised to use a multi-modal approach incorporating a variety of clinical techniques to achieve this goal. Common procedures performed to determine the reproductive cycle stages include vaginal cytology, blood hormonal assays (i.e., plasma progesterone, luteinizing hormone, estrogen), and thorough physical examination of the reproductive organs and associated

structures. The reproductive cycle begins with the onset of the proestrus. Clinically, vaginal bleeding, vulva enlargement, and attraction of males due to pheromone secretion are indicative of the start of proestrus (Goodwin et al, 1979). Females will remain non-receptive to mounting by males during proestrus. This stage is usually 6-11 days in length, with an average of 9 days (Feldman and Nelson, 2004).

The primary influencing hormone during proestrus is estrogen, which is synthesized and secreted by the ovarian follicles (Feldman and Nelson, 2004). This has been demonstrated by administering estrogens to ovariohysterectomized females, leading them into a state of proestrus without vaginal bleeding (Concannon, 1987). Circulating estrogen concentrations in early proestrus are typically above 25 pg/mL, while late proestrus normally are around 60-70 pg/mL (Feldman and Nelson, 2004; Olson, 1982). Serum testosterone levels increase in the bitch in late proestrus, typically obtaining concentrations of 0.3-1.0 ng/mL (Olson et al, 1984). Serum progesterone concentrations throughout proestrus are low (< 0.5 ng/mL) except during the last 24-72 hours leading into estrus (Feldman and Nelson, 2004).

When performing ultrasonography in the bitch during proestrus, evaluation of the ovaries will reveal a gradual increase in overall size due to follicular development. This will appear as focal hypoechoic to anechoic rounded structures (Feldman and Nelson, 2004). Vaginoscopy will reveal a thickened, well rounded, edematous, smooth, and shiny vaginal mucosa (Jeffcoate, 1988). Other changes associated with proestrus include growth of mammary gland ducts and tubules, proliferation of oviductal fimbria, thickening of oviducts and endometrium, elongation of uterine horns, increased myometrial sensitivity, enlargement of the cervix, and proliferation of the vaginal wall (Concannon and DiGregorio, 1986; Concanon 1987). Vaginal cytology during early proestrus often resembles that from a bitch in anestrus with numerous parabasal cells, small

to large intermediate vaginal epithelial cells, neutrophils (± bacteria), and the presence of red blood cells (Feldman and Nelson, 2004). Mid-proestrus typically will contain red blood cells, and an increase in percentage of superficial cells replacing the smaller parabasal and intermediate cells (Feldman and Nelson, 2004). Late proestrus generally will contain, decreased number of red blood cells, with more than 80% of exfoliated superficial vaginal cells, with a nucleus that is vesiculated or pyknotic (Feldman and Nelson, 2004).

Estrus generally is described as the stage during which the bitch allows the male to mount and breed. The duration is usually between 5-9 days. Hormonal changes include a peak of estrogen concentrations 24-48 hours prior to the onset of estrus, followed by decline of estrogen and increase of concentrations of serum progesterone. These hormonal changes are vital for the positive feedback between the hypothalamus and pituitary gland resulting in the secretion of the follicle stimulating hormone (FSH) and the luteinizing hormone (LH) surge in early estrus (Concannon, 1987). The LH surge initiates ovulation within 24-48 hours, after which the corpus luteum forms (Feldman and Nelson, 2004). As the estrus stage progresses, serum progesterone levels continue to rise, additionally doing so through several weeks of diestrus. Testosterone concentrations obtain maximum levels at the time of both the preovulatory LH surge and behavioral receptivity to males (Olsen et al, 1984).

Ultrasonography during estrus can reveal a decrease in the number of visible follicles, an oval shape of the ovaries that becomes more rounded post ovulation, and the presence of cystic-like anechoic structures that are indistinguishable from follicles. These may represent nonovulatory follicles, corpora hemorrhagica, fluid-filled copora lutea, or cystic luteinized follicles (England and Allen, 1989; Wallace et al, 1992). All ovarian follicles are believed to rupture within 12-96 hours (Jeffcoate, 1998). Throughout estrus the uterus is preparing for

implantation. Vaginal bleeding has typically stopped at the start of the estrus phase and the uterus can normally begin to be palpable during abdominal examination due to its increased size and overall thickness. The vulva becomes soft and flaccid, allowing easy access for the male to penetrate. Vaginal discharge is often reported as a straw-colored to pink fluid (Feldman and Nelson, 2004). Vaginal cytology throughout standing (early estrus) includes >80% superficial cells and anuclear squames with no neutrophils (Feldman and Nelson, 2004). Red blood cells may be present on vaginal cytology. Vaginal endoscopy typically will reveal a reduction in mucosal vascularity and edema, and the luminal surface of the vagina then becomes more "crenulated" (Concannon, 1987). Crenulation is the development of angulated folds of vaginal mucosa with sharp edges (Feldman and Nelson, 2004).

Diestrus is defined as the stage of progesterone dominance. It begins with the cessation of standing heat and ends when concentrations of serum progesterone return to baseline levels, typically < 1 ng/mL (Feldman and Nelson, 2004). During this phase, the bitch will abruptly refuse to allow the male to breed. The duration of this stage is 56-58 days in the pregnant bitch and 60-100 days in the nonpregnant bitch (Feldman and Nelson, 2004). Corpora lutea regression is associated with the gradual decrease of serum progesterone concentration and changes to vascular supply (Feldman and Nelson, 2004). Studies have reported the presence of lymphocytes (e.g., CD4, CD8, MHCII-antigen expressing cells) within corpora lutea at early and late diestrus (Hoffman et al., 2004) suggesting an immune-mediated response may also play a role in regression of corpora lutea. Prolactin is also referenced as a key hormone of this stage in the pregnant bitch. As progesterone decreases, prolactin concentrations increase in the late stages of diestrus. Mammary enlargement and secretory activity during diestrus are presumed to be initiated and maintained by prolactin, causing lactation in preparation for offspring (De Coster et

al, 1983; Concannon, 1986). Prolactin is also required for luteal function in the dog (Concannon et al. 1987). Administration of dopamine agonists induces significant declines in progesterone and terminates luteal function (Concannon et al. 1987). Serum immunoreactive relaxin concentrations differ between pregnant (>3.0 ng/mL) and non-pregnant dogs (<0.25 ng/mL) approximately 6-7 weeks into gestation (Steinetz et al, 1989; Buff et al, 2000). Relaxin is produced primarily by the placenta (Tsutsui and Stewart, 1991). The uterus is highly glandular and vascular during diestrus. On vaginal cytology an abrupt change from superficial cells to intermediate and parabasal cells is usually one of the first indicators of diestrus (Feldman and Nelson, 2004).

Anestrus is the phase in which the bitch is in a state of "reproductive rest" and the uterus involutes undergoing self-repair. It can be difficult to determine the start of anestrus in the non-pregnant bitch. Typically, this phase is considered to last an average of 4.5 months (Feldman and Nelson, 2004). Complete repair of the endometrium to a basal state requires ~120 days after decline of progesterone to basal levels in the non-pregnant dog and ~140 days after a fertile cycle (Talwar et al, 1985; Johnston et al, 1985). Sporadic bursts of LH secretion and increases in FSH can occur throughout anestrus in the bitch. Progesterone is extremely low during anestrus (<1 ng/mL), while estrogen concentrations significantly fluctuate (Feldman and Nelson, 2004). Vaginal cytology in anestrus reveals parabasal and intermediate vaginal epithelial cells (± neutrophils and bacteria).

1.2 Infertility in the Bitch

Understanding infertility in any species can be a daunting challenge. When confronted with infertility in the bitch, several attributing factors must be considered. It is crucial for any practicing veterinarian to obtain a thorough history of the bitch's breeding and reproductive

background. Additional questions to address include: the bitch's environment, signalment, and pre-existing health conditions. For instances, bitches housed together overtime may begin to have a synchronous cycle inducing a condition referred to as "dormitory effect" (Johnston et al., 2001). The most common reported cause of female infertility is the failure to perform proper timed breeding (Feldman and Nelson, 2004; Johnston, 2001).

Abnormalities of the reproductive cycle that can cause infertility include persistent anestrus or primary anestrus. This is a condition in which the bitch lacks having a normal reproductive cycle by 2 years of age (Johnston, 1991). Primary causes for persistent anestrus in an intact bitch include: silent heat, abnormalities of sexual differentiation, hypothyroidism, drug-induced, systemic disease, ovarian cyst, ovarian aplasia, and immune-mediated oophoritis (Johnston, 2001). Silent heat in the bitch is described as ovarian activity with no associated vulva swelling, vulvar discharge, and/ or attraction of males (Johnston, 1991). This can be diagnosed by frequent assessment of serum progesterone concentrations yielding results greater than 2 ng/mL and large percentages of cornified cells on vaginal cytology (Johnston, 1991). In one study, 18 bitches with silent heat were successfully induced into estrus after treatment with FSH injections given intramuscularly (IM) or subcutaneously (SC) (Arbeiter and Dreier, 1972).

Abnormalities of sexual differentiation involve abnormal chromosome complements and karyotypes that may lead disorders of sex development, resulting in primary anestrus (Johnston, 1991; Bosu et al., 1978; Johnston et al., 1985). Hypothyroidism is one of the most common endocrine disorders of dogs and is believed to cause infertility by interfering with gamete maturation (Johnston, 1989). Granulosa cell function is supported by thyroid hormones and is required for placental trophoblast function once conception has occurred (Johnson et al., 1997). Approximately 75% of bitches with hyperadrenocorticism have reported persistent anestrus

(Reimers, 1983). It is believed that elevated serum cortisol levels cause a reduction in LH synthesis and secretion from the pituitary gland result in infertility (Kemppainen, 1983). LH is also a hormone required for luteal function in the dog. Hypophysectomy leads to an immediate decline in concentrations of blood progesterone and ends luteal function; whereas administration of LH elevates blood progesterone (Concannon, 1980).

Bitches with other systemic diseases, such as renal azotemia and cancer (transmissible venereal tumors, adenocarcinomas, leiomyomas), are less likely to cycle than normal bitches (Johnston et al., 2001). Progesterone-secreting ovarian cyst can lead to a negative feedback to the pituitary gland, decreasing gonadotropin release and preventing normal cycling (Johnston et al., 2001). Ovarian aplasia is a rare congenital anomaly in the dog result of a defect in prenatal germ cell migration, resulting in elevated serum gonadotropin levels (Johnston, 1989; Johnston, 1991). Another reported rare causes of infertility in the bitch is oophoritis. This is described as the diffuse infiltration of mononuclear inflammatory cells within the ovary, typically accompanied by fibrosis of the surrounding tissues (Johnston, 1989; Andersen and Simpson, 1973; Nickel et al. 1991). The cause is still unknown, but speculated to have an auto-immune pathogenesis. Secondary anestrus is a condition described as prolongation of the interestrus interval. In the bitch this may be caused by endocrinopathies such as hypothyroidism and hyperadrenocorticism or luteal cyst that secretes progesterone (Kemppainen, 1983).

If a bitch has a combined proestrus and diestrus phase greater than 6 weeks, this is defined as persistent estrus (Jeffcoate, 1991). Clinically these animals will present with a persistent sanguineous vaginal discharge and prolonged attraction to males, leading to an associated vaginitis (Freshman, 1991; Allen and Renton, 1982). Most commonly this is caused by presence of ovarian granulosa cell tumors or follicular cysts; however, an association with

hepatic portosystemic shunts, thought to alter metabolism of circulating hormones, and idiopathic lymphocytic oophoritis have been reported (Johnston et al. 2001; Freshman, 1991; Nickel et al., 1991).

Physical abnormalities to the reproductive tract and associated structures may also play a role in infertility. The most common vaginal abnormality is typically circumferential strictures, resulting in failure of natural copulation by the male penis (Wykes and Soderberg, 1983). Other anatomic structural abnormalities that could result in infertility include vaginal prolapse, hyperplasia. This is commonly seen in large (giant) breed bitches during proestrus and estrus and will commonly reoccur (Johnston, 1989).

Uterine infections and other alterations must also be ruled out if infertility is suspected. They can potentially create a harmful environment for both the sperm and egg. Common pathogens to cause an infertile uterus include aerobic bacteria (Enterobacteriaceae, gram-positive cocci), *Brucella canis*, canine herpesvirus, and *Mycoplasma canis* (Johnston et al. 2001). These can lead to pyometra in the bitch and cause pregnancy loss or infertility. Another common alteration of the uterus is cystic endometrial hyperplasia (CEH), leading to failure of implantation post conception (Freshman, 1991). The breeding age of the bitch must also be considered in causes of infertility. Dogs do not undergo a true cessation of the reproductive cycle coinciding with old age, but a decrease in fertility is often seen in bitches great than 5 years of age, typically with an increased interestrus interval (Johnston et al, 2001; Perkins and Thomas, 1993; Johnson et al., 1987). Other miscellaneous causes of infertility speculated in the literature involve production of anti-sperm antibodies produced by the cervix in the bitch (Feldman and Nelson, 2004). Male dogs infected with *Brucella canis* have also been reported to have anti-sperm antibodies (George and Carmichael, 1984). There is additionally one report of bitches

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producing anti-egg zona pellucida antibodies post immunization techniques with isolated and solubilized swine or canine zonae pellucidae, resulting in an infertile female (Mahi-Brown et al, 1982).

Hypoluteoidism is also reported as a cause of infertility in the bitch. It is described as a reproductive condition in which insufficient levels of endogenous progesterone are synthesized and secreted by corpora lutea, resulting in failure to maintain a functional secretory endometrium (Feldman and Nelson, 2004; Johnston et al., 2001). This condition can prevent normal embryo implantation, development, and ultimately end in total loss of pregnancy. If serum levels fall less than 2 ng/mL for greater than 48 hours, pregnancy can be lost (Feldman and Nelson, 2004; Johnston et al., 2001). More recently, it has been reported that pregnant bitches that underwent abortion between four and six weeks of gestation had a decrease in serum progesterone below 10 ng/mL beginning at the third week of pregnancy (Thuróczy et al., 2016). The etiology of hypoluteoidism in the bitch is still not fully understood. Many have speculated this condition is result of an associated gene mutation and poor breeding schemes (e.g., inbreeding), suggesting a hereditary component. Others hypothesize a potential signaling defect between receptors within the bitch reproductive tract and the hypothalamic-hypophyseal-gonadal axis, while others propose an autoimmune response against progesterone (Dockweiler et al., 2017; Krachudel et al., 2013). Studies investigating the correlation between preterm labor, myometrial activity, and cervical changes as potential etiologies for hypoluteoidism are currently in progress (Davidson, 2015). Hypoluteoidism in the breeding bitch is relatively uncommon, but presently there are no safe and effective therapies FDA-approved for use in the dog, indicating the need for further scientific investigations.

1.3 Current Medical Therapies for Treating Infertility in the Bitch

The treatment modality of choice for infertility in the bitch will depend primarily on the suspected cause as previous discussed in section 1.2. It is vital that both a thorough history be obtained and an extensive physical examination be performed using routine diagnostic modalities. This will assist the clinician in ruling out any breeding management, infectious, or non-infectious causes of infertility (Figure 1.1).
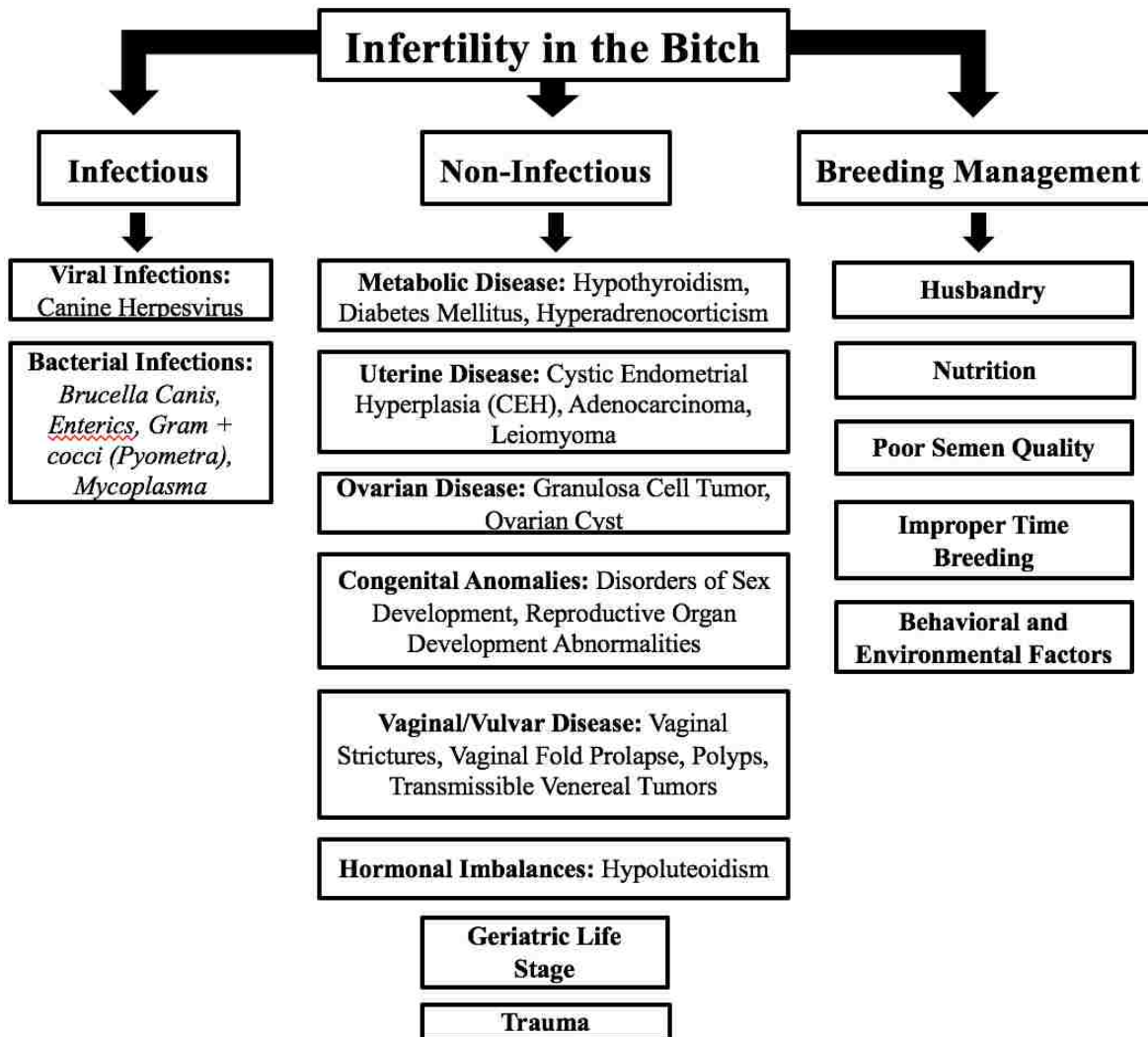

Figure 1.1. Common causes of infertility in the bitch.

It is also important not to lose site of the relationship of the breeding male and its role in the process of canine reproduction. Abnormalities within the male reproductive organs, decrease in sperm production, abnormal sperm morphology, and infectious diseases can all decrease the pregnancy rate in the bitch (Wilborn and Maxwell, 2012).

Even though significant advances have been made within the practice of small animal reproduction, there is still a need for continued investigation into safe alternatives in treating the numerous causes of infertility in the bitch. Current treatments include properly timed breeding and management, hormonal therapy and replacement, assisted reproductive technology (i.e. vaginal, transcervical, and surgical insemination), surgical intervention, and the use of antibiotics when warranted (Feldman and Nelson, 2004; Johnston et al., 2001; Wilborn and Maxwell, 2012; Chastant-Maillard, 2010). Alternative technologies using embryo transfer and intracytoplasmic sperm injection are potential therapies; however, further investigation into developing protocols are required. A recent study successfully reported the birth of seven healthy puppies after *in vitro*-derived embryo transfer in beagles, suggesting an efficacious alternative in assisted reproduction technology (Nagashima et al., 2015).

Medical induction of estrus has historically been used to treat infertility resulting from prolonged inter-estrus intervals, and primary and secondary anestrus. This has been clinically achieved by using commercially available equine chorionic gonadotropin (eCG) from pregnant mares and human chorionic gonadotropin (hCG) from the urine of pregnant women (Feldman and Nelson, 2004). eCG provides primarily an FSH-like action, stimulating initial follicular development, and hCG has a similar effect to LH, serving as an initiator of ovulation (England, 1998). One study reported ovulation in ~50% of bitches after nine consecutive days receiving intramuscular or subcutaneous administration of pregnant mare serum gonadotropin (PMSG).

These subjects exhibited behavioral estrus 10-15 days after the start of treatment (Archbald et al, 1980). Another study using a combination of PMSG and hCG sequentially administered subcutaneously resulted in excess serum concentration of estrogen levels causing several adverse reactions including bone marrow suppression, failure of embryo implantation, and death (England and Allen, 1991).

Protocols using FSH with or without estrogen priming have been described in the literature. Estrogen pretreatment using diethylstilbestrol (DES) for 7 or more days has been proven to successfully induce proestrus (Moses and Shille, 1988). This protocol followed by an intramuscular injection of FSH, 9-11 days after the initial observation of vaginal bleeding, resulted in pregnancy of all treated subjects (Moses and Shille, 1988); However, when using the same protocol and replacing FSH with hCG the onset of behavioral estrus was significantly decreased (Shille et al., 1989).

Protocols using gonadotropin-releasing hormone or GnRH agonist to induce a fertile ovarian cycle have also been described. This of course can only take place with a normal intact pituitary-ovarian axis. One protocol evaluated the use of a surgically implant infusion pump releasing small amount of GnRH every 90 minutes for 6-12 days resulting in successfully induction of proestrus, followed by fertile estrus (Vanderlip et al., 1987; Cain et al., 1988). Historically these studies made significant advancements for new therapies, but the overall high cost to use them made it inaccessible to practitioners (Feldman and Nelson, 2004). An alternative therapy was then evaluated using subcutaneous injections of a GnRH agonist (deslorelin) three times daily for 3 consecutive days. This resulted in the induction of estrus within 9-11 days post starting the treatment in ~80% of subjects, each of which successfully became pregnant (Cain et al 1990). Another study used cabergoline, a dopamine receptor agonist, in 6 bitches diagnosed

with primary or secondary anestrus. Five of the dogs resulted in normal proestrus and estrus, which all were successfully bred and whelped normal litters with no adverse effects reported (Gobello et al, 2002).

Other therapies for non-infectious causes of infertility in the bitch include the use of synthetic progestins for diseases such as hypoluteoidism. There is currently no FDA-approved source of progesterone for supplementation during pregnancy in the dog. Therapies for treating hypoluteoidism in the bitch have presented unique challenges primarily because the definitive cause of the condition is unknown, but the literature reports success using injectable and oral (Regu-Mate®) synthetic progestins in bitches (Tsutsui, 1983; Purswell, 1991; Eilts et al., 1994). Caution must be taken, however, with the use of synthetic progestins due to their numerous reported adverse effects on the overall health of the bitch and the prevention of natural whelping (Eilts et al., 1994; Maddison, 2008).

1.4 The Role of Progesterone in the Bitch

The endocrine system in the bitch plays a vital role in the regulation and ultimately success of pregnancy in the domestic canine. The Beagle dog is currently the most common evaluated breed as it pertains to the correlation between hormone profiles and pregnancy; however, a few reports that have produced similar data have found no differences among other breeds (Johnston et al. 2001; Concannon and Lein, 1989). Progesterone is considered to be the primary hormone during pregnancy and the diestrus phase and is synthesized and secreted by the corpora lutea within the ovary (Figure 1.2).
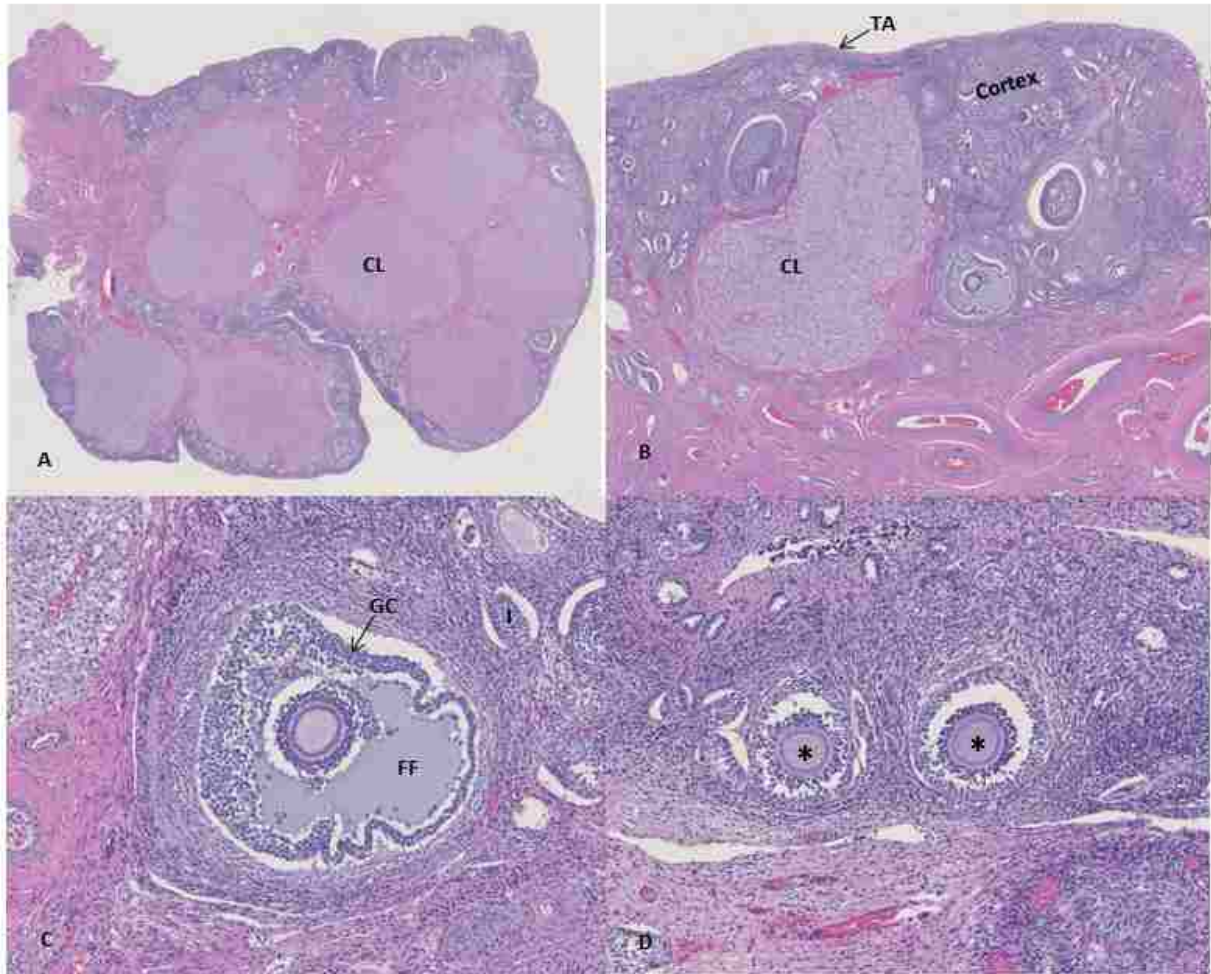
Figure 1.2. Histology images representing the normal microscopic anatomy of the canine ovary: (A) canine ovary with multiple corpora lutea. (B) Corpus luteum, 20x. (C) Tertiary follicle, 10x. (D) Secondary follicles, 10x. FF=antrum filled with follicular fluid, CL=corpus luteum, GC=granulosa cells, I=interstitial gland, TA=tunica albuginea, *=secondary follicles

Its secretion is heavily regulated by luteotropic and luteolytic parameters which is primarily processed by the pituitary gland. Typically, 24-48 hours prior to parturition in the bitch there is a gradual decline of serum progesterone concentrations (Feldman and Nelson, 2004; Johnston et al., 2001). The decline of progesterone prior to parturition takes place coincidently with an increase of prostaglandin F2a levels as result of cyclooxygenase 2 production in fetal trophoblast (Kowalewski et al., 2010). It is generally assumed that plasma progesterone concentrations must be ≥ 2ng/mL to maintain pregnancy (Verstegen-Onclin and Verstegen, 2008). Progesterone

secures the differentiation of both the endometrium and endometrial gland secretion, and also placental attachment (Verstegen-Onclin and Verstegen, 2008). Suppression of uterine contractions is achieved as result of progesterone preventing uterogenic activity of estrogens (Verstegen-Onclin and Verstegen, 2008).

There is no difference between pregnant and non-pregnant bitches in progesterone concentrations during diestrus (Net et al., 1975; Reimers et al., 1978). Therefore, it cannot be used as a sole indicator of pregnancy confirmation in the bitch. Concentrations levels of progesterone in pseudopregnant bitches compared to pregnant animals is still under investigation (Johnston et al. 2001). The number of corpora lutea should be equal to the number of fetuses (Johnston et al, 2001). If there is not a similar correlation of fetus number within a horn and the number of corpora lutea in the ipsilateral ovary, this may be the result of transuterine migration of embryos (Shmizu et al., 1990).

There currently has never been a study to evaluate the total amount of progesterone secreted by each corpora lutea amongst different breeds. There additionally has never been a study to evaluate progesterone secreted by multiple corpora lutea in dogs that produce large litters compared to smaller litters. We understand the importance of the role progesterone plays in the bitch during gestation and we believe answering these previous questions will assist in not only diagnosing, but treating hormonal related causes of infertility in the bitch. This is why our laboratory is particularly interested in investigating safe alternatives to synthetic progestins and evaluating the correlation between progesterone, the ovary, and the uterus.

1.5 Objectives

The objective of this project was to evaluate the efficacy and safety of human-labeled micronized progesterone therapies for use as an alternative treatment of hypoluteoidism in the bitch.

Specific Aims

1. Utilize a dog model in anestrus to measure plasma progesterone concentrations in the bitch after single administration of vaginally (Crinone[®]) and oral (Prometrium[®]) delivered micronized progesterone.

2. Determine and compare the pharmacokinetics (PK) of vaginally (Crinone[®]) and orally (Prometrium[®]) delivered micronized progesterone in the bitch. Data collected will be utilized to devise therapeutic protocols for use in dogs at risk of hypoluteoidism and pregnancy failure. Key pharmacokinetic parameters to determine will include area under the curve (AUC), maximum plasma concentration (C_{max}), time elapsed to reach C_{max} (T_{max}), and elimination half-life ($t_{1/2}$).

3. Evaluate the most efficacious progesterone concentration and administration route that will be capable of maintaining plasma progesterone levels above 1-2 ng/mL when given once daily.

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Chapter 2. Pharmacokinetics of Micronized Progesterone following Oral or Intravaginal Administration in Female Dogs

2.1 Introduction

Progesterone is a vital hormone for both implantation and pregnancy maintenance in both humans and animals. Progesterone secreted from the canine corpus luteum is needed throughout the canine pregnancy until term. It is thought to be necessary for endometrial glandular development, secretion of uterine fluids, endometrial growth, and maintenance of placental attachments (Feldman and Nelson, 2004; Verstegen-Onclin and Verstegen, 2008). Concentrations of plasma progesterone increase coincidentally with LH surge during early estrus, peak at ~20-30d post the luteinizing hormone (LH) surge, and slowly decrease by 60 to 70 days of the non-pregnant diestrus or late pregnancy. Plasma progesterone levels must be ≥ 2 ng/mL to successfully maintain pregnancy in the bitch (Verstegen-Onclin and Verstegen, 2008). Some dogs may experience frank abortion or fetal resorption as result of decreased plasma progesterone concentrations, a phenomenon known as hypoluteoidism.

Hypoluteoidism, also referred to as luteal insufficiency in the literature, is a rising concern in small animal theriogenology with limited treatment options (Practice Committee of the American Society of Reproductive Medicine, 2015; Jones, 1949). It is described as a reproductive condition in which insufficient progesterone is secreted by the corpora lutea resulting in failure to maintain a functional secretory endometrium, thus preventing normal embryo implantation, and development, ultimately ending in loss of pregnancy (Practice Committee of the American Society of Reproductive Medicine, 2015; Johnston et al., 2001; Görlinger, 2005).

Potential etiologies for the condition remain to be elucidated, but both primary and secondary (e.g. pituitary defect) ovarian problems have been described (Hayer, 1997).

The objective of this study was to determine the pharmacokinetics (PK) of vaginally (Crinone®, Watson Laboratories, Inc., Salt Lake City, UT) and orally delivered micronized progesterone (Prometrium®, AbbVie Inc, North Chicago, IL) in the bitch. Our primary focus was to evaluate these progesterone formulations as a possible treatment for low plasma progesterone levels as result of hypoluteoidism. Current synthetic progestins (i.e., medroxyprogesterone, megestrol acetate, proligestone) available to treat bitches pose a risk to their uterine and overall health. Side effects associated with progestin supplementation in dogs and humans include cystic endometrial hyperplasia, mammary tissue dysplasia and potential development of metabolic disorders (Practice Committee of the American Society of Reproductive Medicine, 2015; Fitzpatrick and Good, 1999; Maddison et al., 2008).

Prometrium® and Crinone® are natural micronized progesterone supplements that are currently used in women as a hormone supplement or replacement as part of an assisted reproductive treatment. Prometrium® is available in both 100 mg and 200 mg capsules for oral administration only. Crinone® is available as a 45 mg (4%) or 90 mg (8%) bio adhesive gel emulsion system for intravaginal (IVa) administration.   Hormones that are not water soluble, such as progesterone, have poor absorption and poor bioavailability when administered orally. Previously reported studies have verified that micronized preparations of progesterone yield greater solubility when administered orally and resulted in fewer if no clinical side effects in humans (Chaumeil, 1998). Micronized progesterone is bioidentical to the natural progesterone produced by the corpus luteum, providing a safe alternative to synthetic progestins with significantly less side effects, as it has been shown in women (Practice Committee of the American Society of Reproductive Medicine, 2015; Fitzpatrick and Good, 1999).

We hypothesized that both vaginal and oral treatments would result in a dose-dependent increase in concentrations of plasma progesterone in the bitch. We further hypothesized that oral dosing of micronized progesterone would result in greater, sustained, plasma progesterone concentrations than would be seen with intravaginal micronized progesterone gel. Understanding the half-life and clearance of micronized progesterone will enable practitioners to effectively prescribe it for progestational support, as well as appropriate withdrawal prior to whelping.

2.2 Materials and Methods

Animals

The study protocol was approved by the Louisiana State University Institutional Animal Care and Use Committee. The work was performed in an animal facility accredited by the United States Department of Agriculture and AAALAC International, and assured by the Office of Laboratory Animal Welfare in accordance with *The Guide for the Care and Use of Laboratory Animals* (ILAR, 2011). A total of eight adult sexually intact female dogs were enrolled. All bitches used were screened for any preexisting health conditions by thorough health record evaluation, baseline complete blood counts (CBC), blood chemistries and urinalysis assessments. To prevent interference from other medications a two-week wash out period was instituted prior to the start of the study.

Experiment 1

Eight bitches in anestrus were arranged in a 4x4 Latin square cross over experimental design (Table 2.1).

Table 2.1. Experimental Design: 4x4 Latin square cross over experimental design.

Animal #	Treatment 1	Treatment 2	Treatment 3	Treatment 4
1,2	100 mg, PO	200 mg, PO	45 mg, IVa gel	90 mg, IVa gel
3,4	200 mg, PO	100 mg, PO	90 mg, IVa gel	45 mg, IVa gel
5,6	45 mg, IVa gel	90 mg, IVa gel	100 mg, PO	200 mg, PO
7,8	90 mg, IVa gel	45 mg, IVa gel	200 mg, PO	100 mg, PO

Micronized progesterone; PO-oral administration using tablets of 100 or 200 mg; IVa-intravaginal administration of progesterone bioadhesive gel at 4% and 8% strength to deliver 45 mg and 90 mg of micronized progesterone, respectively.

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Each subject rotated through four different progesterone treatment groups with a minimum seven day-wash out period between treatments: 100 mg oral micronized progesterone, 200 mg oral micronized progesterone, 45 mg intravaginal micronized progesterone and 90 mg intravaginal micronized progesterone (Figures 2.1-2.4).
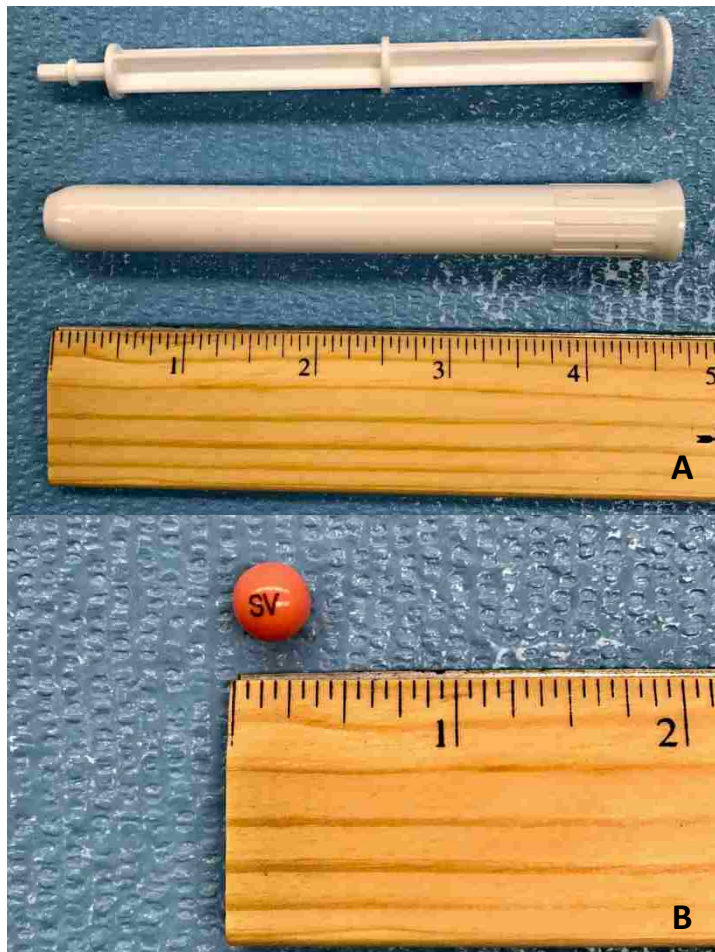

Figure 2.1. (A) Crinone® intravaginal bioadhesive gel applicator. (B) Prometrium® oral capsule.

Figure 2.2. Oral administration of Prometrium® capsule.

Figure 2.3. Intravenous blood collection from cephalic catheter (18G x 1 ¼ in)

Figure 2.4. (A) Parting the vulvar labia to introduce the intravaginal device. (B) Craniodorsal deviation of Crinone® applicator into vagina. (C) Placing the applicator plunger to dispense bioadhesive gel. (D) Bitch hindquarters are raised at a 45°angle for 5 minutes directly after administration of Crinone® intravaginal gel.

Blood samples from each subject were obtained at time points 0, 0.5, 2, 1.5, 2, 4, 6, 8, 12, 24, 36, 48 and 72 hours following one initial dosing of each treatment. Subjects were fed a standard laboratory diet (LabDiet® 5006, Land O' Lakes, Inc., St. Louis, MO) 10 to 15 minutes prior to administration of each respective progesterone treatment and maintained on routine daily

feeding schedule throughout the study. Blood samples were collected with an 18 gauge x 1 inch intravenous cephalic catheter (Sur-Vet®, Terumo Medical Corporation, Somerset, NJ), transferred into 3-mL lithium heparinized plastic sterile tubes (BD Vacutainer®, Becton Dickinson and Company, Franklin Lakes, NJ), and centrifuged for plasma separation for 10 minutes at 1520 g. Plasma samples were placed in duplicate cryotubes and stored at -20° C until analyzed. Concentrations of plasma progesterone were determined by radioimmunoassay (ImmuChem Double Antibody, 125I RIA Kit, MP Biomedicals, Costa Mesa, CA).

Experiment 2

After analyzing data from our first study, an additional pilot study was performed using 4 adult sexually intact bitches in anestrus. Our goal was to evaluate which micronized progesterone formulation and route would best maintain plasma progesterone levels above 1-2 ng/mL when given once daily. Subjects received one daily dose of the respective treatment groups every 24 hours for five days: oral 100 mg micronized progesterone, 200 mg oral micronized progesterone, 4% (45 mg) intravaginal micronized progesterone and 8% (90 mg) intravaginal micronized progesterone. Only one dog was used for each of the four treatment groups. Serial blood collection was then performed at the following time points during five consecutive days: 0 (baseline), 4, 12, 24, 28, 36, 48, 52, 60, 72, 76, 84, 96, 100, and 108 hours. Blood samples were collected from 18 gauge x 1 inch intravenous cephalic catheter (Sur-Vet®, Terumo Medical Corporation, Somerset, NJ), transferred into 3-mL lithium heparinized plastic sterile tubes (BD Vacutainer®, Becton Dickinson and Company, Franklin Lakes, NJ), and centrifuged for plasma separation for 10 minutes at 1520 g. Plasma samples were placed in duplicate cryotubes and stored at -20° C until analyzed. Concentrations of plasma progesterone were determined by fluorescence enzyme immunoassay using an Automated Immunoassay Analyzer (AIA-360, Tosoh Bioscience, Inc., San Francisco, CA).

2.3 Results

Pharmacokinetic analysis was carried out using commercially available software (Phoenix WinNonlin 6.4, Certara Inc., Princeton, NJ). One-compartmental (intravaginal) and non-compartmental (oral administration) modeling were performed to analyze data using the mean concentrations for each dosing to calculate the area under the curve (AUC), maximum plasma concentration (C_{max}), time elapsed to reach C_{max} (T_{max}), and elimination half-life ($t_{1/2}$).

Results for the 100 mg and 200 mg oral doses and 45 mg and 90 mg IVa doses were as follows: AUC, 30.86, 187.96, 90.64, and 226.68 ng.h.mL^{-1}, respectively; C_{max}, 13.47, 169, 8.68, and 13.24 ng.mL^{-1}, respectively; T_{max}, 0.5, 0.5, 0.84, and 1.67 h, respectively, and half-life, 5.87, 6.76, 6.6, and 10.65 h, respectively (Table 2.2).

Table 2.2. Pharmacokinetics of oral and intravaginal (IVa) micronized progesterone administered to eight anestrus dogs.

Pharmacokinetics parameters	100 mg oral	200 mg oral	45 mg IVa	90 mg IVa
AUC (ng.h.mL^{-1})	30.86	187.96	90.64	226.68
C_{max} (ng.mL^{-1})	13.47	169	8.68	13.24
T_{max} (hr)	0.5	0.5	0.84	1.67
Half-life (hr)	5.87	6.76	6.6	10.65

Pharmacokinetic analysis was carried out using commercially available software (Phoenix WinNonlin 6.4, Certara Inc., Princeton, NJ). One-compartmental (intravaginal) and non-compartmental (oral administration) modeling were performed to analyze data using the mean concentrations for each dosing to calculate the area under the curve (AUC), maximum plasma concentration (C_{max}), time elapsed to reach C_{max} (T_{max}), and elimination half-life ($t_{1/2}$).

No adverse reactions were seen during the entire duration of the both experiments.

Progesterone exposure over time, as indicated by the area under the curve, was greater when intravaginal micronized progesterone was used (Table 2.2). There was no present "carry over" effect amongst treatment groups, validating the efficacy of the seven-day washout period. Figure 2.7 distinctly supports this argument showing that by the 72 h mark, all treatment groups have return to baseline levels, also verifying each subject was maintained in the anestrus phase of their

reproductive cycle. Furthermore, baseline concentrations of plasma progesterone were confirmed

immediately before the start of subsequent treatments throughout the study.

Micronized progesterone delivered orally or intravaginally was readily absorbed in bitches

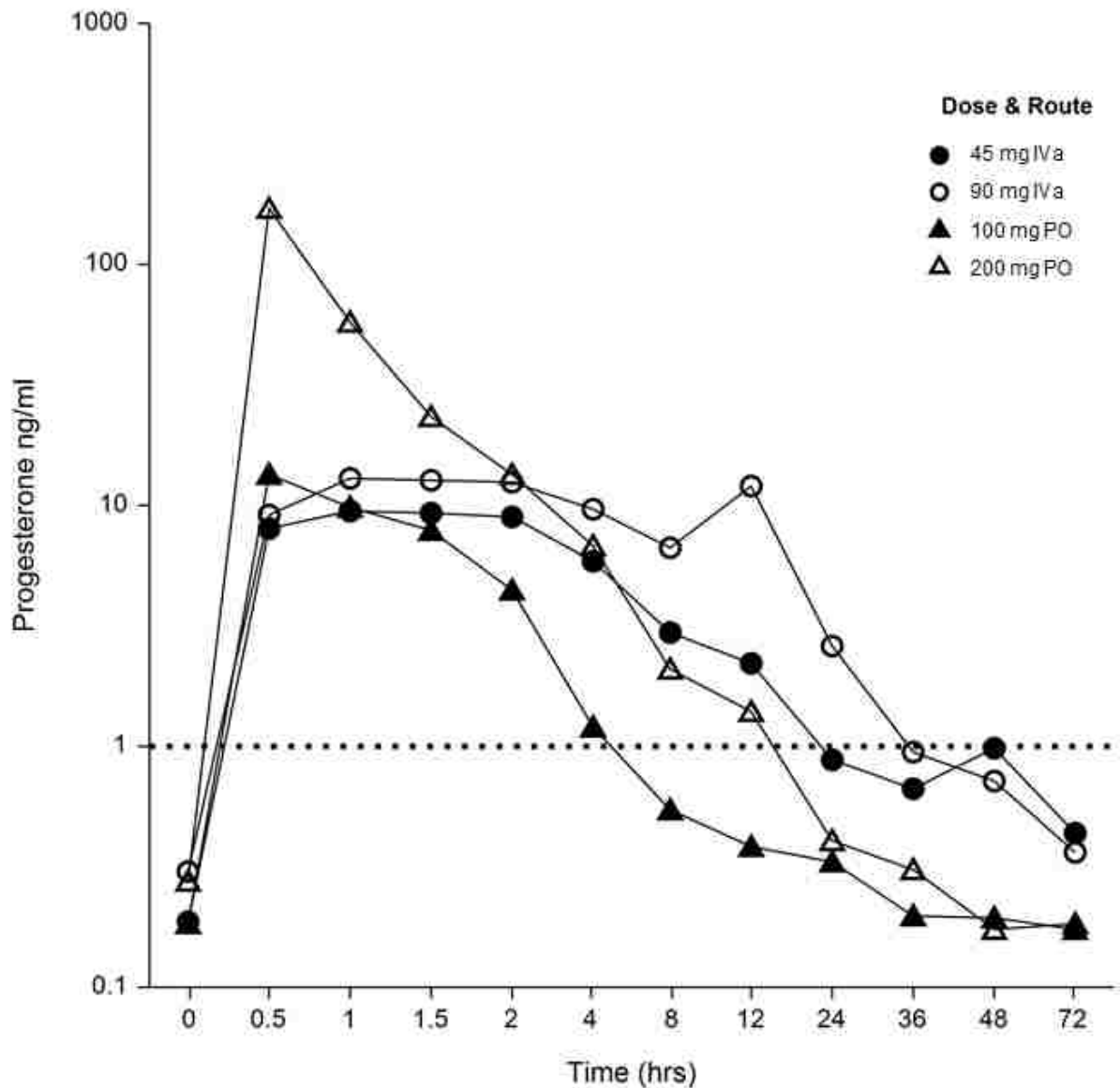following a single or daily dose treatment (Figures 2.5-2.7).

Figure 2.5. Line graph comparing concentrations of plasma progesterone over time (72 hours) after a single administration of micronized progesterone. The dashed line represents target value to maintain plasma progesterone levels above 1 ng/mL with micronized progesterone therapy. PO-oral; IVa-intravaginal.
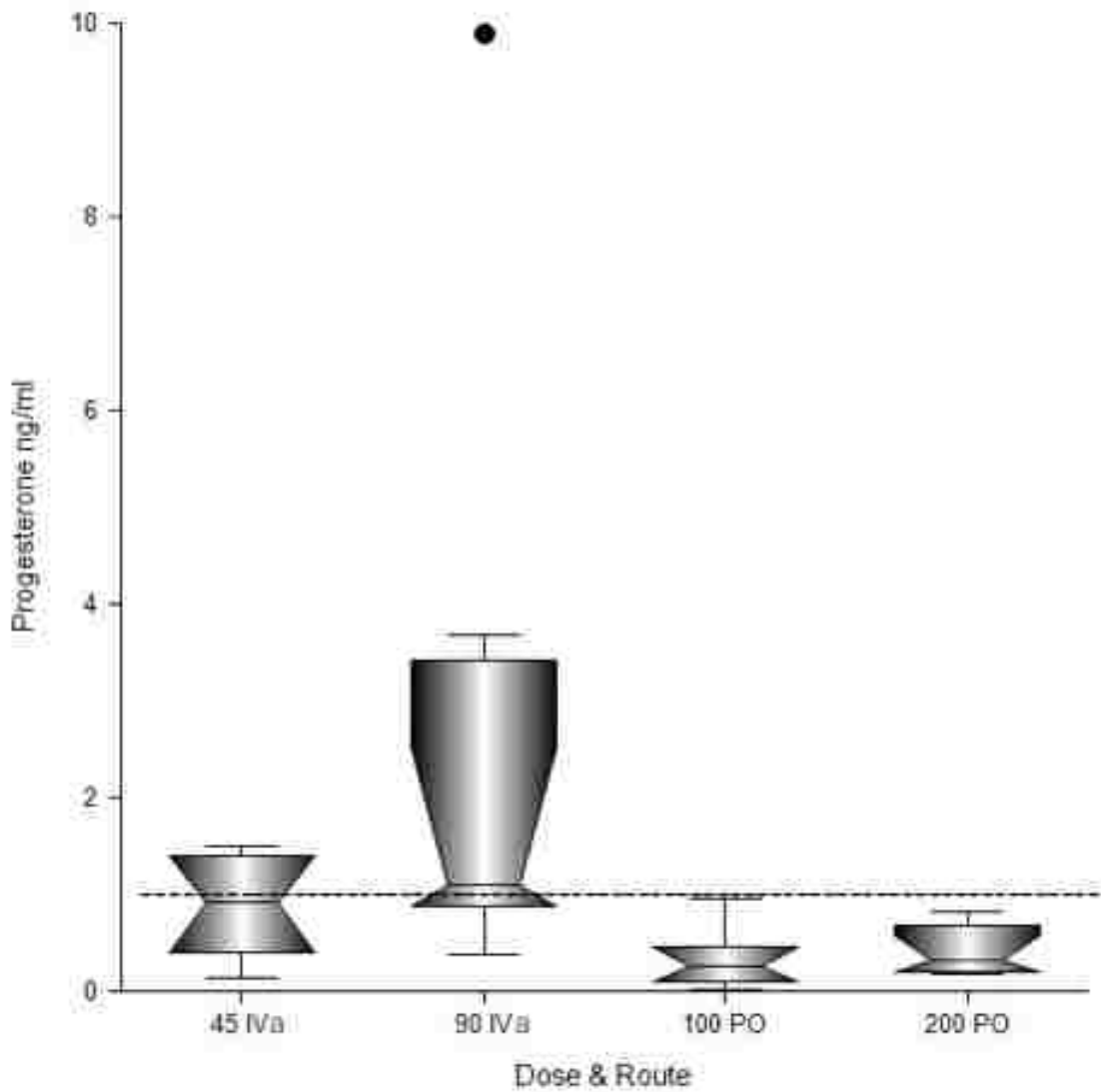
Figure 2.6. Box and whisker plot (at 24 hours) after a single administration of micronized progesterone. The dashed line represents target value to maintain plasma progesterone levels above 1 ng/mL with micronized progesterone therapy. PO-oral; IVa-intravaginal.
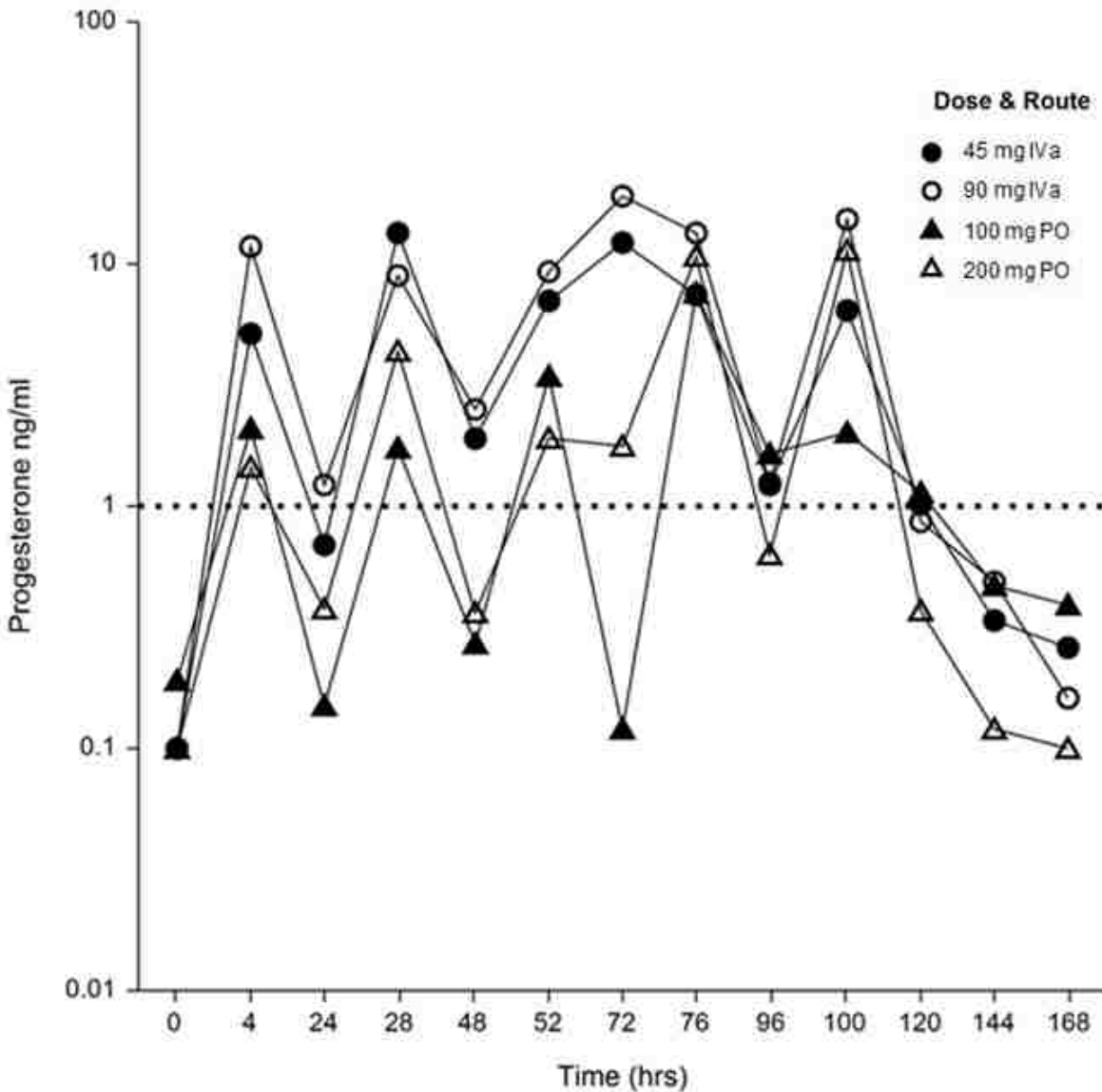
Figure 2.7. Line graph showing concentrations of plasma progesterone following administration of micronized progesterone given once daily for five days. The dashed line represents target value to maintain plasma progesterone levels above 1 ng/mL with micronized progesterone therapy. PO-orally; IVa-intravaginally.

Experiment 1 revealed that intravaginal formulations successfully maintained plasma progesterone levels ≥ 1 ng/mL for 24 hours (Figure 2.6). The oral progesterone formulations yielded the greatest values, but fell below 1 ng/mL between 12-24 hours. Concentrations of plasma progesterone returned to pre-treatment levels by 72 hours (Figure 2.5). Experiment 2

revealed that intravaginal formulations given once daily successfully maintained plasma progesterone levels ≥1 ng/mL for the duration of the experiment (5 days; 120 hours). Concentrations of plasma progesterone returned to pre-treatment levels 72 hours after the final daily dose was administered (Figure 2.7). No adverse reactions were seen during the entire duration of the study. Additionally, blood chemistry panels performed before and after the study revealed no significant findings

2.4 Discussion

Hypoluteoidism is a reproductive condition in which insufficient levels of endogenous progesterone are synthesized and secreted by the corpus luteum. Whereas true hypoluteoidism in bitches during pregnancy is relatively uncommon, declining concentrations of blood progesterone normally seen during canine late gestation may raise concerns for dog breeders and veterinarians, especially if patients have experienced infertility or pregnancy losses in the past. Low (< 5 ng/mL) or declining blood levels of progesterone may prompt veterinarians to prescribe progesterone supplementation. Pre-natal examinations have grown popular among dog breeders where they often request multiple ultrasound examinations to determine fetal well-being. In addition, in bitches with a history of infertility, determination of concentrations of blood progesterone is often performed. Pregnant bitches that present with fluctuating, consistently declining, or overall low plasma progesterone levels prompt clinicians to prescribe progesterone supplementation. This approach is warranted as bitches with a pattern of declining blood progesterone levels may be at risk for infertility and pregnancy loss. There are several case reports where hypoluteoidism has been diagnosed in dogs because clinicians were unable to definitively identify any other common pathogens that cause infertility and abortion in the bitch (Görlinger et al., 2005; Hayer, 1997; Root Kustritz, 2001; Purswell, 1991; Estill, 1998; Johnson,

2008; Günzel-Apel et al., 2012; Tibold and Thuróczy, 2009). The etiology of hypoluteoidism in the bitch is still not fully understood. Many have speculated this condition is result of an associated gene mutation and poor breeding schemes (i.e., inbreeding), indicating a hereditary component. Others hypothesize a potential signaling defect between receptors within the bitch reproductive tract and the hypothalamic-hypophyseal-gonadal axis (Dockweiler et al., 2017; Krachudel et al., 2013). Further studies are needed to fully characterize hypoluteoidism in the dog.

There is currently no FDA-approved source of progesterone for supplementation during pregnancy in the dog. There are a few reports of injectable (oil based) progesterone preparations that have been used in the anestrus and ovariectomized pregnant dog model yielding positive results (Tsutsui, 1983; Scott-Moncrieff et al., 1990; Eilts et al., 1994). The most recent case reports described in the literature have empirically used either oral (i.e., medroxyprogesterone acetate, 0.1 mg/kg, PO SID and altrenogest, 0.088 mg/kg PO SID) or injectable (in oil) synthetic progestogen and natural progesterone (Luteosan®; Alvetra and Werfft AG, Vienna, Austria) to treat hypoluteoidism (Root Kustritz, 2001; Purswell, 1991; Estill, 1998; Johnson, 2008; Günzel-Apel et al., 2012; Tibold and Thuróczy, 2009). These therapies allowed the bitch to give birth to viable puppies, after multiple failed attempts of breeding. Though these reports revealed successful outcomes, current synthetic progestins available to treat dogs pose significant risk to the reproductive tract and the overall health of the bitch. Many adverse effects associated with synthetic progestin use have been reported and include: cystic endometrial hyperplasia, mammary dysplasia, development of metabolic disorders (i.e. diabetes mellitus), masculinization of female fetuses, and prevention of spontaneous parturition (Practice Committee of the

American Society of Reproductive Medicine, 2015; Fitzpatrick and Good, 1999; Maddison et al., 2008).

To our knowledge, this is the first reported study to investigate the pharmacokinetics of micronized progesterone in the bitch. The progesterone used in these products are extracted from a plant root and as such are labeled as a natural product. The progesterone in Crinone® and Prometrium® is converted from diosgenin (extracted from wild yams, *Dioscorea spp)* to progesterone using standard laboratory techniques (Applexweig, 1969). The chemical formula and associated structure ($C_{21}H_{30}O_2$) for both Crinone® and Prometrium® are identical to that of endogenous progesterone produced by the corpus luteum. They also possess the same molecular weight; 314.5 g/mol (Crinone, 2014; Prometrium, 2013). Historically, progesterone given orally was considered unfeasible due to its poor absorption; however, studies have now shown that this can be improved with the appropriate vehicle and particle size (Hargrove et al., 1989). Micronized progesterone formulations possess two major benefits: (1) increased bioavailability with oral use and (2) significantly decreased adverse effects for both short and long term use, compared to synthetic progestogens (Chaumeil, 1998; Hargrove et al., 1989; de Lignieres, 1999). Progesterone is metabolized primarily by the liver and excreted by the kidney.

The ability of intravaginal preparations of micronized progesterone to induce sustained progesterone exposure may provide an alternative strategy for treating pregnant dogs whenever hypoluteoidism is being suspected. We propose that the longer half-life of the intravaginal product is a result of bypassing first pass metabolism in the liver, as well as a slower absorption rate. This same phenomenon termed "first uterine pass effect" has previously been described in women treated with vaginally administered micronized progesterone (Bulletti et al., 1997; Levine and Watson, 2000). Similar findings have been documented in women after vaginal use

of drugs such as terbutaline and danazol (Kullander and Syanberg, 1985; Misutani et al., 1995).

Further investigation is warranted for both the oral and intravaginal preparations in pregnant

dogs to evaluate efficacy, safety, and to determine the optimum time to stop supplementation

prior to whelping. Additionally, alternative strategies to avoid first-pass prehaptic and hepatic

metabolism such as vaginally administered suppositories should be considered.

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Chapter 3. Conclusions

The intent of our study was to determine if the use of micronized progesterone will indeed have a positive effect on the bitch and increase the plasma progesterone levels during anestrus. After establishing this to be true, we set out to determine through a pilot study which route and concentration may be efficacious in maintaining plasma progesterone levels above 1-2 ng/mL. Our initial hypothesis was discovered to be invalid. Intravaginal micronized progesterone was a more effective method than oral administration in the anestrus bitch in both experiments (figures 2.5-2.7).

Our findings raise many questions about variables that pertain to owner compliance. We suspect that adequate training would be required in order to assist owners in becoming comfortable with using the Crinone® gel applicator device. This is why initially the concept of using oral micronized progesterone was appealing. We were unable to extensively evaluate any toxic effects of micronized progesterone use in the bitch. Additionally, we were limited in our sample size, and therefore strongly encourage further studies evaluating a similar protocol in a larger population of bitches. It also would be interesting to evaluate the effects of micronized progesterone in bitches in diestrus and complete a retrospective study of clinical cases in small animal hospitals.

If progesterone levels fall below 2 ng/mL for more than 48 hours during pregnancy it is often terminated (Johnston et al., 2001). Thus the need to discover safe alternatives for treating hypoluteoidism in the bitch as most synthetic progestins pose significant risk to the bitch's health. Our study was originally adapted from the protocols used in women comparing pharmacokinetics of Crinone® and Prometrium® in postmenopausal subjects (Levine and Watson et al., 2000; Simon et al., 1993). Other concepts to explore involve developing technologies to

produce slow-releasing injectable drugs or surgically placed osmotic pumps to deliver effective

doses of micronized progesterone over time. In future investigations of alternative therapies for

infertility in the bitch, it may be judicious to continue to integrate techniques used in other

mammalian species and human reproductive medicine.

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Vita

Raphael Anthony Malbrue is a native of Baton Rouge, Louisiana. He graduated from Catholic High School in 2007. After graduation, he attended Tuskegee University where he graduated in 2011 with a Bachelor of Science in animal, poultry, and veterinary sciences. After undergraduate school, he continued his studies at Tuskegee University School of Veterinary Medicine. He graduated from veterinary school in 2014. Immediately following graduation, Raphael began a residency program in Laboratory Animal Medicine within the Division of Laboratory Animal Medicine at Louisiana State University School of Veterinary Medicine under the guidance of Dr. Rhett Stout and Dr. David Baker. During the residency, he also began his graduate studies under the mentorship of Dr. Carlos Pinto through the Theriogenology Department. Upon completion of his residency training and Master's degree Raphael will begin his career as an assistant professor (Department of Veterinary Preventative Medicine) and clinical laboratory animal veterinarian (University Laboratory Animal Resources) with The Ohio State University Laboratory.