GENE EXPRESSION IN ENDOMETRIAL TISSUES OF NORMAL MARES AND MARES WITH DELAYED UTERINE CLEARANCE

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(ABSTRACT)

Delayed uterine clearance (DUC) is a significant problem contributing to subfertility and infertility in the mare, characterized by an accumulation of fluid and inflammatory debris in the uterine lumen following breeding events, venereal disease or an estral cycle. This syndrome is typically seen in older, multiparous mares and mares with poor reproductive tract conformation. The etiopathogenesis of DUC has not been fully elucidated but suggested causes include poor genital conformation, a cranioventrally tilted uterus, defective myometrial contractions, decreased intrauterine immune activity, inappropriate lymphatic drainage or mucus overproduction. The objective of this research was to evaluate gene expression of selected genes in endometrial tissue samples taken from three categories of mares (young fertile [YF], older clinically normal [ON] and older susceptible [OS]). The genes assayed in this research were oxytocin receptor, $PGF_{2\alpha}$ receptor and progesterone receptor. The expression of each of these genes was normalized using the expression of two housekeeping genes, beta actin and ribosomal 18S RNA. Quantitative real-time polymerase chain reaction (QPCR) was used to evaluate gene expression of the selected genes. Results indicated that there was no statistically significant difference in the expression of any of the three experimental genes among any of the three categories of mares. From this research, the direction of further research regarding the pathogenesis of DUC can be made: myometrial tissues can be assayed for similar genes, the expression of other genes regulating myometrial contraction can be assayed or the expression of uterorelaxants can be studied.

Dedication

To my wife, Kerri, for her support, inspiration and toleration of me throughout my program and my job, during both highs and lows.

To my loving parents for their prayers and encouragement in all of my academic, personal and equine undertakings.

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List of Abbreviations

bps – Base Pairs
Ca^{2+} - Calcium
cAMP – Cyclic Adenosine Monophosphate
cDNA – Complementary Deoxyribonucleic Acid
CEM – Contagious Equine Metritis
Ct- Threshold Cycle
DNA – Deoxyribonucleic Acid
DUC – Delayed Uterine Clearance
EMG – Electromyogram
EPM – Equine Protozoal Myeloencephalitis
G3PDH – Glyceraldehyde-3-Phosphate Dehydrogenase
IgA – Immunoglobulin Type A
IgG – Immunoglobulin Type G
iNOS – Inducible Nitric Oxide Synthase
IP_3 – Inositol 1,4,5-Triphosphate
IU – International Unit
IV – Intravenous
Mg^{2+} - Magnesium
mL - Milliliter
MLCK – Myosin Light Chain Kinase
MLCP – Myosin Light Chain Phosphatase
mRNA – Messenger Ribonucleic Acid
ng - Nanogram
NO – Nitric Oxide
OT-NP – Oxytocin-Neurophysin
PCR – Polymerase Chain Reaction
pg - Picogram
$PGF_{2\alpha}$ – Prostaglandin $F_{2\alpha}$
PHF – Potomac Horse Fever
PMIE – Post-Mating Induced Endometritis
QPCR – Quantitative Real-Time Polymerase Chain Reaction
RNA – Ribonucleic Acid
ROK – Rho A-Associated Kinase
RT– Reverse Transcriptase
SR – Sarcoplasmic Reticulum
µL - Microliter

Chapter 1: Introduction

Delayed uterine clearance (DUC) and subsequent endometritis are major problems contributing to subfertility and infertility in the mare. The clinical syndrome of DUC is characterized by an accumulation and retention of fluid within the uterine lumen. This often occurs following breeding events, during the estral cycle, or with venereal infection. DUC is most commonly recognized in older, multiparous mares and those mares with poor reproductive tract conformation. Post-mating induced endometritis (PMIE) is a naturally occurring event following insemination. The inflammatory by-products and fluid that are generated in response to spermatozoa and contaminant microbes in semen are cleared in a timely fashion by normal mares, but are often retained for prolonged periods of several hours to several days in mares with DUC[1]. The resultant inflammatory fluid creates an inhospitable environment for an embryo to enter five to six days following ovulation and fertilization. Fluid within the uterus prior to breeding, likewise, creates an unfavorable condition for equine sperm and can be deleterious to sperm motility and survival, lending to infertility. Intrauterine fluid can be identified during routine pre-breeding transrectal palpation and ultrasound examination or during a breeding soundness examination.

There are three physical barriers that prevent entry of foreign substances into the equine uterus: (1) a tight seal of the vulvar lips, (2) the vagino-vestibular fold and (3) the cervix. Several factors have been implicated in the mechanics of intrauterine fluid accumulation. Poor anatomical conformation of the mare is a large contributor to uterine fluid pooling. A vulva that is tilted forward with the dorsal commisure recessed into the pelvic canal along with the anus allows for fecal contamination of the reproductive tract, providing a source for pathogens (most commonly *Streptococcus zooepidemicus* or *E. coli*) to colonize the structures of the reproductive tract internal to the vulva. An incomplete seal of the vulvar lips and/or an incompetent vaginovestibular junction allows contaminants and air to be aspirated into the vagina. This condition, also known as ”windsucking”, frequently accompanies this abnormal perineal conformation. Poor

perineal conformation as well as a uterus that has sunk into the abdomen ventral to the pelvis occurs with increasing age and parity, contributing to poor uterine clearance. Other factors in the origin or retention of intrauterine fluid include defective myometrial contractility, lowered immune defenses within the uterus, overproduction of mucus, inadequate lymphatic drainage, or a combination of these factors[2].

Mares with normal uterine clearance evacuate accumulated fluid by sequential contractions of the myometrium, moving the fluid caudally and eventually pushing it out through the cervix[3]. DUC mares may have deficient myometrial contractions or may have cervical pathology (ex. fibrosis, scarring, adhesions) that prevents fluid from being expelled normally[4].

Therapy for DUC is aimed at eliminating the intrauterine fluid by both mechanical and pharmacologic means. Uterine lavage using sterile isotonic fluids can be performed to eliminate fluid. Intrauterine antibiotics may be administered following uterine lavage, depending on the existence of a bacterial infection. Exogenous oxytocin therapy has historically been the cornerstone medical therapy for DUC. This therapy is provided to cause contraction the myometrium, thereby augmenting mechanical clearance of intrauterine contents[2,5]. Prostaglandins are also known to have ecbolic properties and can be used to help expel intraluminal uterine contents as well as cause lysis of the corpus luteum and a return to estrus [4,6]. The use of ecbolic drugs such as fenprostalene or the synthetic prostaglandin analogue cloprostenol has been suggested because of the prolonged duration of action as compared with oxytocin or other prostaglandins. However, the potential ill effects of prostaglandins on the development and function of luteal tissue is concerning and has lead to hesitation in using these drugs for uterine contractile properties in late estrus or within the first 36 hours of diestrus[7].

The research presented in this thesis is aimed at analyzing gene expression in the endometrial tissues of normal mares without delayed uterine clearance versus those mares with the clinical condition. The expression of three important hormonal receptors, oxytocin, progesterone and prostaglandin $F_{2\alpha}$, was evaluated to determine if DUC could be explained by over- or under-expression of particular genes in equine endometrial

tissue. Two additional genes, beta actin and ribosomal 18S RNA, were selected as "housekeeping" genes, genes that should be expressed uniformly in all tissue types of normal and diseased animals. The experimental gene expressions were evaluated with respect to beta actin and ribosomal 18S RNA internal control expression using quantitative real time polymerase chain reaction (QPCR).

1.1: Molecular Biology in Equine Practice

Recent advances in molecular biology in the last few decades have contributed new diagnostic and therapeutic tools to the arenas of equine medicine, surgery and reproduction. The polymerase chain reaction (PCR) is now widely used in the diagnosis of many equine bacterial, protozoal and viral diseases such as salmonellosis, equine protozoal myeloencephalitis, contagious equine metritis, herpesviral diseases and Potomac horse fever. PCR allows for the detection of the causative microorganisms of these diseases in equine blood, spinal fluid, feces and tissue[8]. Many breed registries are using PCR, restriction enzyme digestions, agarose gel electrophoreses and Southern Blotting to determine parentage of horses to verify their breed before allowing them to be registered[8]. Cloning of equines has recently been accomplished successfully in Idaho with the aid of molecular biological technology; injecting enucleated horse ova with DNA from mule fetuses has resulted in healthy cloned mules. Molecular biology has also led to the development of new recombinant vaccines in the equine industry.

Gene expression is another useful molecular biological tool that has been recently developed and utilized. Equine tissues can be homogenized, the RNA extracted, and then be subjected to reverse transcriptase (RT) to synthesize cDNA. That cDNA is then amplified and quantified using real time PCR (QPCR, also known as kinetic PCR) and specific, selected primer sequences. QPCR is a faster and more sensitive form of PCR based on the fundamentals of traditional PCR and provides more accurate and reproducible results than standard, end-point amplicon analysis. QPCR can use specialized markers that fluoresce when bound to double-stranded DNA or use quenchers, specialized fluorogenic probes that bind and cause fluorescence. This binding-induced fluorescence is captured and recorded by a camera; the greater the amount of target DNA in the sample, the greater the fluorescence. Results are transferred to a computer and the DNA quantified. QPCR quantification is based on a threshold value of fluorescence rather than an endpoint value of copy numbers of PCR product. The threshold value, also known as the Ct value, is defined as the PCR cycle at which the reaction shifts from baseline to exponential DNA production. Early in the PCR

amplification process, the exponential phase, conditions are optimal and the amount of double-stranded DNA theoretically doubles at every cycle. Therefore, during this exponential phase of the reaction, the fluorescent signal increase is directly proportional to the amount of PCR product that is produced. The Ct value is related directly to the initial number of cDNA copies present at the onset of the reaction; the greater the initial number of cDNA present in the sample, the earlier the exponential phase is achieved. This method of determining quantity of cDNA is more accurate than traditional PCR and allows for comparison of amounts of cDNA between experimental samples as the reaction proceeds rather than at the end of the reaction. Real-time PCR also eliminates the need for post-reaction processing of DNA such as gel electrophoresis or blotting. It is with this technology that the experiment outlined in this thesis was performed. The ultimate goal of this experiment was to lay the foundation for future experiments in reproductive molecular biology that may benefit the equine field practitioner in making diagnostic and therapeutic decisions during breeding soundness examinations.

Chapter 2: Literature Review

2.1: Delayed Uterine Clearance Defined; Historical Work Involving Delayed Uterine Clearance

The accumulation of fluid within the lumen of the uterus is an established, well-defined clinical problem occurring following breeding in the affected mare. This fluid can interfere with pregnancy if it persists until the conceptus enters the uterus at day five or six post-ovulation. The cellular and inflammatory by-products within the uterus are lethal to the early embryo[2]. Zent et al proposed that the prevalence of delayed uterine clearance (DUC) in a Thoroughbred mare population could reach as high as 15%[9]; LeBlanc et al stated that, "…recurrent bouts of endometritis after mating represent the majority of infertility and subfertility problems in equine practice"[5]. A survey of over 1000 equine veterinarians in the Unites States revealed that endometritis was the third most frequently occurring problem in adult horses[10]. Mares naturally respond to intrauterine deposition of foreign, antigenic material, such as bacteria or spermatozoa, with an inflammatory reaction. Mares with normal uterine clearance remove the by-products of this reaction via coordinated contractions of the myometrial smooth muscle and by immune system phagocytosis. Mares with DUC retain this fluid for greater than 24-36 hours resulting in persistent mating-induced endometritis (PMIE). Several groups of researchers have performed experiments to examine the phenomenon of uterine inflammation following uterine challenge. Substances such as *Streptococcus* bacteria, charcoal, chromium-labeled microspheres and technetium-albumin radiocolloid have been infused into mares' uteruses to evaluate the response and rate of clearance[5,11-13]. Evans et al performed one of the earliest studies in 1987 in which estrus and diestrus intrauterine environments were created using exogenous, intramuscular administration of estradiol and progesterone. These mares, of varying ages and parity, were then challenged by intrauterine inoculation of *Streptococcus zooepidemicus*, charcoal and ^{51}Cr-labeled microspheres. The mares then had their uteruses flushed after five hours. The results of this work showed that there was a significant positive correlation between

age and stage of estrous and numbers of bacteria and microspheres retained in the uterus[14]. This study suggested that the estrogen-influenced uterus has decreased physical clearance but that younger mares have increased ability to evacuate uterine contents. In a similar experiment, Troedsson and Liu used non-antigenic chromium microspheres followed by *Streptococcus zooepidemicus* intrauterine infusions to demonstrate the uterus' capacity for physical clearance of foreign material and inflammatory products. This group found that mares potentially susceptible to chronic uterine infections had a significant delay in physical clearance of microspheres from the uterus compared to resistant mares[11].

Nikolakopoulos and Watson showed that uterine contractility is important in the clearance of uterine fluid, but not necessarily for the elimination of bacteria[15]. This group infused bacteria into the uteruses of five normal estral mares during a control heat and during a heat cycle in which the mares were treated with a uterine-relaxing drug (a beta2-agonist, clenbuterol). During the period of pharmacologic-induced uterine relaxation, all mares accumulated fluid whereas none of the mares accumulated fluid during the control estrus. The cellularity, color, percentage of neutrophils and bacterial content of the fluid did not differ between the two cycles[15]. This work supports the theory that impaired contractility and subsequent decreased mechanical clearance leads to persistent mating-induced endometritis. LeBlanc et al designed an experiment to examine uterine clearance mechanisms in the early post-ovulatory period in nine mares, four that were considered resistant to endometritis and five considered susceptible to endometritis. This study also used an inoculum of *Streptococcus* organisms, charcoal and ^{51}Cr-microspheres instilled into the uterus. At ovulation, the uterus was cultured and then lavaged with saline. The effluent was then analyzed to determine the amount of retained charcoal and microspheres. The group found that mares susceptible to endometritis had more positive bacterial cultures and accumulated more fluid within the uterine lumen after ovulation than did resistant mares, leading the group to conclude that the uterine cellular defense mechanisms were dysfunctional in the early post-ovulatory period, but that no decrease in mechanical clearance was apparent[12]. A 1994 study by LeBlanc et al, building on the

information and data collected from previous work, used radiolabeled technetium infused into the uterus and measured by scintigraphy. This experiment demonstrated that normal mares in estrus, those resistant to post-mating induced endometritis, clear greater than 50% of the radiocolloid within two hours of infusion whereas mares with poor cervical dilation or those susceptible to endometritis and DUC did not adequately clear a significant amount of radiocolloid[13]. In a follow-up experiment, this same group looked at the effect of exogenous oxytocin administration on clearance of technetium radiocolloid and found that 20 IU of oxytocin given intravenously immediately following scintigraphy caused evacuation of >90% of the radiocolloid within 30 minutes of administration[5]. This supports the current use of exogenous oxytocin as a treatment for DUC.

In 2001, Rigby et al hypothesized that DUC was a result of an intrinsic contractile defect in myometrial smooth muscle[4]. This group performed an in vitro experiment using strips of myometrium harvested following hysterectomy of young nulliparous mares, older reproductively normal mares, and older mares with delayed uterine clearance. The tension generated in the circular and longitudinal strips was measured as the muscle strips were subjected to potassium chloride, oxytocin or $PGF_{2\alpha}$. The intracellular calcium concentration of the muscle strips was also determined by laser cytometry. It was found that mares with DUC consistently generated less tension in the myometrium when exposed to any of the contractile agents but that the intracellular calcium levels were the same for all groups. This led to the supposition that the failure to contract in DUC was not related to calcium transport but may be related to intracellular second messengers further downstream[4]. In a related experiment, von Reitzenstein et al studied the patterns and strength of uterine contractions in normal and DUC mares treated with oxytocin and detomidine, an alpha-2 agonist used a sedative. It was shown that normal mares had an increased number of contractions, the contractions lasted longer and were of greater intensity than in DUC mares when given either of three treatments: (1) 0.001 mg/kg intravenous (IV) detomidine alone, (2) 0.001 mg/kg IV detomidine followed in ten minutes by 10 IU oxytocin IV and (3) 0.5mL IV saline followed by 10 IU oxytocin IV.

When detomidine was given before oxytocin, normal mares demonstrated an increase in the number and strength of myometrial contractions but no similar, significant effect in contractions was measured in DUC mares. Detomidine also led to an increased number and percentage of total propagating contractions as opposed to non-propagating contractions in normal mares but caused no change in propagating contraction patterns in DUC mares[3]. This data suggested that mares with DUC have a defect in myoelectrical signaling and a decrease in the contractile strength of the uterine muscle[3].

Another significant factor in the clearance of accumulated intrauterine fluid is the position of the uterus within the mare's abdomen. With increasing age and parity, the uterus of the mare becomes more cranioventrally located within the abdomen. LeBlanc et al used scintigraphy during estrus in 44 mares, 24 reproductively normal and 20 with DUC. This group infused radiocolloid into the uteruses of these primiparous and nulliparous mares and found that the angle formed between the cervix and uterus of normal mares was more horizontal in position than the more vertically oriented angle formed in DUC mares. This suggests that failure to evacuate intrauterine contents may be due to the cranioventral location of the uterus[17]. Impaired lymphatic drainage of intrauterine contents has also been suggested to play a role in the pathogenesis of delayed uterine clearance. LeBlanc et al used intrauterine India ink to evaluate clearance by lymphatics in normal mares and mares susceptible to endometritis. Her group found that lymphatic clearance of India ink was impaired in the endometritis mares compared to the normal mares based on the post-mortem identification of reduced amounts of ink recovered in the regional lymph nodes that drain the uterus[18].

A 2005 study by Alghamdi et al investigated the role of nitric oxide (NO), a known smooth muscle relaxant, and an inducible form of nitric oxide synthase (iNOS) in the etiopathogenesis of PMIE and DUC. This group took endometrial biopsy samples and uterine secretion samples from susceptible and resistant mares at 13 hours post-insemination and used PCR technology to assay these two components (iNOS and NO), respectively. Their data suggested a possible role for these two compounds in the cause of DUC by showing that susceptible mares had higher levels of both NO and iNOS than

did resistant mares[19]. Fang et al used ovariectomized rats and inoculated their uteruses with *E.coli*. The rats were then euthanized and their uteruses assayed for multiple forms of NOS. This group found that there was a localized increase in type II nitric oxide synthase expression and nitric oxide production in response to intrauterine infection and that the nitric oxide system may play a role in host response to restrict the infection[20].

2.2: Physiology of Smooth Muscle Contraction

Myometrial smooth muscle plays a very important role in the evacuation of uterine contents. This muscle is capable of stretching to accommodate a growing fetus, is strong enough to cause the expulsion of the fetus and fetal membranes and then is able retract to normal, non-gravid size within seven to ten days of parturition. A failure of contraction of this muscle would lead to the retention of any accumulated fluid or debris in the nongravid uterus. Calcium plays a critical, essential role in the contraction of all muscles within the body, including the smooth muscle of the myometrium. The contraction of smooth muscle differs from that of skeletal muscle. Skeletal muscles store large quantities of calcium ions in their sarcoplasmic reticulum and are stimulated to release it by action potentials generated by the somatic nervous system. Released calcium binds to a protein molecule called troponin, resulting in a shift in the configuration of this protein and starting a cascade of sliding of actin and myosin filaments. Smooth muscle cells behave differently. When stimulated by the autonomic nervous system or by extracellular agonists (ex. oxytocin, $PGF_{2\alpha}$), smooth muscle cells release calcium ions that bind to a molecule called calmodulin instead of troponin. This calcium-calmodulin complex activates myosin directly and initiates the swiveling or sliding of the myocytes, which then contract as a unit[16]. A schematic drawing of the events leading to smooth muscle contraction is shown in Figure 1.

The Events Involved in Smooth Muscle Contraction

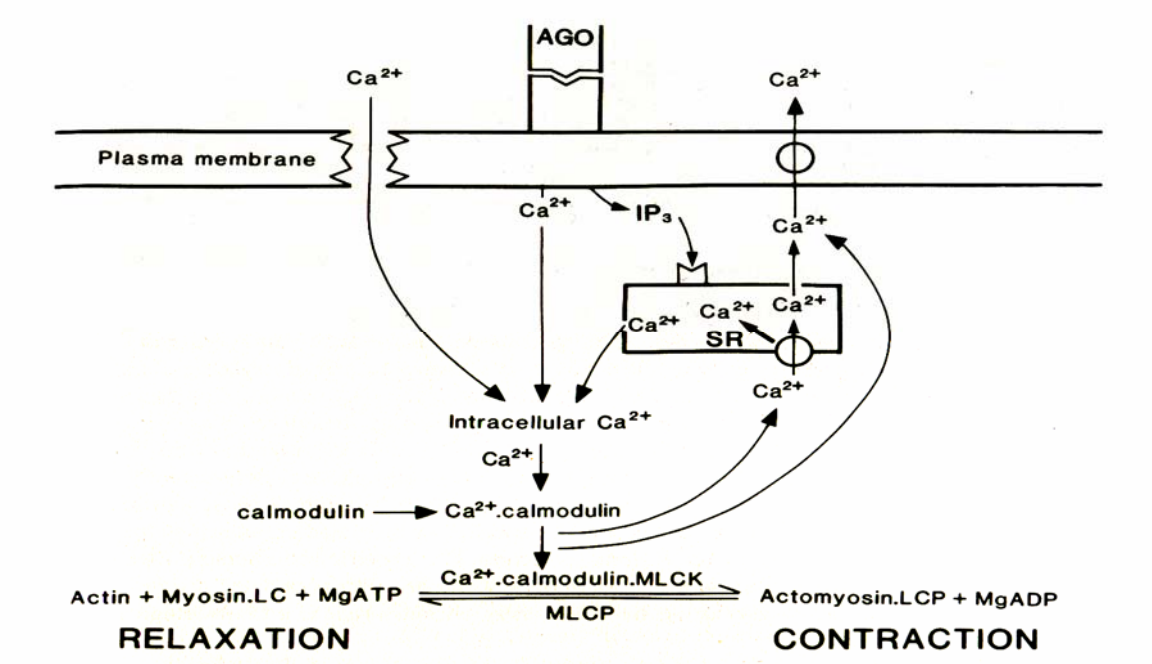

Figure 1: Schematic illustrating the events leading to uterine myometrial contraction or relaxation. The increase in intracellular [Ca^{2+}] can result from influx from extracellular sources through voltage- or receptor-gated channels or via release from intracellular sarcoplasmic reticulum stores. AGO=agonist; IP_3=inositol 1,4,5-triphosphate; SR=sarcoplasmic reticulum; MLCK=myosin light chain kinase; MLCP=myosin light chain phosphatase; LCP=phosphorylated myosin light chain; Mg=magnesium[24].

2.3: Physiologic Compounds Involved in the Pathogenesis and Treatment of Delayed Uterine Clearance

There are numerous hormones and circulating substances that cause a change in the tone and environment within the mare's tubular reproductive tract. Substances that increase uterine tone are known as ecbolic substances and include oxytocin, prostaglandins (especially $PGF_{2\alpha}$) and, anecdotally, progesterone. Oxytocin is a hormone that is synthesized and packaged into secretory vesicles by the hypothalamus and eventually secreted from the posterior pituitary into the general circulation to effect smooth muscle contraction. In circulation, oxytocin is transported bound to a protein carrier called a neurophysin[21,22]. Oxytocin is associated with myometrial contractions and mammary gland milk "let-down" in the female and has been shown to be involved in semen transit in the testes of the male. It has been recently shown that oxytocin can be produced in sites other than the hypothalamo-neurohypophyseal axis. Bae and Wilson found that oxytocin was localized in the endometrium of the mare at the light microscopic and ultrastructural level[21]. This group used immunostaining and immunogold labeling of endometrial biopsy specimens collected from mares during estrus[21]. The local production of oxytocin has great implications in the autocrine and paracrine control of myometrial contraction in the mare. To investigate the abundance of oxytocin-neurophysin (OT-NP) in mare's endometrium, Behrendt-Adam et al performed a molecular biological research study in which endometrial biopsy samples were taken from nonpregnant mares during estrus and at five, ten, and 15 days post-ovulation and from pregnant mares at days ten, 15 and 20 post-ovulation[22]. Biopsy samples were subjected to RT-PCR and Southern blotting to determine the amounts of OT-NP and glyceraldehyde-3-phosphate dehydrogenase (G3PDH, a constitutively-produced enzyme found in most tissues and frequently used in molecular biology as a reference for expression, commonly referred to as a "housekeeping" gene) mRNA. The data collected from this research demonstrated that the amount of OT-NP was positively correlated with serum estradiol levels in open mares and negatively correlated with serum progesterone levels in all mares. This suggests that uterine oxytocin may be involved in regulation of

reproductive tract function during the estrous cycle and/or establishment of pregnancy in horses[22]. In 1999, Cadario et al concluded that oxytocin caused a dose-dependent increase in intrauterine pressures in both normal and DUC mares[23]. This group used intrauterine catheters with pressure sensors connected to a physiograph to determine the intrauterine pressure and direction of myometrial contractions following injections of oxytocin[23].

Prostaglandin$F_{2\alpha}$ is another important compund in uterine contraction. Prostaglandin$F_{2\alpha}$ is one of a host of eicosanoids produced by the metabolism of arachadonic acid by the enzyme cyclooxygenase. Prostaglandins are liberated by most tissues within the body and serve important roles in the inflammatory cascade as well as effecting vasoactive and smooth muscle changes such as contraction or relaxation of vascular endothelial or gastrointestinal smooth muscles. Prostaglandin $F_{2\alpha}$ has been shown to cause a dose-dependent elevation of calcium in vascular smooth muscle cells, calcium being intimately involved in the generation of smooth muscle contraction[24]. Prostaglandin$F_{2\alpha}$ released from the uterine endometrium is known to cause regression of a mature corpus luteum in most domestic species, thereby allowing the animal to return to estrus. Prostaglandin$F_{2\alpha}$ also has ecbolic or uterotonic effects, causing myometrial contraction, and plays a role in ovulation at the follicular level. Exogenous $PGF_{2\alpha}$ administered intramuscularly to mares will cause physical and behavioral changes such as sweating, apparent abdominal pain from myometrial smooth muscle contractions, diarrhea and/or anorexia. These effects are transient and short-lived when natural $PGF_{2\alpha}$ is used. Synthetic $PGF_{2\alpha}$-analogues such as cloprostenol (Estrumate®) appear to have fewer adverse side effects and exert their ecbolic activity over a longer period of time. Obstetrical manipulations such as manual cervical dilation or intrauterine infusion of materials will also cause endogenous $PGF_{2\alpha}$ release. Cadario et al measured serum concentrations of a metabolite of $PGF_{2\alpha}$ in normal and DUC mares following intrauterine infusion of radiocolloid with subsequent administration of exogenous oxytocin. The data from this experiment showed that DUC mares had an increase in $PGF_{2\alpha}$ metabolite levels after oxytocin administration whereas normal mares had no such increase[25]. Jones et al

used electromyography (EMG) and intraluminal catheters to measure electrical activity of the myometrium and intrauterine pressure when mares were treated with a variety of ecbolic and uterorelaxant substances. It was found that oxytocin and the synthetic prostaglandin cloprostenol caused prolonged EMG activity followed initially by a short burst pattern that was most pronounced in estrus and least in diestrus, suggesting that uterine motility is stimulated to a greater extent during estrus[26]. A related study was done by Troedsson et al using EMG and increased durations of myometrial activity during estrus similar to the results found by Jones et al were obtained[27].

Progesterone and estrogen are the steroid reproductive hormones and are intimately involved in the physical, behavioral and immunologic components of the estrous cycle. Estradiol, the principal circulating estrogenic compound in the mare, is synthesized in the ovary by granulosa and thecal cells of the ovarian follicles. Estradiol is responsible for the behavioral signs of sexual receptivity as well as physical changes in the tubular reproductive tract, including relaxation of the cervix and uterus, endometrial edema and increased secretions from the tract. Estrogens have been shown to stimulate local immunity within the endometrium so that the intrauterine environment is less susceptible to infection[14]. Estrogens are at their highest level during estrus and decline during diestrus. Progesterone is produced by the corpus luteum and by the placenta during pregnancy. In the mare, early luteal cell formation occurs prior to ovulation so that the progesterone levels begin to rise before the end of estrus[7]. Progesterone levels are highest during diestrus and are barely detectable during estrus (<1 ng/mL). This steroid hormone has activities antagonistic to those of estrogen. Progesterone suppresses behavioral signs of estrus, inhibits endometrial edema, suppresses reproductive secretions, causes an increase in tone of the uterus and cervix, and causes reduction of the cervical canal diameter[28]. It has been shown in murine models that progesterone causes a decrease in local production of the immunoglobulins IgG and IgA by the endometrial immune cells, therefore giving the hormone an immunosuppressive effect within the uterus[29]. Progesterone , although having a known effect on increasing tone of the myometrium, has been shown to reduce myometrial contractility in humans, causing

uterine quiescence[30]. This suggests that progesterone's effects are not influential on propulsive contractions but more on tonic contraction.

The three genes assayed in this research (oxytocin receptor, $PGF_{2\alpha}$ receptor and progesterone receptor) were selected based on their respective clinical relevances. Oxytocin and $PGF_{2\alpha}$ possess ecbolic properties that make them essential for evaluation in DUC mares. Progesterone, although not an ecbolic hormone, has a clinical effect on uterine tone and levels begin to rise in the early pre-ovulatory period. Therefore, progesterone receptor was selected because the expression of the gene encoding this protein may influence the development of DUC.

Materials and Methods

Subjects

Endometrial biopsy samples from 25 mares were used in this study. These mares were utilized in a previous research project conducted at the Texas A&M College of Veterinary Medicine[4]. The mares were categorized into three groups: (1) six were young fertile, nulliparous mares (non-DUC), (2) seven were older, reproductively normal mares (non-DUC) and (3) twelve were age-matched, older, susceptible mares (DUC). Mares were determined to be susceptible or resistant to DUC based on results of breeding soundness examinations, reproductive history and measurements of uterine function. The measurements of uterine function included: (1) maximal height and location of free fluid within the uterus during estrus, (2) free fluid within the uterus during diestrus, (3) uterine fluid 72 hours following insemination, (4) uterine clearance of a radiocolloid using nuclear scintigraphy, (5) uterine fluid, and (6) positive endometrial culture 96 hours following bacterial challenge with *Streptococcus zooepidemicus*.

Endometrial Tissue Collection

One month following categorization and uterine challenge studies, each mare was inseminated with equivalent volumes of fresh semen. Eighteen hours post-breeding, the mares were anesthetized and hysterectomized via a ventral midline celiotomy. Endometrial and myometrial tissues were harvested from the surgically removed uteri. Tissue samples were flash frozen in liquid nitrogen and stored at –70° C until the time of RNA extraction.

Extraction of RNA from Endometrial Biopsy Samples

Frozen endometrial biopsy samples from each mare were mechanically disrupted and homogenized in liquid nitrogen with a mortar and pestle. Qiagen's RNeasy Maxi Kit[a] was used to extract RNA from each tissue sample and to digest genomic DNA. This kit utilizes a silica-gel-based membrane and high-salt buffer to adsorb RNA. Samples were lysed and homogenized in the presence of a denaturing guanidine isothiocyanate

buffer; the sample was then applied to a membrane-containing column that bound RNA and washed away contaminants. All RNA molecules greater than 200 base pairs (bps) are recovered with this technique. This procedure allows for enrichment of mRNA because most RNA molecules less than 200 bps are ribosomal, transfer or mitochondrial RNA.

Quantification of RNA

RNA was quantified using Tecan's GENios[b] machine with the Magellan[b] fluorointensity program and Invitrogen's RiboGreen[c] dye. A dilution series of experimental RNA samples was made and the fluorointensity of these serial dilutions was compared to a known standard RNA fluorointensity (standard curve) to determine experimental RNA concentration. The concentration of RNA was obtained so that the proper volume of sample could be added to the following reactions to get optimal results based on manufacturer's suggestions of quantity of sample to be added per reaction.

Complementary DNA (cDNA) Synthesis Using Reverse Transcriptase (RT)

Bio-Rad's iScript cDNA Synthesis[d] kits were used to synthesize cDNA from a standard amount of each endometrial RNA sample. These kits use a two-step RT reaction with oligo dT nucelotides and random hexamer primers to generate cDNA. An Eppendorf Master Cycler thermal cycler[e] was used for RT reactions. The reaction was run using the following three-step synthesis protocol: five minutes at 25° C for primer annealing, 30 minutes at 42° C for RT-mediated chain elongation and 5 minutes at 85° C for inactivation of reverse transcriptase enzyme. To verify reaction results (ie cDNA synthesis), agarose gel electrophoresis reactions were performed on multiple experimental samples alongside no-reverse transcriptase (no-RT) and no template controls. Gels were stained with ethidium bromide and viewed with ultraviolet light to check for appropriate sized fragment bands in the experimental sample lanes.

Primer Design and DNA Sequencing

Custom oligonucleotide primers were designed for each gene sequence of interest (beta actin, ribosomal 18S RNA, oxytocin receptor, progesterone receptor and prostaglandin F2α receptor) using the commercial primer design program Primer 3[f]. Gene sequences for equine beta actin (AF035774), ribosomal 18S RNA (AJ311673) and progesterone receptor (AF007798) had been previously determined and published and were located using NCBI's PubMed Nucleotide Search Engine[g].

Equine gene sequences for oxytocin and $PGF_{2\alpha}$ had not been published, so a consensus sequence was constructed using known human, bovine and murine sequences. SeqWeb's online consensus design software[h] was used for construction of the equine consensus sequence. Once a consensus sequence was produced, primers were designed using "intron-spanning" criteria such that left and right primers were located on different exons, as extrapolated from intron and exon breaks found on the human genome. By having forward and reverse primers located on different exons, genomic DNA amplification from potential, inadvertent genomic DNA contamination of RNA samples was lessened during QPCR. These custom primers were then constructed by Operon[i] and rehydrated according to manufacturer's recommendations. The first set of primers was designed such that a 400-500 base pair region of the gene was produced during the PCR reaction. This amplified portion was then sequenced at the Virginia Bioinformatics Institute[j] (VBI) at Virginia Tech. The sequence produced by VBI was then compared to known, published gene sequences using NCBI Blast Engine[k] to confirm that the proper sequence had been amplified. Then, a second set of internal primers was designed to produce a 100-150 base pair segment of DNA that spanned the previously identified intron. Once all primers had been designed and constructed, optimization PCR reactions were performed using temperature and cDNA concentration gradients to determine optimal annealing temperatures and sample concentrations for each primer. Reactions were determined to be optimal when their efficiencies exceeded 90% and correlation coefficients met or exceeded 0.99.

Plasmid Standard Preparation

cDNA for each specific gene of interest (beta actin, ribosomal 18S RNA, oxytocin receptor, progesterone receptor and $PGF_{2\alpha}$ receptor) was isolated from agarose gel electrophoresis and was ligated into pCR2.1 plasmid vectors using TOPO TA cloning kits[c]. These plasmids were then purified using Qiagen's QiaPrep MiniPrep System[a]. Top10 *E.coli* [c] bacteria from Invitrogen were transformed using the prepared plasmids.

Quantitative Real Time Polymerase Chain Reaction (QPCR)

Once cDNA and plasmid standards were synthesized and primers were designed for each tissue sample, Bio-Rad's iQ SYBR Green Supermix kits[d] were used to prepare samples for QPCR. A Bio-Rad iCycler QPCR machine[d] was used to run each reaction. Samples were run in 96 well plates using 25 μL reaction volumes. Based on the results of optimization reactions using temperature and concentration gradients, the optimal temperature for amplification of each gene and its respective primer set was determined. The following annealing temperatures were selected for each respective gene: Beta actin and oxytocin receptor at 65°C, ribosomal 18S RNA at 63.3°C, progesterone receptor at 51°C, and $PGF_{2\alpha}$ receptor at 60°C.

The following three-step protocol was used for each QPCR reaction:

Step 1: 95°C for 3 minutes to activate polymerase and denature double-stranded cDNA

Step 2: 45 cycles at 95°C for 30 seconds each to denature DNA and primers

1 cycle at selected temperature for each gene as determined during optimization reactions (51, 60, 63.3 or 65°C) for 30 seconds to allow primer annealing

1 cycle at 72°C for 20 seconds for chain extension

Step 3: 1 cycle at 95°C for 1 minute for final denaturation of double-stranded

structures

Step 4: 1 cycle at 55°C for 1 minute to prepare for melt curve synthesis; allows double-stranded products to re-anneal

Step 5: 80 cycles at 10 seconds each, starting at 55°C and increasing by 0.5°C each cycle for melt curve construction

SYBR Green is a marker dye that does not bind to single-stranded nucleic acids (ie. mRNA) but that binds with great affinity to double-stranded nucleic acids. Once bound to the double-stranded molecules, the dye fluoresces. A camera records the fluorescence and displays the results as threshold cycle or Ct (Figure 2). All experimental samples were run in triplicate along with their respective no-reverse transcriptase (no RT) controls and a no-template (no cDNA) negative control. Each QPCR plate also contained triplicate plasmid standards. Melt curve analysis was performed on each plate to verify product purity. During melt curve construction, the temperature of the thermal cycler is increased incrementally until double stranded products denature and the SYBR Green dye is released, causing a sharp decrease in fluorescence. An example of a melt curve graph is depicted in Figure 3.

Example of Quantitative Real-Time PCR Display

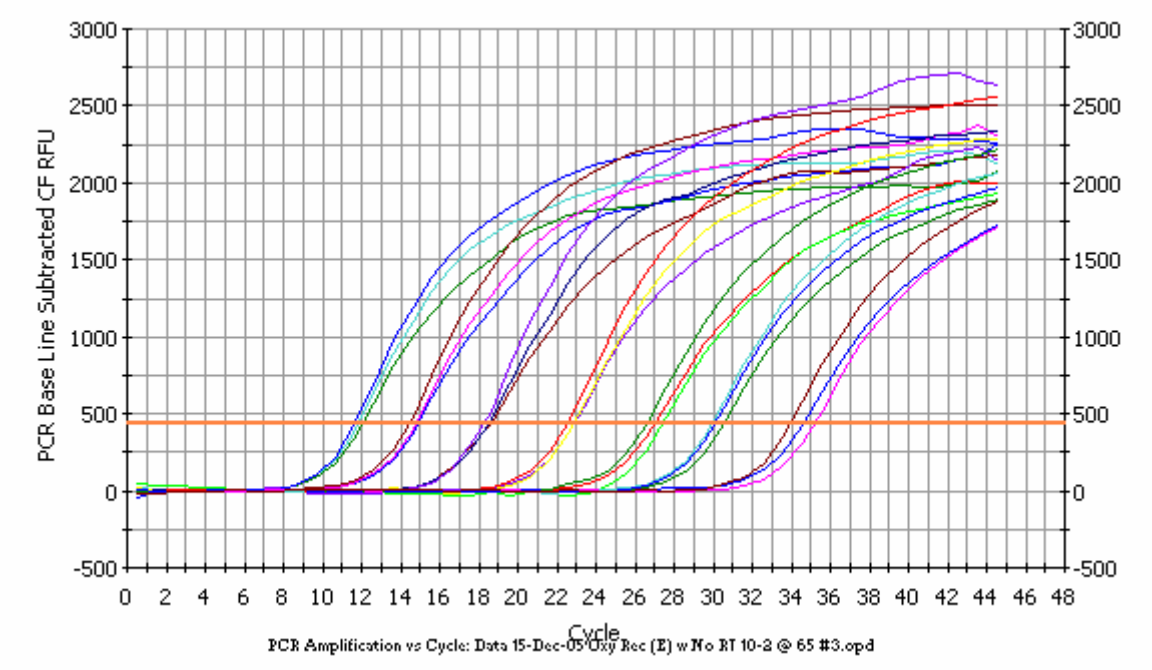

Figure 2: Illustration of computer-generated results of a QPCR reaction showing the Ct value. This graph illustrates cDNA standard samples that have been sequentially diluted tenfold. This example was from an oxytocin receptor plate.

Example of QPCR Melt Curve

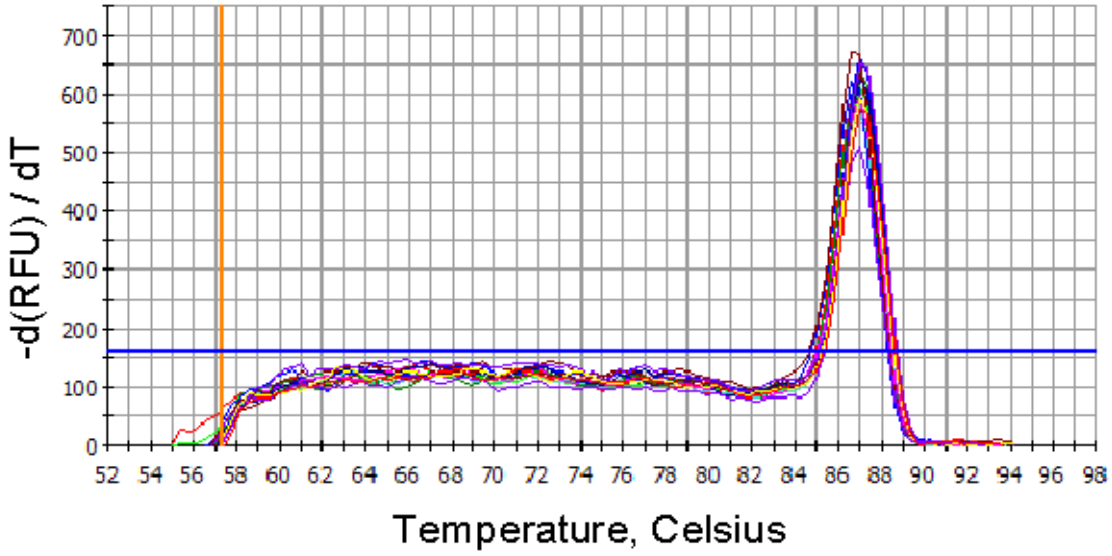

Figure 3: Graph depicting melt curve analysis obtained following QPCR reaction of oxytocin receptor cDNA. The melt curve is obtained by plotting the negative first derivative values of the fluorescence intensity as a function of the temperature, which results in a peak. The single peak verifies product purity.

Once the Ct values were produced by QPCR in triplicate for each mare in the experiment, a spreadsheet was constructed. The Ct values for each mare were entered and the arithmetic mean of each triplicate was calculated. The plasmid standard Ct values were used to construct a linear regression plot with the average Ct value plotted on the y-axis and the plasmid concentration on the x-axis. From this logistic regression plot, an R^2 value was determined based on the slope of the generated line. A slope-intercept equation (y=mx+b) was then made and solved for "x" for each mare. This "x" value represents the cDNA concentration in the initial sample of each mare. An example of one of the regression plots is shown in Figure 4.

Regression Analysis Plot Constructed from Plasmid Standards

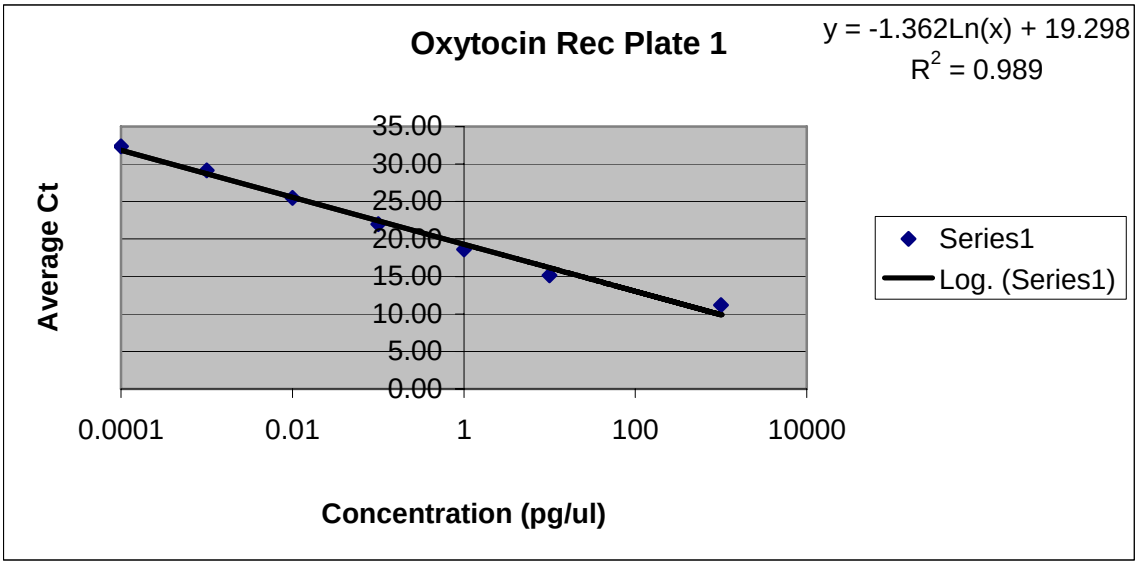

Figure 4: Example of regression analysis plot constructed from plasmid standard Ct values and used to determine initial cDNA concentration for each horse. This example was taken from an oxytocin receptor plate.

Statistical Analysis

SAS statistical software[1] was used for generation of statistical results. Within this program, a Kruskal-Wallis test was used for analysis of the data. This method of analysis is a non-parametric test used to compare three or more independent groups of sampled data. Unlike parametric tests, the Kruskal-Wallis makes no assumptions about the distribution of the data (e.g., normality); the test uses the ranks of the data rather than their raw values to calculate the statistic. Statistical significance was defined as $p \leq 0.05$.

Results

The expression of the experimental genes oxytocin receptor, $PGF_{2\alpha}$ receptor and progesterone receptor was normalized using each of two "housekeeping" genes, ribosomal 18S RNA and beta actin. Regardless of which housekeeping gene was used for normalization, there was no statistically significant difference discovered among expression levels of each of the three experimental genes in any of the three categories of mares examined in this research. The mean Ct values and initial cDNA concentrations for YF, ON and OS mares were calculated and are reported in Tables 1 and 2. Figure 5 shows graphically the average Ct values $\pm$ standard deviation for each of the five genes assayed. Figure 6 shows graphically the calculated initial cDNA concentrations for each gene.

Average Ct Values

Mare Category	Beta Actin	Ribosomal 18S RNA	Oxytocin Receptor	$PGF_{2\alpha}$ Receptor	Progesterone Receptor
Young Fertile (YF)	26.32 ± 2.86	15.64 ± 2.45	30.91 ± 2.69	34.06 ± 2.01	28.87 ± 2.64
Older Normal (ON)	26.62 ± 3.05	16.65 ± 0.56	31.41 ± 2.69	29.47 ± 13.06	29.88 ± 3.19
Older Susceptible (OS)	26.81 ± 2.51	15.65 ± 2.36	30.71 ± 2.76	34.08 ± 2.74	29.05 ± 2.49

Table 1: Average Ct ± standard deviation values for each of the three categories of mares for each of the five genes assayed.

Calculated Average Initial cDNA Concentration (pg/µL)

Mare Category	Beta Actin	Ribosomal 18S RNA	Oxytocin Receptor	$PGF_{2\alpha}$ Receptor	Progesterone Receptor
Young Fertile (YF)	0.14 ± 0.26	144.38 ± 261.71	.0061 ± .0093	.000042 ± .000055	.0094 ± .014
Older Normal (ON)	0.20 ± 0.41	9.69 ± 5.26	.0011 ± .0019	.000039 ± .000039	.0031 ± .0064
Older Susceptible (OS)	0.16 ± 0.38	71.80 ± 136.78	.0022 ± .0037	.00025 ± .00054	.0054 ± .011

Table 2: Average initial cDNA concentration (pg/µL) ± standard deviation for each of the three categories of mares for each of the five genes assayed. Standard deviations are reported in parentheses.

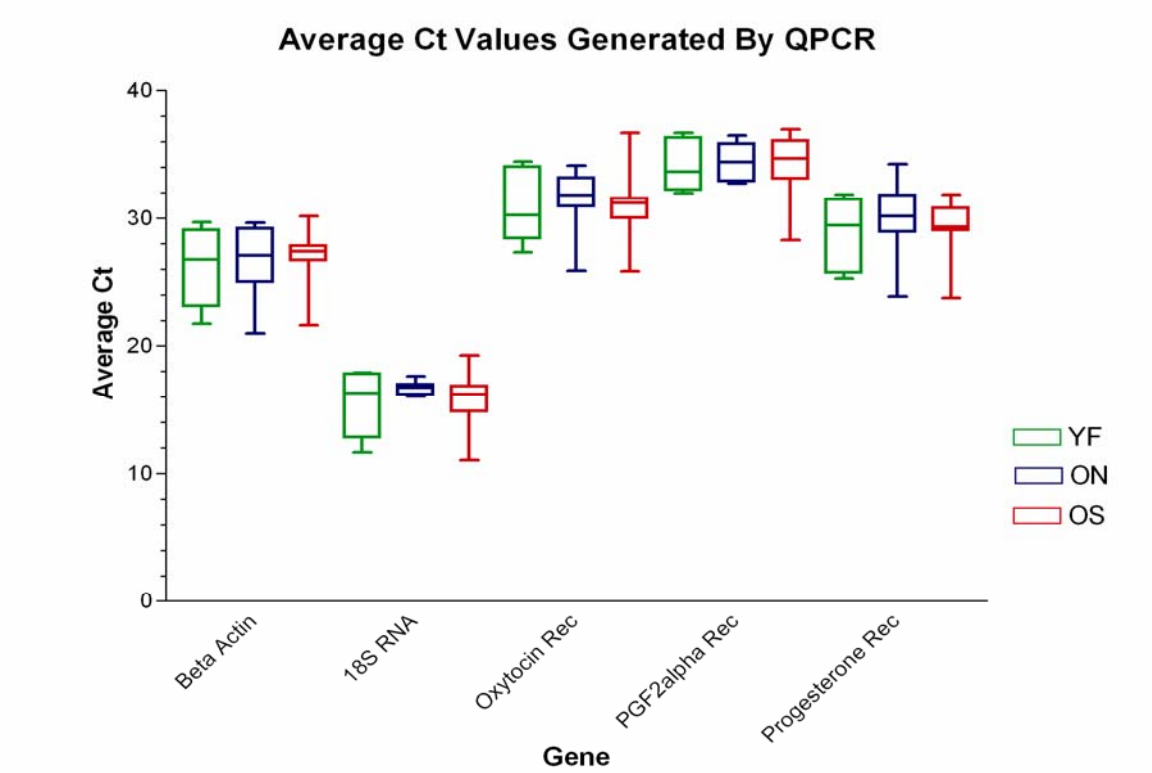

Figure 5: Graphical representation of average Ct values for each category of mare for each of the two housekeeping genes and three experimental genes assayed.

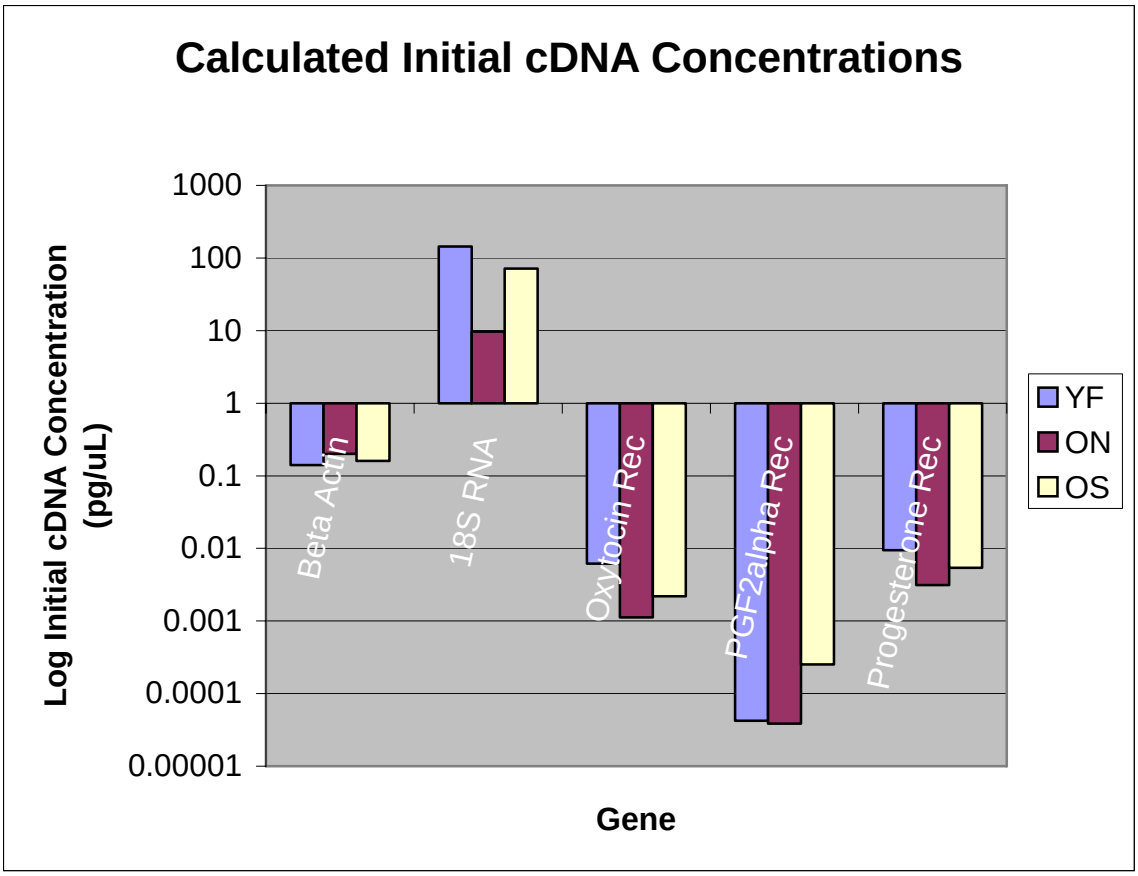

Figure 6: Graphical representation of initial cDNA concentration (pg/µL) for the two "housekeeping" genes and three experimental genes assayed.

The data for each of the three experimental genes was normalized using the housekeeping gene data. This was accomplished by dividing each mare's experimental value by the corresponding mare's value for either beta actin or ribosomal 18S RNA (ie. calculated initial cDNA concentration of experimental gene divided by concentration of beta actin or 18S RNA). When the expression of the three experimental genes was normalized using beta actin, the difference was not statistically significant at the $p \leq 0.05$ level; for oxytocin receptor, $p=0.0841$, for $PGF_{2\alpha}$ receptor, $p=0.3881$ and for progesterone receptor, $p=0.1507$. When the expression was normalized using ribosomal 18S RNA, statistical significance was also not found; for oxytocin receptor, $p=0.6674$, for $PGF_{2\alpha}$ receptor, $p=0.5866$ and for progesterone receptor, $p=0.3746$.

There was an apparently large degree of variability in the expression of ribosomal 18S RNA among the three categories of mares. This variability reduces the validity of this gene as a true housekeeping gene that, by definition, should be expressed at similar levels regardless of age or clinical condition. The large standard deviations reported indicate the highly variant, non-normally distributed data. For example, the standard deviation of the average Ct for $PGF_{2\alpha}$ receptor for the older normal mare group was aberrantly high (13.06) because two of the seven mares' samples did not recover any cDNA product and therefore had no average Ct value associated with it. This spuriously low value caused a large standard deviation to be calculated. The data was re-analyzed after exclusion of these two mare's data; statistical significance was still not found at the $p \leq 0.05$ level.

Discussion and Conclusions

The pathogenesis of delayed uterine clearance was investigated at the molecular level using real-time PCR to assess the expression of the receptors of the uterotonic substances oxytocin, progesterone and $PGF_{2\alpha}$ in the endometrium of normal and clinically affected mares. No significant differences were discovered among any of the three categories of mares included in this experiment (YF, ON, OS). However, there remains a great deal of investigation that can be pursued using this same technology using different intermediates and different tissue types.

The uterus is a myogenic organ, meaning that its smooth musculature can contract in the absence of hormonal or neural input. This, however, does not imply that agonists such as oxytocin and $PGF_{2\alpha}$ do not play critical roles in modulating the activity of the organ. The contraction of myometrial smooth muscle is dependent on calcium, the release of which is stimulated by action potentials, oxytocin, prostaglandins and other stimuli[31]. Molnar and Hertelendy used rat myometrium exposed to oxytocin, $PGF_{2\alpha}$, endothelin and inositol 1,4,5-triphosphate (IP_3, an intracellular second messenger) to show that the primary action of $PGF_{2\alpha}$ in myometrial cells is to enhance extracellular Ca^{2+} influx through gated channels, whereas oxytocin and endothelin receptors are coupled to phospholipase C, generating IP_3 and raising the intracellular concentration of free Ca^{2+} from intracellular (sarcoplasmic reticulum) as well as extracellular sources[32]. Wray also corroborates this finding in her review on the physiologic mechanisms of uterine contractility[33]. Endothelin is a potent vasoconstrictor and has been shown to cause uterine contractions in multiple species[34]. The rise in intracellular calcium initiates the next phases of contraction: the formation of the calcium-calmodulin molecule complex, activation of myosin light-chain kinase (MLCK), myosin phosphorylation and, finally, the myosin head's interaction with actin [35]. This work identifies several other intermediates in the contractile pathway that could be assayed using QPCR (ie MLCK, MLCP, calmodulin, IP_3, endothelin).

Although there were no statistically significant differences among oxytocin, prostaglandin and progesterone receptors in the mares assayed in this study, the expression of mRNA for oxytocin and progesterone receptors was approaching significance at the $p \leq 0.05$ level ($p=0.0841$ and $p=0.1507$, respectively). In future work using similar study design and molecular techniques, the inclusion of greater numbers of mares may lead to the finding of statistically significant results with less influence of the non-normally distributed data. In addition to increasing sample size, the inclusion of other genes of interest encoding such proteins as MLCK, iNOS, MLCP, relaxin, endothelin and others as well as the analysis of myometrial tissues may uncover important factors in the etiology or development of delayed uterine clearance.

It has been suggested, based on the work of Rigby et al, that DUC is ultimately a result of defective myometrial contraction[4]. This group determined that intracellular calcium concentrations of myometrial cells were not altered either in health, with increasing age or with DUC. This leads to the speculation that downstream calcium intermediates may play a significant role in the pathogenesis of DUC. After calcium concentrations have risen, the calmodulin, MLCK and myofibrillar protein molecules become the integral intermediates leading to myometrial contraction. Future applications of molecular biological technology should focus on the expression of these molecules in normal and DUC mares to evaluate their contribution to the disease process.

Rigby's group looked at myometrial samples whereas our research was focused on cell surface receptors for uterotonic agonists found in the endometrium. This may have lead to the absence of significant results in this research. While the endometrium plays an important role in uterine activity and health, the myometrium is ultimately responsible for the contractions that lead to the expulsion of inflammatory debris. Although no significant differences were found in the expression of mRNA encoding the oxytocin, progesterone and $PGF_{2\alpha}$ receptors, there may indeed be differences in the expression of these molecules in the myometrium or in the cell-to-cell signaling between endometrial and myometrial cells. Cell-to-cell signaling molecules and endometrium/myometrium interactions are sources of future investigation into the

pathogenesis of DUC. Work by Carsten and Miller has shown that prostaglandin receptors are found on the membrane of the sarcoplasmic reticulum in addition to the cell membrane and that prostaglandin-mediated rises in intracellular calcium may be a result of a direct intracellular action in combination with a cell surface membrane receptor-coupled action[24]. However, the current study is assaying mRNA production for receptors, regardless of their eventual site of placement and should still be an adequate gauge of up- or down-regulation of these receptors in normal and DUC mares.

The converse of Rigby's group's hypothesis that inappropriate myometrial contraction causes DUC may also need to be investigated; that is, does excessive relaxation of the myometrium become a significant contributor to the pathogenesis of DUC? Uterorelaxants such as nitric oxide[19], which is formed by the inducible enzyme nitric oxide synthase [19,20], the hormone relaxin[36], or substances that potentiate myosin light-chain phosphatase (MLCP) may be over-expressed in DUC mares. MLCP is responsible for the dephosphorylation of MLCK, turning off the stimulus for contraction[35]. MLCP activity is influenced by another phosphorylating molecule, rho A-associated kinase (ROK); ROK phosphorylates MLCP and renders it inactive[37]. Therefore, down-regulation of ROK or a defect in its production may lead to increased MLCP activity, reducing MLCK-mediated myosin phosphorylation and, hence, inhibition of myometrial contraction. Cyclic AMP (cAMP) is generated from the enzyme adenylate cyclase and this intracellular second-messenger has been shown to cause a relaxation of myometrial and other smooth muscles via the activation of cAMP-dependent protein kinase[38]. Up-regulation of adenylate cyclase may lead to excessive uterine relaxation and DUC. The uterus is also endowed with adrenergic receptors, both α and β; α receptors, particularly α_1, are primarily associated with uterine contraction whereas β receptors are associated with relaxation[34]. The expression of these β receptors may be aberrantly over-expressed in DUC mares, leading to myometrial relaxation. Myometrial relaxation can be assessed *in vivo* using pressure transducers or *in vitro* via myometrial muscle biopsy tension generation as described by Rigby[4]. Herein lie other arms of the myometrial

regulatory pathway that must be investigated at the molecular or ultrastructural level to help elucidate the pathogenesis of DUC in mares.

The role of magnesium in DUC mares may be an important avenue to investigate. Magnesium is known to be a co-factor necessary for proper oxytocin/oxytocin receptor interaction[39] Magnesium is a potent tocolytic used for uterine relaxation in pre-term labor in humans[35]. Hurd et al demonstrated the antagonistic effect that magnesium has on oxytocin-induced myometrial contraction. This group showed that pretreatment of cultured myometrial cells with magnesium caused a reduction in intracellular calcium in response to exposure to oxytocin[40]. Laser flow cytometric analysis of fluorescent intensity using magnesium-sensitive dyes to assay magnesium levels from mares' myometrium could be performed to see if the amount of magnesium in DUC mares differs from that found in normal mares. This flow cytometry technology has been used to assay magnesium in other tissues and could be applied to uterine tissues, as well[41].

The hypothesis that DUC mares would under-express receptors for oxytocin was made prior to this investigation; the results of this research did not support this hypothesis. This could mean that the receptors are constitutively expressed in heath or diseased uteruses and the oxytocin secretion or binding is impaired with increasing age or with disease. The work of Sharp et al suggests that the affinity of oxytocin receptors for oxytocin is reduced in pregnant mares, which are under the influence of progesterone[42]. Murata et al has shown that oxytocin receptor mRNA is up-regulated in the presence of increased circulating estrogen concentrations in the rat[43]. However, in this research project, the oxytocin receptor mRNA is ubiquitously expressed in these mares which were in late estrus or early diestrus, and should have been under the influence of high estrogen concentrations.

Myometrial cell prostaglandin production stimulated by oxytocin has not been reported for the mare as it has been in other species[4]. Nikolakopoulos et al showed that DUC mares had reduced circulating levels of $PGF_{2\alpha}$ following both endogenous oxytocin release and after exogenous oxytocin administration[44]. In the face of normal, ubiquitous $PGF_{2\alpha}$ receptor production as demonstrated in the current project, this may explain why

DUC mares do not generate appropriate myometrial contractions—the receptors exist but the circulating levels of $PGF_{2\alpha}$ are inadequate to cause signal transduction and initiation of the influx of intracellular calcium.

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Endnotes

[a] Qiagen Inc., Valencia, CA 91355

[b] Tecan Group Ltd, Männedorf, Switzerland

[c] Invitrogen Corporation, Carlsbad, California 92008

[d] Bio-Rad Laboratories, Hercules, CA 94547

[e] Eppendorf North America, Westbury, NY 11590

[f] http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi

[g] http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide&itool=toolbar

[h] SeqWeb v.3; Accelrys Inc., San Diego, CA 92121

[i] Operon Biotechnologies, Inc., Huntsville, AL 35805

[j] https://www.vbi.vt.edu/

[k] http://www.ncbi.nlm.nih.gov/BLAST/

[l] SAS Institute Inc., Cary, NC 27513

Vita

Giles Anthony Gray is the son of Mr. and Mrs. Giles Gray, Jr. of Castlewood, VA. Anthony was born in Bristol, TN and raised outside of Lebanon, VA. He graduated valedictorian of Lebanon High School in 1992 and then attended the Virginia Military Institute on a full academic scholarship. He graduated from VMI in 1996 with a B.S. in Biology with minors in Chemistry and Psychology. He attended graduate school at the University of Virginia before coming to veterinary school at the Virginia-Maryland Regional College of Veterinary Medicine in 1997. He graduated from VMRCVM in 2001 and completed a rotating internship in Equine Field Services and Large Animal Medicine at VMRCVM in 2002. He worked as an associate veterinarian at Davie County Large Animal Hospital in Mocksville, NC for a year before accepting a position as a resident in Equine Field Services at VMRCVM. Anthony has passed the certification exam to become a Diplomate in the American Board of Veterinary Practitioners as an Equine Specialist. Anthony is married to Kerri Carico Gray, a senior veterinary student at VMRCVM, class of 2006. The couple will be moving to Bristol, TN where Anthony will be a partner at Abingdon Equine Veterinary Services and Kerri will be a small animal emergency vet at the Airport Pet Emergency Clinic in Blountville, TN.