N-acetyl cysteine as a potential treatment for persistent breeding-induced endometritis in the mare

by
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ABSTRACT

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Dr. Tracey Chenier

Persistent breeding-induced endometritis (PBIE) is a major cause of infertility in mares. PBIE susceptible mares do not clear inflammation post-breeding in a timely fashion, ultimately resulting in early embryonic death. N-acetyl cysteine (NAC), a mucolytic, has anti-inflammatory properties, affects inflammatory cytokines and is a mild inhibitor of nitric oxide synthase. Increased nitric oxide (NO) levels and alterations in inflammatory cytokines have been documented in PBIE mares. The objective of this study was to determine if NAC treatment of PBIE-susceptible mares would lower NO and inflammatory cytokine levels, thereby resulting in the resolution of clinical signs associated with PBIE. The effects of NAC histologically on the endometrium were also determined. A randomized, blinded, cross-over design clinical trial was performed utilizing PBIE susceptible mares (n=9). Intrauterine infusion of 180mls of 3.3% NAC was performed 12 hours prior to insemination. Mares were sampled for endometrial cytology and intra-uterine fluid to determine interleukin-6 concentration (ELISA) and nitric oxide (Colorimetric assay) levels at 12 and 60 hours post-insemination. Endometrial biopsies were taken at the same time points post-insemination for scoring based on the degree of inflammation.
present and to determine gene expression of inflammatory cytokines by qPCR. Clinical signs of endometrial edema and intra-uterine fluid volumes were assessed at 12 and then every 24 hours post breeding. Statistical analysis was performed using a repeated measures ANOVA and a Mann Whitney Wilcoxon Test. Pre-breeding intrauterine treatment with NAC did not improve clinical signs in PBIE-affected mares, nor did it affect NO levels, IL-6 concentration or cytokine gene expression. Differences across time points post-AI were noted for IL-6 gene expression, which was highest at 12 hours post-AI (p=0.003). Expression of other cytokines evaluated did not change over time. Treatment with NAC did have a significant effect on the degree of endometrial inflammation noted histologically, with treated cycles displaying more diffuse and severe neutrophil infiltration compared to control cycles. The effects of NAC on the endometrium of PBIE- susceptible mares seen in this study is concerning. Further research is required to evaluate the safety of NAC in the treatment of PBIE-susceptible mares.
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Firstly, I would like to thank my supervisor, Dr. Tracey Chenier for her guidance and endless support throughout the DVSc program. Research was a newer concept to me when I began, but Dr. Chenier provided me with everything I needed to complete this project. Starting with the basics, progressing through to the more difficult elements, she helped me to grow as a researcher. She was consistently available for feedback and assistance, even late at night or on weekends. She had a unique ability to create open and constructive brainstorming sessions while making me feel comfortable to ask questions, however simple they may have been. When the program became overwhelming, she knew exactly what to say to push me outside of my comfort zone and to move my progress forward. Dr. Chenier was not only a great mentor for research, she also was a great mentor as a clinician and as a teacher. During my first season as a resident in Theriogenology, Dr. Chenier allowed me to grow as a clinician as I learned to manage my own research mares, while providing me with the guidance and support I needed. Having such a knowledgeable and skilled clinician to learn from was an invaluable opportunity for me, and one that I will be forever grateful for. I thoroughly enjoyed working with her, both in the clinic, and on this research project.

The past three years could also not have been possible without my other mentor, Dr. Cathy Gartley. Dr. Gartley has been there for me, providing valuable insight on everything from to how to deal with the most difficult of cases, to feedback regarding my research and, even advice on life outside the clinic. She has been encouraging, approachable, and extremely knowledgeable. I was always excited to see cases with her, not only for the learning
opportunities but also for the byproducts of conversation and laughter that would emerge from spending time together in the clinic. Dr. Gartley also took the time to discuss various conditions with me outside the classroom and agreed to coordinate a special topics course to help prepare me for boards. I cannot express my gratitude for her dedication to teaching.

In addition to the mentorship that both Dr. Chenier and Dr. Gartley have provided me, I will forever cherish their friendship.

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Many thanks go to Dr. Monica Antenos for teaching me everything there is to know about pipetting and running ELISAs. I would not have been able to complete this section of my research project without her. I thoroughly valued her teaching, knowledge, opinion, and patience. I enjoyed my experience working with her and am forever thankful for her help. A special thank you to Dr. Allison MacKay and Dr. Liz St. John for their encouragement and help while working in the lab as well.

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This project would not have been possible without the generous contributions from Equine Guelph and the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA) and to them I owe many thanks.

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only did he live through the day to day with me, he also helped me master Microsoft Word and Excel and helped me with my work, so we could spend time together. To my parents, Dave and Diane, they have been there from the beginning: from encouraging me to follow my dreams, to talking me through panicking about an undergraduate physics 100 course, to talking me through the final weeks of my DVSc and telling me to never give up. I am especially appreciative to my dad, who reminded me to take things day by day, and to my mom for always looking out for my well-being, and telling me it’s okay to take a break. To my siblings, Danielle and Matt, my brother-in-law Rick, and my niece and nephews, Spencer, Hailey and Koen, thank you for understanding that I could not come home to spend as much time with you as I wanted. To my partner’s family, the MacKenzie, Bill, Heather, Laura, Peter, Candace, Emma and Grace, thank you for being a great support system and for giving me a home away from home. Lastly, I am thankful for my furry friends, Cooper and Oreo who have been my constant study companions.
DECLARATION OF WORK PERFORMED

I declare that I performed all the work described in this thesis with the exception of the following:

Bacterial culture of endometrial swabs was performed at the Animal Health Laboratory at the University of Guelph.

Real-time quantitative PCR on endometrial tissue was performed by Dr. Bienzle’s research laboratory at the University of Guelph in the Department of Pathobiology.

The preparation and staining with Hematoxylin and Eosin (H&E) of endometrial tissues for histopathological evaluation was performed by the Animal Health Laboratory at the University of Guelph.

Histological evaluation and scoring of inflammation for these samples was performed by Dr. Tracey Chenier in the Department of Population Medicine, with the input of Dr. Rob Foster in the Department of Pathobiology at the University of Guelph.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AHL</td>
<td>Animal Health Laboratory</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AIC</td>
<td>Akaike Information Criterion</td>
</tr>
<tr>
<td>AI</td>
<td>Artificial Insemination</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CDE</td>
<td>Chronic Degenerative Endometritis</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disease</td>
</tr>
<tr>
<td>CUI</td>
<td>Chronic Uterine Infection</td>
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<tr>
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</tr>
<tr>
<td>CD8+</td>
<td>Cluster of Differentiation 8</td>
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</tr>
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<td>Complement Component 5b</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>C_{T}</td>
<td>Cycle Threshold</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>CRISP-3</td>
<td>Cysteine Rich Secretory Protein 3</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DUC</td>
<td>Delayed Uterine Clearance</td>
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<tr>
<td>EMG</td>
<td>Electromyography</td>
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<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>EeF2</td>
<td>Eukaryotic Translation Elongation Factor 2</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
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<tr>
<td>hCG</td>
<td>Human Chorionic Gonadotropin</td>
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<tr>
<td>Hpf</td>
<td>High Power Field (400x)</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>Ig(T)</td>
<td>Immunoglobulin T</td>
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<tr>
<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
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<tr>
<td>IL-1α</td>
<td>Interleukin-1α</td>
</tr>
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<td>IL-1RA</td>
<td>Interleukin 1 Receptor Agonist</td>
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<td>KCl</td>
<td>Potassium chloride</td>
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<tr>
<td>LRS</td>
<td>Lactated Ringer’s Solution</td>
</tr>
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<td>LPS</td>
<td>Lipopolysaccharides</td>
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<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>mOsm</td>
<td>Milliosmole</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
</tr>
<tr>
<td>MCWE</td>
<td>Mycobacterial Cell Wall Extract</td>
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<tr>
<td>NAC</td>
<td>N-acetyl Cysteine</td>
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<tr>
<td>NET</td>
<td>Neutrophil Extracellular Trap</td>
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<tr>
<td>nNOS</td>
<td>Neuronal Nitric Oxide Synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric Oxide Synthase</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor-κB</td>
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<tr>
<td>NOD</td>
<td>Nucleotide oligomerization domain</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PBIIE</td>
<td>Persistent Breeding Induced Endometritis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PRP</td>
<td>Platelet Rich Plasma</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear neutrophils</td>
</tr>
<tr>
<td>PGF2α</td>
<td>Prostaglandin F2α</td>
</tr>
<tr>
<td>PGFM</td>
<td>Prostaglandin F2α metabolite</td>
</tr>
<tr>
<td>RQ</td>
<td>Relative Quantification</td>
</tr>
<tr>
<td>qPCR (qRT-PCR)</td>
<td>Reverse Transcriptase Quantitative PCR</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RPS23</td>
<td>Ribosomal Protein S23</td>
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CHAPTER ONE: LITERATURE REVIEW

1.1 INTRODUCTION AND SIGNIFICANCE OF PERSISTENT BREEDING INDUCED ENDOMETRITIS (PBIÉ)

In 2010, horses contributed 19.6 billion dollars to the Canadian economy (Evans 2012). At that time, the horse population in Canada was 963 500, of which 41% were part of the breeding sector. Twenty-two percent of the national equine population resides in Ontario, representing the second largest horse population within Canada, following Alberta. In Ontario, 14% of horses are within the breeding sector (Evans 2012). The health of the breeding industry is therefore critical to the overall success of the horse industry. Despite this, reproductive efficiency in horses is generally low, for a variety of reasons, including infertility caused by uterine disease. Endometritis is the third most common medical condition encountered overall in equine veterinary practice (Traub-Dargatz et al. 1991), and approximately 60% of mares affected by endometritis are barren (Overbeck et al. 2011). Causes of endometritis include acute bacterial infections, chronic infections, and persistent breeding-induced inflammation. Estimates of the prevalence of PBIÉ vary depending upon the study and the method used to define the condition. When defined as “any fluid accumulation within the uterus one day following breeding”, PBIÉ was present in 13.7% of intensively managed Thoroughbred broodmares (Zent et al. 1998). Another study identified PBIÉ in 43% of a mixed population of mares (Newcombe 1997). Given the fairly large number of horses in the breeding sector both in Ontario and Canada as a whole, endometritis and more specifically PBIÉ, causes a significant economic impact to both the Canadian and Ontario breeding industries.
Mares affected by endometritis have impaired fertility, leading to decreased pregnancy rates and the requirement for repeat breedings. Endometritis impairs fertility by two mechanisms: premature luteolysis caused by inflammatory-driven prostaglandin release, and by the direct toxic effects of uterine inflammation on the embryo (Neely et al. 1979; Troedsson 1997; Troedsson et al. 2001b). A short-lived inflammatory reaction is a normal response following breeding in all mares (Kotilainen et al. 1994; Katila et al. 1995) and is necessary since non-viable spermatozoa, inflammatory debris and other components of the ejaculate must be cleared from the female’s reproductive tract prior to embryo descent on day 5 or 6 post-breeding (Betteridge et al. 1982; Freeman et al. 1992). A subset of mares fail to clear the normal inflammatory response in a timely fashion, leading to persistent inflammation and early embryonic death at the time of embryo descent. Mares in which this occurs are considered susceptible to PBIE. Understanding this post-breeding immune response and susceptibility to PBIE is key to understanding methods that may reduce the impact of PBIE on mare reproductive performance.

1.2 THE IMMUNE SYSTEM & THE NORMAL INFLAMMATORY RESPONSE

The immune system is divided into two broad categories: the innate immune system and the adaptive immune system. Response by the innate immune system does not require previous exposure to an antigen; the same response will occur regardless of the inciting cause. The innate immune system consists of physical barriers, phagocytic cells and plasma proteins. By comparison, the adaptive immune system has a memory component, which primes its response to specific agents such as a certain species of bacteria or virus, following initial exposure (Tizard 2013). Adaptive immunity is further divided into humoral and cell-mediated immunity, where the
former is targeted against extracellular pathogens and the latter is targeted against intracellular pathogens. In the uterus, the innate immune system plays the most important role in the defense against equine endometritis (Snyder 2017). This can be explained by the fact that the innate immune response allows for a more rapid response than the adaptive immune response. While the adaptive immune response does play a small role in uterine defense, it requires diligent regulation since the formation of antibodies towards antigenic material, in this instance semen, would be disastrous for future fertility (Troedsson 2006; Lyle 2011; Foster 2017).

Researchers first believed that endometritis was due to bacterial contamination at the time of breeding (Pycock and Allen 1990; Asbury et al. 1980; Mitchell et al. 1982; Liu et al. 1985; Watson et al. 1987a; Watson et al. 1988; Williamson et al. 1987; Troedsson 1991). Pycock et al. (1990) characterized the inflammatory response in mares following bacterial inoculation with *Streptococcus zooepidemicus*. A massive influx of neutrophils into the uterine lumen within 30 minutes of inoculation occurred in all mares. Since that study, the inflammatory pathway following bacterial inoculation has been further elucidated. An increase in arachidonic acid metabolites leads to increased vascular permeability, causing neutrophil infiltration (Watson et al. 1987b; LeBlanc and Causey 2009). Prostaglandin E$_2$, leukotriene B$_4$ as well as other serum proteins are present within the uterine lumen at this time and may act as chemoattractants (Watson et al. 1987a; Watson et al. 1987c; Watson et al. 1988; Pycock et al. 1990). Antibodies, such as IgG or complement (C3) then attach to the surface of the bacteria, providing binding sites for neutrophil attachment, which allows opsonisation to occur. This leads to bacterial phagocytosis by the neutrophils. Complement (C3) and IgG levels decrease within the first 24 hours, which is likely a result of increased consumption during the inflammatory response.
This initial response is followed by a secondary peak in neutrophil numbers twelve hours following inoculation. This secondary peak is without a concurrent increase in serum proteins, but instead is accompanied by an increase in local immunoglobulin secretions (IgA) (Williamson et al. 1987). Immunoglobulins identified in the uterus of mares following bacterial inoculation consist of IgA, IgG, IgG(T) and IgM (Kenney and Kahleel 1975; Asbury et al. 1980), where IgA is produced by the endometrium and IgG passively diffuses into the uterine lumen from the peripheral circulation (Mitchell et al. 1982; Liu et al. 1985). Inflammatory exudate is removed within 48 hours following inoculation via uterine contractions caused by prostaglandin F2α release, as evidenced by lack of intra-luminal fluid visible on ultrasound. Excess fluid is also removed via lymphatics following closure of the cervix.

The inflammatory response to deposition of semen in the uterus is believed to be essentially identical to that described above, and has been better characterized (Kotilainen et al. 1994; Katila et al. 1995). Spermatozoa cause post-breeding inflammation via neutrophil chemotaxis and complement activation (Troedsson 1995). More specifically, once sperm enter the uterus, they activate complement present within uterine secretions. This leads to cleavage of C5 factor into C5a and C5b, which creates a chemotactic signal to neutrophils. As in bacterial endometritis, prostaglandins, leukotriene B4 and other serum proteins may also act as chemoattractants (Watson et al. 1987a; Watson et al. 1987c; Watson et al. 1988; Pycock et al. 1990). Neutrophils enter the uterine lumen, become activated and bind to sperm via complement C3b factor or complement-independent factors, allowing for the initiation of the inflammatory response. Furthermore, neutrophil extracellular traps (NETs) and traditional ligand binding may enhance this neutrophil attachment, ultimately leading to phagocytosis of the
spermatozoa by neutrophils (Dahms and Troedsson 2002; Alghamdi and Foster 2005). Concurrently, prostaglandin $F_{2\alpha}$, a metabolite of arachidonic acid via the cyclooxygenase pathway, is released within the uterine lumen, initiating uterine contractions to assist with physical evacuation of fluid and debris (Troedsson et al. 1995). Activated neutrophils are responsible for prostaglandin release, and this is part of the normal response to breeding in an attempt to clear the uterus of physiologic inflammation prior to embryo descent (Troedsson 1991).

The immediate contractile response following breeding is mediated via adrenergic nerves and locally via prostaglandins in seminal plasma (Bae et al. 2001). Concurrent oxytocin release further heightens the response of the myometrium. Alexander et al. (1995) demonstrated that oxytocin release occurs following interaction with a stallion, including stallion vocalization and physical contact, as well as following artificial insemination. Madill et al. (2000) showed that such breeding stimuli also causes uterine contractions in teased mares. As measured by myometrial electrodes and a Grass polygraph, these contractions were found to be almost immediate following the stimuli, and almost always coincided with oxytocin release (Madill et al. 2000). Oxytocin is released from the pituitary gland and binds to endometrial receptors, which are upregulated while estrogen levels rise and progesterone levels decrease, on days 14-17 of the estrus cycle (Stull and Evans 1986; Jones et al. 1991). Therefore, the response to oxytocin release is greatest during estrus. This is also true when exogenous oxytocin is administered during estrus and diestrus. Myometrial contractions are more frequent but short lived in estrus compared to diestrus, likely due to an increased number of receptors (Troedsson et al. 1995). Oxytocin binding to up-regulated receptors leads to prostaglandin synthesis and contributes to increased uterine
contractions (Sharp et al. 1997). In addition, prostaglandin F$_{2\alpha}$ is known to up-regulate oxytocin receptors, creating a positive feedback loop. Interestingly, oxytocin can lead to myometrial contractions via activation of direct and indirect pathways. When activation takes place via the direct pathway, myometrial contractions occur regardless of prostaglandin levels; however, prostaglandin levels must be elevated for myometrial contractions to take place via the indirect pathway. This was demonstrated through the administration of phenylbutazone to block prostaglandin synthesis. Normal mares treated with oxytocin and phenylbutazone did have myometrial contractions but these were without concurrent prostaglandin F$_{2\alpha}$ metabolite (PGFM) plasma elevations. Additionally, when these mares were treated with phenylbutazone only, they showed decreased uterine clearance (Cadario et al. 1995). Therefore, phenylbutazone blocks prostaglandin-mediated uterine contractions, but does not have an effect on oxytocin-mediated clearance. Conflicting results were noted by the same researchers in 1999, when PGFM levels were only elevated in mares with intra-uterine fluid following treatment with oxytocin, but not in normal mares. These workers hypothesized that uterine inflammation primed the uterus for PGF$_{2\alpha}$ release (Cadario et al. 1999).

Regardless of the mechanisms involved, myometrial contractions lead to clearance of inflammatory products from the uterus. Uterine contractions, in combination with uterine folds, contribute to clearance via the cervix. In addition, clearance via lymphatics is dependent upon myometrial contractions (LeBlanc et al. 1995). If the pressure within the uterus is not sufficient, lymphatic drainage cannot occur, leading to fluid collection in the lumen instead of its removal via the lymphatics (Causey 2006). A mucociliary apparatus also exists within the uterus, where
mucus is produced by endometrial cells and then propelled by cilia. This, combined with uterine contractions, minimizes bacterial attachment and maximizes removal of inflammatory cells and debris from the uterus (Causey 2007). The combination of innate immune response and mechanical clearance result in the inflammatory response being short-lived following breeding in normal mares (Troedsson 1999).

1.3 RISK FACTORS FOR PBIE

Factors associated with susceptibility to PBIE in mares include poor perineal conformation, age, anatomic location of the uterus, and fluid retention during diestrus (Ricketts and Alonso 1991; Troedsson et al. 1993a; LeBlanc et al. 1994a; LeBlanc et al. 1998; Brinkso et al. 2003; Woodward et al. 2012; Canisso et al. 2016). Reproductive anatomy is an important component of the mare’s uterine defense system. The three anatomical barriers that protect the uterus from external contamination are: apposition and vertical angulation of the vulvar lips (perineal conformation), the vestibulo-vaginal sphincter and the cervix (Pascoe 2007; Pycock 2009; Bergfelt 2009). Mares with normal perineal conformation have at least two-thirds of the vulvar length below the pelvic brim, less than 10-degree anterior tilt to the vulva, and vulvar lips that are well apposed to prevent fecal contamination of the vagina (Pascoe 2007; Pycock 2009 McCue 2008; Canisso et al. 2016). Perineal conformation may be altered from normal in some instances. For example, lean athletic mares with very little perineal fat may have a recessed anus leading to cranial angling of the vulva, therefore pulling more than one-third of the vulva above the pelvic brim (Hurtgen 2006; Pascoe 2007). Similar changes may occur in older mares with increased parity as seen by lengthening of the vulva and increase in the angle of declination
(Pascoe 1979). The vestibulo-vaginal sphincter is formed by mucosal folds at the demarcation of the vestibule from the vagina at the level of the external urethral orifice (Bergfelt 2009). Mares with an incompetent vestibulo-vaginal sphincter aspirate air into the vagina (pneumovagina) during exercise, or following urination, a term commonly referred to as “wind-sucking”. Pneumovagina leads to irritation and inflammation of the vagina, vestibule and cervix, predisposing the mare to secondary bacterial infection and endometritis (Pascoe 2007; McCue 2008; Canisso et al. 2016).

Abnormalities affecting the cervix include fibrosis, tortuosity and incompetence. Cervical malfunction can occur in older maiden mares, mares with repeated embryo recovery attempts or following abortion or dystocia-induced damage (Pycock 2007; McCue 2008; LeBlanc and Causey 2009). An inability to dilate the cervix following breeding, as seen with fibrosis post dystocia or in older maiden mares, can lead to decreased uterine clearance (Pycock 2007). Reilas et al. (2016) showed that occlusion of the cervix by way of a catheter for 25 hours following artificial insemination (AI) leads to increased intra-uterine fluid volumes and increased neutrophil number in normal mares. In addition, they saw a worsening of fibrosis on endometrial biopsies in study mares where fluid was retained for either 25 hours or 24 hours with some fluid removed at 6 hours, and speculated that retention of fluid in the uterus may lead to alterations in the inflammatory response.

Other anatomical abnormalities include stretching of the broad ligament with cranial displacement of the uterus over the pelvic brim, which leads to decreased uterine clearance due to simple gravitational effects (LeBlanc and Causey 2009). In fact, uterine clearance of a radiocolloid as assessed by scintigraphy demonstrated that mares with a more ventral uterine
cervical angle cleared <30% of the radiocolloid in two hours, compared to normal mares that cleared >50%. Mares with delayed clearance also tended to be over the age of 12, were pluriparous and had a history of endometritis (LeBlanc et al. 1998). Therefore, many of these risk factors are affected both by age of the mare and parity.

Carnevale and Ginther (1992) first showed that older pony mares in diestrus had less uterine contractility as assessed by trans-rectal ultrasound prior to and following breeding, compared to younger mares under the same conditions. The older mares also had a higher incidence of endometritis, as assessed by endometrial biopsy, and increased intra-uterine fluid. Woodward et al. (2012) examined the association between mare age, uterine fluid retention and endometrial biopsy scores. In their study, mares over 9 years of age were more likely to accumulate fluid than younger mares and even more so when mares were older than 17 years. A higher percentage of older mares also had Kenney biopsy scores of IIb or III. Age is therefore likely associated with a more severe endometrial biopsy score. Age and biopsy score appear to lead to increased fluid retention and PBIE susceptibility. However, this study did not assess the effects of parity as a confounding variable, as the reproductive history of the mares in this study was unknown. Similar results were noted for mare age in a study by Barbacini et al. (2003), where the incidence of uterine fluid accumulation post-breeding was higher in mares aged 10-16 years old compared to younger mares. This study also revealed that barren mares were more likely to demonstrate uterine fluid retention compared to maiden and post-parturient mares. Increased levels of uterine fluid (>2cm) post-Al were associated with lower pregnancy rates; per cycle pregnancy rates were 41.9% in mares with fluid retention compared to 56.2% in mares without fluid. Clearly, pluriparous mares tend have an increased likelihood of abnormal perineal
conformation, an incompetent vestibulo-vaginal sphincter and a fibrotic cervix, all of which can contribute to susceptibility to PBIE (Hurtgen 2006; LeBlanc and Causey 2009; Christoffersen and Troedsson 2017).

The association between uterine biopsy grade and susceptibility to chronic uterine infections (CUI) was assessed by Troedsson et al. (1993a). In this study, mares were divided into four categories based on severity of histologic findings (1=normal to 4=severe) and were challenged with a bacterial inoculation of *Streptococcus zooepidemicus* during estrus. Mares were considered susceptible to CUI if endometrial cytology revealed >1 neutrophil/5hpf and endometrial culture had >20 bacterial colonies at 96 hours post challenge. Mares with grade I endometrial biopsies were more likely to be resistant to the inoculation, whereas mares with a biopsy score of grade III were more likely to be susceptible to CUI, indicating an association between severity of endometrial biopsy score and susceptibility to CUI. However, there was no association between biopsy grades IIa or IIb and susceptibility, limiting the application of biopsy grade to predict susceptibility. In a similar study by Williamson et al. (1989), mares were classified as susceptible or resistant to CUI based on reproductive history and endometrial biopsy scores. Following inoculation, mares were re-classified based on their ability to clear an inoculation of *Streptococcus zooepidemicus* after 10 days. Susceptibility was more highly correlated with reproductive history than endometrial biopsy. However, a study design limitation existed in that mares were only classified as susceptible ten days following bacterial inoculation, allowing considerable time for less susceptible mares to clear the infection prior to the sampling time point. In addition, biopsy scores were not defined using the current endometrial biopsy grading
system described by Kenney and Doig (1986), limiting the capability to compare with other studies.

1.4 PATHOPHYSIOLOGY OF PBIE

1.4.1 Humoral and Innate Immunity

Much research has focused on mare susceptibility versus resistance to acute bacterial endometritis and PBIE. Possible differences between mares with respect to their ability to clear intra-uterine infection were first described by Hughes and Loy (1969) and Peterson et al. (1969), where some mares appeared to be resistant to infections, while others did not. The initial inflammatory reaction appeared to be the same amongst these mares, but differed with time in that some mares (susceptible) maintained the infection, and others (resistant) cleared it. This suggested that an altered inflammatory defense mechanism may be involved. Subsequent work focused on possible differences in the humoral immune response, specifically differing levels of immunoglobulins between susceptible and resistant mares. Asbury et al. (1980) found increased intra-uterine levels of IgA, IgG and IgT in mares susceptible to endometritis following bacterial inoculation with *Streptococcus zooepidemicus*, compared to normal mares. In addition, Mitchell et al. (1982) noted higher total immunoglobulin, IgA and IgG in susceptible mares, as defined by endometrial biopsies. Similarly, Williamson et al. (1983) found elevated levels of IgA in mares with active infections, compared to resistant mares. Conversely, Troedsson et al. (1993c) found no significant differences between susceptible and resistant mares. This study examined IgA, IgG and complement cleavage factor C3 expression in mares that were either resistant or susceptible to a bacterial inoculation of *Streptococcus zooepidemicus*. In both groups, levels of IgA, IgG and
C3 decreased in the first 24 hours following inoculation. Levels of IgG and C3 rose in resistant mares by 36 hours, however they remained low in susceptible mares, suggesting continued bacterial opsonisation. No differences were detected in IgA levels between groups. The susceptibility of endometritis was thus not attributed to deficiencies in immunoglobulins.

Watson and Dixon (1993) showed upregulation of MHC Class II expression in the endometrial epithelium of susceptible mares, compared to normal mares in estrus. The majority of cells with MHC Class II expression consisted of T lymphocytes, but also included some epithelial cells. When assessed histologically, endometrial T lymphocytes were increased in non-bred mares susceptible to endometritis, compared to resistant mares, both in estrus and diestrus (Fumoso et al. 2007). Following artificial insemination, PBIE-susceptible mares had higher levels of T lymphocytes in diestrus only, compared to resistant mares (Fumoso et al. 2007). This is interesting to the understanding of PBIE, since no differences in T lymphocyte number were observed in normal mares regardless of stage of cycle (Watson and Thomson 1996). Tunon et al. (1999) characterized the type of T lymphocyte expression in normal mares. They found that CD4+ T cells were elevated at 6 hours following insemination, but not at 48 hours. No differences in CD8+ T cells were noted at either time point. Susceptible mares also had more CD4+ expression than CD8+ expression and more CD8+ cells compared to resistant mares (Watson and Thomson 1996), likely due to persistent antigenic stimulation in affected mares.

Alterations in uterine neutrophil chemotaxis, phagocytosis, and bactericidal activity in susceptible mares have been other areas of research in understanding PBIE. Decreased opsonins from uterine secretions of mares susceptible to bacterial endometritis was noted by Asbury et al. (1982). In 1985, Liu et al. demonstrated that uterine secretions of mares infused with bacterial
inoculum, with grade III uterine biopsies and a history of uterine infections, had less neutrophil elasticity and chemotaxis compared to mares with grade I biopsies and normal reproductive histories. They then showed that neutrophil migration was delayed following inoculation with *Streptococcus zooepidemicus* in susceptible mares compared to resistant mares. In a follow-up study, Lui *et al.* (1986) found that neutrophil levels in susceptible mares were elevated and remained as such from 15 to 25 hours post inoculation compared to resistant mares. Interestingly, neutrophil migration was decreased in susceptible mares at 20 hours; however, by 25 hours, migration had resumed in these mares. Watson *et al.* (1987c) showed further neutrophil dysfunction, in that uterine-derived neutrophils displayed decreased phagocytic capabilities in susceptible mares compared to resistant mares pre-inoculation. Following induction of inflammation with 1% oyster glycogen, susceptible mare neutrophils displayed decreased bactericidal activity when incubated for 24 hours with a genital strain of *Streptococcus zooepidemicus*, as assessed by percent survival of bacteria. The mechanisms by which neutrophil function could be affected in susceptible mares were investigated by Troedsson *et al.* (1993d), who compared phagocytic ability of neutrophils in susceptible and resistant mares following bacterial inoculation in estrus with *Streptococcus zooepidemicus*. The authors concluded that the decreased phagocytic ability of neutrophils seen in susceptible mares was caused by differences in uterine secretions and not by dysfunctionality of the neutrophils themselves. This was made evident by the demonstration of adequate phagocytosis when neutrophils from susceptible mares were placed in an optimal environment. In addition, susceptible mares in this study actually had improved chemotaxis compared to resistant mares. This is in contrast to Liu *et al.* (1985) who did not see chemotaxis improvement when uterine lavage fluid containing
neutrophils taken from mares with grade III biopsies were placed in the presence of chemoattractants. Troedson (1993d) suggested that differences in components of uterine secretions could result in variations in opsonisation by complement and antibodies (Troedsson et al. 1993d). This was supported by the decreased bactericidal activity of neutrophils seen in vitro when factors required for the classical and alternate complement pathways were removed (Watson et al. 1988). Troedsson (1999) suggested that stationary aged uterine fluid, as is often the case with PBIE-susceptible mares, can lead to inactivation of complement proteins, decreasing neutrophil phagocytic success. Furthermore, neutrophils must have a surface (the endometrium) on which to perform phagocytosis, and this surface may not be available when there is intra-luminal fluid present (Troedsson 1999). In summary, susceptible mares have functional neutrophils and the same rate of phagocytosis as resistant mares; however, the functional and phagocytic abilities of those neutrophils appear to be reduced when exposed to the altered uterine fluid of PBIE-susceptible mares. Susceptible mares also have increased numbers of neutrophils for prolonged periods of time following induction of inflammation, compared to resistant mares (Liu et al. 1986; Watson et al. 1987c; Nikolakopoulos and Watson 1997; Woodward and Troedsson 2013). Therefore, the uterine fluid of susceptible mares causes altered neutrophil functionality, which could contribute to differences in both the timing of their presence within the uterine lumen and their levels.

Increased intra-uterine neutrophil number and altered uterine secretions can have a direct effect on spermatozoa, further contributing to decreased fertility. Spermatozoa incubated in the uterine secretions of inflamed uteri in vitro demonstrated altered velocity and progressive motility, which was attributed to the presence of neutrophils binding to the spermatozoa.
Some of these effects could be mediated by the presence or absence of seminal plasma, whose effects on fertility and the post-breeding inflammatory response are controversial. In large concentrations, seminal plasma has been shown to be detrimental to the quality of thawed semen (Morrell et al. 2014); however, it also may shorten the duration of post-breeding inflammation when present in smaller volumes (Troedsson et al. 2000; Troedsson et al. 2001). Mares inseminated with sperm, milk extender and seminal plasma demonstrated decreased uterine contractions and increased intrauterine neutrophil concentrations, compared to AI with sperm and extender alone. This suggests both an increased intensity and duration of inflammation post-breeding when seminal plasma is used (Portus et al. 2005). However, Troedsson et al. (2000) showed that seminal plasma decreases neutrophil phagocytosis of sperm and Alghamdi et al. (2004) demonstrated that seminal plasma suppresses neutrophil sperm binding in a dose-dependent manner, in vitro. Fifteen-day pregnancy rates were also higher in mares inseminated with spermatozoa resuspended in seminal plasma suspension without semen extender compared to spermatozoa resuspended in semen extender alone (Alghamdi et al. 2004). It has thus been suggested that seminal plasma or some of its components could be beneficial in the management of PBIE in susceptible mares (Troedsson et al. 2000; Alghamdi et al. 2004).

1.4.2 Uterine Clearance

Failure to clear uterine fluid post-breeding has become the most important and most commonly used definition for PBIE in mares (Brinsko et al. 2003). This inability to clear intra-uterine fluid is characteristic of the condition, and occurs due to defects in the normal uterine clearance mechanisms, which vary by stage of the estrous cycle. Evans et al. (1986) demonstrated
the influence of reproductive steroid environment on uterine clearance in anestrous maiden mares using uterine infusion of *Streptococcus zooepidemicus*, chromium-labeled microspheres, and charcoal. Mares in the study were treated with either estradiol or progesterone and were infused with *Streptococcus zooepidemicus*, chromium-labeled microspheres, and charcoal. Estrogen-treated mares had rapid uterine clearance of microspheres and bacteria by 3 days post-inoculation. By comparison, uterine clearance of progesterone-treated mares was poor and produced persistent bacterial infections following inoculation, demonstrating the inhibitory effects of progesterone on uterine clearance. Subsequently, in 1987 his group demonstrated a correlation between decreased uterine clearance and age, where young mares cleared non-antigenic markers more efficiently than old mares even in an estrogen-dominated environment (Evans *et al.* 1987). Troedsson and Liu (1991) demonstrated that mares resistant to bacterial infections cleared a significantly greater amount of intra-uterine non-antigenic chromium-labelled microspheres ($^{51}$Cr) than susceptible mares. Mares were classified as potentially susceptible or resistant to uterine infection based on reproductive history, biopsy score and per rectum examination. They were challenged with an intra-uterine infusion of *Streptococcus zooepidemicus* and inoculated with microspheres. Resistant mares had a negligible number of microspheres recovered by uterine lavage 24 hours post inoculation whereas a large number of microspheres were recovered in susceptible mares at 96 hours post inoculation.

Uterine contractility also appears to be different between susceptible and resistant mares. Troedsson *et al.* (1993b) used electromyography (EMG) following surgical implantation of myometrial electrodes to assess myometrial activity. Resistant mares showed greater intensity, duration and frequency of myometrial contractions compared to susceptible mares, following
intrauterine bacterial challenge. Furthermore, LeBlanc et al. (1994a) infused the uterus of resistant and susceptible mares with a radiocolloid and monitored clearance via scintigraphy. Resistant mares cleared the product within two hours of infusion, whereas susceptible mares displayed delayed clearance. The same group (Neuwirth et al. 1994) showed that susceptible mares only cleared less than 15% of the radiocolloid within two hours of infusion, compared to over 50% of the product cleared by this time point in resistant mares. These findings could in part be explained by susceptible mares possibly having inherently abnormal patterns of propagation of myometrial contractions, leading to retention of the fluid. Mares with delayed uterine clearance (DUC) were more likely to have an opposing pattern of propagation compared to normal mares. More specifically, instead of the uterine contractions commencing in the uterine horns followed by the uterine body, contractions in affected mares were initiated in the uterine body and then the uterine horns (von Reitzenstein et al. 2001; LeBlanc and Causey 2009). In addition, Rigby et al. in 2001 showed that susceptible mares may have decreased uterine clearance due to an intrinsic contractile defect of the myometrium. Reproductive tracts were removed from young nulliparous mares as well as from older resistant and susceptible mares, and both longitudinal and circular strips of myometrium were utilized in isometric tension studies. Strips were exposed to KCl, oxytocin and PGF$_{2\alpha}$ and responses were compared between the three groups. Results of this in-vitro study showed that the myometrium of susceptible mares generated less tension than that of both young nulliparous and older resistant mares; however, they were not able to determine the exact mechanism by which this occurred.

Differing levels of nitric oxide (NO) may further contribute to decreased myometrial contractility and therefore decreased uterine clearance (Alghamdi et al. 2005). Nitric oxide is
formed from L-arginine via the nitric oxide synthase (NOS) enzyme. Three isoforms of NOS have been identified and include neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NO (iNOS). Both iNOS and eNOS have been identified in the uterus and their patterns of expression vary; eNOS is constitutively produced whereas iNOS is induced by inflammatory conditions (Moncada and Higgs 1993). It is likely that the persistence of inflammation seen in PBIE susceptible mares leads to increased levels of NO (Woodward et al. 2013b). Nitric oxide is a smooth muscle relaxant, and high levels could account for the diminished myometrial contractility seen in PBIE-susceptible mares. In support of this theory, susceptible mares had higher levels of NO in their uterine secretions 13 hours following artificial insemination, as well as higher NOS mRNA expression in their biopsies, compared with resistant mares (Alghamdi et al. 2005). Differences in nitric oxide levels between susceptible and resistant mares were further classified by Woodward et al. (2013b). Specifically, total intrauterine NO in susceptible mares increased at six and 12 hours post-Al, whereas levels remained stable in resistant mares over 24 hours. Uterine iNOS mRNA expression was not different between susceptible and resistant mares at any time point. Khan et al. (2018) performed in vitro experiments whereby myometrium obtained from susceptible mares was treated with a nitric oxide synthase inhibitor to determine its effects on contractility. Results showed that myometrial contractility improved when treated with the inducible NO synthase inhibitor 1,400 W dihydrochloride.

Additional mechanisms for the delayed uterine clearance in susceptible mares may include abnormalities in the electrical pattern for contractility, smooth muscle fatigue, and unsuitable hormone signaling (LeBlanc and Causey 2009). Mares with Grade III biopsy scores have up to 40% less uterine perfusion compared to normal mares, due to endometrial vessel elastosis,
leading to decreased amounts of hormones, neurotransmitter and inflammatory mediators delivered to the uterus (Esteller-Vico et al. 2007). In 1999, Nikolakopoulos and Watson showed that susceptible and resistant mares have similar patterns of systemic oxytocin secretion following AI; however, susceptible mares have lower levels of PGFM compared to resistant mares following breeding. Prostaglandin metabolite (PGFM) is used as a marker for levels of PGF$_{2\alpha}$, and decreased levels are attributed to a possible defect in its production or release at the oxytocin receptors. As noted above, when administered exogenous oxytocin, susceptible mares produce minimal levels of PGFM compared to resistant mares. It is therefore possible that differences in prostaglandin play a role in the intrinsic abnormalities in uterine contractility and that these may be caused by decreased uterine perfusion.

1.4.3 Mucociliary Apparatus and Lymphatics

Some mares susceptible to PBIE may have an altered mucociliary apparatus and poor lymphatic drainage. Freeman et al. (1990) showed that mares with urine pooling, a source of inflammation, have greater histological staining for carboxylated acid mucins in the uterus, as compared to normal anestrus and transitional mares. Furthermore, Causey et al. (2000) showed that mares with DUC have increased mucus production compared to normal mares, as assessed by endometrial biopsy staining. Increased mucus production is believed to be in response to uterine inflammation or irritation (Freeman et al. 1990). This mucus also tends to be localized in fibrotic nests and in areas of increased inflammation. This is important, as mucus flow can be affected by increases in volume and due to an increase in viscosity. Dilution of mucus with intraluminal fluid can similarly impact the effectiveness with which mucus is able to bind and move particles (Causey 2007). Therefore, while excess mucus can be detrimental, insufficient mucus
allows adhesion of substrates to the endometrial epithelium, leading to decreased clearance. The issue is further compounded as increased fluid volume within the uterus leads to separation of the endometrial folds, which not only leads to a poor environment for neutrophil phagocytosis, but also affects mucociliary clearance. Causey (2006, 2007) speculated that mucociliary flow is best achieved when the endometrial walls appose each other closely, and that the cilia become ineffective when they are further apart due to fluid accumulations. Some mares susceptible to endometritis demonstrate loss of cilia from the endometrial epithelium, leading to decreased movement of particles and fluid (Ferreira-Dias et al. 1994; Ferreira-Dias et al. 1999). More specifically, mares with grade II uterine biopsies demonstrated patchy areas of microvilli loss, and mares with grade III uterine biopsies had areas totally devoid of microvillus cells and ciliated cells on scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Ferreira-Dias et al. 1994; Ferreira-Dias et al. 1999). Mares with impaired lymphatics, a pendulous uterus, or with impaired cervical drainage show increased fluid volumes within the uterus, further contributing to the problem (LeBlanc et al. 1995). While the exact mechanisms by which uterine mucociliary clearance affects PBIE have not been elucidated, it is evident that it likely contributes significantly.

The movement of spermatozoa to the oviduct following breeding is caused not only by uterine contractions, but also by endometrial epithelial ciliary flow (Troedsson et al. 2005). Failure of ciliary movement could also affect sperm transport, further contributing to decreased fertility. In fact, Causey (2006) stated that sperm transport is altered in the oviducts of susceptible mares, leading to lower numbers of spermatozoa in the caudal isthmus, another mechanism by which fertility may be diminished in susceptible mares.
1.4.4 Cytokine Profiles of Susceptible and Resistant Mares

Cytokines are cell-derived polypeptides involved in inflammatory mediation, which recently have been found to be different between PBIE susceptible and resistant mares. Cytokines are produced at sites of inflammation by stromal, endothelial and immune cells, and once present, manage the inflammatory response (Feghali and Wright 1997; Tizard 2013). They can be categorized into pro-inflammatory cytokines, which as their name implies, promote inflammation, and anti-inflammatory cytokines, which control inflammation and prevent its persistence. Cytokines mediate the type of inflammation that takes place, how long it lasts and what other cells may become involved, by having both agonistic and antagonist functions on different types of cells (Feghali and Wright 1997). Some of the most commonly identified pro-inflammatory cytokines in the uterus include those of the interleukin-1 family (IL-1α and IL-1β), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and interleukin 8 (IL-8). IL-1α and IL-1β are involved in the recruitment of other pro-inflammatory cytokines, such as TNF-α. IFN-γ aids in neutrophil migration from the vasculature into the site of inflammation. TNF-α is involved in prostaglandin production, apoptosis and aids neutrophils, which have been recruited by IL-8, in their ability to neutralize pathogens (Tizard 2013). Anti-inflammatory cytokines such as interleukin-10 (IL-10) and interleukin-1RA (IL-1RA), where interleukin-1RN (IL-1RN) is the gene which encodes for IL-1RA, are important for the regulation of inflammation. In fact, IL-10 has been shown to inhibit the production of IL-1α and IL-1β, IL-6, IL-8 and TNF-α in humans (Tizard 2013). Finally, interleukin-6 (IL-6) can act as both a pro-inflammatory and anti-inflammatory cytokine (Tizard 2013) and can be present in the uterus of mares following an inflammatory
stimulus (Woodward et al. 2013a). A simplified schematic of interactions between cytokines can be found in Figure 1.

Differing levels of endometrial mRNA expression of these cytokines can be noted between resistant and susceptible mares at different time points following insemination or bacterial inoculation (summarized in Tables 1 and 2). Briefly, PBIE-susceptible mares appear to have elevated baseline mRNA expression of pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α) and decreased mRNA expression of anti-inflammatory cytokines (IL-10) in estrus compared to normal mares (Fumoso et al. 2003; Fumoso et al. 2007). Following AI, PBIE susceptible mares have persistent elevations of pro-inflammatory cytokine expression (IL-1β, IL-8 and TNF-α) at day 7 post-AI (Fumoso et al. 2003; Fumoso et al. 2007). Increased mRNA expression of IL-8 at 3 hours post-inoculation with E. coli was found in normal mares compared to PBIE mares (Christoffersen et al. 2012), and mRNA expression of the anti-inflammatory cytokine, IL-10 was elevated by day 7 post-AI in susceptible mares (Fumoso et al. 2007). By comparison, however, Woodward et al. (2013a) found that susceptible mares had lower levels of anti-inflammatory IL-1RN and IL-10 and pro-inflammatory IL-6 at six hours post-AI, and found no differences at 24 hours post-AI compared to resistant mares. In contrast to Fumoso et al. (2007), other studies did not find differences in IL-8 expression between susceptible and resistant mares. Disparities amongst studies could be attributed to differences in the study design including mare inclusion criteria, sample size and time points. Increased mRNA expression of pro-inflammatory cytokines occurs in normal and susceptible mares following AI as well as post infusion of extender, phosphate buffered saline (PBS) and seminal plasma in normal mares (Palm et al. 2008).
1.5 DIAGNOSIS OF PBIE

As understanding of PBIE pathophysiology evolved over the past four decades of scientific investigation, so has the definition and diagnosis of this condition. Early studies (Hughes and Loy 1969; Petersen 1969) identified that prolonged and excessive fluid retention occurred following bacterial inoculation of some mares. These studies highlighted the impact of acute bacterial endometritis on the fertility of broodmares. However, mares with PBIE may or may not have a bacterial component to the disease (Kotilainen et al. 1994; Troedsson 1995). Over time, failure of uterine clearance became the focus of study, and the term DUC was used to describe the condition (Evans et al. 1986; LeBlanc et al. 1998; Rigby et al. 2001). Methods to identify DUC mares included failure to clear radiocolloid, India ink or chromium-labelled microspheres. However, these methods were expensive and impractical for identifying mares in clinical practice. Brinsko (2003) recommended that mares be defined as having DUC/PBIE if they retained greater than 2 cm of intrauterine fluid as assessed by trans-rectal ultrasound examination at 72 hours post-AI. For the first time, practitioners had a practical method available to identify PBIE mares for treatment. However, this method only identified a lack of clearance and not persistent inflammation within the uterus, also typical of PBIE, limiting its clinical application. Troedsson et al. (1993a) demonstrated correlations between histological endometrial lesions and a mare’s clinical response to challenge with Streptococcus zooepidemicus, suggesting mares could be identified as “potentially susceptible” on the basis of an endometrial biopsy score of III. However, correlation of susceptibility to PBIE with grades of IIa or IIb were poor. Woodward et al. (2013a, 2013b) and Alghamdi et al. (2005) demonstrated a similar approach using insemination with killed or live sperm, respectively. Mares were first screened as “potentially susceptible” on the
basis of biopsy score (Woodward et al. 2013a; Woodward et al. 2013b) as well as a clinical history of low fertility rates, poor perineal conformation, and endometritis (Alghamdi et al. 2005). Selected mares were inseminated and then monitored by trans-rectal ultrasound for fluid retention up to 96 hours later. In addition, endometrial cytology at 96 hours was used to demonstrate persistence of the inflammatory response. Only mares with both persistence of fluid and cytological evidence of inflammation at 96 hours post-insemination were defined as being susceptible to PBIE. This approach has since been used in several research studies to select and classify mares susceptible to PBIE, although the time point used for determination has varied between 48, 72 and 96 hours across studies (Troedsson et al. 1991; Alghamdi et al. 2005; Woodward et al. 2013a; Woodward et al. 2013b; Khan et al. 2018). From a clinical standpoint, this strict criterion might exclude mares with less severe disease that would be subfertile and may progress to more severe disease over time. In addition, insemination with a dose of killed semen prior to breeding to diagnose PBIE may define the condition clearly for experimental studies, however it is not practical for practitioners in the field. Inclusion of additional criteria such as perineal conformation, persistent endometrial edema (Pycock 2009), and breeding history (subfertility, repeated endometritis and history of fluid accumulation in previous cycles) would likely further improve this definition and classification methodology. In other words, mares could be classified as likely to be susceptible based on their reproductive history and perineal conformation. This would allow practitioners to plan for increased monitoring post-breeding and to have a possible treatment plan prepared in the event that the mare showed signs of PBIE such as any accumulation of fluid, persistence of edema near ovulation or the development of edema post-ovulation.
1.6 TREATMENT OF PBIE

Treatment of PBIE can be divided into three broad categories: improving mechanical uterine clearance, diluting and removing debris, and modifying and improving the immune response.

1.6.1 Ecbolic Therapy

Ecbolic therapies including oxytocin and prostaglandin are shown to increase uterine clearance by increasing myometrial activity (Troedsson 1997; LeBlanc and Causey 2009). The effects of oxytocin on uterine clearance were shown when both susceptible and resistant mares cleared >90% of radiocolloid following its administration (20U IV). This is in comparison to susceptible mares only clearing negligible amounts and normal mares clearing 50-80% of radiocolloid when not treated with oxytocin (Leblanc et al. 1994b). Oxytocin is a widely used treatment for PBIE (LeBlanc and Causey 2009), with recommended dosages ranging from 10-25U given in the first 48 hours post-breeding. Cadario et al. (1999) showed that both susceptible and resistant mares have dose-dependent responses to oxytocin administration, with doses of 10U resulting in the strongest and most prolonged uterine contractions as compared to 5U and 2.5U. Administration of oxytocin results in uterine pressure changes within 30-65 seconds of administration, and high amplitude uterine contractions for 30 minutes, resulting in almost immediate voidance of the uterus (Cadario et al. 1999; LeBlanc et al. 2009). Propulsion of uterine fluid was described by Allen (1991) in one mare following treatment in estrus with oxytocin (5U IV). Intra-uterine fluid moved from the cranial uterus, to the caudal uterus and into the cranial vagina by 1-hour post administration, as assessed by trans-rectal ultrasound. Furthermore, administration of oxytocin (10U SC, 5U IV; 10U IV) in the peri-ovulatory period does not result in a reduction of fertility (Allen 1991). However, due to its short duration of action, oxytocin must
be given every few hours. Unfortunately, some mares do not respond to oxytocin administration, and continue to accumulate uterine fluid despite treatment (Hurtgen 2006; LeBlanc and Causey 2009). Factors suspected to affect the response to oxytocin treatment include decreased or non-functional oxytocin receptors in the endometrium, a pendulous uterus, or a closed cervix (LeBlanc and Causey 2009). Moreover, inappropriately high dosages of oxytocin result in an abnormal propagation of uterine contractions, thereby rendering the treatment unsuccessful. In fact, Campbell and England (2002) determined that doses above 30U caused decreased uterine contractions compared to lower doses. In addition, Rasch et al. (1996) determined that pregnancy rates were improved when 15U of oxytocin was administered IV compared to 25U IV seven to eight hours following AI (78% versus 52%) in mares with any intra-uterine fluid accumulation prior to breeding.

Oxytocin treatment causes prostaglandin release from the endometrium, potentially prolonging its duration of action. Administration of prostaglandins such as cloprostenol alone also promotes uterine clearance, with weaker but more prolonged contractions noted following its administration compared to oxytocin (LeBlanc and Causey 2009). Low amplitude uterine contractions last up to four or five hours, which not only leads to physical clearance of the fluid but also aids with lymphatic flow (Combs et al. 1996; Troedsson 1997; LeBlanc and Causey 2009). Prostaglandins may also cause cervical relaxation, further aiding mechanical clearance. Prostaglandin E2 (misoprostol) applied to the cervix has also been administered in the treatment of PBIE, as it too leads to dilation of the cervix due to its direct ability to break down collagen and decrease total collagen content in the cervix (Tang et al. 2007; LeBlanc and Causey 2009). Care must be taken when administering prostaglandins for the treatment of PBIE. If given in the first
48 hours following ovulation, mares could have decreased pregnancy rates, due to luteolytic effects and decreased progesterone production from the early developing corpus luteum (Troedsson et al. 2001a; Nie et al. 2002; Brendemuehl 2002; Nie et al. 2003). However, when prostaglandin was given no less than 12 hours prior to ovulation, no effects on luteal lifespan were seen. Therefore, if PBIE mares are to be treated with prostaglandins, treatment should be limited to within this time frame (Troedsson et al. 2001a; Nie et al. 2003).

1.6.2 Uterine Lavage

The use of ecbolic drugs is often combined with therapeutic uterine lavage to dilute irritants, as well as to clear inflammatory debris and excessive fluid from the uterus. Post-breeding lavage induces uterine contractions, attracts new neutrophils and stimulates serum opsonins to aid in the inflammatory response, due to the mildly irritating nature of the infusion (Brinsko et al. 1990). Performing uterine lavage at ≥ 4 hours post-breeding is not detrimental to fertility (Brinsko et al. 1990; Brinsko et al. 1991) and has been shown to increase pregnancy rates in PBIE susceptible mares, when combined with ecbolic therapy 4-8 hours post-breeding (Knutti et al. 2000). Furthermore, pregnancy rates doubled in the group lavaged at 4-8 hours compared to those lavaged at 18-20 hours post-breeding; although this effect was not statistically significant.

The effect on fertility of pre-breeding uterine lavage with Lactated Ringer’s Solution (LRS) immediately prior to insemination with fresh-chilled semen was explored by Vanderwall and Woods (2003). In this study, six out of ten mares had residual fluid as detected by trans-rectal ultrasound immediately following the procedure and five out of six of these mares became pregnant. Three of the four mares that did not accumulate fluid following lavage became
pregnant. None of the pregnant mares had residual fluid any greater than 39mm in depth. This study therefore provides another option for the treatment of PBIE, especially in cases where more than one breeding is performed in an attempt to clear any inflammation caused by the first breeding.

1.6.3 Immunomodulation

Additional treatments for PBIE include approaches to correct the altered inflammatory response of susceptible mares. These include administration of anti-inflammatory agents such as glucocorticoids, and intrauterine infusion of agents such as Mycobacterial cell wall extract (MCWE), platelet rich plasma (PRP) and chelating agents such as tris-EDTA (LeBlanc 2010; Woodward and Troedsson 2015; Canisso et al. 2016).

Two main studies evaluated the efficacy of glucocorticoid treatment on the clinical signs of PBIE (Dell’ Aqua Jr et al. 2006; Bucca et al. 2008). Five doses of 0.1mg/kg of prednisolone acetate every 12 hours, beginning in estrus, with the last treatment given at the time of AI, resulted in reduction of uterine fluid volume and improved clarity in mares with a history of uterine fluid accumulation. However, a reduction of uterine neutrophil function in all mares was also seen (Dell’ Aqua Jr et al. 2006). Similarly, treatment with a single dose of 50mg of dexamethasone IV at the time of breeding decreased uterine edema, uterine fluid volume and uterine fluid turbidity in susceptible mares (Bucca et al. 2008). In both studies, pregnancy rates were improved in treated mares having a history of intra-uterine fluid accumulation (Dell’ Aqua Jr et al. 2006) or those mares with more than three PBIE risk factors (Bucca et al. 2008), but not in normal mares. Interestingly, no differences in neutrophil function were noted between control and treated cycles (Bucca et al. 2008). Reduction of neutrophil function may occur by blocking
the cyclo-oxygenase and 5-lipoxygenase pathways of inflammation (Woodward and Troedsson 2015). Glucocorticoids may be beneficial due to their effects on NO production and cytokine expression, as they reduce mRNA expression of IL-1, which plays a role in the activation of iNOS (Barnes 1998); however, when measuring the direct effects of dexamethasone in PBIE on NO production, no effect was noted in both iNOS mRNA expression and total intrauterine NO (Woodward et al. 2013b). Woodward et al. (2015) showed a decrease in IL-1β mRNA expression following treatment with dexamethasone in susceptible mares, but no differences in other cytokines were noted. Overall, it appears that the post-breeding inflammatory response is less severe and resolves more rapidly when glucocorticoids are used.

Other treatments aimed at altering the inflammatory response include MCWE or Propionibacterium acnes, which enhances the immune response by causing cytokine release and by activating immune cells (Phillips and Filion 2001; Fumoso et al. 2003; Woodward et al. 2013b; Woodward et al. 2015). Treatment of PBIE susceptible mares with MCWE returned intra-uterine pro-inflammatory cytokine levels to those more typical of resistant mares, more specifically with significant down-regulation of IL-1β (Fumoso et al. 2003; Woodward et al. 2015). Treatment of PBIE-susceptible mares with MCWE 24 hours prior to breeding also decreased intrauterine NO levels compared to non-treated control mares (Woodward et al. 2013b). This can be explained by the fact that reducing the up-regulation of pro-inflammatory cytokines leads to less inflammation and therefore fewer inflammatory products, such as NO.

More recently, intrauterine infusion of PRP has been investigated as a potential therapy for PBIE. Plasma is thought to enhance phagocytosis by increasing the amount of complement present in uterine fluid (Liu and Troedsson 2008). Metcalf et al. (2012) examined the effect of
PRP treatment prior to AI on endometrial mRNA expression of cytokines in PBIE susceptible mares. Interleukin-1β, IL-6 and IL-8 were down-regulated and iNOS expression reduced, in PRP-treated cycles, compared to untreated cycles. These results were attributed to the renewing effects of PRP by the formation of growth factors, cytokines and chemokines. The effect of PRP on pregnancy rates was assessed in PBIE-susceptible barren mares (Metcalf 2014). Treatment with PRP resulted in decreased retention of intra-luminal fluid when compared to untreated cycles, which may have led in part to significantly increased pregnancy rates during treatment cycles (67% pregnancy rate in the treated group compared to 19% in the non-treated group).

Reghini et al. (2016) examined the effect of PRP treatment four hours post-AI in mares with chronic degenerative endometritis (CDE). Percentage of neutrophils as well as depth of intra-uterine fluid accumulations were both reduced 24 hours post-AI in CDE-affected mares treated with PRP when compared to control cycles. However, no significant effects of PRP treatment on NO levels were noted in this study. Effects of treatment with PRP prior to AI compared to treatment following AI in PBIE-susceptible mares was assessed in a study by Segabinazzi et al. (2017). Both treatment protocols resulted in decreased neutrophils on endometrial cytology, higher conception rates and lower COX-2 immunostaining when compared to untreated cycles. However, no effect of PRP treatment on intra-uterine fluid levels was observed. Although there are some inconsistencies in results between studies, the overall effect that PRP decreases uterine inflammation is clear. More studies are required in order to determine the exact mechanisms of action and optimal treatment protocol.

Two proteins identified in seminal plasma also appear to have beneficial effects on breeding-induced inflammation: lactoferrin and CRISP-3 (Doty et al. 2011; Troedsson et al. 2014),
and research has focused on whether these proteins could be of benefit in the treatment of PBIE. More specifically, lactoferrin has been shown to enhance neutrophil phagocytosis of non-viable spermatozoa, assisting with clearance of sperm from the uterus prior to embryo descent (Troedsson et al. 2014). In contrast, CRISP-3 decreases neutrophil binding to live spermatozoa, thereby improving chances of fertilization (Doty et al. 2011). Fedorka et al. (2016) examined the effects of infusion of one billion spermatozoa alone, or in combination with seminal plasma, CRISP-3 or lactoferrin on mRNA expression of select cytokines in PBIE mares. A similar study was repeated in PBIE-resistant mares (Fedorka et al. 2017). Results showed that only lactoferrin decreased IL-1RN mRNA expression when compared to other treatments and no other effects of infusion with lactoferrin or CRISP-3 were noted in PBIE-susceptible mares (Fedorka et al. 2016). However, infusion of PBIE-resistant mares with seminal plasma and spermatozoa resulted in increased mRNA expression of the pro-inflammatory cytokines IL-8 and IL-1β, and decreased levels of TNF-α (Fedorka et al. 2017). Infusion of lactoferrin alone suppressed TNF-α expression, but no effects of CRISP-3 were noted. These results were in comparison to the mRNA expression noted in the other treatment cycles. Due to the promising effects of lactoferrin on inflammatory cytokine expression, its use as a post-breeding treatment was explored (Fedorka et al. 2018).

Intra-uterine infusion of three different doses of human recombinant lactoferrin (50µg/ml, 250µg/ml and 500µg/ml diluted in 10ml of LRS) in PBIE mares 6 hours following AI resulted in a decreased neutrophil:epithelial cell ratio at 18 hours post treatment, but did not change post-breeding intra-uterine fluid accumulation compared to the control cycle. Increased mRNA expression of IL-1RN and decreased mRNA expression of IFNγ were also seen in treated cycles.
Given these results, treatment with lactoferrin seems promising, yet more research is needed prior to its clinical usage.

1.6.4 N-Acetyl Cysteine

N-acetyl cysteine (NAC) is a mucolytic agent used in the treatment of bacterial endometritis when the presence of a biofilm is suspected, or in cases where excess uterine mucus is present in horses (Ferris et al. 2016). N-acetyl cysteine disrupts disulphide bonds between mucin polymers, reducing mucus viscosity (LeBlanc 2010). In addition to its mucolytic effects, NAC has demonstrated antioxidant and anti-inflammatory properties in multiple models of disease in both human and veterinary medicine. This may be in part due to its metabolism, as it is de-acetylated to cysteine. Cysteine is a precursor of glutathione, which decreases free radical formation (Bisseling et al. 2004). Increased levels of glutathione were detected in circulating neutrophils in a human clinical trial evaluating NAC for the treatment of cystic fibrosis. Airway neutrophil counts significantly decreased following high-dose NAC treatment in these patients (Tirouvanziam et al. 2006). In a human chronic obstructive pulmonary disease (COPD) in vitro model, NAC was evaluated for both its mucolytic properties and role as an anti-oxidant (Cazzola et al. 2017). The study found that higher concentrations of the drug reduced pro-oxidative factors and enhanced anti-oxidative factors, in addition to inhibiting pro-inflammatory cytokines (IL-1β, IL-8, TNF-α), suggesting a dose-dependent effect of NAC.

N-acetyl cysteine affects cytokine and LPS-induced production of NO in rat peritoneal macrophages, C₆ glial cells and primary astrocytes (Pahan et al. 1997). This in vitro study demonstrated that when these cells were incubated with NAC two hours prior to the addition of an LPS stimulus, NO production was inhibited at the level of iNOS. This was attributed to the
inhibition of nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB). Similar effects were noted in a murine LPS-induced preterm labor model, where multiple aspects of the inflammatory response were reduced by NAC pre-treatment (Paintlia et al. 2008). The anti-inflammatory effects of NAC were further investigated in a rat multiple sclerosis model. Here, treatment with NAC blocked the induction of monocyte/macrophage produced TNF-α, IFN-γ, IL-1β and iNOS as well as splenocyte production of IFN-γ. In addition, splenocyte production of the anti-inflammatory cytokine IL-10 was increased in the NAC-treated group, demonstrating a clear impact of NAC on cytokine production (Stanislaus et al. 2005). Finally, studies have examined the effects of NAC on NO production specifically (Bergamini et al. 2001; Majano et al. 2004). In both studies, inflammation was induced by LPS in rat models. Treatment with NAC resulted in inhibition of iNOS, and Majano et al. (2004) were able to demonstrate that this inhibition may have occurred partially at the level of NF-κB.

N-acetyl cysteine has been effective for treatment of inflammation in horses in experimental studies, specifically in cases of mucosal injury in the right dorsal colon, and corneal ulceration (Haffner et al. 2003; Rotting et al. 2003). These studies show that NAC has more than just mucolytic effects. Its ability to alter cytokine expression, iNOS induction and neutrophil levels provides evidence that it may be useful in cases of uterine inflammation, such as PBIE.

Multiple recent studies have examined the utility of NAC for treatment of endometritis (Witte et al. 2012; Gores-Lindholm et al. 2013; Melkus et al. 2013). Potential adverse effects of NAC on the uterus have been assessed (Witte et al. 2012; Melkus et al. 2013). First, oral administration of NAC was evaluated in normal mares during estrus. The effects on mucus viscosity were determined one- and five-days following treatment, in addition to evaluation of
endometrial epithelial integrity, neutrophil infiltration and COX-2 staining on endometrial biopsies taken on day five. Oral NAC treatment had no effect on endometrial epithelial integrity or mucus viscosity, but did result in decreased infiltration of neutrophils and COX-2 staining compared to untreated mares (Witte et al. 2012). Further studies consisted of healthy mares treated with intra-uterine infusion of 5% NAC during estrus (Melkus et al. 2013). Endometrial biopsies were taken at 24- and 72-hours following treatment in order to determine epithelial integrity, neutrophil infiltration and COX-2 staining. Results showed that no damage to the epithelium was sustained, and that at 72 hours post-treatment, fewer neutrophils and less COX-2 staining was present in treated mares compared to controls. This demonstrated that NAC was safe, and that it may also have an anti-inflammatory effect in the uterus (Melkus et al. 2013). In a more clinical study, Gores-Lindholm et al. (2013) assessed endometrial structure, neutrophil activity and reproductive performance in both fertile and barren mares treated with intra-uterine NAC. They showed that peripheral neutrophil release of oxygen radicals was reduced by 3.0% NAC solution, thereby possibly diminishing excessive intra-uterine neutrophil-induced damage, and that endometrial epithelial architecture was maintained. Decreased mucus was noted at the histological level in fertile mares but not in barren mares treated with NAC. These results conflicted with those by Witte et al. (2012) and could be explained by differing routes of administration of NAC. These studies suggest that intrauterine infusion or oral treatment with NAC is not detrimental on the endometrium, may or may not affect mucus production in fertile, healthy mares, and likely reduces inflammation in the uterus, as evidenced by decreased neutrophil infiltration and COX-2 staining.
Based on its anti-inflammatory, anti-oxidant and NO modulating effects, the utility of NAC as an adjunct for the treatment of PBIE in susceptible mares is worthy of further study. This research was therefore undertaken with the goal of furthering our understanding of the effects of using NAC in the management PBIE in mares.

1.7 RESEARCH OBJECTIVES

The specific objectives of this research were:

1. To determine if mares susceptible to PBIE treated with N-acetyl cysteine will resolve their clinical signs more rapidly in treated cycles compared to control cycles, and

2. To determine if mares susceptible to PBIE will have lower concentrations of nitric oxide and a modified inflammatory cytokine profile following treatment with N-acetyl cysteine compared to control cycles.
1.8 REFERENCES


**Figure 1:** Simplified flow chart displaying cytokine interactions (Tizard 2013)
<table>
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<tr>
<th>Study</th>
<th>Time Points</th>
<th>Mares</th>
<th>Cytokines</th>
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</table>
| Fumoso et al. 2003            | Baseline (estrus & diestrus), 24 hours post Al & 7 days post Al              | PBIE susceptible & resistant mares | IL-1β, IL-6 & TNFα                | **Baseline:** IL-1β, IL-6, TNFα elevated in susceptible mares in estrus; IL-1β & TNFα elevated in susceptible mares in diestrus  
**24 hours post Al:** no differences between susceptible & resistant  
**7 days post Al:** IL-1β & TNFα elevated in susceptible mares |
| Fumoso et al. 2007            | Baseline (estrus & diestrus), 24 hours post Al & 7 days post Al              | PBIE susceptible & resistant mares | IL-8 & IL-10                       | **Baseline:** IL-8 elevated & IL-10 reduced in susceptible mares in estrus; IL-8 & IL-10 elevated in susceptible mares in diestrus  
**24 hours post Al:** IL-8 elevated & IL-10 reduced in susceptible mares  
**7 days post Al:** IL-8 & IL-10 elevated in susceptible mares |
<p>| Palm et al. 2008              | Pre-treatment (baseline) &amp; 12 hours following treatment (PBS, seminal plasma, skim-milk based semen extender) | Normal mares                  | IL-1β, IL-6 &amp; TNFα                | IL-1β, IL-6 &amp; TNFα all increased post Al for all treatments compared to pre-treatment levels |
| Nash et al. 2010              | 12 hours pre-treatment &amp; 24 hours (frozen/thawed semen)                     | Normal mares                  | IL-8                               | IL-8 levels remained the same pre &amp; post treatment                                                                                                                                 |
| Christoffersen et al. 2010    | 0, 3, 12, 24, 48 &amp; 72 hours following intra-uterine inoculation with <em>E. coli</em> | Normal mares                  | IL-1β, IL-8, IL-10 &amp; TNFα         | IL-1β, IL-8, IL-10 &amp; TNFα increased at 3 hours post inoculation                                                                                                                                 |
| Woodward et al. 2013          | Baseline, 2, 6, 12 &amp; 24 hours post Al                                       | PBIE susceptible &amp; resistant mares | IL-1β, IL-6, IL-8, IL-10, IFN-γ, IL-1RN &amp; TNFα | IL-6, IL-1RN &amp; IL-10 decreased at 6 hours post Al in susceptible mares |</p>
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<td></td>
<td>No differences</td>
<td>24 hours post AI</td>
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<td></td>
<td>↑ in susceptible mares</td>
<td>7 days post AI</td>
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<td></td>
<td>↑ for all treatment types</td>
<td>12 hours post AI compared to pre-treatment</td>
<td>Palm et al. 2008</td>
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<td></td>
<td>↑</td>
<td>3 hours post inoculation</td>
<td>Christoffersen et al. 2010</td>
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<tr>
<td></td>
<td>No differences</td>
<td>Baseline, 2, 6, 12 &amp; 24 hours post AI</td>
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<tr>
<td>IL-8</td>
<td>↑ in susceptible mares</td>
<td>Baseline estrus</td>
<td>Fumoso et al. 2003</td>
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<td>↑ in susceptible mares</td>
<td>Baseline diestrus</td>
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<td>24 hours post AI</td>
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<td>Change</td>
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<td></td>
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<td>Baseline in diestrus</td>
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<td>3 hours post inoculation</td>
<td>0, 3, 12, 24, 48 &amp; 72 hours following intra-uterine inoculation with E.coli in normal mares</td>
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<td>Baseline, 2, 12 &amp; 24 hours post AI</td>
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<td>Woodward et al. 2013</td>
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Table 2: mRNA expression of select cytokines in equine endometritis literature, organized by cytokine
CHAPTER TWO: THE EFFECTS OF TREATMENT WITH N-ACETYL CYSTEINE ON CLINICAL SIGNS IN PBIE SUSCEPTIBLE MARES

2.1 ABSTRACT

Persistent breeding induced endometritis (PBIE) is a major cause of infertility in mares. Uterine inflammation at the time of embryonic descent ultimately results in early embryonic death. A severe endometrial biopsy grade (IIb or III) has been identified as a risk factor for PBIE. Intra-uterine fluid accumulation (>2cm in depth), pathologic endometrial edema and elevated intra-uterine neutrophil levels are all clinical features of PBIE. N-acetyl cysteine (NAC), a mucolytic used to treat bacterial endometritis in mares, has anti-inflammatory properties, and was investigated to determine if intra-uterine infusion before breeding would result in the improvement of clinical signs associated with PBIE. In addition, the effects of NAC at the histological level on the endometrium were determined. A randomized, blinded, cross-over design clinical trial was performed utilizing PBIE susceptible mares (n=9). Intrauterine infusion of 3.3% NAC was performed 12 hours prior to insemination, and endometrial cytology and endometrial biopsy samples were obtained at 12 and 60 hours post-insemination. Endometrial biopsies were scored based on the degree of inflammation (neutrophils) present. Clinical signs of endometrial edema and intra-uterine fluid volumes were assessed by trans-rectal ultrasound at 12 and then every 24 hours post breeding. Statistical analysis utilized repeated measures ANOVA and a Mann Whitney Wilcoxon Test. Treatment with NAC did not improve clinical signs in PBIE-affected mares. However, treatment with NAC had a significant effect on the degree of endometrial inflammation noted histologically, with treated cycles displaying more diffuse and
severe neutrophil infiltration compared to control cycles. The effects of NAC on the endometrium of PBIE-susceptible mares seen in this study is concerning. Further research is required to evaluate the safety of NAC in the treatment of PBIE-susceptible mares.

2.2 INTRODUCTION

Uterine inflammation is a normal response to breeding and is believed to clear the uterus of debris, spermatozoa and inflammatory products prior to embryo descent. In some mares, the inflammatory response is prolonged, leading to early embryonic death and infertility. Affected mares are considered susceptible to persistent breeding-induced endometritis (PBIE) (Troedsson 2006; Woodward and Troedsson 2015; Christoffersen and Troedsson 2017).

Considerable research has focused on the reasons some mares are susceptible to PBIE, with the majority of studies focusing on the immune system and mechanical clearance of the uterus (Nikolakopoulos and Watson, 1999; Watson 2000; LeBlanc and Causey 2009; Troedsson 2006; Woodward and Troedsson 2015; Christoffersen and Troedsson 2017). Multiple aspects of immune dysfunction have been investigated as potential contributors to PBIE. An altered humoral immune response, specifically differing levels of immunoglobulins between susceptible and resistant mares, has been identified (Asbury et al. 1980; Mitchell et al. 1982; Williamson et al. 1983; Troedsson 1991). In contrast, Troedsson et al (1993b) found that PBIE-susceptible mares did not have immunoglobulin deficiency at 24 hours following AI, disproving the previous belief that immunoglobulin alterations were the cause of PBIE. Troedsson et al. (1993c) and other researchers demonstrated that PBIE-susceptible mares had elevated numbers of intra-uterine neutrophils with reduced functionality and phagocytic properties (Lui et al. 1986; Watson et al. 1986; Xue et al. 1990).
Additional studies have recently furthered our understanding of the role of inflammation in PBIE. Differences in mRNA expression of both pro- and anti-inflammatory cytokines have been identified either at baseline levels, or at multiple time points following AI, between normal and affected mares. Persistent expression of pro-inflammatory cytokines for prolonged periods of time, as well as reduced expression of anti-inflammatory cytokines following breeding, may contribute to the manifestation of PBIE (Fumoso et al. 2003; Fumoso et al. 2007; Christoffersen et al. 2012; Woodward et al. 2013a; Christoffersen and Troedsson, 2017). PBIE-susceptible mares are also known to have an underlying myometrial defect, causing decreased uterine contractility (Troedsson et al. 1993a; LeBlanc et al. 1994a; Neuwirth et al. 1994; Rigby et al. 2001; von Reitzensein et al. 2001; LeBlanc and Causey 2009). In addition, intra-uterine nitric oxide levels are elevated in PBIE-susceptible mares, further contributing to reduced uterine contractions (Alghamdi et al. 2005; Woodward et al. 2013b; Khan et al. 2018). A reduction in uterine contractility leads to decreased uterine clearance, a feature of PBIE. From these studies, it is clear that the mechanisms involved in uterine health following breeding, including the immune response, persistent inflammation and uterine clearance, are complex and interdependent. Dysregulation of any of these mechanisms have the potential to lead to PBIE in mares.

Clinical signs of PBIE include abnormal endometrial edema (Samper 2007), persistence of a >2cm depth of intra-uterine fluid beyond 48 hours post-AI detected on trans-rectal ultrasound (Brinsko 2003; Pycock 2007), presence of inflammatory cells on endometrial cytology and
endometrial histopathology consistent with uterine inflammation (LeBlanc 2010; Troedsson 2011; Canisso et al. 2016).

Treatment modalities for PBIE do exist, yet these are not without limitations. Ecbolic therapies, including oxytocin and prostaglandin, increase uterine clearance by increasing myometrial activity, and can be highly effective at improving uterine clearance (LeBlanc et al. 1994b, Combs et al. 1996; Troedsson 1997; Cadario et al. 1999; LeBlanc and Causey 2009). However, some mares are refractory to oxytocin and continue to accumulate fluid despite therapy (Hurtgen 2006; LeBlanc and Causey 2009). Additionally, prostaglandins administered as ecbolic therapy post-ovulation may interfere with progesterone production from the corpus luteum, potentially resulting in early embryonic death. Prostaglandins are therefore only recommended in the pre-ovulatory period, limiting their use in treatment of PBIE (Troedsson et al. 2001; Nie et al. 2002; Brendemuehl 2002; Nie et al. 2003). Immunomodulators, such as glucocorticoids, are useful in mares with sterile inflammation but should be avoided in mares with concurrent bacterial involvement or other medical conditions such as laminitis (LeBlanc 2010). Therefore, alternative treatment modalities for PBIE are needed.

N-acetyl cysteine (NAC) is a mucolytic agent that disrupts disulphide bonds linking mucin polymers (LeBlanc 2010) and has anti-inflammatory and anti-oxidant properties (Bergamini et al. 2001; Majano et al. 2004; Cazzola et al. 2017). N-acetyl cysteine inhibited the enzyme responsible for NO synthesis and blocked pro-inflammatory cytokine production in a multiple sclerosis (MS) model using rats, and enhanced anti-oxidative factors in in vitro human chronic obstructive pulmonary disease (COPD) research (Stanislaus et al. 2005). N-acetyl cysteine is currently used as an intra-uterine infusion in mares with excessive uterine mucus production or in cases of
bacterial endometritis complicated by a biofilm (LeBlanc and Causey, 2009; LeBlanc 2010; Troedsson 2012). Infusion of NAC improved fertility rates of barren mares when used during the prior cycle or 48 hours pre-breeding (Gores-Lindholm et al. 2009). Oral (Witte et al. 2012) and intra-uterine (Melkus et al. 2013) NAC administration decreased neutrophil number and COX-2 staining in uterine histological samples in normal mares. N-acetyl cysteine appears to be safe for use in horses: it was reported to cause no adverse effects on normal mare endometrium following intra-uterine infusion of either a 3.3% or 5% solution (Gores-Lindholm et al. 2013; Melkus et al. 2013) or following oral administration (10mg/kg) (Witte et al. 2012). No studies to date have examined the effects of NAC on the resolution of clinical signs of PBIE in susceptible mares. Based on its anti-inflammatory and anti-oxidant effects, we investigated NAC as a potential treatment for PBIE in susceptible mares.

The objective of this study was to determine the efficacy of intra-uterine infusion of NAC as a treatment for PBIE in susceptible mares. We hypothesized that intra-uterine treatment of PBIE-susceptible mares with NAC would result in more rapid resolution of excessive endometrial edema, percent neutrophils on endometrial cytology, intra-uterine fluid volume and inflammation as assessed by endometrial biopsy, compared to untreated cycles.

2.3 MATERIALS AND METHODS

All the procedures performed on animals in this study were approved by the University of Guelph Animal Care Committee (Animal Utilization Protocol number 3213), in accordance with the Canadian Council on Animal Care guidelines.
2.3.1 Selection of PBIE Susceptible Mares

Mares previously identified as susceptible to PBIE (Khan et al. 2018) were utilized in this study. Mares were selected in a manner similar to that of Alghamdi et al. (2005), and Woodward et al. (2013a, 2013b; 2015). Potentially PBIE-susceptible mares were identified on the basis of a Kenney biopsy score of IIb or III, negative bacterial culture and <2 neutrophils per high power field (hpf) on endometrial cytology. Study mares were then confirmed to be PBIE-susceptible by failure to clear intra-uterine fluid following intra-uterine challenge with killed spermatozoa (i.e. persistence of greater than 2cm of intrauterine fluid 96 hours following insemination). Immediately prior to the commencement of the clinical trial, study mares were again screened to ensure they were negative for bacterial endometritis, and pre-trial endometrial biopsies were obtained. Bacterial cultures were submitted to the Animal Health Laboratory (AHL, Guelph, ON, Canada). Only mares without bacterial endometritis were enrolled in the trial.

2.3.2 Clinical Trial

A blinded, randomized, cross over designed clinical trial was performed using ten PBIE-susceptible mares (Figure 1). Mares were housed at the Arkell Equine Research Station at the University of Guelph and were of various breeds (Standardbred and mixed breed) and ages (7-19 years). The order of Treatment (N-acetyl cysteine intra-uterine infusion) and Control (saline) cycles for each mare were assigned via random number allocation, with a washout period between experimental cycles that consisted of at least one estrous cycle. The primary investigator conducting clinical examinations and all personnel involved in the daily care of the mares were blinded to treatment and control assignment of the mares during data collection. Only cycles which fit the definition of PBIE, (>2cm of intra-uterine fluid for at least 48 hours post
AI) were included in the analysis. For this reason, one mare was removed from the statistical analysis for both treatment and control cycles.

Mares were monitored by trans-rectal palpation and ultrasound and estrus was induced with an intra-muscular dose (125mcg) of cloprostenol (Estrumate®, Intervet Canada Corp., Kirkland QC, CAN) as needed. Once in estrus with a 35mm or larger follicle identified, 2,500 IU of human chorionic gonadotropin (hCG, Chorulon®, Intervet Canada Corp., Kirkland, QC, Canada) was administered intravenously to induce ovulation. During treatment cycles (TX), 180ml of a 3.3% NAC solution consisting of 30ml of N-acetyl cysteine (Acetylcysteine Injection 20% solution, Alveda Pharmaceuticals Inc., Toronto, ON, Canada) diluted in 150ml of 0.9% sodium chloride (0.9% Sodium Chloride Injection USP, Baxter Corporation, Mississauga, ON, Canada) was infused into the uterus using a 21-inch insemination pipette (Partnar Animal Health, Ilderton, ON, Canada). During control cycles (CONTROL), 180ml of sterile saline (0.9% Sodium Chloride Injection USP) was infused into the uterus. Uterine infusions for both treatment and control cycles were performed at the time as the hCG injection. Twelve hours following infusion, mares were artificially inseminated with 1 x 10^9 killed spermatozoa in 30ml of semen extender prepared as previously described (Woodward et al. 2013a; Woodward et al. 2013b; Woodward et al. 2015; Khan et al. 2018). Mares were monitored via trans-rectal ultrasound beginning 12 hours post-Al, and this was repeated at 24 hour intervals through to and including 84 hours post-Al (12, 36, 60 and 84 hours post-Al), to determine endometrial edema score (0= none; 1= minimal; 2= moderate; 3= normal maximal and 4= excessive or pathological; Diel de Amorim et al. 2016) and intra-uterine fluid volume (IUF). The IUF volume was calculated using the formula IUF (ml)=length*width*height, each measured in centimeters, where the IUF was the mean value
derived from 2-5 calculations of fluid as assessed in the uterine body as well as from within the left and right uterine horns.

Endometrial cytology, endometrial fluid (this sample was used for a different experiment) and endometrial biopsy samples were taken, in that order, at 12 and 60 hours post-AI. Endometrial cytology samples were aseptically obtained using a guarded swab (Kalayjian Industries Inc., Signal Hill, CA, USA) (Khan et al. 2018). Briefly, the guarded swab was passed through the cervix, the swab was advanced beyond the sheath and then rotated several times before being left in place for a minimum of 30 seconds, and then the swab was retracted into the sheath. The outer sheath was then rotated to collect endometrial cells in the cap, and the entire unit was then removed. Endometrial biopsies were taken aseptically at the base of the right uterine horn, by trans-rectal guidance of a Pilling-Weck biopsy punch (Jorgensen Laboratories, Loveland, CO, USA), through the cervix, as previously described (Kenney 1978). Endometrial biopsy samples were placed in 10% formalin solution (Surgipath® 10% Neutral Buffered Formalin, Leica Biosystems Richmond, Inc., Richmond, IL, USA) and submitted to the Animal Heath Laboratory (University of Guelph) for embedding in paraffin wax, sectioning and routine staining with Hematoxylin and Eosin (H&E).

2.3.3 Inflammation as Assessed by Endometrial Biopsies

Endometrial biopsies were examined for the degree and distribution of neutrophils, lymphocytes and plasma cells (‘inflammatory cells’) by a Board Certified Theriogenologist (TC), blinded to mare treatment assignments. A semi-quantitative inflammation score between 1-4 (1=mild; 2=moderate; 3=marked and superficial; 4= severe and diffuse) was assigned to endometrial biopsies, and was based on the compilation of three separate observations made
across a minimum of 10 high power fields (Figure 2; Table 1). The first observation consisted of the number of neutrophils per high power field as adapted from Alvarenga et al. (2016) (1 = 0-3 neutrophils/hpf); 2 = (4-10 neutrophils/hpf); 3 = 10-30 neutrophils/hpf); 4 = >30 neutrophils/hpf). The second observation consisted of the depth of inflammatory cell infiltration, with scores of 1-3 having inflammatory cells present in the most superficial areas only (stratum compactum) whereas those with diffuse infiltration of inflammatory cells deep into the endometrium (stratum spongiosum) were given a score of 4. The third observation consisted of the presence of endometrial luminal epithelium erosion, as defined by loss of epithelium with evidence of cell proliferation on both the edges and below the area of erosion (cells becoming thin and stretched on the edges; presence of hyperchromatic cells below). The presence of erosion contributed to a more severe score (3 or 4).

2.3.4 Endometrial Cytology (% Neutrophils)

Endometrial cytology slides were prepared and stained with modified Wright Giemsa stain (PROTOCOL™, Hema3™ Stat Pack, Fisher Brand®, Pittsburg, PA), as previously described (Khan et al. 2018). The percentage of neutrophils to total cells was determined (endometrial cells and neutrophils combined) across multiple fields of view, at 400x. This accounted for any variability of cell distribution. The mean percentage of neutrophils was calculated after a minimum of 500 total cells were counted.

2.3.5 Statistical Analyses

Statistical analyses were performed using the SAS software program (SAS Institute, Inc., Version 9.3, Cary, NC, USA). Differences in IUF, % neutrophils and uterine edema scores were compared between NAC-treated (TX) and saline (CONTROL) cycles, as well as across time points
using repeated measures ANOVA. Mare identification (ID) was included as a variable in the statistical model, but was removed, as no significance was detected. Differences in biopsy inflammation score between NAC-treated (TX) and saline (CONTROL) as well as across time points were analyzed using the Mann Whitney Wilcoxon test. The Akaike information criterion (AIC) was utilized to determine the best fit model. Normality of the data was tested using the Shapiro-Wilk test. Data for estimated intrauterine fluid volume (IUF) required logarithmic transformation, however all other outcome parameters were normally distributed. Results are presented as medians +/- lower limit (LL) and upper limit (UL) when log transformation was required and as means +/- standard error (SE) when no data transformations were performed. Where there was no effect of treatment, data for NAC-treated (TX) and saline (CONTROL) cycles were combined to then examine the effect of number of hours post-AI. Similarly, where there was no effect of hours post AI, data for 12 and 60 hours post-AI were combined to then examine the effects of treatment. Correlation between % neutrophils and biopsy inflammation score was analyzed using the Spearman correlation coefficient, to account for the significance in the model and distribution of the data. Correlation between IUF and uterine edema score was assessed using the Pearson correlation coefficient, since the model had no effects and data was normally distributed. The p value was set at <0.05 for statistical significance for all statistical analyses.

2.4 RESULTS

2.4.1 Biopsy Inflammation Score

Pre-trial biopsies were available for 7 of the 9 PBIE-susceptible mares used in this study. The mean pre-trial (baseline) biopsy inflammation score was 1.14 +/- 0.65. Biopsy inflammation
score was significantly greater in the NAC treated cycle (TX mean = 3.0 +/- 0.5) compared to the saline treated cycle (CONTROL mean = 2.2 +/- 0.5), when biopsy inflammation scores at 12 and 60 hours post-Al were combined for each treatment cycle (p=0.01) (Figure 3). The endometrial effects of NAC included erosions of the luminal epithelium and marked infiltration of neutrophils in the stratum compactum (Figures 2d, 4 and 5). One of the differences in criteria between a biopsy inflammation score of 1-3 compared to a biopsy inflammation score of 4 was the presence of deep-seated neutrophil infiltration. The incidence of an inflammation score of 4 was much greater in NAC treated cycles with time points combined (8/18) in comparison to saline treated cycles (1/18) with time points combined (Table 1). There was no significant time effect on biopsy inflammation score from 12 to 60 hours post-Al when both treatment cycles were combined; mean (12 hours post Al) was 2.8 +/- 0.5 whereas mean (60 hours post-Al) was 2.3 +/- 0.5 (p=0.12) (Figure 6).

2.4.2 Estimated Intra-Uterine Fluid Volumes (IUF)

Treatment with NAC did not affect calculated IUF when time points were combined within each treatment cycle (p=0.78; TX median = 3.9cm³; range = 1.1-14.7 cm³; CONTROL median = 4.5 cm³; range = 1.2-16.8 cm³) (Figure 7). In contrast, the effect of time was significant (p=0.005), with more intra-uterine fluid present at 36 hours post-Al (median = 9.8cm³; range = 2.6-36.2cm³) compared to 12 hours post-Al (median = 3.4 cm³; range = 1-11.6 cm³), 60 hours post-Al (median = 3.9cm³; range = 0.9-17.5cm³) and 84 hours post-Al (median = 2.5cm³; range = 0.6-9.5cm³) (Figure 8).
2.4.3 Endometrial Edema

Treatment with NAC did not affect endometrial edema scores (p=0.90; TX mean = 1.7 +/- 0.6; CONTROL mean = 1.7 +/- 0.6) (Figure 9). Endometrial edema score was significantly higher (p=0.04) at 12 hours (mean = 2.1 +/- 0.6) and 36 hours (mean = 2.0 +/- 0.6) compared to 60 hours post-AI (mean = 1.3 +/- 0.6) and 84 hours (mean = 1.3 +/- 0.6) post-AI (Figure 10). The estimated IUF and uterine edema scores were not significantly correlated (r = -0.14; p = 0.45).

2.4.4 Endometrial Cytology (% Neutrophils)

Treatment with NAC did not affect % neutrophils on endometrial cytology (p=0.39; TX mean = 48.4% +/- 12.5%; CONTROL mean = 40.9% +/- 12.5%) (Figure 11). Evidence of inflammation decreased over time post-AI (p=0.005). The % neutrophils was significantly higher at 12 hours post-AI (mean = 57.7% +/- 12.5%) compared to 60 hours post-AI (mean = 31.6% +/- 12.5%) (Figure 12). Additionally, % neutrophils and biopsy inflammation score were positively correlated (r=0.43; p = 0.006).

2.4.5 Interactions Between Treatment Cycles and Hours Post-AI (Appendix 1)

There was no significant interaction between treatment cycle and hours-post-AI for biopsy inflammation score (p=0.12; Appendix 1: figure 1), IUF (p=0.84; Appendix 1: figure 2), endometrial edema (p=0.65; Appendix 1: figure 3), nor % neutrophils (p=0.98; Appendix 1: figure 4).

2.5 DISCUSSION

Intra-uterine infusion of PBIE-susceptible mares with a 3.3% NAC solution 12 hours pre-AI resulted in more inflammation on endometrial biopsy compared to treatment with saline 12
hours pre-Al. This is in contrast to previous studies where treatment with NAC did not appear to be detrimental to the endometrium, and the endometrial luminal epithelium remained intact (Witte et al. 2012; Gores-Lindholm et al. 2013; Melkus et al. 2013). Melkus et al. (2013) reported that intra-uterine infusion of 5% NAC in normal mares resulted in less endometrial inflammation on biopsy at 72 hours post-treatment compared to control mares treated with saline. However, NAC treated and control normal mares both had higher numbers of neutrophils/hpf 24 hours following treatment (10.9 neutrophils/hpf in NAC group; 8.3 neutrophils/hpf in saline group). This resolved by 72 hours, with treated mares having significantly less neutrophils than controls. Normal mares treated orally with NAC for four days had fewer neutrophils present in the endometrium one day following treatment, compared to untreated controls (Witte et al. 2012). It is interesting that in the Witte et al. (2012) and Melkus et al. (2013) studies, normal mares in estrus had more neutrophils without provocation of inflammation, such as AI. In the studies mentioned above, mares were simply treated in estrus and biopsies were obtained. Untreated mares or those treated with saline had greater than 2 neutrophils/hpf, which is considered to be indicative of endometritis (Woodward et al. 2013a) (4.8 neutrophils/hpf with no treatment; 3.9 neutrophils/hpf with saline infused intra-uterine at 72 hours post treatment). The reasons for this increase in supposedly normal mares without an inflammatory challenge are not known, but could include differences in the original classification as normal mares, posing as a possible limitation to their study, or to the irritating nature of the infusion of NAC itself. Gores-Lindholm et al. (2013) reported that the degree of acute inflammation in endometrial biopsies was not different three days following intra-uterine infusion of a 3.3% NAC solution as compared to pre-treatment within a mixed group of 20 mares (10 barren treated; 6 fertile treated; 6 fertile
control). However, mares in that study were treated with uterine lavage with lactated Ringer’s solution (LRS) daily for two days between the initial infusion and the procurement of the biopsy, possibly aiding resolution of any inflammation resulting from the NAC infusion by the time the biopsy was taken. In contrast to the above studies, which utilized either normal or mixed populations of mares, our trial involved only PBIE-affected mares, selected partially on the basis of poor endometrial biopsy scores (Grade IIb and III). Normal mares are likely more capable of clearing any inflammatory reaction caused by infusion of NAC or other irritating substances. However, this inflammatory reaction may not be cleared as rapidly in mares that are already susceptible to uterine inflammation, such as those used in our study.

Inflammatory scores of 4 were almost exclusively assigned to mares in the NAC treated cycles (Table 1). The inflammation noted in these biopsies consisted almost entirely of neutrophils and was diffusely deep-seated, extending into the stratum spongiosum and occasionally into the glandular lumen (Figures 2d and 5). Additionally, severely affected mares, with a score of 3 or 4, often displayed signs of a reactive luminal epithelium indicative of cell injury. The biopsies in our study also had some areas of erosion or complete loss of the endometrial luminal epithelium (Figure 4) and these changes were taken into consideration for the biopsy inflammation score (score of 3 or 4). These changes, as well as those mentioned above, are all suggestive of a marked endometrial response to the irritating nature of NAC. Witte et al. (2012) reported that treatment with NAC caused increased epithelial cell proliferation and suggested it to be evidence of endometrial regeneration. Gores-Lindholm et al. (2013) mentioned an increase in epithelial cell height in saline treated fertile mares compared to NAC treated fertile mares. The changes in these studies could suggest endometrial reactivity; however, the
epithelium remained intact. The biopsies with areas of erosion were very similar in appearance to those seen in mares treated with intra-uterine kerosene, which was previously reported to cause severe inflammation (R. Foster, Personal communication; Bracher et al. 1991). Mares with Grade III biopsy scores that received an intra-uterine infusion of 50ml of commercially available kerosene demonstrated a more prolonged and severe inflammatory response histologically, compared to similarly treated mares with Grade I or II biopsy scores (Bracher et al. 1991). However, in that study, mononuclear cells were the predominant inflammatory cell type present, compared to the predominance of neutrophils seen in our study following the NAC infusion. One previous study using intra-uterine NAC (Gores-Lindholm et al. 2013) demonstrated an increase in lymphocytes and plasma cells only on endometrial biopsy following NAC infusion in barren mares, but the authors noted that these changes were too chronic in nature to be due to NAC infusion.

The osmolality of 3.3% NAC solution used in the present study was considerably higher than the 0.9% saline used in the control cycles (saline = 280mOsm; NAC = 668mOsm). While no studies have examined the effects of hyperosmotic solutions on the endometrium specifically, multiple studies have shown that hyperosmotic stressors are damaging to cells and are pro-inflammatory in nature (Nemeth et al. 2002; Luo et al. 2005; Schwartz et al. 2008; Brocker et al. 2012). Exposure of cells to hyperosmotic solutions leads to an efflux of fluid out of the cell, shrinkage of the cell, alteration in protein synthesis, and damage to both the cytoskeleton and nucleus. This can lead to cellular apoptosis (Brocker et al. 2012). It is possible that the inflammatory reaction seen in our study was further exacerbated by insemination 12 hours following NAC infusion, limiting the ability of the already compromised endometrium to clear the
inflammation. In previous studies that administered intra-uterine NAC, treatment was often given 24-36 hours prior to breeding (LeBlanc and Causey 2009; Gores-Lindholm et al. 2009; LeBlanc 2010; Lyle 2012; Troedsson 2012; Gores-Lindholm et al. 2013; Buczkowska et al. 2015), perhaps allowing for some recovery prior to another insult. Given the biopsy findings in our study, one could speculate that intra-uterine infusion of NAC may not be as inert as previously believed.

Treatment with NAC did not improve clinical signs of PBIE, including IUF. Intrauterine fluid retention occurs in PBIE mares for several reasons, including lack of cervical dilation, pendulous uterus and poor uterine contractility. Cervical patency is known to improve uterine drainage (Pycock 2007, LeBlanc and Causey 2009, Relias et al. 2016). Some study mares had fibrotic and tortuous cervices that resulted from use as repeated embryo donors in previous research studies. Therefore, some mares retained considerable fluid, leading to a significant amount of variability in IUF levels between mares. This may have limited the apparent efficacy of intra-uterine NAC treatment in our study.

The PBIE-susceptible mares in our study demonstrated the expected inflammatory response over time, including changes in IUF, uterine edema scores and % neutrophils. IUF was most elevated at 36 hours and diminished thereafter. Myometrial contractions are one of the main mechanisms of uterine clearance (Troedsson 2006; LeBlanc and Causey 2009; Troedsson and Woodward 2016; Christoffersen and Troedsson 2017). Normal mares clear intra-uterine fluid within 24 hours following AI (Katila 1995); it is considered abnormal to find any intra-uterine fluid greater than 24 hours post-ovulation and into diestrus (Newcombe 1998). However, PBIE-susceptible mares do not demonstrate normal uterine clearance. LeBlanc et al. (1994a; 1995) found that susceptible mares cleared negligible amounts of radiocolloid 4 hours following
infusion; furthermore, India ink was maintained for up to 72 hours post-infusion. Furthermore, Troedsson et al. (1993a) showed that susceptible mares inoculated with *Streptococcus zooepidemicus* had a marked decrease in myometrial activity between 7-17 hours post-inoculation compared to resistant mares. In our study, IUF was greatest at 36 hours but not at 12 hours post-Al. Troedsson et al. (1993a) demonstrated that the pattern of uterine contractility of PBIE-susceptible and normal mares was similar initially following a challenge, however the duration and intensity of myometrial contractions rapidly declined in PBIE-susceptible mares, whereas in resistant mares it did not. This may explain the increase in IUF seen from 12 to 36 hours in our study mares.

Endometrial edema and estimated IUF were not significantly correlated in this study. This is contradictory to Kotilainen et al. (1994) where pre-treatment (Al, extender, seminal plasma, PBS) uterine fluid level was positively correlated with uterine edema. In addition, Samper (2007), found that presence of fluid at the time of ovulation was more commonly noted in mares with grade three, four and five edema scores. These differences could be accounted for by the fact that pre-treatment fluid levels could vary from post-treatment fluid levels and that the group of mares in those studies were normal and not screened as PBIE-susceptible.

Percent neutrophils on cytology were highest at 12 hours post-Al in our study when treatment cycles were combined. An influx of neutrophils into the endometrium occurs following infusion with spermatozoa in normal mares, and was first documented by Kotilainen et al. (1994). In normal mares, neutrophils appear at 4 hours, peak at 8 hours and are absent by 48 hours post-breeding (Katila 1995). This response is very similar to that noted in normal mares subjected to intra-uterine infusion of *Streptococcus zooepidemicus* four hours following breeding (Troedsson
A similar pattern of neutrophil infiltration occurs in PBIE-susceptible mares, but is much more severe. Neutrophils were significantly elevated in susceptible mares as early as 2 hours post AI, compared to 6 hours post AI in resistant mares (Woodward et al. 2013a). Endometrial neutrophil infiltration peaked in both groups at 6 hours post AI, and decreased thereafter, however neutrophils were consistently elevated in susceptible mares throughout (Woodward et al. 2013a). Overall, persistent inflammation was a feature of our PBIE-susceptible study mares, consistent with previous findings.

Dose and timing of administration of NAC may have affected efficacy in the present study. A 3.3% solution was selected based on previous studies using intra-uterine NAC in mares as a mucolytic (LeBlanc and Causey 2009; LeBlanc 2010; Lyle 2012; Troedsson 2012; Gores-Lindholm et al. 2013; Buczkowska et al. 2015; Scoggin 2016). Sub-fertile mares with a history of mucus hyper-secretion that received an intrauterine infusion of 3.3% NAC pre-breeding, attained a pregnancy rate of 77% (Gores-Lindholm et al. 2013). However, research in other fields, such as in cystic fibrosis (humans) and rodent pulmonary studies, suggests that the dose of NAC needs to be substantial enough to create an anti-inflammatory effect (Pahan et al. 1997; Tirouvanziam et al. 2006). It is therefore possible that the selected dose of NAC in our study was adequate for its previously known mucolytic effects, but not high enough to cause direct anti-inflammatory effects. NAC was administered pre-breeding in our clinical trial, in the same fashion as previous studies (Gores-Lindholm et al. 2013). Rat intra-peritoneal, cell culture and tissue model studies have demonstrated the anti-inflammatory effects of NAC when administered as a pre-treatment prior to induction of inflammation (Pahan et al. 1997; Majano et al. 2004; Cazzola et al. 2017). Together, this evidence provided the basis for the timing of NAC infusion in our study. However,
since no other studies have examined NAC treatment specifically in PBIE mares, treatment following the inflammatory response induced by breeding might prove more beneficial. In fact, oral NAC treatment of patients clinically ill with cystic fibrosis led to modulation of the pulmonary inflammation seen with this condition (Tirouvanziam et al. 2006).

In conclusion, intra-uterine treatment with 3.3% NAC did not improve clinical signs in PBIE mares. NAC infusion was associated with a significant and prolonged acute inflammatory reaction of the endometrium, which was suspected to be due to the hyperosmotic nature of NAC. Further studies are needed to explore the effects of hypertonic solutions on the endometrium in both normal and PBIE-susceptible mares. Additional studies evaluating alternative non-irritating immunomodulatory treatments such as platelet-rich plasma, which might normalize the inflammatory response of PBIE mares, would be of value.
2.6 REFERENCES


**Figure 1.** Graphical representation of the clinical trial timeline. Intrauterine infusion with N-acetyl cysteine (TX) or saline (CONTROL) was performed at time of hCG injection, with AI of $1 \times 10^9$ killed spermatozoa performed 12 hours later. Mares were monitored by ultrasound 12 hours post-Al and then every 24 hours. Sampling time points indicate when endometrial cytology, fluid samples and biopsies were taken (Abbreviations: hCG, human chorionic gonadotropin; NAC, N-acetyl cysteine).
Figure 2. Photomicrographs of endometrial biopsies from PBIE-susceptible mares. Inflammation biopsy scores were assigned by a blinded, board certified Theriogenologist. Scoring was based on degree and distribution of inflammatory cells and presence or absence of endometrial epithelial erosion (a: inflammation biopsy score of 1; b: inflammation biopsy score of 2; c: inflammation biopsy score of 3; d: inflammation biopsy score of 4). Hematoxylin and Eosin (H&E), x 200.
Figure 3. Differences in mean biopsy inflammation score (+/- SE) on histopathology of uterine biopsies from 9 PBIE-susceptible mares treated by intra-uterine infusion with N-acetyl cysteine (TX) and saline (CONTROL) cycles. Maximum biopsy score possible was 4. A significant treatment effect was present when both biopsy time points (12 and 60 hours post-AI) within treatment cycles were combined (p=0.01).
Figure 4. Photomicrograph of endometrial biopsy (score=4) displaying erosion of endometrial luminal epithelium following intra-uterine 3.3% NAC administered 12 hours prior to AI in some PBIE-susceptible mares. Hematoxylin and Eosin (H&E), x 200.
Figure 5. Photomicrograph of endometrial biopsy (score=4) displaying neutrophil infiltration of the stratum spongiosum and glandular lumen following intra-uterine 3.3% NAC administered 12 hours prior to AI in some PBIE-susceptible mares. Hematoxylin and Eosin (H&E), x 400.
Figure 6. Mean biopsy inflammation score (+/- SE) on histopathology of uterine biopsies from 9 PBIE-susceptible mares at 12 and 60 hours post AI when both intra-uterine infusion treatment cycles (TX=N-acetyl cysteine, CONTROL=saline) were combined within each time point. Maximum biopsy score possible was 4. No significant time effect was present (p=0.12).

Figure 7. Median intra-uterine fluid volume (cm³) (+/- LL and UL) of 9 PBIE-susceptible mares in N-acetyl cysteine treated (TX) and saline (CONTROL) cycles when the time points 12, 36, 60 and 84 hours post AI were combined within each treatment cycle. No significant effect of treatment was present (p=0.78).
Figure 8. Median intra-uterine fluid volume (cm$^3$) (+/- LL and UL) of 9 PBIE-susceptible mares at 12, 36, 60 and 84 hours post AI when treatment cycles (TX=N-acetyl cysteine, CONTROL=saline) were combined within each time point. Differing superscripts between time points were significantly different at p<0.05.

Figure 9. Mean endometrial edema score (+/- SE) of 9 PBIE-susceptible mares in N-acetyl cysteine treated (TX) and saline (CONTROL) cycles when the time points 12, 36, 60 and 84 hours post AI were combined within each treatment cycle. Maximum edema score possible was 4. No significant effect of treatment was observed (p=0.90).
Figure 10. Mean endometrial edema score (+/- SE) of 9 PBIE-susceptible mares at 12, 36, 60 and 84 hours post AI when treatment cycles (TX=N-acetyl cysteine, CONTROL=saline) were combined within each time point. Maximum edema score possible was 4. Between time points, differing superscripts were significantly different at p<0.05.

Figure 11. Mean % neutrophils (+/- SE) on cytology of uterine swabs obtained from 9 PBIE-susceptible mares treated by intra-uterine infusion with N-acetyl cysteine (TX) or saline (CONTROL). No significant treatment effect was present when both cytology time points (12 and 60 hours post-AI) within treatment cycles were combined (p=0.39).
Figure 12. Mean % neutrophils (+/- SE) on cytology of uterine swabs obtained from 9 PBIE-susceptible mares at 12 and 60 hours post AI when treatment cycles (N-acetyl cysteine=TX, saline=CONTROL) were combined within each time point. Between time points, differing superscripts were significantly different at p<0.05.

Table 1. Inflammation biopsy score assignment for each PBIE-susceptible mare in treated and control cycles at 12 and 60 hours post-Al. Score assignment was performed by a blinded, board certified Theriogenologist and was based on degree and distribution of inflammatory cells as well as presence or absence of endometrial epithelial erosion.
3.1 ABSTRACT

Persistent breeding-induced endometritis (PBIE) is a major cause of infertility in mares. Transient uterine inflammation is a normal response to breeding, however, PBIE susceptible mares do not clear this inflammation in a timely fashion. Uterine inflammation at the time of embryonic descent from the oviducts ultimately results in early embryonic death and is seen clinically as infertility. N-acetyl cysteine (NAC), a mucolytic used to treat endometritis in mares, has anti-inflammatory properties, affects inflammatory cytokines post-treatment and is a mild inhibitor of nitric oxide synthase. Increased nitric oxide (NO) levels and alterations in inflammatory cytokines have previously been documented in PBIE mares. The objective of this study was to determine if NAC treatment of PBIE-susceptible mares would lower nitric oxide and inflammatory cytokine levels. A randomized, blinded, cross-over design clinical trial was performed utilizing PBIE susceptible mares (n=9). Mares were screened negative for bacterial endometritis prior to enrolment in the study. Intrauterine infusion of 180ml of 3.3% NAC was performed 12 hours prior to insemination, when a follicle > 35 mm was present. Interleukin-6 concentration (ELISA) and nitric oxide level (Colorimetric assay) in intra-uterine fluid samples were determined at 12 and 60 hours post-insemination. Gene expression of inflammatory cytokines (Interleukin-6, Interleukin-10, Interleukin-1β and Tumor Necrosis Factor-α) were determined by qPCR from endometrial biopsy samples taken at 12 and 60 hours post-insemination. Statistical analysis was performed using a repeated measures ANOVA. In this
clinical trial, pre-breeding intrauterine treatment with NAC did not affect NO levels, IL-6 concentration or cytokine gene expression. Differences across time points post-AI were noted for IL-6 gene expression, which was highest at 12 hours post-AI (p=0.003). Expression of other cytokines evaluated did not change over time. While treatment with NAC pre-breeding did not have an effect on the cytokines evaluated, the assessment of inflammatory cytokines both at the gene and protein level at different time points post artificial insemination will add to the understanding of the alterations in inflammatory cytokine levels in mares susceptible to PBIE.

3.2 INTRODUCTION

Persistent breeding induced endometritis (PBIE) in mares is characterized by a prolonged inflammatory response following breeding, ultimately leading to early embryonic loss and infertility. A normal and transient inflammatory response occurs post-breeding; however, in a subset of susceptible mares, this inflammation persists (Troedsson, 2006; Woodward and Troedsson 2015; Christoffersen and Troedsson, 2017). Susceptibility to PBIE is associated with an altered inflammatory response as well as poor mechanical clearance of the uterus (Nikolakopoulos and Watson 1999; Watson 2000; Troedsson 2006; LeBlanc and Causey 2009; Woodward and Troedsson 2015; Christoffersen and Troedsson, 2017; Khan et al. 2018).

The inflammatory response of the endometrium can be characterized by alterations in cytokines. Baseline levels of pro-inflammatory cytokines are different between PBIE-susceptible versus resistant mares. Specifically, during estrus, PBIE-susceptible mares have elevated endometrial mRNA expression of IL-1β, TNF-α, IL-6 and IL-8 compared to resistant mares (Fumoso et al. 2003; Fumoso et al. 2007). Additionally, endometrial mRNA expression of the
anti-inflammatory cytokine IL-10 is lower during estrus in PBIE-susceptible mares, and higher in diestrous, compared to normal mares (Fumoso et al. 2007). Differences in the endometrial inflammatory response between normal and PBIE-susceptible mares are also evident following artificial insemination (AI). Expression of IL-1β, TNF-α, IL-8 and IL-10 mRNA is elevated seven days post-AI in PBIE-susceptible mares compared to resistant mares (Fumoso et al. 2003; Fumoso et al. 2007). Other differences include reduced IL-6, IL-1RN and IL-10 in susceptible mares compared to resistant mares 6 hours post Al (Woodward et al. 2013a).

Treatment approaches used in the management of mares with PBIE include ecbolic therapy such as oxytocin, to enhance uterine clearance, and immunomodulators such as dexamethasone and mycobacterial cell wall extract (MCWE) to suppress inflammation (LeBlanc 2010; Woodward and Troedsson 2013). The effect of immunomodulation on mRNA expression of inflammatory cytokines has been explored in both susceptible and resistant mares (Fumoso et al. 2003; Fumoso et al. 2007; Woodward et al. 2015). It appears that correction of the altered inflammatory response associated with susceptibility to PBIE may be an effective approach to treatment.

In addition to an altered inflammatory response post-breeding, researchers have demonstrated that elevated levels of nitric oxide (NO), a smooth muscle relaxant, may contribute to the decreased mechanical clearance of the uterus reported in PBIE mares (Alghamdi et al. 2005; Woodward et al. 2013b; Khan et al. 2017). Inducible nitric oxide synthase (iNOS) is the enzyme responsible for the production of induced NO in the equine uterus. PBIE susceptible mares had elevated NO levels in their uterine secretions and increased iNOS mRNA expression at 13 hours following Al (Alghamdi et al. 2005). A dose-dependent inhibition of NO on myometrial
contractility was demonstrated in vitro (Khan et al. 2017), suggesting that altered NO levels in PBIE-susceptible mares play a role in reduced myometrial contractility and uterine clearance.

Given what is currently understood about the pathophysiology of PBIE, use of a compound exhibiting both anti-inflammatory and NO-suppressive effects should be an effective approach to treatment. N-acetyl cysteine (NAC) is a mucolytic agent used in treatment of equine bacterial endometritis where a biofilm is suspected, or in cases where excess uterine mucus is present (LeBlanc and Causey 2009; Gores-Lindholm et al. 2009; LeBlanc 2010). Intra-uterine treatment with a 3.3% NAC solution in mares with a history of endometritis, either during the previous cycle or 48 hours prior to breeding, leads to higher than anticipated fertility rates (85%) (Gores-Lindholm et al. 2009; LeBlanc 2010). N-acetyl cysteine (NAC) reduces mucus viscosity by disrupting disulphide bonds (LeBlanc 2010), and has mild anti-oxidant and anti-inflammatory effects in multiple models of disease (Stanislaus et al. 2005; Cazzola et al. 2017). In horses, NAC inhibited serum-derived neutrophil oxidative burst when infused as a 3% solution and did not have an adverse effect on the endometrium of either fertile or barren mares (Gores-Lindholm et al. 2013). Intra-uterine and oral administration of NAC in mares led to decreased neutrophil and COX-2 staining on histologic endometrial samples from normal mares, confirming an anti-inflammatory effect (Witte et al. 2012; Melkus et al. 2013). However, no studies to date have examined effects of intrauterine infusion of NAC on NO levels, cytokine mRNA expression, or on cytokine concentration in uterine fluid.

The objective of this study was to determine if intra-uterine infusion of PBIE-susceptible mares with a 3.3% NAC solution in saline would decrease NO levels and alter the endometrial cytokine profile compared to control cycles.
3.3 MATERIALS AND METHODS

All the procedures performed on animals in this study were approved by the University of Guelph Animal Care Committee (Animal Utilization Protocol number 3213), in accordance with the Canadian Council on Animal Care guidelines.

3.3.1 Mare Selection, Study Design and Sampling

A randomized, blinded, cross over designed clinical trial was performed with a washout period (minimum one estrous cycle) between experimental cycles. The primary investigator, who performed all clinical evaluations, was blinded to mare assignment (treated versus control cycles) during data collection. In order to randomize the order of treatment and control cycles, mares were assigned a number, with those numbers subsequently randomly sorted into treated and control cycles.

Mares susceptible to PBI (n=10) were selected for inclusion in the clinical trial based on an endometrial biopsy score of IIb or III, normal cytology and negative bacterial culture on endometrial swab, and persistent accumulation of intra-uterine fluid 96 hours following challenge by insemination with killed semen (Alghamdi et al. 2005; Woodward et al. 2013a; Woodward et al. 2013b; Woodward et al. 2015). Mares were housed at the Arkell Equine Research Station at the University of Guelph for the duration of the study and were of various breeds (Standardbred and mixed breed) and ages (7-19 years).

Mares were monitored for estrus by trans-rectal palpation and ultrasound. Mares in diestrus, determined by presence of a corpus luteum (CL), were given an intra-muscular injection of cloprostenol (125mcg) (Estrumate®, Intervet Canada Corp., Kirkland, QC, Canada) to induce estrus. Once a follicle >35mm was identified during estrus, mares were given 2,500 IU of human
chorionic gonadotropin (hCG, Chorulon®, Intervet Canada Corp., Kirkland, QC, Canada) intravenously, to induce ovulation. Using a 21-inch insemination pipette (Partnar Animal Health, Ilberton, ON, Canada), mares were trans-cervically infused with either a 3.3% solution of NAC consisting of 30ml of N-acetyl cysteine (Acetylcysteine Injection 200mg/ml, Alveda Pharmaceuticals Inc., Toronto, ON, Canada) added to 150ml of 0.9% sodium chloride (0.9% Sodium Chloride Injection USP, Baxter Corporation, Mississauga, ON, Canada) (treated cycle) or 180ml of 0.9% sodium chloride (0.9% Sodium Chloride Injection USP, Baxter Corporation, Mississauga, ON, Canada) (control cycle). Twelve hours following infusion, the mares were inseminated with $1 \times 10^9$ killed spermatozoa in 30ml of semen extender, as previously described (Woodward et al. 2013a; Woodward et al. 2013b; Woodward et al. 2015; Khan et al. 2018).

Trans-rectal ultrasonography was performed beginning twelve hours after AI, and was repeated every 24 hours thereafter until 84 hours post-AI (12, 36, 60 and 84 hours), to evaluate endometrial edema grade (0-4; 0 = no edema; 4 = pathologic edema) and endometrial fluid volume. Fluid volume was calculated using the formula $V=length\times width\times depth$, each measured in cm. Multiple locations were measured in the uterine body and horns, and an average of 2-5 locations were used in the calculation. Additional samples taken at 12 and 60 hours post-AI consisted of an endometrial swab for cytology, collection of uterine fluid and an endometrial biopsy. Cytology samples were aseptically obtained using a guarded uterine swab (Kalayjian Industries Inc., Signal Hill, CA, USA) as previously described by Khan et al. (2018). Endometrial biopsy specimens were procured in an aseptic manner using a Pilling-Weck biopsy punch (Jorgensen Laboratories, Loveland, CO, USA), as described by Kenney (1978). The endometrial biopsy sample was immediately placed in 655µL of RNAlater (Sigma-Aldrich®, Oakville, ON,
Canada) and stored overnight at 4°C. The following morning, it was removed from the RNALater and stored at -80°C until analysis, as per the manufacturer’s instructions.

3.3.2 Real-Time Quantitative PCR

A pre-trial endometrial biopsy sample was obtained from a subset of mares (n=4), in addition to endometrial biopsies obtained at 12 and 60 hours post-Al for all mares. Total RNA from endometrial tissue was isolated with the Qiagen RNeasy™ kit (©QIAGEN, Toronto, CAN), according to the manufacturer's protocol. First strand cDNA was synthesized from 200 ng of RNA with 50 μm oligo primer and reverse transcriptase enzyme. The reference genes SDHA, β-actin, EeF2, RPS23 and GAPDH were amplified from different concentrations of template cDNA. Most reference genes were found to be inconsistent among mares, therefore, a specific set of reference genes was optimized for each mare and used to determine the relative expression of the gene of interest for each mare (Table 1). Reverse transcription of mRNA was initiated to inactivate the reverse transcriptase and simultaneously activate the thermostable DNA polymerase. cDNA was amplified in triplicate by qRT-PCR in a LightCycler 480 instrument (Roche Life Science, Laval, PQ) using SYBR Green (Roche) and primers to equine IL-1β, IL-6, IL-10, and TNF-α (Table 2). Cycle threshold (Ct) values were calculated and normalized to each mare’s specific reference gene pair (calibrator normalized relative quantification).

3.3.3 Intra-Uterine Fluid Collection

Intra-uterine fluid was collected in one of two ways. In mares with low-to-moderate fluid volume, a Bivona catheter (Partnar Animal Health) was passed aseptically through the cervix and into the mare’s uterus. After inflating the cuff, a hand held suction pump (Nalgene™ hand-operated vacuum pump, Sigma-Aldrich® Oakville, ON, Canada) was attached to the catheter and
an assistant applied external suction until fluid was obtained in the tube. In mares with a large volume of intra-uterine fluid, a sample was readily obtained by free-flow during endometrial swab sampling, through the outer plastic guard. The fluid sample was immediately placed in a polypropylene centrifuge tube (Corning® Incorporated, Corning, NY, USA) and held at room temperature until processing. Once returned to the laboratory, the fluid was centrifuged at 1500 x g for 12 minutes to remove cellular debris. The supernatant was aspirated, placed in 1ml aliquots (Fisherbrand®, Cryogenic Vial 1.2mL, Fisher Scientific, Markham, ON, Canada) and stored at -20°C for further analyses.

3.3.4 ELISA Determination of Interleukin-6 Concentration in Uterine Fluid

Intrauterine IL-6 concentration was determined using the Equine IL-6 DuoSet®ELISA (R&D Systems™, Minneapolis, MN, USA) according to manufacturer instructions. Samples were diluted 1:20 in reagent diluent (1% BSA in PBS) and run in duplicate. The intra-assay coefficient of variation was 7.4% and the inter-assay coefficient of variation was 10.9%. The kit was validated for equine intrauterine fluid by determination of percent recovery and linearity, according to R&D Systems™ Spike, Recovery & Linearity Protocol. The detailed validation process can be found in Appendix 2.

3.3.5 Nitric Oxide Concentration

Nitric oxide is unstable and has a short half-life in vivo, necessitating the measurement of its metabolites, nitrate and nitrite, as alternatives. The concentration of nitrate and nitrite present in intrauterine fluid was measured to provide an estimate of NO concentration, using the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical Co., Ann Harbor, MI, USA), according to
the manufacturer’s instructions. Samples were diluted 1:5 with buffer solution, and run in duplicate.

3.3.6 Statistical Analyses

Statistical analyses were performed using the SAS software program (version 9.3, SAS Institute, Inc., Cary, NC, USA). Only estrous cycles fitting the definition of PBIE were included in the analysis (n=9 saline control; n=9 NAC treatment cycles). Differences in endometrial mRNA expression of IL-6, IL-10, TNF-α and IL-1β, as well as IL-6 and NO concentrations in uterine fluid, were compared between control and treated cycles over time (12h and 60h post-Al) by repeated measures ANOVA. The Akaike Information Criterion (AIC) was utilized to determine the best-fit model. The Shapiro-Wilk test determined that the data was not normally distributed and required logarithmic transformation prior to analysis, therefore results are presented as medians (back transformed) +/- upper and lower confidence limits.

Interaction effects between treatment and time were examined in the model, and the p value was set at <0.05 for statistical significance. When there was no statistically significant treatment-by-time interaction seen, the data from both treatment groups were combined to examine the effect of time post-Al, and data from both time points were combined within each treatment group to evaluate for any main effects of treatment.

For the subset of mares with baseline biopsies available (T=0; n=4), differences in IL-6, IL-10, TNF-α and IL-1β mRNA expression were also compared between baseline, 12 hours and 60 hours post-Al using repeated measures ANOVA.
3.4 RESULTS

3.4.1 Effect of Intra-Uterine Treatment of 3.3% NAC in PBIE Susceptible Mares

When both time points were combined within each treatment group, intrauterine infusion with 3.3% NAC 12 hours prior to AI did not affect endometrial mRNA expression of IL-6 (p=0.86) (Appendix 3: Figure 1), IL-10 (p=0.58) (Appendix 3: Figure 2), TNF-α (p=0.92) (Appendix 3: Figure 3), or IL-1β (p=0.65) (Appendix 3: Figure 4) compared to untreated cycles (Table 3). NAC infusion had no effect on the concentration of IL-6 in uterine fluid as determined by ELISA (p=0.083) (Figure 1), nor did it affect the amount of NO in uterine fluid (p=0.46) (Figure 2).

3.4.2 Changes in Endometrial Inflammation in Response to AI Over Time

a) mRNA expression – 12 versus 60 hours (n=9 mares)

Endometrial IL-6 mRNA expression was significantly greater at 12 hours post AI compared to 60 hours post AI, when both treatment groups were combined within each time point (p=0.02) (Figure 3). In the control cycle, IL-6 mRNA expression at 12 hours post AI was significantly greater than at 60 hours post AI (p=0.02) (Appendix 3: Figure 5). Endometrial mRNA expression did not change from 12 to 60 hours for IL-10 (p=0.73) (Appendix 3; Figure 6), TNF-α (p=0.86) (Appendix 3: Figure 7) and IL-1β (p=0.6) (Appendix 3: Figure 8), when both treatment groups were combined within each time point (Table 4).

b) mRNA expression – baseline versus 12 and 60 hours post-AI (n=4) (Table 5)

For the subset of four mares with baseline biopsies, there was an overall significant main effect of time on IL-6 mRNA expression (p=0.0004) (Figure 4) as well as an overall significant effect of time on IL-1β mRNA expression (p=0.0030) (Figure 5). In contrast, mRNA expression of IL-10
(p=0.1779) (Figure 6) and TNF-α (p=0.0744) (Figure 7) did not change over time. No significant treatment main effect was seen for the subset of mares with baseline biopsies.

### 3.4.3 IL-6 Concentration as Measured by ELISA

IL-6 concentration in intrauterine fluid (ELISA) did not change over time post AI, when both treatment groups were combined within in each time point (p=0.42) (Figure 8). There was no time by treatment interaction.

### 3.4.4 Nitric oxide

Although NO increased from 12 to 60 hours post-AI, the change in NO concentration over time did not reach significance, when both treatment groups were combined within in each time point (p=0.056) (Figure 9). There was no time by treatment interaction.

### 3.5 DISCUSSION

The anti-inflammatory effects of NAC, including cytokine modulation, have been shown in multiple models of animal and human disease. In a human chronic obstructive pulmonary disease (COPD) in vitro study, NAC inhibited pro-inflammatory cytokines (IL-1β, IL-8, TNF-α) (Cazzola et al. 2017). Similarly, in a multiple sclerosis (MS) study, treatment with NAC led to inhibition of monocyte/macrophage induced TNF-α, IFN-γ, IL-1β production and stimulated IL-10 production (Stanislaus et al. 2005). However, the results of the current study suggest that the anti-inflammatory effects of intra-uterine infusion of a 3.3% NAC solution were too mild to result in alterations of cytokine expression. By comparison, other immunomodulatory therapies have demonstrated the potential to alter the inflammatory cytokine profile of PBIE-susceptible mares. Some treatment modalities (lactoferrin, Mycobacterium Cell Wall Extract (MCWE) and
dexamethasone) lower pro-inflammatory cytokine mRNA expression, such as TNF-α (Fedorka et al. 2016) and IL-1β (Fumoso et al. 2003; Woodward et al. 2015) and increase mRNA expression of IL-10, an anti-inflammatory cytokine (Fumoso et al. 2007) to levels more typical of normal mares. However, many of the other cytokines evaluated in these studies (IL-6, IL-1RN, IL-8) were unaffected by these treatment methods. It is possible that a treatment effect following NAC infusion was not apparent due to the choice of cytokines evaluated in the current study; further studies examining the effects on other endometrial cytokines may elicit additional information. Other possible reasons for the lack of treatment effect of NAC include the selection of time points for sampling, differences in the definition of PBIE mares between studies and differences in qPCR methodology, including the selection of reference genes.

Methodologies used in qPCR differ across studies and may account for variability in endometrial cytokine mRNA expression. Specifically, the choice of reference gene(s) appears to vary. In most studies, only one reference gene is selected to determine relative gene expression, and limited reporting of the validation process is given (Fumoso et al. 2003; Fumoso et al. 2007; Christoffersen et al. 2012a; Christoffersen et al. 2012b; Woodward et al. 2013a; Woodward et al. 2013b; Woodward et al. 2015; Fedorka et al. 2016; Fedorka et al. 2017; Fedorka et al. 2018). Recently, it has been suggested that more than one reference gene should be selected, in order to increase the validity of the analysis (Bustin et al. 2009). In addition, reference genes previously believed to be stable have since been shown to have a large degree of variation across studies (Kozera et al. 2013). Therefore, validation of chosen reference genes specific to the species, experimental design, and tissue of interest would improve repeatability across studies (Jacob et al. 2013; Kozera et al. 2013; Bustin et al. 2017). Significant variability is known to occur in equine
gene expression across different tissues (Tessier et al. 2017). In the present study, two stable reference genes were selected and validated for each mare, and seven of the nine mares shared at least one gene within the reference gene pairs (EeF2) (Table 1). Given that cytokine mRNA expression is measured relative to that of selected reference genes, it is therefore possible that some of the differences noted across studies with respect to relative cytokine gene expression in equine endometritis, are due to the differences in research methodologies and the selection of reference genes between studies.

Intra-uterine infusion with NAC did not alter NO levels post-Al in PBIE-susceptible mares. Elevated intra-uterine NO levels in PBIE susceptible mares are suspected to play a role in decreased uterine contractility and uterine clearance (Alghamdi et al. 2005; Woodward et al. 2013b; Khan et al. 2017; Khan et al. 2018). Alghamdi et al. (2005) showed that PBIE susceptible mares had higher NO in their uterine secretions and higher iNOS mRNA expression 13 hours post-Al compared to resistant mares. In addition, Woodward et al. (2013b) showed that susceptible mares had elevated mRNA expression of iNOS at 2 and 6 hours post-Al. Nitric oxide production and iNOS synthesis were reduced by treatment with NAC in rat in-vitro studies, murine pre-term labor studies, and multiple sclerosis studies (Pahan et al. 1997; Bergamini et al. 2001; Majano et al. 2004; Stanislaus et al. 2005; Paintlia et al. 2008). Mycobacterium cell wall extract (MCWE), an immunomodulator, given IV 24 hours prior to AI decreased uterine NO in susceptible mares. In the same study, administration of 50mg of dexamethasone IV 24 hours prior to AI, did not decrease uterine NO concentrations in PBIE- susceptible mares (Woodward et al. 2013b). In-vitro treatment of endometrial tissue samples with 1400 W, a highly specific iNOS inhibitor, resulted in decreased endometrial NOS activity in both susceptible and resistant mares (Khan et
al. 2018). It appears that NAC is a very mild iNOS inhibitor, and the dose and timing of NAC administration in this study may explain the lack of effects seen.

The preparation and dose of 3.3% NAC intra-uterine infusion was chosen for several reasons. This dose was first described by LeBlanc and Causey (2009) as being mucolytic and able to dislodge inspissated uterine secretions in mares with a history of infertility. The effects of a 3.3% NAC intra-uterine infusion were further characterized by Gores-Lindolhm et al. (2009) and LeBlanc (2010), who showed improved pregnancy rates (85%) in mares with a history of endometritis. Further, Gores-Lindholm et al. (2013) demonstrated that treatment with NAC at this dose led to decreased uterine mucus thickness, and had no adverse effects on the endometrium in barren and healthy mares with Kenney biopsy scores ranging from I to Iib. Pregnancy rates of 77% were attained when using this dose combined with uterine lavage, oxytocin treatment and as-needed intra-uterine antibiotics. Since that time, 3.3% NAC has been recommended for cases of mucus hypersecretion or in mares with suspected biofilm (Lyle 2012; Troedsson 2012; Buczkowska et al. 2015; Scoggin 2016). Recently, an in vitro study demonstrated that 3.3% NAC disrupted E. coli biofilm and readily killed E. coli within the biofilm (Ferris et al. 2016). However, it is possible that the dose chosen was too low to affect cytokine expression and NO concentrations. Tirouvanziam et al. (2006) showed that only high doses of NAC affected cytokine concentration in a human cystic fibrosis study. In addition, NO release from rat peritoneal macrophages was significantly reduced when higher doses of NAC were utilized (Pahan et al. 1997). In most of these studies, NAC was administered pre-breeding or prior to induction of inflammation, justifying the timing of administration in our study. However, it is possible that treatment during an inflammatory response may be more beneficial than pre-
treatment. In fact, Tirouvanziam et al. (2006) showed clinical improvement in already ill patients treated with oral NAC.

Interleukin-6 was the only cytokine showing significant differences in relative expression across time points. Increased expression of IL-6 at 12 hours compared to 60 hours post-AI, is similar to the findings of Woodward et al. (2013a), who showed elevated IL-6 mRNA expression between 2 and 12 hours post-AI, and Fumoso et al. (2003) who showed higher expression of IL-6 mRNA at 24 hours compared to 7 days post-AI. While these timelines are not identical to those chosen in our study, the trends noted between studies are similar in that IL-6 mRNA expression is elevated at time points nearest AI and decreases over time.

The lack of differences observed for endometrial IL-10, TNF-α and IL-1β mRNA expression between 12 and 60 hours post-AI is in contrast to other studies, where some of these cytokines changed in their expression over time. Interleukin-10 was elevated in susceptible mares at 2 and 24 hours, and IL-1β mRNA expression was increased at 2 and 6 hours compared to baseline levels, following AI (Woodward et al. 2013a). Interleukin-10 mRNA expression in susceptible mares was lower at 24 hours compared to 7 days post-AI (Fumoso et al. 2007). Interestingly, these studies also showed no changes for other cytokines. No changes in TNF-α mRNA expression were noted in Woodward et al. (2013a), nor did IL-1β mRNA expression change between 24 hours and 7 days post-AI (Fumoso et al. 2003).

Baseline cytokine mRNA expression is reportedly different in PBIE susceptible mares compared to normal mares (Fumoso et al. 2003; Fumoso et al. 2007). Comparisons to cytokine mRNA expression at baseline have also been made in PBIE susceptible mares following AI, with IL-1β, IL-6, IL-8, IL-10 and IFN-γ being elevated from baseline at various time points following
insemination (Woodward et al. 2013a). In our study, although only a subset of mares had baseline biopsies taken for qPCR analysis, baseline mRNA expression was lower for all cytokines tested, and increased at 12 hours following AI. This elevation in cytokine expression decreased by 60 hours post-AI, but did not return to baseline levels. The changes observed in IL-6 and IL-1β mRNA expression over time are reflective of the inflammatory response to AI. However, the lack of significant changes across time for IL-10 and TNF-α mRNA expression from baseline was unexpected. Tumor Necrosis Factor -α is pro-inflammatory and would be expected to rise near the time of AI to evoke endometrial inflammation. Interleukin-10 is anti-inflammatory and would be expected to rise sometime after AI, to mediate the inflammatory response. Our results are similar to Woodward et al. (2013a) who found no differences in TNF-α following AI in susceptible mares and a much lower expression of IL-10 in susceptible mares in comparison to resistant mares. IL-10 and TNF-α may play less of a role in PBIE than previously suspected.

Despite an increase in IL-6 mRNA expression 12 hours post-AI, intra-uterine IL-6 concentration as measured by ELISA, was not significantly different across time points post-AI in this study. However, IL-6 concentration was greatest at 12 hours post-AI, even though not statistically significant, demonstrating that IL-6 concentration follows the same pattern as mRNA expression. In the present study, considerable variability in IL-6 concentration across mares made detection of differences difficult, and the lack of significance could be attributed to the large variability in our data.

In conclusion, treatment with 3.3% NAC as an intrauterine infusion 12 hours prior to AI did not affect mRNA expression of IL-6, IL-10, TNF-α and IL-1β, nor did it affect intra-uterine IL-6 concentration and NO levels, in PBIE-susceptible mares. In general, when comparing mRNA
expression to baseline values for the subset of mares with pre-trial biopsies, it appears that AI in itself causes an increase in mRNA expression across time points post-AI. Subsequent differences for all mares between 12 and 60 hours post-AI are less evident, notably for IL-10, TNF-α and IL-1β, but are clear for IL-6. IL-6 mRNA expression is greatest at 12 hours compared to 60 hours post-AI, implicating it as likely playing a role in the manifestation of PBIE in susceptible mares. Future studies should be aimed at characterizing the inflammatory cytokine mRNA expression pattern post-AI and how it differs from normal in PBIE susceptible mares. From there, what is known of the effect of different treatment modalities on these cytokines at specific time points could be utilized accordingly in treating PBIE susceptible mares.
3.6 REFERENCES


Figure 1. Median IL-6 concentration in intra-uterine fluid (including lower and upper confidence limits) of PBIE-susceptible mares (n=9) in N-acetyl cysteine treated (TX) and saline (CONTROL) cycles when time points (12h, 60h post-Al) were combined for each cycle. Differing superscripts are significant at p<0.05.

Figure 2. Median nitric oxide in intra-uterine fluid (including lower and upper confidence limits) of PBIE-susceptible mares (n=9) in N-acetyl cysteine treated (TX) and saline (CONTROL) cycles when time points (12h, 60h post-Al) were combined for each cycle. Differing superscripts are significant at p<0.05.
Figure 3. Median IL-6 mRNA expression (including lower and upper confidence limits) of PBIE-susceptible mares (n=9) at 12 and 60 post AI when treatment cycles were combined within each time point. A significant time effect was observed (p=0.02). Differing superscripts are significant at p<0.05 (Abbreviations: RQ, relative quantification).

Figure 4. Median IL-6 mRNA expression (including lower and upper confidence limits) in a subset of PBIE-susceptible mares with baseline biopsies (n=4). Differences between baseline, N-acetyl cysteine treated (TX) at 12 hours and (TX) at 60 hours post-AI are depicted by differing upper case letters. Differences between baseline, saline treated (CONTROL) at 12 hours and (CONTROL) at 60 hours are depicted by differing lower case letters. Significance was defined as p<0.05 (Abbreviations: RQ, relative quantification).
**Figure 5.** Median IL-1β mRNA expression (including lower and upper confidence limits) in a subset of PBIE-susceptible mares with baseline biopsies (n=4). Differences between baseline, N-acetyl cysteine treated (TX) at 12 hours and (TX) at 60 hours post-AI are depicted by differing upper case letters. Differences between baseline, saline treated (CONTROL) at 12 hours and (CONTROL) at 60 hours are depicted by differing lower case letters. Significance was defined as p<0.05 (Abbreviations: RQ, relative quantification).

**Figure 6.** Median IL-10 mRNA expression (including lower and upper confidence limits) in a subset of PBIE-susceptible mares with baseline biopsies (n=4). Differences between baseline, N-acetyl cysteine treated (TX) at 12 hours and (TX) at 60 hours post-AI are depicted by differing upper case letters. Differences between baseline, saline treated (CONTROL) at 12 hours and (CONTROL) at 60 hours are depicted by differing lower case letters. Significance was defined as p<0.05 (Abbreviations: RQ, relative quantification).
Figure 7. Median TNF-α mRNA expression (including lower and upper confidence limits) in a subset of PBIE-susceptible mares with baseline biopsies (n=4). Differences between baseline, N-acetyl cysteine treated (TX) at 12 hours and (TX) at 60 hours post-AI are depicted by differing upper case letters. Differences between baseline, saline treated (CONTROL) at 12 hours and (CONTROL) at 60 hours are depicted by differing lower case letters. Significance was defined as p<0.05 (Abbreviations: RQ, relative quantification).

Figure 8. Differences in median IL-6 concentration in intra-uterine fluid (including LL and UL) of PBIE-susceptible mares at 12 and 60 hours post AI. Differing superscripts are significant at p<0.05.
Figure 9. Differences in median NO in intra-uterine fluid (including LL and UL) of PBIE-susceptible mares at 12 and 60 hours post AI. Differing superscripts are significant at p<0.05.
Table 1. Pairs of reference genes used in clinical trial for each PBIE-susceptible mare.

<table>
<thead>
<tr>
<th>Reference genes</th>
<th>DAISY</th>
<th>GERTRUDE</th>
<th>DRAGON</th>
<th>ASTER</th>
<th>LEAH</th>
<th>DAPHNEE</th>
<th>JANIE</th>
<th>Betty Boop</th>
<th>FERN</th>
</tr>
</thead>
<tbody>
<tr>
<td>EeF2/B-actin</td>
<td></td>
<td></td>
<td>✔</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPS23/SDHA</td>
<td>✔</td>
<td></td>
<td>✔</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EeF2/GAPDH</td>
<td>✔</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EeF2/RPS23</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EeF2/SDHA</td>
<td></td>
<td></td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Primer sequences for amplification of reference genes and selected cytokines.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>EeF2</td>
<td>CCAACGGCAAGTTCAGCAAG</td>
<td>GCTTCTCGATCAGCTTTGCC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GTTTGTGATGGGCGTGAAACC</td>
<td>TTGGCAGCACCAGTAGAACG</td>
</tr>
<tr>
<td>SDHA</td>
<td>GCAGAAGAAGGCATTTGAGG</td>
<td>CCTGTCGATTACGGGTCTGT</td>
</tr>
<tr>
<td>RPS23</td>
<td>ACGAGATCAGAAGTGCCAGC</td>
<td>ACCTAAAGCGGACTCCAGG</td>
</tr>
<tr>
<td>β-actin</td>
<td>GACCCAGATCATGTTTGAGACCT</td>
<td>TGATGGAGTTGAAGTAGTTTCG</td>
</tr>
<tr>
<td>TNF-α</td>
<td>AGCCCATGTTGTAGCAAACC</td>
<td>CACTTTGGGGTGATCGGC</td>
</tr>
<tr>
<td>IL-6</td>
<td>CCCGTGACCAAACCTGCAAAA</td>
<td>TGTTGTGTTTCCAGGCACTCA</td>
</tr>
<tr>
<td>IL-10</td>
<td>CCAGTCTGAGAACAGCTGCA</td>
<td>GCTCCACTGCCTGCTCTTA</td>
</tr>
<tr>
<td>IL-1β</td>
<td>GGGTGTGAACATGCTGCAGG</td>
<td>CTCATCAGAAAGCTGGGTGCA</td>
</tr>
</tbody>
</table>

Table 3. Table of median mRNA expression with lower (LL) and upper (UL) limits of IL-6, IL-10, TNF-α and IL-1β in PBIE-susceptible mares, following intra-uterine treatment with 3.3% NAC treated (TX) and saline (CONTROL) cycles (n=9) when both time points (12h, 60h post-Al) were combined within each treatment cycle.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Median mRNA Expression: NAC Treatment Cycle</th>
<th>LL</th>
<th>UL</th>
<th>Median mRNA Expression: Saline Control Cycle</th>
<th>LL</th>
<th>UL</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>0.28</td>
<td>0.11</td>
<td>0.7</td>
<td>0.26</td>
<td>0.1</td>
<td>0.64</td>
<td>0.86</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.018</td>
<td>0.008</td>
<td>0.04</td>
<td>0.024</td>
<td>0.01</td>
<td>0.052</td>
<td>0.58</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.09</td>
<td>0.05</td>
<td>0.15</td>
<td>0.09</td>
<td>0.05</td>
<td>0.16</td>
<td>0.92</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.011</td>
<td>0.004</td>
<td>0.026</td>
<td>0.013</td>
<td>0.005</td>
<td>0.032</td>
<td>0.65</td>
</tr>
</tbody>
</table>
Table 4. Table of median mRNA expression with lower (LL) and upper (UL) limits of IL-6, IL-10, TNF-α and IL-1β at 12 and 60 hours post-AI in PBIE-susceptible mares (n=9) when treatment cycles were combined at each time point.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Median mRNA expression 12 hours post-AI</th>
<th>LL</th>
<th>UL</th>
<th>Median mRNA expression 60 hours post-AI</th>
<th>LL</th>
<th>UL</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>0.5</td>
<td>0.2</td>
<td>1.25</td>
<td>0.14</td>
<td>0.06</td>
<td>0.36</td>
<td>0.02</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.023</td>
<td>0.01</td>
<td>0.05</td>
<td>0.019</td>
<td>0.009</td>
<td>0.04</td>
<td>0.73</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.091</td>
<td>0.05</td>
<td>0.16</td>
<td>0.086</td>
<td>0.05</td>
<td>0.15</td>
<td>0.86</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.013</td>
<td>0.005</td>
<td>0.03</td>
<td>0.010</td>
<td>0.004</td>
<td>0.026</td>
<td>0.60</td>
</tr>
</tbody>
</table>

Table 5. Table of median mRNA expression with lower (LL) and upper (UL) confidence limits of IL-6, IL-10, TNF-α and IL-1β at baseline, 12 and 60 hours post-AI for the N-acetyl cysteine treated (TX) and saline (CONTROL) cycles for a subset of PBIE-susceptible mares (n=4).
CHAPTER FOUR: GENERAL DISCUSSION

4.1 POSSIBLE LINKS BETWEEN MRNA EXPRESSION AND PBIE CLINICAL SIGNS

Persistent breeding induced endometritis continues to be a source of infertility in the equine breeding industry (Newcombe 1997). PBIE is caused by mechanical factors, such as poor myometrial contractility, and by differences in the uterine immune response following breeding (Christoffersen and Troedsson 2017). Altered inflammatory cytokine expression is believed to play a role in PBIE, with susceptible mares having increased and persistent mRNA expression of pro-inflammatory cytokines and decreased mRNA expression of anti-inflammatory cytokines following AI (Woodward and Troedsson 2015; Christoffersen and Troedsson 2017). Many studies have examined cytokine mRNA expression in both PBIE resistant and susceptible mares, but none have related mRNA expression to clinical signs associated with PBIE. N-acetylcysteine, used as an intra-uterine mucolytic in mares, has also been shown to have anti-inflammatory properties, such as affecting cytokine mRNA expression in other models of disease (Stanislaus et al. 2005; LeBlanc 2010; Cazzola et al. 2017).

The clinical trial reported in this work was based on our hypothesis that treatment of PBIE susceptible mares with NAC would alter inflammatory cytokine mRNA expression, leading to the resolution of PBIE clinical signs. The results of our study did not reveal any beneficial effect of NAC treatment on either PBIE clinical signs or cytokine mRNA expression, making it difficult to assess any improvement of clinical signs by means of altered mRNA expression, following treatment. However, some speculations can still be made regarding this concept by examining patterns of cytokine mRNA expression and comparing them to the clinical signs observed at the
different time points post-Al. IL-6 mRNA expression was significantly greater at 12 hours compared to 60 hours post Al. This is similar to % neutrophil levels and endometrial edema scores, both of which were significantly elevated at time points nearest Al (12 hours post Al for % neutrophils; 12 and 36 hours post Al for uterine edema scores). Similar, though slightly different trends were noted for intra-uterine fluid (IUF) volume, with significantly larger fluid volumes at 36 hours compared to 12, 60 and 84 hours post Al. This pattern of inflammation was expected as neutrophils are recruited following breeding (Kotilainen et al. 1994) starting at 4 hours and peaking at 8 hours after Al (Katila 1995). In PBIE susceptible mares, this pattern is similar but is even more pronounced (Woodward et al. 2013). From this, one could speculate that IL-6 plays a role in promoting inflammation early on in the uterine response, and that this in turn results in the clinical signs observed in PBIE mares. Interleukin-6 has both pro- and anti-inflammatory properties. It is involved in the initiation of the inflammatory response but also controls inflammation by means of decreasing neutrophil recruitment (Tizard 2013). The effects of IL-6 on neutrophils were apparent in this study, where % neutrophils decreased significantly by 60 hours post Al, possibly as a result of earlier IL-6 mRNA up-regulation.

The persistence of clinical signs in our PBIE study mares, albeit reduced at 60 hours from 12 hours, could be the result of a lack of IL-10 mRNA up-regulation, as relative expression of IL-10 did not change between 12 and 60 hours post-Al. Interleukin-6 promotes expression of IL-10, which is an anti-inflammatory cytokine (Tizard 2013). It is possible that altered IL-6 expression also led to aberrations in IL-10 expression. We hypothesize that the failure of increased IL-10 expression needed to modulate the inflammatory response to breeding, could account for some of the persistence of clinical signs seen with PBIE. One would expect that under normal
circumstances, levels of IL-10 mRNA expression would be highest following inflammation. Yet, if this increase in IL-10 does not occur, as is suspected in PBIE mares, persistent inflammation, and thereby persistence of clinical signs, results. This supports the notion that anti-inflammatory cytokine expression is altered in PBIE susceptible mares, and that there may in fact be lack of an anti-inflammatory response, not just an exaggerated pro-inflammatory response, playing a role in this condition.

Conclusions on the effects of the pro-inflammatory cytokines TNF-α and IL-1β, on clinical signs cannot be made due to a lack of change noted across time points following AI. It is possible that IL-1β and TNF-α are in fact affected in PBIE susceptible mares, but that the time point of significant increase was not examined in this study. Further investigations are required to determine how these cytokines may impact clinical signs associated with PBIE. Moreover, in an attempt to better link clinical outcomes with cytokine mRNA expression, future studies should examine how treatment methods known to alter cytokine mRNA expression, such as MCWE, dexamethasone, platelet rich plasma (PRP) and lactoferrin, affect PBIE clinical signs.

4.2 LIMITATIONS

One of the largest limitations of our study was the inability to measure IL-10, TNF-α, and IL-1β concentration in uterine fluid. While equine ELISA kits exist for these cytokines, no validations have been published specifically for equine uterine fluid. As such, validation was attempted for these kits but was unsuccessful. The most common reason for failure of validation was suspected to be matrix interference due to an unidentified compound or substance within uterine fluid that caused either false increases or decreases of measured cytokine
concentrations. Many of the samples were viscous in nature, possibly contributing to matrix interference of the assay. The viscosity of the sample was suspected to be due to the high protein content in fluid obtained from an inflamed uterus. Some ELISA kits can be validated despite this by simply diluting the sample appropriately with the reagent diluent provided in the kit (MC personal communication, R&D systems technical support). This was the case for IL-6, the only successfully validated cytokine, as the sample was diluted 1:20; however, the appropriate dilution factor varies for each individual cytokine. The dilution factor should be great enough to overcome matrix interference, but not so much so that the cytokine of interest can no longer be detected. Various dilutions were attempted for IL-10, TNF-α, and IL-1β, as reported in Appendix 4.

Although IL-6 was successfully validated, the process itself had limitations, notably in terms of the negative control. For validation, a positive and negative control were utilized and consisted of PBIE-susceptible mares enrolled in the study (positive controls) and normal mares (negative controls) (Appendix 2). Undiluted uterine fluid was readily available for PBIE-study mares, however, normal mares did not have any uterine fluid. The procurement of endometrial fluid from these mares was through a low-volume lavage, ultimately diluting the sample. While the exact volume of infused fluid was known for this procedure, the amount recovered was not 100%, leading to an unknown dilution factor. This made comparisons between negative and positive control mares difficult. Since fluid could not be collected in any other way in normal mares, this represented another limitation for the validation process.

Other validation methods that could have been attempted include purification or washing of the sample with spin columns and adding fetal bovine serum (FBS) to the reagent diluent. The
quantity of FBS to be added to the reagent diluent varies greatly based on the type of sample, and can range from 10-50% (MC personal communication, R&D systems technical support). FBS and spin columns could have either bound the viscous component of the sample or filtered it out. Neither of these methods were performed due to the limited volume of sample fluids available from the study mares, since each cytokine would require its own validation attempts with either FBS, spin columns or both. Further studies should be performed to determine if the addition of FBS to reagent diluent contributes to the validation process and if so, what the appropriate FBS concentration is when running these kits on equine uterine fluid. In the same regard, perfecting the methodology for endometrial fluid to be filtered through spin columns could also be a topic of research.

The equine species has a great deal of variability in its gene expression (Tessier et al. 2017), making the selection of reference genes a challenge. Consequently, each study mare had her own set of reference genes. This method is valid as the reference genes selected were expressed in a stable manner for each individual mare, allowing for comparisons to be made across them. This method is superior to choosing two reference genes for all mares, that are not expressed in a stable manner. The latter could be accomplished by employing software methodology such as GeNorm or NormFinder, which are designed to choose the two best overall reference genes for all subjects in a study. This was attempted in our clinical trial, however, large variability in the data meant that no two best reference genes could be identified, leading to the selection of the best two for each individual mare. To confirm that reference genes were not affecting result outcomes, the variable “reference gene” was entered into the repeated measures ANOVA model to confirm that they were non-significant.
An additional limitation of this study was the repeated biopsies and procurement of fluid at multiple time points during each estrous cycle. The effect of previous time point sampling on subsequent samples is unknown. Watson and Sertich (1992) and Christoffersen et al. (2012), demonstrated that repeated biopsies were not detrimental to uterine health or pregnancy rates; however, no studies have specifically examined the effects of procurement of fluid, endometrial cytology and biopsy combined on the level of inflammation 48 hours later. Ideally, one sampling time point would have taken place per cycle, with multiple cycles of treatment and control, although the variability across cycles becomes greater with this approach.

4.3 OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

Despite significant amounts of research, the mechanisms underlying the pathophysiology of PBIE in mares remain poorly understood. This study provided more information on mRNA expression of IL-6, IL-10, TNF-α and IL-1β in PBIE-susceptible mares at both 12 and 60 hours post-AI. Baseline mRNA expression of these cytokines was also obtained for a subset of mares. While statistically significant differences were not often noted between these time points, the information and trends found in the study contributed to defining the general cytokine profile and alterations in the immune response of PBIE-susceptible mares. Knowing more about different cytokine expression at various time points post-AI and how these differ from normal mares may help target the development of new treatment modalities. Furthermore, integrating the immune response with the clinical picture of PBIE, by assessing both cytokine expression and clinical signs at various time points, could help complete our understanding of PBIE. For example, PRP shows promise in its effects on IL-6, IL-8 and IL-1β and iNOS mRNA expression 24 hours post-
AI in PBIE-susceptible mares (Metcalf et al. 2012). Elevated NO is believed to play a role in delayed uterine clearance. Other time points could be examined following treatment with PRP and clinical impacts could be assessed, such as fluid accumulation, as it has been shown that iNOS is affected. Further research is needed to determine how other cytokines would be affected at different time points by various treatments and how clinical signs would thereby be impacted.

Woodward et al. (2013) demonstrated that many cytokines change significantly in their mRNA expression at 6 hours post AI. This is not to say however, that cytokine mRNA expression also does not change at other time points post AI, or that other cytokines are altered at different time-points. Up-regulation of mRNA expression of inflammatory cytokines can be extremely short-lived and could easily be missed if the appropriate time points are not selected for each cytokine of interest. Increased expression may also be extremely mild in nature, making differences difficult to detect, even in extreme inflammatory models, such as septic shock (MC personal communication, Dr. Wood). In future studies, additional time points both immediately after AI and later in the immune response, should be examined in order to capture changes to cytokine mRNA expression across a broader timeline of the immune response. Additional cytokines of interest include IL-8, IL-1RA and IFN-γ. IL-8 is involved in neutrophil chemotaxis and it is well-known that neutrophils are elevated in PBIE-susceptible mares. IL-1RA, encoded by IL-1RN gene, is an anti-inflammatory cytokine involved in the regulation of IL-1β whereas IFN-γ is pro-inflammatory. IL-1RN and IFN-γ changed across certain time points in the study by Woodward et al. (2013) and recently, PBIE-susceptible mares treated with CRISP-3 and lactoferrin had altered endometrial IL-1RN and IFN-γ expression (Fedorka et al. 2018). The
additional examination of these cytokines in future studies would certainly add to our understanding of the pathophysiology of PBIE.

Treatment with NAC had no effect on the resolution of clinical signs or on endometrial cytokine mRNA expression in PBIE susceptible mares. However, a significant and unexpected finding was the severe detrimental effects of treatment with NAC on the endometrium in PBIE-susceptible mares. Loss of endometrial luminal epithelium (erosion), diffuse, deep-seated neutrophil infiltration and presence of neutrophils in the glandular lumen were some of the changes noted in NAC treated cycles, and are similar to changes noted following intra-uterine irritants such as kerosene. These results were particularly surprising given that NAC is widely used in mares that could be susceptible to PBIE, such as those with bacterial endometritis with a biofilm or in mares with abnormal mucus accumulation. The results from this study should prompt re-evaluation of the practice of NAC intrauterine infusion in mares with a history suggestive of PBIE.

This is the first study demonstrating the adverse effects of NAC on the endometrium of PBIE mares. Previous studies suggested NAC did not appear to have an effect on the endometrium, however those studies included normal mares or mares with histories of barrenness, but not necessarily PBIE. As was found for kerosene (Bracher et al. 1991), it is possible that the detrimental effects of NAC are exacerbated in PBIE mares or those with poor biopsy grades. Furthermore, it is possible that NAC in itself is not detrimental to the endometrium histologically, but when it is combined with AI in PBIE-susceptible mares, the adverse effects are severe, and that similar changes could be seen with other irritants combined with NAC. Further studies are needed to confirm the adverse effects of NAC on mares with different endometrial
biopsy grades and/or when given in combination with AI or other treatments. This would allow for clinicians to better target their patients when considering NAC infusion and allow for appropriate measures to be taken, such as performing uterine lavage between NAC infusion and breeding when NAC treatment is necessary. It is evident that more research needs to be undertaken before treating any PBIE-susceptible mare with NAC.

Finally, much is left to learn regarding the overall pathophysiology of PBIE. Risk factors for PBIE-susceptible mares, such as endometrial biopsy grade or extensive fibrosis, could be evaluated for their effects on the expression of inflammatory genes. Studies have shown that mares with severe endometrial fibrosis associated with endometrosis have altered uterocalin and uteroferrin expression patterns as well as altered hormone receptors, suggesting that normal uterine functionality is altered in cases of endometrial pathology (Lehmann et al. 2011; Rebordao et al. 2014). It is possible that some of these changes also play a role in the development of PBIE. Specific treatments could then be developed for more targeted immunologic abnormalities. Inflammation is a tightly regulated process and begins with recognition of foreign material, such as spermatozoa. This is followed by the release of inflammatory mediators for cellular signaling to recruit inflammatory cells. It is well known that some of these processes are altered in PBIE-susceptible mares, however much remains unknown. Studies moving forward could focus on differences in inflammatory gene sequencing in response to AI in PBIE-susceptible mares compared to resistant mares. Marth et al. (2015, 2016) demonstrated the importance of pathogen recognition and NOD-like receptors as well as induction of chemokines in normal mares following bacterial inoculation with E.coli (3, 12, 24, 48 and 72 hours). The inflammatory response was very rapid, occurring within 3 hours post-inoculation (Marth et al. 2015). Similar studies of
PBIE resistant and susceptible mares at multiple and early (1-3 hours) time points following AI would provide us with new insight into how and why PBIE develops. It is possible that susceptible mares also have altered recognition of pathogens, in this case spermatozoa, leading to the up-regulation of chemokines, cytokines and other mediators of inflammation, such as NO. Additional information on the altered immune response of PBIE mares, including the initial recognition of pathogens and cell signaling, will clarify the pathophysiology of this important cause of infertility.

In conclusion, PBIE remains poorly understood in mares despite extensive past research, likely due to the multifaceted pathophysiology of this disease process that includes risk factors, altered mechanical clearance mechanisms, potentially abnormal pathogen recognition, persistent inflammation post-breeding, and altered response to traditional uterine therapies. Future research to better understand the early post-breeding response in particular may help to identify key time points and mechanisms for therapy that can impact the immune response of PBIE-susceptible mares.
4.4 REFERENCES


Appendix 1. Interactions between treatment cycles and hours post-AI for outcomes measured

Figure 1. Mean biopsy inflammation score (+/- SE) on histopathology of uterine biopsies from 9 PBIE-susceptible mares in N-acetyl cysteine treated (TX) and saline (CONTROL) cycles at 12 and 60 hours post AI. Maximum biopsy score possible was 4. There was no significant interaction between treatment and hours-post-AI (p=0.12).
Figure 2. Median intra-uterine fluid volume (cm$^3$) (+/- LL and UL) of 9 PBIE-susceptible mares in N-acetyl cysteine treated (TX) and saline (CONTROL) cycles at 12, 36, 60 and 84 hours post AI. There was no treatment by hours-post-AI interaction (p=0.84).

Figure 3. Mean endometrial edema score (+/- SE) of 9 PBIE-susceptible mares in N-acetyl cysteine treated (TX) and saline (Control) cycles at 12, 36, 60 and 84 hours post AI. Maximum edema score possible was 4. There was no treatment by hours-post-AI interaction (p=0.65).
Figure 4. Mean % neutrophils (+/- SE) on cytology of uterine swabs obtained from 9 PBIE-susceptible mares at 12 and 60 hours post AI in N-acetyl cysteine treated (TX) or saline (CONTROL) treated cycles. No significant treatment by time interaction was present (p=0.98).
Appendix 2. Validation process for IL-6 ELISA kit

The validation process for the Equine IL-6 DuoSet®ELISA (R&D Systems™, Minneapolis, MN, USA) was based on manufacturer instructions (Spike, Recovery, and Linearity & ELISA Development Guide by R&D systems). A spiked neat cytokine solution (A) was prepared using the cytokine standard to a concentration of 45 ng/ml (approximately 10 times the recommended high standard of the standard curve).

Three base samples of 1000µL were prepared: unspiked, spiked and control. The unspiked sample consisted only of 1000µL sample material. The spiked sample consisted of 20µL of spiked neat cytokine solution (A) and 980µL of sample solution. The control sample had 20µL of spiked neat cytokine solution (A) and 980µL of reagent diluent (1% BSA in PBS), which was provided by the manufacturer.

Serial dilutions were made from the neat sample: 1:2, 1:4 and 1:8 with reagent diluent. Positive and negative control samples were included in the validation process. Positive controls consisted of study mares (PBIE susceptible) with excessive fluid, allowing for multiple validation attempts. Negative controls consisted of endometrial fluid obtained by low-volume lavage from normal mares. While our negative controls are not ideal due to dilution of endometrial samples in order to collect fluid, there was no other method to collect endometrial fluid from normal mares that have no intra-uterine fluid.

Percent recovery and linearity of samples during validation runs were calculated as per the Spike, Recovery and Linearity & ELISA Development Guide provided by the manufacturer (R&D Systems Inc.) Methodology was validated when percent recovery and linearity of all samples fell within 80-120%.
Several validation runs were required. The positive control mare samples (spiked and unspiked) were run at different dilution factors (1:20, 1:30 and 1:50). The 1:20 dilution factor allowed for percent recovery and linearity values to fall within 80-120%, validating this kit. Samples for the study were therefore conducted at a 1:20 dilution with reagent diluent.
<table>
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**Table 1.** Calculated values of percent recovery and linearity for final IL-6 validation. Values of percent recovery and linearity for the positive control (1:20) spiked and unspiked are bolded and fall between 80-120%.
Appendix 3. Main effects of treatment and hours post-Al on cytokines evaluated

**Figure 1.** Median IL-6 mRNA expression (including lower and upper confidence limits) of PBIE-susceptible mares in N-acetyl cysteine treated (TX) and saline (CONTROL) cycles when time points (12h, 60h post-Al) were combined for each cycle. Differing superscripts are significant at p<0.05 (Abbreviations: RQ, relative quantification).

**Figure 2.** Median IL-10 mRNA expression (including lower and upper confidence limits) of PBIE-susceptible mares in N-acetyl cysteine treated (TX) and saline (CONTROL) cycles when time points (12h, 60h post-Al) were combined for each cycle. Differing superscripts are significant at p<0.05 (Abbreviations: RQ, relative quantification).
Figure 3. Median TNF-α mRNA expression (including lower and upper confidence limits) of PBIE-susceptible mares in N-acetyl cysteine treated (TX) and saline (CONTROL) cycles when time points (12h, 60h post-AI) were combined for each cycle. Differing superscripts are significant at p<0.05 (Abbreviations: RQ, relative quantification).

Figure 4. Median IL-1β mRNA expression (including lower and upper confidence limits) of PBIE-susceptible mares in N-acetyl cysteine treated (TX) and saline (CONTROL) cycles when time points (12h, 60h post-AI) were combined for each cycle. Differing superscripts are significant at p<0.05 (Abbreviations: relative quantification).
Figure 5. Median IL-6 mRNA expression (including lower and upper confidence limits) in PBIE-susceptible mares in N-acetyl cysteine treated (TX) and saline (CONTROL) cycles at 12 and 60 hours post AI. No significant treatment by time interaction was seen ($p=0.41$). A significant main effect of time was present ($p=0.02$), whereas no significant main treatment effect was seen ($p=0.86$). Different uppercase letters show significant differences between 12 and 60 hours post AI. Significance was defined as $p<0.05$ (Abbreviations: RQ, relative quantification).

Figure 6. Median IL-10 mRNA expression (including lower and upper confidence limits) of PBIE-susceptible mares at 12 and 60 hours post AI, when TX and CONTROL cycles were combined. Differing superscripts are significant at $p<0.05$ (Abbreviations: RQ, relative quantification).
**Figure 7.** Median TNF-α mRNA expression (including lower and upper confidence limits) of PBIE-susceptible mares at 12 and 60 hours post AI, when TX and CONTROL cycles were combined. Differing superscripts are significant at p<0.05 (Abbreviations: RQ relative quantification).

**Figure 8.** Median IL-1β mRNA expression (including lower and upper confidence limits) of PBIE-susceptible mares at 12 and 60 hours post AI, when TX and CONTROL cycles were combined. Differing superscripts are significant at p<0.05 (Abbreviations: RQ, relative quantification).
**Appendix 4.** Processes for validation attempts for IL-10, TNF-α and IL-1β ELISA kits

The validation attempts for IL-10, TNF-α and IL-1β ELISA kits (R&D Systems) followed the same principles as that described in Appendix 1 for IL-6.

Briefly, four validation attempts were conducted for IL-10. The spiked solution had a concentration of 92.5ng/ml of IL-10. For the first validation, positive controls were diluted 1:20, 1:30 and neat, based on successful results from the IL-6 1:20 dilution validation. Results fell below the standard curve, therefore in the second attempt samples from a suspected truly positive control mare (normal mare post-breeding) were utilized, yet results continued to fall below the standard curve. The standard curve was thus shifted to range from 5ng/ml to 0.078ng/ml and all neat positive control samples were utilized. However, the IL-10 kit was never successfully validated.

For TNF-α, four validation attempts were conducted. The spiked solution had a concentration of 37.5ng/ml of TNF-α. The first validation consisted of neat, 1:20 and 1:30 dilutions of positive controls. Results were most promising with dilutions near 1:4, resulting in three other validation attempts of 1:4 dilutions with different positive controls being used. Attempts were unsuccessful.

For IL-1β, one single validation attempt was conducted. The spiked solution had a concentration of 45ng/ml of IL-1β. The positive control mare was diluted 1:10, 1:20 and 1:30 for validation. This was unsuccessful. No other attempts could be pursued as samples were depleted.