ARGININE SUPPLEMENTATION DURING ESTRUS HAS NO EFFECT ON UTERINE BLOOD FLOW OR FLUID CLEARANCE IN NON-PREGNANT MARES

By

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To Grandma Shirley
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ARGININE SUPPLEMENTATION DURING ESTRUS HAS NO EFFECT ON UTERINE BLOOD FLOW OR FLUID CLEARANCE IN NON-PREGNANT MARES

By
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Uterine fluid accumulation has been identified as a factor that can negatively impact the reproductive performance of mares by reducing pregnancy and successful fertilization as well increasing early embryonic death rates. L-arginine is an amino acid responsible for various functions in the body and has been identified as an essential amino acid in equine nutrition. L-arginine supplementation has been shown to positively impact reproductive performance in pigs and mice, and alter uterine involution in mares. Acting as a nitric oxide donor, L-arginine has been demonstrated to increase blood flow. An increase in blood flow has been associated with hastened uterine involution and fluid clearance. The objectives of this study were to determine the effect of L-arginine supplementation on uterine arterial blood flow and fluid clearance in non-pregnant mares. It was hypothesized that mares supplemented with L-arginine would have hastened uterine fluid clearance when compared to control. Twelve non-pregnant light horse mares were used in a 3x3 Latin Square design study. The three treatments included dietary supplementation with L-arginine, isonitrogenous amounts of urea, or no supplementation coupled with administration of oxytocin. Treatments were initiated 10 d
post ovulation, and continued until all fluid was absent from the uterus, resulting in a total length of supplementation of approximately 12 days. Mares were examined by transrectal ultrasonography and infused with 880 mL of sterile saline combined with 120 mL of semen extender upon discovery of a 33 mm follicle. Mares were examined by transrectal Doppler ultrasonography at 12-h intervals following infusion and uterine blood flow and fluid presence were recorded until all fluid was absent from the uterus. Mean fluid clearance across treatments was 53.8 ± 4.9 h. Short-term arginine supplementation had no effect on rate or latency of fluid clearance or blood flow measured as pulsatility or resistance indices. Although previous research has demonstrated L-arginine supplementation improved uterine blood flow and hastened uterine fluid clearance in early postpartum mares, results of this study indicate L-arginine supplementation has limited effects on uterine hemodynamics and fluid clearance in nonpregnant mares.
CHAPTER 1
INTRODUCTION

Uterine fluid accumulation has been shown to negatively impact the reproductive performance of the mare, including reduced pregnancy rates as well as increased early embryonic loss (McKinnon and Voss, 2005). The accumulation of fluid in the uterus of the mare is also associated with endometritis and has a negative correlation to the reproductive efficiency of the mare (McKinnon and Voss, 2005).

In the equine industry there are economic incentives that influence breeders to maintain a 12-month breeding schedule (McKinnon and Voss, 2005). Mares are unique in that they have a fertile foal heat occurring approximately 10 d post-foaling (Blanchard et al., 2002). Breeding on foal heat allows the breeder to maximize breeding efficiency and thus maintain an economic advantage. Many breed registries mandate a universal birthday of January 1 for foals. Breeding on foal heat can allow for the foal to be born at or near that date and maintain a competitive advantage. Factors such as uterine fluid accumulation can negatively impact the success achieved when breeding during this foal heat period (McKinnon and Voss, 2005). Delayed uterine involution as well as prolonged endometritis are often responsible for the decline in breeding efficiency during foal heat (McKinnon and Voss, 2005).

Ultrasonography is an extremely valuable tool for estimating the quality and quantity of intrauterine fluid, as well as other key reproductive attributes (McKinnon and Voss, 2005). Blood flow measurements obtained via Doppler ultrasonography can also help determine the reproductive efficiency of the mare (Ginther, 1992). An increase in blood flow has been shown to increase reproductive efficiency in pigs (Mateo et al., 2007) and rats (Greene et al., 2012). Increases in blood flow have also been associated
with increased rates of uterine involution following parturition (Ginther, 1992). Uterine blood flow is measured using indices such as the pulsatility index (PI) and the resistance index (RI; Ginther, 1992). A decrease in RI is associated with an increase in blood flow and there is a strong correlation between RI and PI (Ginther, 1992).

Arginine is one of the most versatile amino acids and serves as a precursor for a multitude of molecules (NRC, 2007). Arginine is recognized as an essential amino acid for horses, but the requirements are not yet known (NRC, 2007). However, as in other species, arginine is presumably synthesized in the horse, but in insufficient quantities and must be supplied in some part by the diet (NRC, 2007).

Arginine supplementation has been investigated for its potential reproductive benefits in rats and pigs and has shown favorable results including increased litter size, number of offspring born alive, and increased placental attachments. (Mateo et al., 2007; Li et al., 2010; Greene et al., 2012). Arginine supplementation has also been associated with an increase in uterine arterial blood flow and hastened uterine involution in postpartum mares (Mortensen et al., 2011).

Amit et al., (1998) stated that arginine is believed to influence blood flow by impacting the production of nitric oxide (NO). The conversion of arginine to citrulline involves the production of NO through the citrulline/NO cycle (Wu and Morris, 1998). Nitric oxide generated from arginine by the vascular endothelium has been shown to be an important regulator of vascular tone and subsequent blood flow (Amit et al., 1998). Arginine has also been shown to influence important angiogenic and vasculogenic factors such as vascular endothelial growth factor (VEGF; Greene et al., 2012).
We hypothesized that mares supplemented with L-arginine would have increased uterine blood flow and consequently hastened uterine fluid clearance when compared to a control. Increased blood flow is associated with increased lymphatic drainage, which is associated with uterine fluid clearance. The objectives of the current study were to investigate the effects of L-arginine supplementation on uterine fluid removal, uterine arterial blood flow and hemodynamics. In addition, this study investigated the effects of arginine supplementation on follicular development and ovulation. To the authors’ knowledge, this is the first study to investigate the effects of L-arginine supplementation on the rate of uterine fluid clearance.
Chapter 2
Review of Literature

Overview of Equine Reproductive Physiology

Reproductive Anatomy

The main reproductive organs of the mare include the ovaries, oviducts, uterus, and cervix. The ovary of the mare consists of three layers including the cortex, medulla, and the hilum (Berne et al., 1998). The ovary of the mare is unlike that of other farm animals. The inner zone is considered the cortex and consists of germinal epithelium, which contains the oocytes (Berne et al., 1998, Senger, 1997). The outer layer, known as the medulla consists of heterogenous cells (Senger, 1997). The hilum is located on the convex side of the ovary and is the area of attachment to the abdominal cavity as well as a passage for blood vessels and nerves (Senger, 1997).

The follicles on the ovary are one of four types. The most immature and undeveloped follicles, primordial follicles contain immature oocytes surrounded by flat, squamous granulosa cells (Senger, 1997). Primordial follicles can lie dormant for up to 50 years in humans and 30 years in horses and have little to no biological activity (Berne et al., 1998). Primary follicles develop from primordial follicles and are characterized by a single layer of cuboidal cells surrounding the oocyte. At this time, primary follicles develop receptors to follicle stimulating hormone (FSH), but are still considered gonadotropin-independent (Hillier, 2001). Primary follicles can then develop into secondary follicles, which have multiple layers of granulosa cells that undergo cytodifferentiation into the theca externa and theca interna (Hillier, 2001). Tertiary follicles, also known as antral follicles are characterized by the development of a fluid filled antrum (Senger, 1997).
Folliculogenesis occurs in a wavelike pattern that results in the selection of a dominant follicle (Senger, 1997). The mare is unique in that ovulation occurs at one point in the ovary known as the ovulation fossa (Senger, 1997). Mares are considered a monotocous species, meaning that they generally ovulate a single oocyte, resulting in a single embryo (Senger, 1997).

**Oviduct**

The oviduct is the passageway for oocytes from the ovary to the uterus, as well as a pathway for spermatozoa from the uterus to the oocyte (Pauerstein *et al.*, 1974). The oviduct is supported in the abdomen of the mare by the mesosalpinx region of the broad ligament (Pauerstein *et al.*, 1974). The oviduct does not directly connect to the ovary, rather it surrounds the ovary with a structure known as the infundibulum (Senger, 1997). The infundibulum has many ciliated projectiles that function to move the ovulated oocyte into the oviduct and towards the uterus (Pauerstein *et al.*, 1974). Following the infundibulum, is a region of the oviduct called the ampulla. The ampulla is a ciliated structure that functions to move the oocyte toward the site of fertilization (Pauerstein *et al.*, 1974). Fertilization occurs at a site termed the ampullary-isthmus junction (Senger, 1997). The distinction between the ampulla and isthmus can be seen histologically, as the isthmus is surrounded by smooth muscle cells and characterized by a smaller luminal area, while the cells lining the ampulla are ciliated to assist in transport of the oocyte towards the site of fertilization (Senger, 1997). This smooth muscle acts in a peristaltic function to push sperm cells toward the oocyte and the site of fertilization (Senger, 1997). The oviduct joins the uterus at the uterotubual junction (Pauerstein *et al.*, 1974).
Uterus

The uterus of the mare is considered a bicornuate uterus with a single body and less-developed horns than that of the sow (Senger, 1997). The uterus lies horizontal in the mare and is suspended by the broad ligament (Ginther, 1992). The arrangement of the uterus in regards to the attachment to the broad ligament allows for palpation of the uterus through the rectum (Blanchard et al., 2002). As the mare ages and parity increases, the uterus is suspended lower in the abdomen as the broad ligament stretches and extends to accommodate gestation (Blanchard et al., 2002).

The uterus is characterized by three distinct layers. The serous layer is continuous with the broad ligament and is the outermost layer of the uterus. The myometrium consists mainly of smooth muscle, and its main function is to push the foal into the birth canal during parturition. In addition, this smooth muscle is responsible for contractions required to clear the uterine lumen of fluid and during uterine involution following parturition (Ginther, 1992). The myometrium is significantly thick and responsible for the changes in tone that are seen throughout the estrous cycle of the mare. During behavioral estrus, under the influence of estrogen, more tone can be expected in the uterus than during anestrus (Hayes and Ginther, 1986). The innermost layer, known as the endometrium is a complex mucosal membrane containing a rich blood supply that houses and supports the developing fetus during pregnancy (Ginther, 1992). The endometrium of the uterus is glandular and secretory in nature (Ginther, 1992).

The uterus of the mare is characterized by a large uterine body anterior to the cervix with two smaller uterine horns that terminate at the oviduct (Blanchard et al., 2002). In a nonpregnant state, the uterine lumen is nearly indistinguishable and defined by dominant endometrial folds, which can be palpated through the rectum. The
endometrial folds lie longitudinally and form a “wagon-wheel” type appearance when viewed on an ultrasound (Blanchard et al., 2002).

Uterine endometrial biopsies can be used to grade the uterus of the mare based on inflammation and fibrosis of the uterus (McKinnon and Voss, 2005). These biopsies are useful in helping to diagnose reasons for infertility in mares and create an accurate prognosis as to the mare’s future reproductive potential (McKinnon and Voss, 2005). Grading is done on a scale from Grade I, which is a normal endometrium to a Grade III, which is indicative of severe inflammation or diffuse fibrosis (Blanchard et al., 2002). A grade of IIB results in a 10-50% less chance of conceiving or carrying a foal to term, while a grade of III indicates less than a 10% chance of carrying a foal to full term (Blanchard et al., 2002). Increased inflammation is also a factor in reduction in uterine fluid clearance or an increase in uterine fluid accumulation (Blanchard et al., 2002).

Cervix

The cervix of the mare is an adaptable organ that is lined with secretory epithelial cells. These epithelial cells secrete a thin mucus for lubrication during estrus and a thicker mucus during diestrus that is less permeable to foreign contaminants and bacteria (Senger, 1997). During estrus under the influence of estrogen the cervix can expand to accommodate the stallion’s penis as well as during parturition to allow for passage of the foal (Senger, 1997). During diestrus as well as pregnancy under the influence of progesterone, the cervix is tightly closed (McKinnon and Voss, 2005). The cervix of the mare is characterized by longitudinal folds that extend from the endometrial folds of the uterus (Blanchard et al., 2002). The cervix of the mare is unique from the cervix of other farm animals in that its lumen can greatly expand and contract because of a thick layer of muscular fibers as well as an absence of transverse cervical rings.
(Blanchard et al., 2002). These unique characteristics allow the uterus to be more easily accessed through the cervix than that of the cow, which allows for ease of artificial insemination as well as other breeding practices (Blanchard et al., 2002). Although the cervix can easily be palpated through the rectum due to its thick-walled nature, it is more readily palpated under the influence of progesterone, which causes the cervix to maintain a more rigid state (Hayes and Ginther, 1986). In contrast, under the influence of estrogen during estrus, the cervix is flaccid and difficult to palpate (Hayes and Ginther, 1986).

Maintaining the integrity of the cervix, and especially the external os of the cervix, is important in regards to fluid clearance as well as fluid accumulation in the uterus of the mare (McKinnon and Voss, 2005). Cervical injuries that can occur during foaling or breeding can compromise the integrity of the uterus as well as impact the overall reproductive efficiency of the mare (McKinnon and Voss, 2005).

**Uterine Vasculature and Blood Flow**

**Reproductive Vasculature**

The reproductive tract of the mare is suspended in the abdomen by the broad ligament (Ginther, 2007). Arteries supplying blood to the uterus and ovaries pass through the broad ligament and connect to the ovaries and the uterus after branching off of the aorta (Ginther, 2007). The broad ligament is broken into three unique areas that are distinctive in their function and can be useful in locating individual blood vessels (Ginther, 2007). The mesometrium attaches to the uterus, while the mesovarium attaches to the ovaries, and the mesosalpinx projects from the mesovarium and supports the oviduct (Ginther, 2007).
The aorta passes along the spinal column dorsal to the reproductive tract in the mare (Bollwein et al., 2002). The ovarian artery branches off of the aorta and runs dorsally along the abdominal wall until it enters through the mesovarium and connects to the ovary (Ginther, 2007). The location and anatomy of the uterine artery is unique in the mare from that of the cow in that it branches off of the aorta at a different origin (Ginther, 2007). The uterine artery extends from the external iliac artery in horses, which is in contrast to cows and heifers where it branches from the internal iliac artery (Ginther, 2007). The uterine artery forms both a caudal and a smaller cranial branch. The uterine artery branches course along the antimesometrial border and gives origin to branches that run over the individual horns (Ginther and Pierson, 1984). The uterus receives blood from the uterine branch of the ovarian artery, the uterine artery, and the uterine branch of the vaginal artery, and all three sources are interconnected to varying degrees, in addition to a variation between mares (Ginther, 2007). In older, multiparous animals, the uterine artery follows a convoluted path as a result of uterine involution and stretching of the broad ligament during gestation (Ginther and Pierson, 1984). The uterine artery is looser than the other arteries in support of physiological changes seen during gestation and uterine involution (Ginther, 2007). In addition, the diameter of the uterine artery changes substantially during gestation (Ginther, 2007).

Ultrasound has been identified as a key tool in identifying arteries as well as measuring blood flow through the arteries (McKinnon and Voss, 2005). The uterine artery is identified on the screen of an ultrasound by determining where the external iliac artery (EIA) branches from the aorta (Ginther and Pierson, 1984). The branching of the EIA is followed closely by the branching of the uterine artery from the EIA. The
branching of the deep circumflex artery (DCA) follows closely after, but may originate directly from the aorta before the branching of the EIA. The uterine artery will always pass above and close to the DCA regardless of its origin (Ginther, 2007). In open mares, the artery may be barely detectable because of its small size and diameter, which ranges from 2-6 mm in mares between the ages of 6 and 13 (Ginther, 2007).

**Arteries and Hemodynamics**

Blood flow is characterized by both systemic and pulmonary circulation (Maton et al., 1993). Pulmonary circulation is defined as the portion of the cardiovascular system which pumps deoxygenated blood from the heart to the lungs via the pulmonary artery and subsequently returns oxygenated blood to the heart via the pulmonary vein (Maton et al., 1993). Systemic circulation is the fraction of the cardiovascular system, which transports oxygenated blood away from the heart through arteries, and returns deoxygenated blood to the heart through veins (Maton et al., 1993).

Doppler ultrasonography has been identified as a noninvasive method of assessing uterine blood flow in women and more recently in horses (Bollwein et al., 1998). The relationship between blood flow and other reproductive characteristics including ovulation, pregnancy, and pregnancy loss is an emerging area of research across species (Bollwein et al., 1998). Research in humans has shown that low uterine blood flow and perfusion is a cause of infertility (Bollwein et al., 1998). Additionally, the quality of the uterine blood flow is important in obtaining a successful embryo transfer or artificial insemination (Bollwein et al., 1998).

Age has been shown to cause a difference in blood flow of the uterine artery. Additionally, parity can influence the blood flow in the uterine artery. Older multiparous mares have been shown to have significantly less blood flow than younger maiden
mares (Bollwein et al., 1998). The period of the estrous cycle is also a significant factor in regards to blood flow, with significantly lower blood flow on day 0-10 as compared to days 15 and 20 (Bollwein et al., 1998).

Increased blood flow in ovarian and uterine arteries has been shown to hasten uterine involution (Mortensen et al., 2011). Mares with increased blood flow to reproductive tissues showed hastened uterine involution when measured as uterine body diameter as well as gravid and non-gravid horn diameter (Mortensen et al., 2011). Additionally, mares supplemented with L-arginine displayed a decrease in days with measurable uterine fluid (3.4 ± 1.5 d) as compared to control mares (7.11 ± 3.1 d) (Mortensen et al., 2011).

**Blood Flow Indices**

Blood flow can be measured by obtaining waveform velocities and reflected through a Doppler index (Ginther, 2007). These indices are ratios of velocity measurements, which make them independent of Doppler angles (Alcazar, 2004). Doppler angle, also known as insonation angle is important in measuring blood flow through the use of Doppler ultrasound (Davé and Milner, 2000). Insonation angle is the angle between the Doppler ultrasound beam and the direction of blood flow in a vessel (Davé and Milner, 2000). The Doppler instrument identifies only the blood flow velocity component being directed straight towards the transducer along the Doppler ultrasound beam (Davé and Milner, 2000). The relationship is equal to $V \cos A$, where $V$ is the true blood flow velocity in the vessel and $A$ is the Doppler angle (Davé and Milner, 2000). An error in the measurement of the Doppler angle causes an error in the estimation of the true blood flow velocity that increases as the error in Doppler angle increases (Davé
Use of indices including pulsatility and resistance indices allows for accurate blood flow measurements without the acquisition of an insonation angle.

Waveform velocities are depicted on ultrasound displays as velocity displays (Ginther, 2007). The changes in the Doppler-shift frequencies and the signal amplitudes are displayed as a waveform, representing a cardiac cycle depicting the pulsatile nature of arterial blood flow (Ginther, 2007). The maximum point in the traced outline of a spectrum represents the peak systolic velocity (PSV), while the low point before the next systolic increase represents the end diastolic velocity (EDV) (Ginther, 2007). An average of the maximum values over a single cardiac cycle is known as the timed-average maximum velocity (TAMV) (Ginther, 2007).

The resistance index (RI) is a routinely used Doppler index (Ginther, 2007). The RI relates the extent of resistance in the tissues and the extent of vascular perfusion (Ginther, 2007). A higher RI will result in less blood flow through the individual vessel. Conversely, a lower RI is indicative of increased blood flow. Resistance index is calculated as follows:

\[
\text{Resistance Index (RI)} = \frac{\text{Peak Systolic Velocity (PSV)} - \text{End Diastolic Velocity (EDV)}}{\text{Peak Systolic Velocity (PSV)}}
\]

Another widely used index in determining blood flow is the pulsatility index (PI). Pulsatility index measures the difference in PSV and ESV as it relates to the timed average mean velocity (TAMV) (Ginther, 2007). An increase in PI is indicative of decreasing perfusion of the distal tissues (Ginther, 2007). Pulsatility index and RI are highly correlated \((r > 0.9)\) and only one is usually necessary when measuring blood flow (Ginther, 2007). Pulsatility index is calculated as follows:
Pulsatility Index (PI) = Peak Systolic Velocity (PSV) - End Diastolic Velocity (EDV)
Timed Averaged Maximum Velocity (TAMV)

Arginine

Arginine Requirement

Arginine is an amino acid that is found in many different proteins in the body. Proteins are a major component of almost all tissues in the body, as well as, enzymes, hormones, and other substances (NRC, 2007). Horses like other nonruminants do not have a protein requirement, rather they have a requirement for individual amino acids (NRC, 2007). However, with the exception of lysine, the requirements for individual amino acids have not been determined in the horse (NRC, 2007).

Arginine is considered one of the ten essential amino acids to the horse in addition to histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (Efron and Barbul, 2000). Essential amino acids cannot be synthesized by the body in sufficient quantities and must be supplied by the diet (NRC, 2007). Arginine is not considered a limiting amino acid, which is an amino acid that if deficient will impact the effectiveness of other amino acids (Efron and Barbul, 2000).

Arginine Properties and General Functions

The amino acid side chain of arginine consists of a 3-carbon aliphatic straight chain, while the distal end is capped by a guanidium group. Arginine imparts basic qualities and allows for the formation of multiple hydrogen bonds (Tapiero et al., 2002).

Arginine is one of the twenty most common natural amino acids, but is considered an essential amino acid and needs to be supplied by the diet (Tapiero et al., 2002). The common form seen in the body is L-arginine (Tapiero et al., 2002). L-arginine has many functions in the body and has many health benefits including functioning as a precursor
for nitric oxide, as a component in collagen and enzymes, as well as the synthesis of important proteins including creatine and insulin (Evoy et al., 1998). L-arginine has also been suggested to play a role as a minor antioxidant (Evoy et al., 1998). L-arginine is a major component of seminal fluid and is important for maintaining a healthy ejaculate volume. As a result, it has been deemed as an important factor in sperm motility (Keller and Polakoski, 1975). Research into the role of arginine and reproductive efficiency of stallions has not been conducted. L-arginine can also function to remove excess ammonia from the body via the urea cycle and help to maintain whole-body nitrogen balance (Visek, 1986).

Arginine supplementation is gaining support in the equine world because of its reputation as a potent vasodilator (Clarkson et al., 1996). Vasodilation is not only important in delivering oxygen-rich blood to muscles, but also in the removal of lactic acid which can have a negative effect on the performance of the horse (Wu and Meininger, 2000). The performance horse industry is also interested in arginine supplementation because of arginine’s role in creatine synthesis in the muscle (Sewell and Harris, 1995). Creatine is a nitrogenous organic acid that aids in the production of energy-rich ATP molecules (Sewell and Harris, 1995). It is important to remember that the daily requirements of L-arginine in the horse are unknown, so supplementation may simply be meeting the requirements necessary for normal function.

**Arginine Synthesis**

In the body, arginine can be synthesized from L-glutamine, proline, citrulline, and ornithine (Wu and Morris, 1998) (Figure 2-1). Many of the enzymes required to synthesize L-arginine are found in cells throughout the body; however certain enzymes are restricted to the liver and intestinal mucosa (Wu and Morris, 1998). Endogenous
arginine synthesis varies by species as well as age, nutritional status and developmental stage (Wu and Morris, 1998). At birth, the intestine is the major site of arginine synthesis, but as the animal ages, the intestine becomes a major site of citrulline production as intestinal arginase production increases (Wu and Morris, 1998). Despite recent interest in arginine, very little is known about the regulation of intestinal citrulline and arginine synthesis (Wu and Morris, 1998).

Approximately 60% of net endogenous arginine synthesis occurs in the kidney (Wu and Morris, 1998). Citrulline is extracted from the blood and converted to arginine by the enzymes arginosuccinate synthase (ASS) and arginosuccinate lyase (ASL) (Wu and Morris, 1998). A correlation between renal citrulline uptake and renal arginine output has been demonstrated in adult humans and rats (Wu and Morris, 1998). This correlation means that in vivo arginine synthesis in the kidney is closely associated to citrulline production in other organs.

The liver is another major site of arginine synthesis (Wu and Morris, 1998). Arginine synthesis occurs in hepatic cells via the hepatic urea cycle (Wu and Morris, 1998). Arginine synthesis by the liver is only accomplished when urea cycle intermediates such as ornithine are produced in sufficient quantities (Wu and Morris, 1998).
Figure 2-1. L-Arginine Synthesis as adapted from Wu and Morris, 1998

**Relationship between Arginine and Citrulline**

A strong relationship exists between the amount of citrulline in the system and the amount of arginine that is produced (Wu and Morris, 1998). Citrulline is co-produced with nitric oxide (NO) as a product of the breakdown of arginine by nitric oxide synthase (NOS; Wu and Morris, 1998). In addition, citrulline can be recycled back to arginine via the citrulline/NO pathway (Figure 2-2). The enzymes ASS and ASL are necessary for this recycling and are present in most cell types.

Figure 2-2 also depicts the products of the catabolism of L-arginine. Arginine is a major precursor for NO (Wu and Morris, 1998). A byproduct of the catabolism of L-arginine is citrulline, which can then be converted back to L-arginine through the citrulline/NO cycle (Wu and Morris, 1998).
Effects of Arginine and Nitric Oxide on Reproduction and Blood Flow

Arginine is a major precursor for NO and is considered a NO-donor molecule (Wu and Morris, 1998). Nitric oxide is produced from L-arginine by a family of enzymes known as nitric oxide synthases (Wu and Morris, 1998; Table 2-1). Nitric oxide is an important cellular signaling molecule that has many functions including acting as a signal to control vascular tone and angiogenesis (Wu and Morris, 1998).

Table 2-1. Nitric Oxide Synthase Profiles and their Actions

<table>
<thead>
<tr>
<th>Name</th>
<th>Location</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuronal NOS (nNOS)</td>
<td>Nervous tissue, Skeletal muscle</td>
<td>Cell communication</td>
</tr>
<tr>
<td>Inducible NOS (iNOS)</td>
<td>Immune system, Cardiovascular system</td>
<td>Immune defense</td>
</tr>
<tr>
<td>Endothelial NOS (eNOS)</td>
<td>Endothelium</td>
<td>Vasodilation</td>
</tr>
</tbody>
</table>

Nitric oxide generated from L-arginine by the vascular endothelium is thought to play a major role in the regulation of vascular tone, which affects blood pressure, blood perfusion, and blood flow (Amit et al., 1998). The endothelium of blood vessels uses NO as a signaling molecule, triggering relaxation of smooth muscle surrounding blood

Figure 2-2. Citulline/Nitric Oxide cycle as adapted from Wu and Morris, 1998
vessels, resulting in dilation of blood vessels and increased blood flow (Amit et al., 1998). Research has shown that an increase in a NO-donor such as L-arginine increases endothelial production of NO (Amit et al., 1998).

The effect of L-arginine supplementation on enhancement of reproductive function is a current topic that is under review by many people. Gilts supplemented with 1.0% L-arginine from d 30 of gestation until parturition had a 22% increase in number of pigs born alive, as well as 24% increase in litter birth weight (Mateo et al., 2007). Although NO was not measured, the authors speculated that these increases could be due to the angiogenic effects of NO on the placenta of pigs during gestation (Mateo et al., 2007).

Supplementation of pregnant mice with 2% L-arginine resulted in an increase in weight gain during the latter one-third of gestation, total litter size, number of pups born alive, litter birth weight, and litter weight of pups born (Greene et al., 2012). Additionally, the transcriptional activity of Vegfr2, a receptor for vascular endothelial growth factor (VEGF), a potent endothelial growth factor was shown to increase in fetoplacental tissues after L-arginine supplementation (Greene et al., 2012). L-arginine supplemented to pregnant gilts at 0.8% of the diet for 25 days enhanced the vascularity of the chorionic and allantoic membranes compared to control groups (Li et al., 2010). Enhancement of vascularity in the placenta has demonstrated improvements in number of animals born alive as well as provides an area to examine in order to fully understand the effects of L-arginine supplementation during pregnancy.

The role of NO on uterine blood flow has been investigated in vivo in the porcine model (Barszczewska et al., 2005). A bolus of NO donor was injected into the uterine artery of pigs during the estrous period. Results from these studies, including blood
pressure measurements, indicate that NO is an important factor in the regulation of blood flow through the porcine reproductive tract during estrus regardless of the period of the estrous cycle. (Barszczewska et al., 2005). Sheep pregnant with multiple fetuses administered L-arginine parenterally, had a 23% decrease in the number of lambs born deceased and a 59% increase in the number of lambs born alive compared to control (Lassala et al., 2011). This enhancement of pregnancy outcome was attributed to the increased circulating levels of arginine, ornithine, cysteine, and proline (Lassala et al., 2011).

Research into the reproductive benefits of arginine in horses has been limited to a few studies. Supplementation of 1.0% L-arginine to pregnant mares beginning 21 d prior to expected foaling date has been shown to increase blood flow in the uterine and ovarian arteries during the early postpartum period (Mortensen et al., 2011). Additionally, postpartum mares displayed a significant reduction in the amount of fluid present in the uterus as well as an increase in uterine arterial blood flow to the formerly gravid uterine horn when fed L-arginine as compared to control mares (Kelley et al., 2011).

**Uterine Fluid**

**Fluid Accumulation**

Endometritis has long been associated with a decrease in the fertility of mares, and free fluid accumulation is a significant indicator of endometritis (McKinnon and Voss, 2005). Approximately 15% of Thoroughbred mares develop endometritis, resulting in accumulation of uterine fluid due to impaired uterine clearance mechanisms (Drost et al., 2002) Reproductively normal mares respond to an infection in the uterus by activation of the immune system as well as increased uterine luminal contractions to
evacuate the contents of the uterus (McKinnon and Voss, 2005). Mares that are unable to do this are generally less reproductively fit and are susceptible to a decrease in fertility (Blanchard et al., 2002).

Ultrasonographic examination is a useful tool in estimating the quantity of fluid in the uterine lumen (McKinnon and Voss, 2005). Ultrasound is a beneficial tool in deciding whether or not to breed a mare given the condition of the uterus. Uterine fluid can easily be identified on the display of an ultrasound and is distinguishable as a dark area inside the lumen of the uterus (McKinnon and Voss, 2005). Uterine fluid can be categorized by appearance on ultrasound with grades ranging from I-IV, with grade I being white (hyperechoic) to grade IV being black (anechoic) on the display (McKinnon and Voss, 2005).

A small amount of anechoic fluid in the lumen of the uterus was once thought to have no impact on reproductive performance and was normal (Blanchard et al., 2002). However, controlled research studies as well as case study observations have determined that even small amounts of uterine fluid can adversely affect fertility (Blanchard et al., 2002). Fluid is most commonly seen during diestrus and immediately following ovulation, however any fluid seen in the uterine lumen should be considered abnormal (Blanchard et al., 2002). Accumulation of fluid is indicative of endometritis, and can identify mares that have impaired ability to mechanically evacuate the uterus (Blanchard et al., 2002).

The degree of fluid echogenicity is a function of the concentration of inflammatory cells and debris (McKinnon and Voss, 2005). Hyperechoic fluid is characterized by a higher concentration of debris and immune cells, normally resulting from an infection.
Some mares also generate anechoic fluid that is sterile in nature (Blanchard et al., 2002). This fluid can reduce fertility and decrease the chances of successful fertilization (McKinnon and Voss, 2005). It has been hypothesized that these mares have deficient lymphatic drainage of endometrial edema that causes it to collect in the uterine lumen (Blanchard et al., 2002). This anechoic fluid can become hyperechoic quickly if not treated and lead to increased endometritis and decreased fertility (Blanchard et al., 2002).

Uterine fluid accumulation has been determined to be associated with increased early embryonic death as well as reduced day 50 pregnancies (McKinnon and Voss, 2005). Additionally, fewer mares become pregnant when uterine fluid is present during the first postpartum ovulatory period also known as foal heat (McKinnon and Voss, 2005).

Methods of Fluid Removal

Prudent management is the preferred way of maintaining a reproductively sound mare with a better chance of conception and maintenance of pregnancy to term (Blanchard et al., 2002). The use of ultrasound is important in identifying mares that may be predisposed to accumulating uterine fluid (Blanchard et al., 2002). Ultrasonography is also useful in determining the quality of the fluid present, which aids in determining the preferred course of corrective action (Blanchard et al., 2002). Additionally, the use of uterine endometrial biopsies as described previously is a key management technique that can help identify mares that may be predisposed to endometritis and fluid accumulation (McKinnon and Voss, 2005).

Uterine lavage is a technique that is routinely used to remove fluid, especially hyperechoic fluid from the uterus (McCue and Hughes, 1990). Uterine lavage is usually
performed before breeding or 4-8 hours after breeding (McCue and Hughes, 1990). Uterine lavage functions by expanding the uterine lumen through the use of sterile fluid and subsequently flushing the infused fluid out of the uterus, along with to the hyperechoic fluid containing neutrophils, antigens, and debris (McCue and Hughes, 1990). Research has shown that uterine lavage performed after breeding does not decrease pregnancy rates or interfere with sperm in the oviduct (McCue and Hughes, 1990). Uterine lavage is normally conducted in conjunction with an infusion of antibiotics post infusion (McCue and Hughes, 1990).

The most routine route of uterine fluid removal is through the use of ecbolics (Blanchard et al., 1991). Ecbolics such as oxytocin or prostaglandins are administered to stimulate uterine contractility and expulsion of uterine contents (Blanchard et al., 1991). Administration of oxytocin has been shown to be safe for 2-3 days following ovulation, but administration of prostaglandins post-ovulation has been shown to reduce the function of the corpus luteum and negatively affect the ensuing estrous cycle (Blanchard et al., 2002). In addition, the use of prostaglandins has been associated with increased embryonic death in subsequent ovulations (Blanchard et al., 2002). Ecbolics are also used to promote uterine clearance without the use of uterine lavage (Blanchard et al., 2002). Mares that are susceptible to uterine fluid accumulation may have an inability to clear the uterine lumen of fluid without the administration of ecbolics (Blanchard et al., 2002). Current industry standards are the administration of 20 IU oxytocin intramuscularly once or twice a day (Blanchard et al., 2002). However, case study reports from Florida have shown that former industry standards of multiple
injections of oxytocin 4-6 hours apart can cause uterine spasms that are unproductive in eliminating uterine fluid (Blanchard et al., 2002).

Naturally, oxytocin is synthesized in the hypothalamus and secreted by the posterior pituitary gland (Gimpl and Fahrenholz, 2001). Smooth muscle cells in the uterus contain receptors that bind to oxytocin (Gimpl and Fahrenholz, 2001). Receptor number is increased late in pregnancy and in some animals oxytocin injections are given to facilitate parturition. However this is not normally the case in horses, as induction of early parturition can lead to foal death (Blanchard et al., 2002). Oxytocin binds to a G-protein coupled receptor and causes contraction through second messengers (Gimpl and Fahrenholz, 2001). The half-life of oxytocin is relatively short in the blood, averaging about three minutes (Gimpl and Fahrenholz, 2001).

The study presented in this thesis was undertaken to determine if an increase in blood flow attributed to L-arginine supplementation would accelerate uterine fluid clearance. Previous research has demonstrated that L-arginine supplementation hastened uterine involution (Mortensen et al., 2011) and increased uterine blood flow (Kelley et al., 2011). The increase in blood flow may permit more rapid clearance of fluid from the uterus. The objectives of the current study were as follows:

- Determine the effect of L-arginine supplementation on uterine artery blood flow
- Determine the effect of L-arginine supplementation on uterine fluid clearance
- Determine the effect of L-arginine supplementation on follicular and ovulatory dynamics.
Animals

Twelve non-pregnant light-horse mares (mean ± SE, 540.5 ± 56.5 kg) were used for this study. The experimental protocol was reviewed and approved by the Institute of Food and Agricultural Sciences (IFAS) Animal Care and Use Committee at the University of Florida. Mares were maintained on two pastures and housed at the IFAS Equine Sciences Center in Ocala, Florida (Latitude 29º 18’ 12” N). Mares remained on pasture throughout the supplementation period. While on pasture, mares had free-choice access to water and salt blocks. Mares were confined in 3.6 x 3.6 m box stalls at 0730 and 1500 h for approximately 30 min to facilitate feeding. Mares scheduled to be examined or weighed were removed from their stalls and placed in small paddocks devoid of vegetation, but with free-choice access to water and Coastal bermudagrass hay for approximately 1 h. During ultrasound examinations, mares were moved into a covered barn equipped with fans to facilitate air circulation and placed in individual stocks.

Experimental Design

The study was designed as a 3x3 Latin Square, evaluating 3 dietary treatments over 3 consecutive periods, permitting all mares to undergo all treatments. Each period consisted of one estrous cycle. To accommodate frequent and timely ultrasound examination, mares were initially blocked by age (10.5 ± 5.8 y; range 3-22 y) and breed (Thoroughbred (n=5), and stock type (n=7)), then randomly assigned to one of four estrous synchronization groups (3 mares per group). Synchronization of mares was accomplished using a single dose of Lutalyse® (dinoprost tromethamine; Pfizer Inc.,
New York City, NY) injected IM followed by daily oral administration of 0.044mg/kg BW Regumate® (altrenogest; Intervet Inc., Summit, NJ) for 2 wk, and finally a second dose of Lutalyse® injected IM on d 15. Synchronization of mare groups was staggered at 3 to 5 d intervals, and each group had equal representation of the three dietary treatments.

**Dietary Treatments**

Mares in each synchronization group were randomly assigned to one of three dietary treatments in a 3X3 Latin Square design: arginine supplementation (ARG), urea supplementation (UREA), or no supplementation coupled with oxytocin administration (OXY). Arginine was supplied as L-arginine (Ajinomoto AminoScience LLC, Raleigh, NC) at a rate of 200 mg/kg BW/d. This amount is based on the rate of supplementation used by Mateo et al. (2007) in pregnant gilts, and represents approximately 1% of estimated DM intake for a non-pregnant mare. A feed grade source of urea was used for the UREA treatment, which served as an isonitrogenous control. Urea was fed at a rate of 114 mg/kg BW/d. No dietary supplementation was provided to mares on the OXY treatment. Supplementation rates were determined from body weights obtained on d 7 post-ovulation in each period. While on the ARG treatment, approximately 75% of daily L-arginine intake originated from the ARG supplement.

The basal diet consisted of *ad libitum* access to mixed bahiagrass pasture and Coastal bermudagrass hay and 0.5% BW/d of a grain mix concentrate formulated for gestating and lactating mares (Ocala Breeder’s Feed and Supply, Ocala, FL). The basal diet was designed to meet or slightly exceed the nutrient requirements of horses at maintenance (NRC, 2007). ARG and UREA supplements were hand-mixed into the concentrate portion of the ration and fed once daily at 1500 h. Dietary treatment began 10 d post-ovulation and continued until all fluid was absent in the uterus as determined
by ultrasound examination (approximately 12 d total of supplementation). Nutrient composition of basal feeds and supplements is presented in Table 3-1.

**Uterine Infusion**

Mares were initially evaluated by transrectal ultrasonography to map follicular development beginning 13 d after ovulation. Examinations were conducted once daily until a 33 mm follicle was observed. Once a follicle of this size was observed, mares received a uterine infusion consisting of 880 mL of sterile saline (0.9% NaCl) mixed with 120 mL of semen extender (E-Z Mixin®-OF, Animal Reproductive Systems, Chino, CA). This fluid mixture was determined based on preliminary studies measuring rate of fluid clearance over a range of fluid volumes. The fluid mixture was prepared on the day of infusion and stored at 4°C until the time of infusion. Sterile tubing was passed through the cervix of the mare and into the uterine body. Tubing was secured through the use of an air-filled bladder, and fluid was gradually infused at a rate of approximately 300 mL/min. Completion of the infusion was considered time=0 h. Mares assigned to the OXY treatment received 20 IU oxytocin (AgriLabs, St. Joseph, MO) administered IM immediately after fluid infusion. Mares were examined via ultrasound at 12-h intervals to record uterine blood flow and fluid disappearance. When all fluid was cleared from the uterus, dietary treatment was terminated, and mares continued to undergo once daily ultrasound evaluation. When no discernible fluid was detected for 48 h, all mares received 20 IU oxytocin administered IM to ensure total fluid clearance before starting the next treatment period. When the next ovulation was confirmed by presence of a corpus luteum, treatments were switched and mares began a new dietary treatment 10 d post-ovulation. The procedures described above were repeated until all mares had completed all treatments.
Doppler Ultrasonography

All uterine and ovarian examinations were conducted transrectally using a digital color Doppler ultrasound with a 10-5 MHz broadband 52 mm-linear probe (Micromaxx®, Sonosite, Bothell, WA). Examinations were conducted by a single operator that was not blinded to treatment. Prior to discovery of a 33 mm follicle, only follicle size and presence of a corpus luteum were evaluated on both the left and right ovary. Once a 33 mm follicle was discovered and the mare had received the uterine infusion, follicle size of only the dominant follicle, amount and location of uterine fluid, and blood flow of the uterine artery were recorded at each exam. The amount of uterine fluid was estimated by measurement of the largest pocket of fluid. Location of the fluid was noted as either uterine body or left or right uterine horn. Spectral-Doppler measurements of both uterine arteries were calculated by the algorithm package in the Micromaxx® ultrasound unit. The sample cursor gate was set at 5 mm and at an initial magnification depth of 7.7 cm. The measurements taken included resistance index (RI) [(peak systolic velocity (PSV) – end diastolic velocity (EDV))/PSV] and pulsatility index (PI) [(PSV-EDV)/ time-averaged maximum velocity (TAMV)] (Ginther, 2007). Uterine arteries were identified as described by Bollwein et al. (1998), with measurements taken near the branching of the external iliac artery or deep circumflex artery or both.

Statistical Analysis

Blood flow and rate of fluid clearance data were analyzed using the PROC MIXED procedure of SAS (Version 9.2, SAS Institute Inc., Cary, NC.) with repeated measures. Latency to corpus luteum development, latency to ovulation, and latency to total fluid clearance were evaluated using the PROC GLM procedure of SAS (Version 9.2, SAS Institute Inc., Cary, NC.). The Kolmogorov-Smirnov for multiple comparisons was used
to test for normal distribution for follicular characteristics. The LS MEANS statement of PROC MIXED was used to compare the treatment means. All data are expressed as the mean ± SE. Differences were considered significant at $P \leq 0.05$ and trends for significance were acknowledged at $P < 0.10$.

One mare completed her first treatment cycle (UREA), but was removed from the study when she failed to produce a 33 mm follicle in the subsequent ovulatory cycle. Data collected from this mare while on the UREA treatment was retained in the statistical analyses, but no data was generated or included for this mare on the ARG and OXY treatments.
Table 3-1. Nutrient composition of basal diet feeds and L-arginine and urea supplements

<table>
<thead>
<tr>
<th>Nutrient&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Grain</th>
<th>Bermudagrass</th>
<th>L-arginine Supplement</th>
<th>Urea Supplement</th>
</tr>
</thead>
<tbody>
<tr>
<td>DE, Mcal/kg</td>
<td>3.47</td>
<td>1.87</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Crude fat, %</td>
<td>4.2</td>
<td>2.3</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Crude protein, %</td>
<td>16.8</td>
<td>9.4</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>NDF, %</td>
<td>20.8</td>
<td>75.4</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>L-arginine, %</td>
<td>1.26</td>
<td>0.01</td>
<td>99.0</td>
<td>--</td>
</tr>
<tr>
<td>Urea, %</td>
<td>--&lt;sup&gt;b&lt;/sup&gt;</td>
<td>--</td>
<td>--</td>
<td>99.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values presented on a 100% DM basis.

<sup>b</sup> Not determined.
CHAPTER 4
RESULTS

Feeding and Supplementation

Mares consumed both the ARG and UREA supplements with little to no initial refusal. In addition, mares maintained BW during the study (data not shown). Total days of supplementation (15 ±1.8 d) did not differ between treatments.

Latency to Total Fluid Clearance

Uterine fluid was absent in all mares prior to uterine infusion, indicating all mares began each treatment period with similar uterine fluid status. On average, total fluid clearance occurred within 53.8 ± 4.9 h (Figure 4-1). Dietary treatment had no effect on average latency to total fluid clearance (P=0.7075) (Figure 4-1).

Rate of Fluid Clearance

Fluid clearance was influenced by time (P<0.0001) and time*treatment (P=0.0088) and tended to be affected by treatment (P=0.0925; Figure 4-2). Mares undergoing the OXY treatment cleared fluid faster (P=0.0388) than those receiving ARG. There was also a trend to clear fluid faster (P=0.0100) when receiving OXY than UREA. Rate of fluid clearance was not different between ARG and UREA. Fluid clearance from the uterus was steady from 0 through 48 h across all treatments, with the amount of fluid remaining at each 12-h interval significantly less than the previous measurement (P<0.0001).

Latency to Follicle Development and Ovulation

On average mares developed a 33 mm follicle within 8.3 ± 1.2 d of treatment initiation. The interval between the development of a 33 mm follicle from one estrus to
the next averaged 21.7 ± 2.1 d across treatments. Dietary treatment had no effect on follicle development (Table 4-1).

On average, mares ovulated within 12.4 ± 1.9 d of treatment initiation. The interval between ovulations during the study period averaged 22.0 ± 1.4 d across treatments. Dietary treatment had no effect on ovulatory dynamics (Table 4-1).

**Blood Flow**

Across treatments, mean resistance index was 0.68 ± 0.03 and 0.70 ± 0.03 for the ovulatory and nonovulatory uterine artery, respectively. Resistance indices in the ovulatory and nonovulatory uterine arteries were unaffected by dietary treatment (Figure 4-3 and Figure 4-4).

The pulsatility index of the ovulatory uterine artery was affected by time ($P<0.0001$), where the PI was higher ($P<0.0001$) immediately after infusion (0 h) compared to all subsequent measurements, regardless of treatment. Similarly, the PI of the nonovulatory uterine artery showed a trend for a time effect ($P=0.0830$), where the PI was higher immediately after infusion (0 h) compared to all subsequent time points ($P<0.05$). The PI of the ovulatory and nonovulatory uterine arteries were not affected by treatment or the time*treatment interaction (Figure 4-5 and Figure 4-6).
Figure 4-1. Mean (± SEM) time (h) to total fluid clearance from the uterus in mares supplemented with L-arginine, urea, or no supplement combined with oxytocin administration. Overall effect of treatment ($P=0.7075$).
Figure 4-2. Mean (± SEM) diameter (mm) of fluid present within the uterus of mares supplemented with L-arginine, urea, or no supplement combined with oxytocin administration. Diameter was measured as the largest width of fluid observable via ultrasound examination. Overall effects of time ($P<0.0001$), treatment ($P=0.0925$), and time*treatment ($P=0.0088$). A pound sign (#) indicates a time*treatment effect as OXY<ARG ($P=0.0388$) and OXY<UREA ($P=0.10$).
Figure 4-3. Mean (± SEM) resistance index of the nonovulatory uterine artery following uterine infusion in mares supplemented with L-arginine, urea, or no supplement combined with oxytocin administration. Overall effects of time ($P=0.9447$), treatment ($P=0.3789$), and time*treatment ($P=0.4070$).
Figure 4-4. Mean (± SEM) resistance index of the ovulatory uterine artery following uterine infusion in mares supplemented with L-arginine, urea, or no supplement combined with oxytocin administration. Overall effects of time ($P=0.6308$), treatment ($P=0.7755$), and time*treatment ($P=0.8952$).
Figure 4-5. Mean (± SEM) pulsatility index of the nonovulatory uterine artery following uterine infusion in mares supplemented with L-arginine, urea, or no supplement combined with oxytocin administration. Overall effects of time ($P=0.0830$), treatment ($P=0.7827$), and time*treatment ($P=0.1644$).
Figure 4-6. Mean (± SEM) pulsatility index of the ovulatory uterine artery following uterine infusion in mares supplemented with L-arginine, urea, or no supplement combined with oxytocin administration. Overall effects of time ($P<0.0001$), treatment ($P=0.3054$), and time*treatment ($P=0.3421$). An asterisk (*) indicates all time points different from 0 h.
Table 4-1. Follicular and ovulatory dynamics in mares supplemented with L-arginine, urea, or no supplement combined with oxytocin administration

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Arginine</th>
<th>Oxytocin</th>
<th>Urea</th>
<th>SEM</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days to develop a 33 mm follicle since treatment initiation</td>
<td>8.3</td>
<td>7.9</td>
<td>9.3</td>
<td>1.2</td>
<td>0.7180</td>
</tr>
<tr>
<td>Days to develop a 33 mm follicle since the previous 33 mm follicle</td>
<td>20.7</td>
<td>22.2</td>
<td>22.3</td>
<td>2.1</td>
<td>0.8603</td>
</tr>
<tr>
<td>Days to ovulation since treatment initiation</td>
<td>12.0</td>
<td>12.2</td>
<td>13</td>
<td>1.2</td>
<td>0.8196</td>
</tr>
<tr>
<td>Ovulatory interval, days</td>
<td>22.0</td>
<td>22.2</td>
<td>21.8</td>
<td>1.4</td>
<td>0.9714</td>
</tr>
</tbody>
</table>
CHAPTER 5
DISCUSSION

The results of the current study demonstrated that uterine arterial blood flow could successfully be measured in nonpregnant mares using Doppler ultrasound. However, short-term dietary supplementation with L-arginine had no effect on uterine arterial blood flow nor the rate and latency of uterine fluid clearance in non-pregnant mares.

The findings of this study contradict previous work in postpartum animals, which is likely related to the use of nonpregnant mares in the present study. For example, Mortensen et al. (2011) began feeding mares L-arginine 21 d prior to expected foaling and documented increases in uterine arterial blood flow, accelerated uterine involution, and more rapid fluid clearance in the early postpartum period.

Significant changes to the uterine environment during and immediately following pregnancy are well-documented (Silva et al., 2011; Ousey et al., 2012). These changes occur in the uterus and the cervix in preparation for parturition, and serve as a repair mechanism for uterine involution following parturition. A study investigating nitric oxide synthase (NOS) isoforms in pregnant rats demonstrated that progesterone played a significant role in regulating NOS expression in the uterus and cervix (Ali et al., 1997). Another study investigated uterine blood flow in pregnant ewes and found an increase in blood flow to ovaries containing a progesterone-producing corpus luteum when compared to ovaries without a corpus luteum (Rosenfeld et al., 1974). The current study evaluated blood flow and fluid clearance during estrus with the presence of a dominant follicle on the ovary. The follicle produces estrogen, which is associated with low progesterone levels until a spike in luteinizing hormone signals for ovulation to occur. The absence of a progesterone-producing corpus luteum in the nonpregnant mares
used in the current study could be one contributing factor as to why no changes in uterine blood flow were observed in response to L-arginine supplementation.

Changes in the vascular architecture are also common during pregnancy (Rosenfeld et al., 1974). In nonpregnant mares, the uterine artery ranges in diameter from 2-6 mm (Ginther, 2007). In pregnant mares, the uterine artery diameter increases significantly (Ousey et al., 2012). The change in uterine artery diameter is related to a decrease in resistance index (RI), which is associated with an increase in blood flow and decreased vascular resistance (Ousey et al., 2012). It may be that the uterus undergoes some type of priming event before pregnancy that allows it to make all necessary changes required to support gestation. The conceptus has also been identified as a key factor in modulating endometrial tissue remodeling and vascular development in mares (Silva et al., 2011). Silva et al. (2011) observed an increase in the area occupied by blood vessels in early pregnancy and preimplantation in mares, as well as an increase in the endometrial mRNA abundance of numerous angiogenic factors. The authors concluded that the conceptus played a role in directing angiogenesis in the uterus. The use of non-pregnant mares in the current study would have had no conceptus-mediated angiogenesis.

Placental development is a characteristic of uterine change during pregnancy. The placenta of the mare is characterized as a diffuse, epitheliochorial placenta (Senger, 1997). The mare’s placenta is the least invasive of placental classifications, with all three maternal uterine layers maintained (Senger, 1997). The placenta is supplied with nutrients through vasculature connections in microcotyledons consisting of highly vascularized chorionic villi, which extend into invaginations of the endometrium (Senger,
Placental development is a process that includes a large amount of angiogenesis and angiogenic factors that are absent in the nonpregnant mare. A study demonstrated that L-arginine supplemented mice had increased transcriptional activity of vascular endothelial growth factor receptor 2, an important angiogenic compound, in the fetoplacental unit (Greene et al., 2012). Changes seen in these studies would not be displayed in the current study due to the use of non-pregnant mares. The vascular remodeling occurring in the placenta downstream of the uterine artery can increase uterine artery blood flow upstream where it is being measured. The lack of placental vascular remodeling in the nonpregnant mares used in this study may be a factor in explaining a failure to influence blood flow with L-arginine supplementation.

One of the objectives of the current study was to determine the effect of L-arginine supplementation on uterine fluid clearance. The authors hypothesized that mares supplemented with L-arginine during the estrous period would display increased uterine arterial blood flow and subsequent hastened uterine fluid clearance. There are numerous factors that can influence the rate of uterine fluid clearance of mares including endometrial quality, cervical integrity, and general arrangement of the uterus in the abdominal cavity. Endometrial biopsy scores are used to help to determine mares that are susceptible to endometritis. Mares susceptible to endometritis are also susceptible to accumulation of uterine fluid (McKinnon and Voss, 2005).

The integrity of the cervix is a major factor in uterine fluid clearance and is influenced by the age and parity of the mare, as well as hormone status. As a mare ages, and parity increases, cervical integrity may decrease due to an increase in the number of parturitions as well as possible injury to the cervix during parturition.
of the estrous cycle is also influential due to the varying levels of reproductive hormones. In this study, uterine infusion occurred during the estrus period of the estrous cycle when the cervix, under the influence of estrogen, was loose and open. This period of the estrous cycle was chosen to simulate post-breeding uterine fluid accumulation, but it may be necessary to evaluate fluid clearance during the diestrus period, when the cervix, under the influence of progesterone, is closed tightly.

A high degree of variability between horses was noted in this study; thus, a larger number of horses are likely required to ascertain the effects of L-arginine supplementation on blood flow to reproductive tissues in nonpregnant mares. Additionally, a larger number of mares may allow for improved understanding of uterine fluid clearance in nonpregnant mares.

Finally, this study demonstrates the effectiveness of the ecbolic oxytocin on uterine fluid clearance. Approximately 33% of uterine fluid was absent 12 h post uterine infusion through the use of a single intramuscular administration of 20 IU oxytocin. Current industry standards recommend administration of 20 IU oxytocin intramuscularly every 12 hours for total fluid clearance. This study confirms that the use of oxytocin to aid in uterine fluid clearance is beneficial and successful.
CHAPTER 6
CONCLUSIONS

The current study demonstrated that supplementation of L-arginine to nonpregnant mares had no significant impact on the rate of uterine fluid clearance or uterine arterial blood flow. Supplementing the diets of pregnant animals with L-arginine has been shown to enhance reproductive function. In contrast to those studies, this study suggests that in order for the effects of L-arginine on uterine blood flow to be observed, the uterus must be primed and the animal must be pregnant.

The main objective of the current study was to investigate the effect of L-arginine supplementation on uterine fluid clearance in mares. Because all mares are different, it is important to understand the different causes of uterine fluid accumulation. Some mares may be more susceptible to uterine fluid accumulation and less able to sufficiently evacuate the uterus of fluid. Additional studies should be attempted to mimic uterine fluid build-up throughout the estrous cycle and to determine whether L-arginine can hasten its removal.

The current study demonstrates that the supplementation of L-arginine to nonpregnant mares may not be an appropriate model for the evaluation of uterine fluid clearance and uterine blood flow compared to the postpartum mare. Supplementation of L-arginine to postpartum mares may be beneficial in improving rebreeding efficiency during the foal heat period as an aid in uterine involution. Any improvement in foal heat rebreeding efficiency would allow the producer to maintain a schedule of one foal/mare/year, resulting in a positive economic impact. The small number of horses investigated in this trial could have impacted the results and future trials should use greater numbers of horses.
Finally, investigation on a molecular scale should be completed to better understand the effect that L-arginine has on transcriptional regulation of nitric oxide synthase as well as other angiogenic factors in reproductive tissues.
LITERATURE CITED


BIOGRAPHICAL SKETCH

Robert Jacobs is a native of Florida, and grew up in Plantation, Florida. From a young age, he was exposed to animals both at home and in his academic career. Robert’s first interaction with horses came at his grandfather’s house, where he would routinely ride and care for the thoroughbred that his grandfather had rescued from the track. Robert began his collegiate education at Purdue University where he hoped to go to vet school. Robert made the decision to transfer to the University of Florida before his junior year and began his education at the University of Florida in August of 2007.

Robert gained an interest in reproductive physiology in his Reproductive Physiology and Endocrinology course taught by Dr. Michael Fields, which sparked his desire to pursue this area of research as a career. Additionally, Robert was introduced to equine nutrition by Dr. Lori Warren whose passion for the topic inspired Robert to seek further knowledge on the topic.

Robert began his master’s program as a non-thesis student with plans to apply to vet school and pursue his interests in reproductive physiology and nutrition as a veterinarian. During the first year of his master’s program, it became clear to Robert that he had a passion for research and he decided to switch to a thesis program. Dr. Christopher Mortensen and Dr. Lori Warren were supportive enough to develop a project that combined Robert’s interests in nutrition and reproductive physiology.

Robert plans on continuing his education at Virginia Tech University and obtaining his doctorate in equine reproductive physiology and he hopes to continue doing research as well as pursue his interests in teaching as a member of academia in the future.