Role of the Nitric Oxide System in Persistent Breeding-Induced Endometritis in the Mare

by

Firdous Ahmad Khan

A Thesis
presented to
The University of Guelph

In partial fulfillment of requirements
for the degree of
Doctor of Veterinary Science
in
Population Medicine and Veterinary Science

Guelph, Ontario, Canada

ABSTRACT

ROLE OF THE NITRIC OXIDE SYSTEM IN PERSISTENT BREEDING-INDUCED ENDOMETRITIS IN THE MARE

Firdous Ahmad Khan
University of Guelph, 2016

Advisor:
Dr. Tracey S. Chenier
Co-advisor:
Dr. Elizabeth L. Scholtz

Persistent breeding-induced endometritis (PBIE) is a major cause of infertility in the mare. Delayed uterine clearance, attributed mostly to impaired myometrial contractility, is recognized as an important factor in the development of PBIE. Nitric oxide (NO) may play a role in determining susceptibility of mares to PBIE through an inhibitory effect on uterine contractility. The purpose of this research was to investigate the role of the nitric oxide system in the pathogenesis of PBIE in mares. First, a randomized controlled experiment was used to evaluate the effect of NO on spontaneous uterine contractility. Using an in-vitro muscle contractility model, uterine tissue strips from euthanized mares were treated with increasing concentrations of S-nitroso-N-acetylpenicillamine (an NO donor) and N-acetyl-D-penicillamine (vehicle and time-matched control) at regular intervals. Results indicated a dose-dependent inhibitory effect of NO on spontaneous uterine contractility. Secondly, differences in endometrial nitric oxide synthase (NOS) activity between PBIE-susceptible and resistant mares, and the effect of a specific inducible nitric oxide synthase (iNOS) inhibitor on endometrial NOS activity, were evaluated. Mares susceptible or resistant to PBIE were selected based on preset criteria and the results of an intrauterine challenge with killed spermatozoa during estrus. Endometrial biopsy samples were

cultured in L-arginine supplemented minimum essential medium with or without a specific iNOS inhibitor. The medium and the endometrial tissue were collected after 24 hours of culture to determine endometrial NOS activity. Susceptible mares had significantly greater endometrial NOS activity than resistant mares. The iNOS inhibitor treatment resulted in a significant reduction in endometrial NOS activity. Coupled with the previously reported presence of increased NO levels in the uteri of susceptible mares, the dose-dependent inhibition of spontaneous uterine contractility by NO and the greater endometrial NOS activity in susceptible mares in the studies described in this thesis suggest that the nitric oxide system may play an important role in the development of PBIE in mares. The reduction of endometrial NOS activity by the iNOS inhibitor treatment provides a basis for clinical testing of NOS inhibitors as preventative or therapeutic options for PBIE in mares.

PUBLICATIONS ARISING FROM THIS RESEARCH

Khan FA, Scholtz EL, Chenier TS. The nitric oxide system in equine reproduction: current status and future directions. J Equine Vet Sci 2015;35:481–7.

Khan FA, Chenier TS, Murrant CL, Foster RA, Hewson J, Scholtz, EL. Dose-dependent inhibition of uterine contractility by nitric oxide: a potential mechanism underlying persistent breeding-induced endometritis in the mare. Theriogenology 2017;90:59–64.

Khan FA, Chenier TS, Foster RA, Hewson J, Scholtz, EL. Endometrial nitric oxide synthase activity in mares susceptible or resistant to persistent breeding-induced endometritis and the effect of a specific iNOS inhibitor. Theriogenology (submitted for publication).

Khan FA, Chenier TS, Murrant CL, Foster RA, Hewson J, Scholtz EL. Nitric oxide's dose-dependent inhibition of uterine contractility: a potential mechanism underlying persistent breeding-induced endometritis in the mare. Clin Theriogenol 2016;8:307 (Abstract).

Khan FA, Murrant CL, Chenier TS, Hewson J, Foster RA, Scholtz EL. The effect of nitric oxide on in-vitro contractility of the ovine uterus: a dose response study. Proc 43rd Annual Southern Ontario Reproductive Biology Meeting, McMaster University, Hamilton, Canada, 2015 (Abstract P27).

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my advisor, Dr. Elizabeth Scholtz, and my co-advisor, Dr. Tracey Chenier, for their excellent mentorship and endless support during the last three years. I really appreciate the freedom they gave me to pursue my research interests. Their patience and encouragement throughout the study program provided me confidence when I needed it the most. The enthusiasm Dr. Scholtz and Dr. Chenier showed whenever I discussed my research with them was inspiring and helped me overcome the hurdles I encountered during the program. It has been an absolute pleasure working with both of them.

I am extremely grateful to my advisory committee members, Dr. Joanne Hewson and Dr. Robert Foster, for their constructive criticism of my research and writing. Their comments helped greatly improve the quality of my research and their encouragement kept me going during some difficult phases of the research. Special thanks are due to Dr. Foster for his help with the histological evaluation of endometrial tissue samples.

I would like to thank Dr. Coral Murrant for the generous access to the in-vitro muscle contractility facilities in her laboratory and her insightful comments on the muscle contractility protocol. I really appreciate the support I got from Dr. Murrant and her research team during my work in her laboratory.

I would like to extend my gratitude to Dr. Cathy Gartley for being very accessible and always willing to help. During the last three years, I have received great advice from her on a wide range of issues related to courses, research, exams, clinical cases and a lot of other things. I consider myself very fortunate to have received training in small animal theriogenology under the supervision of such an amazing person and an excellent mentor.

Special thanks go to the Theriogenology technician, Karen Rutherford. Her excellent technical and interpersonal skills made my life easier as a resident. I really appreciate her advice and encouraging words during the difficult times. I am thankful to Shawn-Elizabeth Maloney, Sarah Dyck, Melanie Harness, Evelyn Helps, Megan Ballantine, and Kate Sweetman for their assistance during my work with mares. I greatly appreciate the help and support provided by Dave Vandenberg, Danielle Watson and other staff members at the Arkell Equine Research Station. Assistance provided by the technicians and the agricultural assistants at the Ontario Veterinary College is also highly appreciated.

I am grateful to Dr. Monica Antenos and Dr. William Allan King for giving me access to their laboratory in order to perform endometrial tissue culture and nitric oxide and total protein assays. Dr. Antenos deserves special thanks for her help with the assays.

I owe my gratitude to Dr. Luis Arroyo, Dr. Judith Koenig and Dr. Donald Trout for their help with the collection of the mare reproductive tracts used in the study. I am grateful to Dr. Daniel Kenney for facilitating allocation of research mares and being helpful whenever he was around. I am also grateful to William Sears for his help with the statistical analyses. Brian McDougall deserves a special mention for generously donating the ovine and porcine reproductive tracts used in optimization of the muscle contractility protocol.

Special thanks are due to Dr. Irwin Liu and Faye Harmon (University of California Davis), and Dr. Gabriela Hirsbrunner (University of Bern), for their advice on the in-vitro muscle contractility protocol.

I thank my fellow graduate students for their camaraderie and for lending me a helping hand during the research and in the clinics. Jeba Gnanadhas, Gonçalo Silva, Rames Salcedo and

Michelle Caissie deserve special mention. I am also thankful to Mariana Diel de Amorim for her helpful suggestions related to courses in the DVSc program and preparation for the ACT exam. This work would not have been possible without funding from the Equine Guelph and in-kind contribution from the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA). I am extremely grateful to both organizations for supporting my research. I am also thankful to the Government of Ontario for awarding me the Ontario Trillium Scholarship that served as a major financial support for me during the last three years.

I have been very fortunate to have excellent research mentors in the past as well and most of them continue to provide me moral support. I am especially indebted to Dr. Goutam Das (Indian Veterinary Research Institute), Dr. Oliver Ginther (University of Wisconsin-Madison) and Dr. Mohammad Amin Beg (King AbdulAziz University) for their continuous encouragement and advice.

Finally, I would like to thank my family for their unconditional love and support. Born and raised in the war zone of Kashmir, I understand the great challenges my parents faced in keeping me safe and providing me education. I owe them a lifetime of gratitude for being simply awesome. I am also grateful to my brothers, Tanveer and Irfan for the many precious moments I have spent with them growing up, and for their continuous encouragement. I thank my amazing wife, Afroza, for her support, patience, encouragement and unwavering love. Being a researcher herself, she provided me some helpful suggestions over the course of this research. I am also thankful to my son, Ammar, for making my life more interesting and fun than it ever was. I must also thank him for being such a good boy, especially at times when I was working at home or needed rest after a long day of hard work.

DECLARATION OF WORK PERFORMED

I declare that I have performed all of the work presented in this thesis except for the items listed below.

Hematoxylin and Eosin stained slides for histological evaluation of endometrial tissue samples were prepared by the Histology service of the Animal Health Laboratory at the University of Guelph. The histological evaluation was performed by Dr. Robert A. Foster in the Department of Pathobiology at the University of Guelph.

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

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

AG	Aminoguanidine	IL	Interleukin
AHL	Animal Health Laboratory	iNOS	Inducible nitric oxide synthase
AIC	Akaike information criterion	KHS	Krebs-Henseleit solution
ANOVA	Analysis of variance	L-NAME	L-NG-nitro arginine methyl ester
AUC	Area under the curve	MCWE	Mycobacterial cell wall extract
cGMP	Cyclic guanosine monophosphate	mRNA	Messenger ribonucleic acid
CD4+ T cell	Helper T cell	NAP	N-acetyl-D-penicillamine
CD8+ T cell	Cytotoxic T cell	nNOS	Neuronal nitric oxide synthase
CL	Corpus luteum	NO	Nitric oxide
cNOS	Constitutive nitric oxide synthase	NOS	Nitric oxide synthase
C5	Complement component 5	PBIE	Persistent breeding-induced endometritis
eNOS	Endothelial nitric oxide synthase	PGE2	Prostaglandin E2
H&E	Hematoxylin and eosin	PGF2α	Prostaglandin F2α
hCG	Human chorionic gonadotropin	SNAP	S-nitroso-N-acetylpenicillamine
IFNγ	Interferon gamma	SNP	Sodium nitroprusside
Ig	Immunoglobulin	TAMV	Time-averaged mean velocity
IHC	Immunohistochemistry	TNF	Tumor necrosis factor

CHAPTER ONE

INTRODUCTION AND REVIEW OF LITERATURE

[A portion of this chapter is a part of a review article published in the Journal of Equine Veterinary Science (Khan et al., 2015)].

1.1 INTRODUCTION

According to a recent industry report (Evans, 2012), the horse industry in Canada contributes about 19.6 billion dollars annually to the national economy. The 2010 Canadian Equine Industry Profile Study estimated the total horse population in Canada to be 963,500. A vast proportion (41%) of this horse population is in the breeding sector (Evans, 2012). Persistent breeding-induced endometritis (PBIE) in mares is a serious problem with a significant economic impact on the horse breeding industry. Recognized as a major reason for pregnancy failure in mares (Rigby et al., 2001; Pycock, 2006; Troedsson, 2011; Woodward and Troedsson, 2013), PBIE causes great economic losses to horse breeders due to the costs of diagnostic and therapeutic procedures and the costs associated with re-breeding. Research has shown that young mares (2 to 8 years old) are less susceptible to PBIE, whereas older mares (9 years or older) are more susceptible (Woodward et al., 2012). Not breeding older mares is, however, not an option as they constitute a sizeable proportion of the broodmare population. In contrast to the bovine industry, where reproductive efficiency is a major factor that determines the age of breeding, most mares are usually bred after they have entered the age zone of greater susceptibility to PBIE. A common reason for breeding mares later in their life is that most of them also have a career other than

breeding (for instance, racing, dressage, show jumping) and are therefore usually bred toward or at the end of their performance/competitive career.

The inflammatory response of the equine uterus to semen and contaminants that enter the uterus during breeding has been extensively reviewed (Watson, 2000; Troedsson et al., 2001; Troedsson, 2006; Katila, 2012; Woodward and Troedsson, 2015; Canisso et al., 2016). This inflammatory response involves a combination of mechanical clearance and innate immune components of the uterine defense mechanism (Troedsson, 2006). An increase in uterine contractility occurs immediately after breeding, most likely as a result of oxytocin release from the pituitary gland caused by mechanical stimulation of the vagina and cervix during breeding (Katila, 2012). This is followed by activation of the complement system, resulting in cleavage of factor C5 into C5a and C5b (Asbury et al., 1982; Watson et al., 1987; Troedsson et al., 1993a; Troedsson et al., 1995). Complement factor C5a acts as a chemotactic signal and causes influx of neutrophils into the uterine lumen (Kotilainen et al., 1994; Katila, 1995; Troedsson et al., 1995). The influx of neutrophils into the uterine lumen has been reported to occur within 30 minutes after breeding (Katila, 1995). Troedsson (2006) suggested that activated neutrophils bind to spermatozoa in the uterus through neutrophil extracellular traps (NETs) and a traditional ligand receptor interaction. The neutrophils engulf spermatozoa and release prostaglandin $F_2\alpha$ ($PGF_2\alpha$), which stimulates myometrial contractions and facilitates clearance of spermatozoa, debris, and fluid from the uterus via the cervix (Troedsson et al., 2001).

Adaptive immune components of the uterine defense mechanism have received relatively less attention in research on breeding-induced endometritis in the mare. It is generally believed that adaptive immunity does not play an important role in the uterine inflammatory response after breeding (Troedsson et al., 2005; Katila, 2012). Troedsson et al. (2005) proposed that the

absence of an adaptive immune response after breeding may be a physiological mechanism to prevent any detrimental effects of antibodies against spermatozoa or a cell-mediated specific immune response on fertility during subsequent breedings. An increase in CD4+ but not CD8+ T cells has been shown to occur in the endometrium at 6 and 48 hours after breeding in normal mares (Tunón et al., 2000). Fumuso et al. (2007) evaluated total uterine T cell numbers at 24 hours after insemination and found no difference in the T cell numbers between mares susceptible or resistant to persistent endometritis. Studies on uterine immunoglobulin levels in mares susceptible or resistant to persistent endometritis have reported conflicting results. Higher amounts of IgA, IgG, and IgG(T) in the uterine fluid of susceptible mares were reported by Asbury et al. (1980). Mitchell et al. (1982) reported greater uterine IgA concentrations in susceptible mares but there were no differences in uterine IgG, and IgG(T) concentrations between susceptible and resistant mares. In contrast, Troedsson et al. (1993a) reported increased IgG concentrations in resistant mares and no difference in uterine IgA concentration between susceptible and resistant mares. Further studies are required to clarify the role of immunoglobulins in equine PBIE.

Cytokines play a key role in modulation of inflammation in all tissues, including tissues in the reproductive tract. An increase in the mRNA expression of pro-inflammatory cytokines interleukin-1beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α) was observed 24 hours after insemination. However, there were no differences in cytokine expression between susceptible and resistant mares (Fumuso et al., 2003). In the same study, treatment with a *Mycobacterium phlei* cell-wall extract (MCWE) preparation at the time of insemination down-regulated IL-1 β mRNA expression. In another study (Fumuso et al., 2007), higher mRNA expression of interleukin-8 (IL-8) and lower mRNA expression of interleukin-10 (IL-10) were

observed 24 hours after insemination in susceptible mares compared to resistant mares. Treatment with an MCWE preparation resulted in higher mRNA expression of IL-10 24 hours after insemination in susceptible mares. More recently, Woodward et al. (2013a) reported lower expression of IL-6, IL-10 and IL-1RA (IL-1RN) 6 hours after insemination in susceptible mares. Susceptible mares showed delayed onset of interferon gamma (IFNγ) expression after insemination and slower return to pre-insemination levels. The authors suggested that altered cytokine response contributes to impaired uterine clearance and infertility in susceptible mares.

Persistent breeding-induced endometritis continues to be a focus of active research in equine reproduction. Mechanisms underlying the development of PBIE in the mare are still not completely understood. Delayed uterine clearance is believed to play an important role in susceptibility to PBIE (Watson, 2000; Katila, 2008; Troedsson and Woodward, 2016). This delay in uterine clearance is mostly attributed to impaired myometrial contractility in susceptible mares (Troedsson et al., 1993b). Regulation of myometrial contractility in the mare is poorly understood. However, studies in women have shown that myometrial contractility is predominantly regulated by calcium (Ca^{2+}) in three phases: resting tone maintained by basal concentrations of Ca^{2+}, agonist stimulated contraction involving a marked increase in Ca^{2+} concentration, and restoration of resting state involving decrease in Ca2+ to basal concentrations (reviewed in Aguilar and Mitchell, 2010). Oxytocin, PGF2α, and potassium chloride have been demonstrated in-vitro to stimulate contractility in the equine uterus (Rigby et al., 2001).

Research has shown that mares susceptible to PBIE have greater amounts of nitric oxide (NO) in the uterus than resistant mares (Alghamdi et al., 2005; Woodward et al., 2013b). Nitric oxide has been suggested to play a role in the development of PBIE in mares based on a possible inhibitory effect on myometrial contractility. The only evidence so far for an inhibitory effect of

NO on equine myometrial contractility is a study in which equine myometrial tissue did not respond to electrical stimulus in the presence of NO (Liu et al., 1997). However, the effect of different concentrations of NO on spontaneous uterine contractility in the mare has not been investigated. Mechanisms underlying effects of NO on equine myometrial contractility are still unknown. However, research in other species indicates that NO causes relaxation of myometrial tissue most likely via a cyclic guanosine 5′-monophosphate (cGMP) independent mechanism (reviewed in Buxton, 2004). The greater amounts of NO in the uteri of susceptible mares have been attributed to a higher expression of inducible nitric oxide synthase (iNOS) in the endometrium (Alghamdi et al., 2005). However, to date, differences in nitric oxide synthase activity between susceptible and resistant mares have not been evaluated. Moreover, the effect of nitric oxide synthase inhibitors on endometrial NO production has not been investigated. Studies on the role of the nitric oxide system in PBIE have the potential to provide novel prophylactic or therapeutic options for PBIE in the future.

1.2 REVIEW OF LITERATURE

A mild transient breeding-induced endometritis is a normal, physiological response in mares (Liu and Troedsson, 2008). Spermatozoal transport into the uterine tubes typically occurs within 4 hours of breeding in the mare (Bader 1982; Scott 2000). The transient inflammatory response of the uterus after breeding is believed to be necessary for the clearance of excess spermatozoa present in the uterus after spermatozoal transport into the uterine tubes and the contaminants that get into the uterus during estrus and at the time of breeding (Troedsson, 2006). In normal mares, the inflammation resolves within 24 to 48 hours after breeding. However, the inflammation persists in a subpopulation of mares beyond 72 to 96 hours after breeding and is defined as

persistent breeding-induced endometritis (Woodward and Troedsson, 2015). Persistent breeding-induced endometritis (PBIE) has been reported in about 15% of 746 estrous cycles in a Thoroughbred broodmare population (Zent et al., 1998) and in as many as 43% of 552 estrous cycles in a mixed population of mares (Newcombe, 1997).

Ultrasonographic detection of intrauterine fluid accumulation post breeding is used as the most common indicator of breeding-induced endometritis (Katila, 2012). Intervals from breeding to clearance of intrauterine fluid have been used in various studies to classify mares as being either resistant (clear fluid within 24 to 48 hours post breeding) or susceptible (retain fluid beyond 72 to 96 hours post breeding) to persistent breeding-induced endometritis (Alghamdi et al., 2005; Woodward et al., 2012; Woodward et al., 2013b). A difference between mares in susceptibility to persistent endometritis was first demonstrated in a study by Hughes and Loy (1969) in which young fertile mares showed more efficient resolution of the inflammatory response to intrauterine challenge with *Streptococcus zooepidemicus* than older subfertile mares. This difference between mares in susceptibility to persistent endometritis was confirmed later by several other studies using intrauterine challenge with *Streptococcus zooepidemicus* (Troedsson et al., 1993b; Troedsson et al., 1993c) or spermatozoa (Alghamdi et al., 2005; Woodward et al., 2013b).

1.2.1 Factors associated with susceptibility to persistent endometritis

Factors associated with susceptibility to persistant endometritis in the mare were recently reviewed by Woodward and Troedsson (2015). These factors included age, endometrial biopsy score, perineal conformation, anatomical location of the uterus, fluid retention during diestrus, and results of a pre-breeding bacterial culture. The effect of age on susceptibility to persistent endometritis was demonstrated in a study (Woodward et al., 2012) in which the percentage of

mares susceptible to persistent endometritis was 0%, 36%, and 88% in age groups 2 to 8 years, 9 to 16 years, and ≥ 17 years, respectively. Although the effect of parity was not investigated, the authors suggested that increased predisposition to persistent endometritis in older mares could be a result of degenerative changes in the endometrium associated with increased age and parity. However, there is evidence indicating that age alone can be a causative factor. Ricketts and Alonso (1991) showed that even maiden mares of increased age exhibit degenerative endometrial changes. Similar results were reported in another study (Grüninger et al., 1998) in which older maiden mares exhibited a higher incidence of angiosclerotic changes in the endometrium than younger maiden mares. Endometrial biopsy scores of IIB or III were shown to be associated with a greater susceptibility to persistent endometritis (Troedsson et al., 1993c; Woodward et al., 2012). The exact mechanism for the association between endometrial biopsy score and susceptibility to persistent endometritis has not been investigated. However, alterations in endometrial vascularity and lymphatic drainage, and fibrotic changes in the endometrium, have been proposed as possible reasons (Woodward et al. 2012)

Poor perineal conformation (Hemberg et al., 2005), a pendulous uterus dropping below the pelvic brim (LeBlanc et al., 1998), excess fluid retention during diestrus (Brinsko et al., 2003), and positive pre-breeding culture (Riddle et al., 2007) were also shown to be associated with greater predisposition to persistent endometritis. Poor perineal conformation results in pneumovagina and repeated ascending contamination of the reproductive tract, both of which predispose mares to persistent endometritis and infertility (Dascanio, 2011). Ventral tilting of uterus in relation to the pelvic brim has been shown to reduce lymphatic drainage (LeBlanc et al., 1995) and uterine clearance (LeBlanc et al., 1998). Cervical patency was suggested to be a factor affecting susceptibility to persistent endometritis, especially in older maiden mares (Pycock,

1993; LeBlanc, 2003). The effect of cervical patency on breeding-induced endometritis was tested very recently using 33-French Bivona catheters inserted into the uteri of mares immediately after insemination (Reilas et al., 2016). The catheter was held in the uterus by inflation of a balloon cuff, and the other end was clamped to prevent evacuation of fluid. The authors reported accumulation of large amounts of intrauterine fluid 25 hours after insemination in mares with cervical blockage due to clamped catheters. However, the absence of a control group with just clamped catheters and no insemination makes it impossible to confidently draw conclusions about the effect of cervical blockage on breeding-induced endometritis, owing to a potential confounding effect of inflammation resulting from just the presence of the catheter in the uterus.

1.2.2 Delayed uterine clearance in mares susceptible to persistent endometritis

A number of studies have shown that mares susceptible to persistent endometritis display impaired uterine clearance. In two studies using intrauterine inoculation with *Streptococcus zooepidemicus*, chromium-labeled microspheres, and charcoal (Evans et al., 1986; Evans et al., 1987), more efficient uterine clearance was observed in resistant mares than in susceptible mares. In another study using intrauterine inoculation with *Streptococcus zooepidemicus* and chromium-labeled microspheres (Troedsson and Liu, 1991), resistant mares cleared the microspheres within 24 hours post inoculation whereas susceptible mares failed to clear microspheres from the uterus before 96 hours post inoculation. Similarly, LeBlanc et al. (1994a), in a study using intrauterine infusion of radiocolloid followed by scintigraphy, reported that susceptible mares show delayed uterine clearance. Resistant mares expelled more than 50% of the radiocolloid within 2 hours, whereas susceptible mares expelled negligible amounts (<5%). This delayed uterine clearance in susceptible mares has been mostly attributed to impaired

myometrial contractility. In a study using multiple site electromyography recordings of uterine activity, Troedsson et al. (1993b) showed that susceptible mares have impaired myometrial activity, characterized by reduced frequency, duration, and intensity of myometrial contractions.

Mechanisms underlying the reduced myometrial activity in susceptible mares are still not completely understood. A possible mechanism was proposed by Rigby et al. (2001) in a study that used an in-vitro model to measure isometric tension generated by longitudinal and circular uterine muscle strips in response to potassium chloride, oxytocin, and PGF2α. Uterine muscle strips from susceptible mares showed a lower increase in active tension in response to each of the agonists tested in the study. This difference in response between the uterine strips from susceptible and those from resistant mares did not appear to result from differences in regulation of intracellular calcium ion concentration. The authors concluded that susceptible mares have an intrinsic contractile defect of the myometrium. However, the impaired uterine clearance could be restored by using ecbolic agents. LeBlanc et al. (1994b) reported that susceptible mares cleared more than 90% of the radiocolloid within 30 minutes of treatment with oxytocin (20 units), which is in sharp contrast to the negligible uterine clearance (less than 5%) in these mares without treatment. Based on these findings, Alghamdi et al. (2005) suggested that the reduced myoelectrical activity in susceptible mares represents an inhibition of contractility or induced relaxation rather than an intrinsic inability to contract. Research indicating that susceptible mares have higher amounts of uterine NO than resistant mares (Alghamdi et al., 2005; Woodward et al., 2013b), and the fact that NO is a well-known smooth muscle relaxant, suggest that the nitric oxide system may be involved in causing delayed uterine clearance in susceptible mares.

1.2.3 The nitric oxide system

Nitric oxide (NO) is synthesized in biological systems from L-arginine (Palmer et al., 1988). The reaction is catalyzed by nitric oxide synthase (NOS), an enzyme that exists in multiple isoforms in nature. Three of these isoforms have been identified in mammals, namely neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). The former two are constitutively expressed and collectively referred to as constitutive NOS or cNOS (Stuehr, 1997). The enzyme activities of nNOS and eNOS are regulated by calcium (Ca^{2+}) and calmodulin whereas the enzyme activity of iNOS is largely or completely Ca^{2+} independent (Förstermann and Sessa, 2012). Nitric oxide synthesis and its regulation, especially in equine reproductive tissues, are not completely elucidated. Nitric oxide is a versatile molecule with an established role in a wide range of biological processes including, but not limited to, vasodilation (Palmer et al., 1987), platelet aggregation (Radomski et al., 1991), neurotransmission (Bredt and Snyder, 1989), and cytotoxicity (Walker et al., 1995). The biological actions of NO are mediated either through the guanosine cyclic 3',5'-monophosphate (cGMP) pathway or occur as a result of the direct effect of NO (Schmidt et al., 1993). Following the initial implication of NO in regulation of penile erection in rabbits by Ignarro et al. (1990), the molecule has been shown to be involved in regulation of several reproductive processes in different species (Rosselli et al., 1998; Dixit and Parvizi, 2001). In horses, NO in reproduction is a relatively recent research area. Studies so far in the mare have mainly focused on investigating the presence of the NO system and its role in the ovaries and uterus.

1.2.3.1 The nitric oxide system in female reproduction

The role of NO as a regulator of female reproduction has been investigated extensively in several laboratory animal species and humans. There is abundant evidence supporting a multifaceted

physiological role of NO in the regulation of gonadotropin secretion, steroidogenesis, follicular development, ovulation, luteal development and regression, pregnancy, parturition/labor, and oviductal, cervical, and vaginal function in these species (Rosselli et al., 1998; Dixit and Parvizi, 2001). However, it is a fairly new area of research in farm animal species in general and horses in particular. In mares, studies so far have focused on investigating the presence of the nitric oxide system in the ovary and uterus and its possible role in the modulation of ovarian and uterine function.

1.2.3.1.1 The nitric oxide system in the ovary

The role of NO in the regulation of ovarian function in mares is not completely understood. However, research findings from studies so far are suggestive of its involvement in various reproductive processes that occur within the ovary.

The role of NO in follicular development and function

Although definitive proof of an intrafollicular NO synthase system in the mare is still lacking, there is some information on the concentration of NO in the preovulatory follicle and how it is influenced by treatments that affect follicular growth and ovulation. Administration of human chorionic gonadotropin (hCG) resulted in an increase in intrafollicular NO concentration (Pinto et al., 2003), whereas that of equine pituitary extract had no effect (Carmo et al., 2010). It has been demonstrated that treatment of mares with L-NG-Nitro Arginine Methyl Ester (L-NAME), a nonspecific inhibitor of NOS, or aminoguanidine (AG), a relatively specific inhibitor of iNOS, delayed ovulation after induction with hCG. The median intervals from hCG administration to ovulation were 84 and 54 hours in L-NAME and AG treated mares, respectively, which were significantly longer than the interval (42 hours) in saline treated control mares (Pinto et al., 2002a). This response is, however, different from the complete blockage of ovulation reported

with the use of NOS inhibitors in rats (Shukovski and Tsafriri, 1994) and rabbits (Hesla et al., 1997) or in NOS knockout mouse models (Jablonka-Shariff and Olson, 1998).

Nitric oxide has also been shown to modulate follicular steroidogenesis in various species (Rosselli et al., 1998; Dixit and Parvizi, 2001). In mares too, there are indications that NO may regulate follicular progesterone and estradiol production. Ovulation induction with hCG was associated with a concurrent increase in NO and progesterone. Administration of NOS inhibitors, L-NAME and AG, blocked the increase in both NO and progesterone (Pinto et al., 2003). This suggests a mediating role of NO in intrafollicular progesterone production induced by hCG. However, in an in-vitro study using granulosa cell culture, neither NOS inhibitors (L-NAME and AG), nor sodium nitroprusside (SNP), an NO donor, had an effect on progesterone production. Instead, a prominent effect was noted on estradiol with L-NAME and AG resulting in increased production and SNP resulting in decreased production (Pinto et al., 2002b). Therefore, further research is needed to clarify a potential modulatory role of NO in equine follicular steroidogenesis. The effects of L-NAME, AG, and SNP on estradiol production by granulosa cells in-vitro are suggestive of an inhibitory effect of higher NO concentrations on estradiol biosynthesis. The mechanism underlying this effect is not known in mares but it has been demonstrated in other species that higher NO concentrations inhibit the activity of aromatase (Van Voorhis et al., 1994), a key enzyme in the steroidogenic pathway involved in conversion of androstenedione to estradiol (Hanukoglu, 1992).

The role of nitric oxide in the regulation of follicular blood flow has been investigated in various species such as humans (Anteby et al., 1996), cattle (Pancarci et al., 2011), and sheep (El-Sherry et al., 2013). In the mare, this is still a highly unexplored research area with only preliminary information available on the stimulatory effect of oral supplementation of L-

arginine, a precursor for NO biosynthesis, on the vascular perfusion of dominant follicles in postpartum mares (Kelley et al., 2013).

In addition to its documented physiological functions, NO has been shown to be involved in several reproductive disorders including abnormalities of follicular development and ovulation. Nitric oxide has been implicated in the development of follicular cysts in mice (Nemade et al., 2002) and buffalo (Khan et al., 2011). Also, there are indications that NO may be an important mediator in the disruption of normal follicular development associated with endometritis in cattle (Sheldon et al., 2002) and buffalo (Pande et al., 2013). Although it is well established that endometritis is an important cause of infertility in mares (Troedsson, 1999), its effects on follicular growth and development and the possible role of NO in mediating those effects in the species are unknown. Abnormalities of follicular development in mares such as hemorrhagic anovulatory follicles have been shown to be associated with aberrant follicular blood flow (Ginther et al., 2006). Moreover, differences in blood flow were noted between anovulatory and ovulatory follicles in mares during spring transition (Acosta et al., 2004).

The role of NO in luteal development and function

The existence of a local NO synthesis system in the equine corpus luteum was demonstrated definitively for the first time by Ferreira-Dias et al. (2011) who immunolocalized eNOS and iNOS in large luteal cells and endothelial cells from all luteal stages. Expression of eNOS protein was significantly different between stages, with the highest expression in the late luteal stage, intermediate expression in the early luteal stage and the lowest expression in the mid luteal stage. However, iNOS protein expression did not differ between the three stages. Regulation of the luteal NO system in the mare is not yet completely understood. Growing evidence suggests cytokines may be important regulators of luteal NOS expression and NO production. Tumor

necrosis factor (TNF), interferon gamma (IFNγ), and Fas ligand were shown to upregulate eNOS mRNA expression in equine luteal cell cultures obtained at mid and late luteal stages (Galvão et al., 2013a). Treatment with a combination of the three cytokines increased NO production in luteal cells from the mid luteal stage (Galvão et al., 2008a) whereas treatment with TNF and IFNγ increased NO production in luteal cells from the early luteal stage (Galvão et al., 2008b). More recently, Galvão et al., (2014) showed that leptin and ghrelin increased NO production in luteal cells from the early and mid luteal stages. Studies on temporal and functional relationships of NO with luteal blood flow and function are lacking in the mare. However, in-vitro studies using spermine NONOate, a nitric oxide donor, in equine luteal explants have suggested that NO has a stimulatory effect on luteal angiogenesis and prostaglandin E2 (PGE2) and progesterone production, especially during the early luteal phase (Ferreira-Dias et al., 2011; Costa et al., 2006). Nitric oxide has been shown to be an important part of the luteolytic process in several species, including laboratory animals, humans, and cattle (Shirasuna, 2010). Based on temporal relationships between plasma concentrations of nitric oxide metabolites and a PGF2α metabolite during and after luteolysis in mares, Ginther et al. (2015) suggested that NO might not have a role in the initiation of equine luteolysis but may be involved in late luteolysis.

1.2.3.1.2 The nitric oxide system in the uterus

Initial evidence indicating the presence of a local NO synthesizing system in equine uterine tissue was provided by Welter et al. (2004) who studied the mRNA expression of eNOS and iNOS in the endometrium of cyclic and early pregnant mares using real time-polymerase chain reaction. Expression was compared between Days 0 (ovulation), 1, 5, 11, and 21 in cyclic mares and between Days 12 and 15 in pregnant mares. In cyclic mares, eNOS mRNA expression was greater on Day 5 compared to the other days of the estrous cycle, whereas iNOS mRNA

expression did not differ between days. In pregnant mares, the mRNA expression of eNOS and iNOS was greater (about 4-fold and 10-fold, respectively) on Day 15 than on Day 12. Another study (Honnens et al., 2011) investigated eNOS mRNA expression on Days 0 (ovulation), 1, 5, 11, 15 and -3 (second day of estrus). Endometrial eNOS mRNA expression varied between the days and showed a biphasic pattern with increased expression on Day 5 and Day -3. More recently, Gebhardt et al. (2012) investigated global gene expression in the equine endometrium, showing that eNOS mRNA expression is upregulated during diestrus.

Apart from mRNA expression, immunolocalization and quantification of NOS proteins in the equine uterus has been attempted in a few studies. Welter et al. (2004) used immunohistochemistry (IHC) to clearly localize eNOS protein in endometrial cells, endothelial cells, and basement membrane during different stages of the estrous cycle as well as in early pregnant mares. Expression of iNOS protein was undetectable during estrus but it could be detected during pregnancy in glandular epithelium with a tendency to be greater at Day 15 compared to Day 12. Roberto da Costa et al. (2005, 2007) localized eNOS and iNOS proteins in the equine uterus by IHC and quantified these proteins using western blotting. Endothelial NOS protein was identified in epithelial cells, endometrial glands, endothelial cells, fibroblasts, blood vessels and lymphatic vessels during both follicular and luteal phases. Endometrial eNOS protein expression was higher in the follicular phase compared to the early and late luteal phases. However, iNOS protein was only localized in endothelial cells by IHC and there was no positive signal on western blot.

Although there is little information on the regulation of uterine NO production, there are a few studies on NO production by equine uterine tissue in-vitro and its regulation by steroids and cytokines. Endometrial NO production was highest in the early luteal phase, intermediate in

the mid luteal phase and lowest in the late luteal phase. Nitric oxide production in the follicular phase was lower than in the early luteal phase but similar to that in the mid and late luteal phases (Roberto da Costa et al., 2007). It has been demonstrated that in-vitro NO production by equine endometrial cells from the mid luteal and follicular phases is stimulated by TNF, estradiol, progesterone, and estradiol plus progesterone treatments (Galvão et al., 2013b). In another study (Roberto da Costa et al., 2007), the luteal phase endometrium produced more NO compared to the follicular phase with or without treatment with exogenous progestins and estradiol.

Association of the NO system with uterine hemodynamics has been investigated to some extent in the mare. Uterine blood flow measured as time-averaged mean velocity (TAMV) was positively correlated with eNOS mRNA expression but not correlated with iNOS mRNA expression in the endometrium (Honnens et al., 2011). Multiple studies in mares have evaluated the influence of supplementation of L-arginine, an NO precursor, on uterine blood flow. An increase in uterine blood flow following supplementation was noted in pre- and postpartum mares (Mortensen et al., 2011). However, there was no effect of L-arginine supplementation on uterine blood flow in mares with delayed uterine clearance during estrus (Jacobs et al., 2013). In two other investigations, Kelley et al. (2011, 2013) reported inconsistent results following L-arginine supplementation in mares beginning 21 days before their expected foaling date. An increased blood flow to the previously gravid horn was reported in one study (Kelley et al., 2011), whereas no effect was seen in the other (Kelley et al., 2013). Little is known about the role of NO in the regulation of angiogenesis in the equine endometrium. A recent in-vitro study (Galvão et al., 2013b) showed that NO may be an important mediator in the regulation of angiogenesis in the equine endometrium by TNF and ovarian steroids. It is clear that regulation of angiogenesis and uterine blood flow by NO in the mare is not yet fully understood.

Nitric oxide may be involved in the regulation of prostaglandin production by the equine endometrium. In an in-vitro study (Roberto da Costa et al., 2008), stimulation of equine endometrial tissue with spermine NONOate, an NO donor, increased PGE2 production during early and mid luteal phases and prostaglandin F2 alpha (PGF2α) production during all the stages (early, mid, and late luteal phases and follicular phase) of the estrous cycle. Nitric oxide production was positively correlated with prostaglandin E synthase and prostaglandin F synthase protein expression. However, the relationship between NO and endometrial prostaglandin production has not yet been investigated in-vivo.

1.2.4 Role of the nitric oxide system in persistent breeding-induced endometritis

Besides its suggested physiological functions, there are indications that the NO system may be involved in the development of persistent breeding-induced endometritis. Greater endometrial iNOS mRNA expression, more iNOS-positive inflammatory cells in endometrial biopsies and higher intrauterine NO content were reported 13h after insemination in mares susceptible to persistent mating induced endometritis than those resistant to it (Alghamdi et al., 2005). In another study (Fioratti et al., 2010), susceptible mares had greater NO concentrations in free uterine fluid 8 h after insemination. More recently, Woodward et al. (2013b), in a set of two experiments, investigated the differences in iNOS mRNA and protein expression and intrauterine NO content between susceptible and resistant mares at multiple time points (2, 6, 12, and 24h) post breeding. In addition, the effect of treatment with dexamethasone or mycobacterial cell wall extract (MCWE) on endometrial iNOS mRNA expression and intrauterine NO content was evaluated. A greater percent increase in intrauterine NO content at 6 and 12h post breeding in susceptible mares was recorded in one experiment. In the other, untreated susceptible mares were found to have greater total intrauterine NO compared to untreated resistant mares at 6h post

breeding. In contrast to the findings of Alghamdi et al. (2005), no difference in iNOS mRNA or protein expression was reported between susceptible and resistant mares at any of the time points. The authors suggested that this disagreement between the two studies could have resulted from differences in methodology (different expression methods and different antibodies) used in the two studies. Treatment with MCWE reduced intrauterine NO content whereas dexamethasone did not have any effect. Similar results were reported in another study (Fioratti et al., 2010) in which dexamethasone treatment 2h before insemination did not influence NO concentration in the uterine fluid collected 8 and 24 h after insemination. Intrauterine infusion of platelet rich plasma (PRP) in susceptible mares resulted in lower expression of iNOS mRNA in endometrial biopsy samples collected 1 day after insemination (Metcalf et al., 2012). It is fairly well established that susceptible mares show delayed uterine clearance (Evans et al., 1987; Troedsson and Liu, 1991; LeBlanc et al., 1994a) resulting, at least in part, from impaired myometrial contractility (Troedsson et al., 1993b). The relaxant effect of NO on smooth muscle tissues is well established in many different species. Moreover, NO has been shown to cause relaxation of the myometrium in other species such as rat (Yallampalli et al., 1993) and human (Bradley et al., 1998). The only documented evidence that NO may have an inhibitory effect on uterine contractility in the mare is the reported inability of the myometrial tissue in vitro to respond to electrical stimulus in the presence of NO (Liu et al., 1997). It is possible that greater NO production in the uterus of susceptible mares reduces myometrial contractility, thereby resulting in delayed uterine clearance.

The above review of literature indicates that the nitric oxide system may play an important role in the development of PBIE in mares. However, there are certain important gaps in the literature that need to be addressed. Information on the effect of different concentrations of

nitric oxide on spontaneous uterine contractility in the mare is lacking. Research is needed to determine the differences in endometrial NOS activity between susceptible and resistant mares and the effect of NOS inhibitors on the endometrial NO production. Studies in this area can lead to novel options for prevention or treatment of PBIE in mares.

1.3 RESEARCH OBJECTIVES

The overall goals of this research were to evaluate the role of the nitric oxide system in the development of PBIE in the mare and to investigate the in-vitro efficacy of iNOS inhibition in reducing NO production from the endometrium of mares susceptible to PBIE.

The objectives of the first study were to test the effect of different concentrations of NO on spontaneous uterine contractility in-vitro and to evaluate whether this effect varied between the longitudinal and circular muscle layers of the uterus.

The objectives of the second study were to investigate the difference in total NOS activity of the endometrium between susceptible and resistant mares and the effect of a specific iNOS inhibitor on the total endometrial NOS activity.

CHAPTER TWO

DOSE-DEPENDENT INHIBITION OF UTERINE CONTRACTILITY BY NITRIC OXIDE: A POTENTIAL MECHANISM UNDERLYING PERSISTENT BREEDING-INDUCED ENDOMETRITIS IN THE MARE

Firdous A. Khan[a], Tracey S. Chenier[a], Coral L. Murrant[b], Robert A. Foster[c], Joanne Hewson[d], Elizabeth L. Scholtz[a]

[a]Department of Population Medicine, [b]Department of Human Health and Nutritional Sciences, [c]Department of Pathobiology, [d]Department of Clinical Studies, University of Guelph, Canada

(Published as an abstract in the 'Clinical Theriogenology' journal as part of the proceedings of the 2016 Society for Theriogenology (SFT) Conference held at Asheville, North Carolina, USA and as a research article in the 'Theriogenology' journal)

2.1 ABSTRACT

Nitric oxide (NO) may have a role in persistent breeding-induced endometritis in mares through an inhibitory effect on uterine contractility. The objectives of this study were to test the effect of NO on spontaneous uterine contractility in-vitro and to evaluate whether this effect varied between the longitudinal and circular muscle layers of the uterus. Reproductive tracts were collected from eight euthanized non-pregnant mares (age 4 to 19 years; body weight 405 to 530 kg). Transrectal examination of the reproductive tract was performed before euthanasia to evaluate stage of the estrous cycle and presence of any apparent abnormality. After euthanasia, one uterine tissue sample was collected for histological evaluation and four full-thickness uterine tissue strips (10–12 mm × 2 mm), two parallel to each muscle layer, were excised for in-vitro

contractility evaluation. Strips were suspended in tissue chambers containing Krebs–Henseleit solution, with continuous aeration (95% O_2–5% CO_2; pH 7.4) at 37°C. After equilibration, spontaneous contractility was recorded (pre-treatment) and strips excised in each direction were randomly allocated to each of two groups: 1) SNAP (S-nitroso-N-acetylpenicillamine, an NO donor); or 2) NAP (N-acetyl-D-penicillamine, vehicle and time-matched control). These were treated at 15 min intervals with increasing concentrations (10^{-7} M to 10^{-3} M) of SNAP and NAP, respectively. Contractility data was recorded throughout the experiment. An interaction effect of group-by-concentration was observed ($P<0.0001$). The mean contractility after treatment with 10^{-4} M and 10^{-3} M SNAP were significantly lower than the pre-treatment contractility and the mean contractility after treatment with lower SNAP concentrations. In contrast, contractility did not change significantly in the NAP treated controls. The effect of treatment on uterine contractility was not influenced by age or weight of the mare, stage of estrous cycle, uterine histology grade, or muscle layer. Secondary findings included significant main effects of stage of estrous cycle (increased contractility in estrus compared to diestrus), uterine histology grade (decreased contractility in grade IIB compared to grade I) and age (decreased contractility in mares aged > 8 years compared to mares aged ≤ 8 years). In conclusion, results of this study indicate that NO has a dose-dependent inhibitory effect on spontaneous uterine contractility irrespective of the muscle layer in the mare.

Keywords: Equine, Nitric oxide, Uterine contractility, Breeding-induced endometritis

2.2 INTRODUCTION

A mild transient endometritis which occurs after breeding in mares is a normal, physiological response and does not warrant any treatment (Liu and Troedsson, 2008). In contrast, persistent breeding-induced endometritis (PBIE), where inflammation and intra-uterine fluid retention persist, has a significant negative impact on fertility. Considered as a major reproductive problem in the mare (Woodward and Troedsson, 2013), PBIE has been reported in about 15% of 746 estrous cycles in a Thoroughbred broodmare population (Zent et al., 1998) and in as many as 43% of 552 estrous cycles in a mixed population of mares (Newcombe, 1997). In the latter study, mares with PBIE had a lower pregnancy rate compared to normal mares (49% versus 62%). It has been suggested that the reduced pregnancy rate in mares with PBIE could result either from a direct negative effect on embryonic survival or indirectly from premature luteolysis due to increased prostaglandin F2alpha (PGF2α) production (Troedsson, 1999).

Mares show variability in their susceptibility to persistent endometritis. In one study, some mares were able to efficiently resolve endometritis after experimental bacterial inoculation of the uterus while others were not (Hughes and Loy, 1969). More recent studies using intrauterine infusion of live (Alghamdi et al., 2005) or killed (Woodward et al., 2013b) spermatozoa have reported similar findings. It has been demonstrated that susceptible mares exhibit delayed uterine clearance (Troedsson and Liu, 1991; LeBlanc et al., 1994a), which is believed to be a major factor in the development of persistent endometritis (Troedsson, 2011). Using multiple site electromyography recordings of uterine activity, Troedsson et al. (1993b) showed that susceptible mares displayed impaired myometrial activity, characterized by reduced frequency, duration, and intensity of myometrial contractions. Mechanisms underlying the reduced myometrial activity in susceptible mares are still not completely understood. A possible

mechanism was proposed by Rigby et al. (2001) who, using an in-vitro model to measure isometric tension generated by longitudinal and circular uterine muscle strips in response to potassium chloride, oxytocin, and PGF2α, showed that susceptible mares have an intrinsic contractile defect of the myometrium. Interestingly, this contractile defect did not result from altered regulation of intracellular calcium ion concentration. The impaired uterine clearance could be restored by using ecbolic agents (LeBlanc et al., 1994b), leading Alghamdi et al. (2005) to suggest that the reduced myoelectrical activity in susceptible mares represents an inhibition of contractility or induced relaxation rather than an intrinsic inability to contract.

A possible role of the nitric oxide system in the development of persistent endometritis has been suggested by Alghamdi et al. (2005) and Woodward et al. (2013b) based on their findings that susceptible mares have higher amounts of nitric oxide (NO) in uterine secretions (Alghamdi et al., 2005; Woodward et al., 2013b) and greater endometrial expression of inducible nitric oxide synthase (iNOS) after insemination (Alghamdi et al., 2005). Nitric oxide is synthesized in the body from L-arginine by the enzyme nitric oxide synthase that has three isoforms: endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS), and iNOS (reviewed in Khan et al., 2015). Two of the isoforms, eNOS and iNOS, have been shown to be expressed in the equine endometrium (Welter et al., 2004; Alghamdi et al., 2005; Roberto Da Costa et al., 2007). The eNOS isoform is constitutively expressed and regulates vascular function whereas iNOS expression is typically upregulated during an inflammatory process (Moncada and Higgs, 1993). In a study involving collection of uterine secretions and endometrial biopsy samples 13 hours after insemination, it was observed that susceptible mares have higher amounts of NO in uterine secretions and greater expression of iNOS in the endometrium than resistant mares (Alghamdi et al., 2005). Higher amounts of uterine NO in

untreated susceptible mares compared to resistant mares and a significantly greater increase in uterine NO production at 6 and 12 hours post-insemination in susceptible mares were reported in a more recent study (Woodward et al., 2013b). Considering the well-established relaxant effect of NO on smooth muscle tissues in general, and the previously documented relaxant effects on myometrium in other species such as rat (Yallampalli et al., 1993), monkey (Kuenzli et al., 1998), and human (Bradley et al., 1998), it seems reasonable to speculate that NO may reduce uterine clearance in susceptible mares through an inhibitory effect on uterine contractility, leading to the development of persistent endometritis. The only documented evidence that NO may have an inhibitory effect on uterine contractility in the mare is the reported inability of myometrial tissue in vitro to respond to electrical stimulus in the presence of NO (Liu et al., 1997). To our knowledge, the effect of different NO concentrations on spontaneous uterine contractility in the mare has not been investigated.

The objectives of this study were to test the effect of NO on spontaneous uterine contractility in-vitro and to evaluate whether this effect varied between the longitudinal and circular muscle layers of the uterus. It was hypothesized that NO would have a dose-dependent inhibitory effect on spontaneous uterine contractility irrespective of the muscle layer.

2.3 MATERIALS AND METHODS

All animal procedures in this study were conducted in accordance with the guidelines of the University of Guelph Animal Care Committee and conformed to the recommendations of the Canadian Council on Animal Care. The method of collection and processing of uterine tissue samples and the basic protocol for measuring uterine contractility in this study were similar to

those used previously by Hirsbrunner et al. (2006) for investigating spontaneous uterine contractility in the mare.

2.3.1 Collection and transportation of uterine tissue

Reproductive tracts were collected from clinically healthy non-pregnant light breed mares (N=8) within 30 min of euthanasia using a standard protocol involving an intravenous overdose of pentobarbital sodium (Euthansol, Merck Animal Health Intervet Canada Corp, Kirkland, QC, Canada) at the Ontario Veterinary College, University of Guelph. The mares ranged in age from 4 to 19 years and in body weight from 405 to 530 kg. Transrectal palpation and ultrasonography of the reproductive tract were performed prior to euthanasia to evaluate stage of the estrous cycle and presence of any apparent abnormality. A mare was considered to be in estrus if she had a relaxed cervix, uterine edema, at least one follicle with diameter greater than or equal to 35 mm and no corpus luteum (CL) and in diestrus if she had a firm cervix, tonic uterus, no uterine edema and a CL. After euthanasia, the reproductive tract was visually examined for any gross abnormality and a tissue sample was collected from the base of the right uterine horn for histological evaluation. An 8 to 9 cm long, full thickness circumferential segment close to the base of the right uterine horn was excised and transported within 15 min to the muscle contractility laboratory in a flask containing Krebs-Henseleit solution (KHS) consisting of (in mM): NaCl, 118; KCl, 4.75; $CaCl_2$, 2.54; $MgSO_4$, 1.18; $NaHCO_3$, 24.8; KH_2PO_4, 1.18; Glucose, 10. All chemicals used in the preparation of KHS were purchased from Fisher Scientific, Waltham, MA, USA. The solution was kept at room temperature and pre-aerated with 95% O_2– 5% CO_2 mixture to reach a pH of 7.3–7.4.

2.3.2 Preparation of uterine tissue strips and experimental protocol

The circumferential uterine segment was incised along its longitudinal axis and pinned flat in a dissecting dish containing Krebs-Henseleit solution continuously aerated with 95% O_2–5% CO_2. Full thickness uterine tissue strips of about 10–12 mm length and 2 mm width were dissected, using a custom designed scalpel with two parallel blades. Two strips were excised parallel to the direction of longitudinal muscle fibers and two strips were excised parallel to the circular muscle fibers. Each strip was tied at the ends with 5.0 gauge suture silk and suspended in an individual organ bath containing 10 mL of warm (37°C) Krebs-Henseleit solution continuously aerated with 95% O_2–5% CO_2. The strips were attached to a fixed point at one end and an isometric force transducer (model FT03, Grass Medical Instruments, Quincy, MA, USA) at the other. After a 15 min equilibration, 1g tension was applied, followed 15 min later by another 1g tension. Fifteen min after application of the second 1g tension, spontaneous contractility data was recorded for 30 min in order to determine the pre-treatment contractility of the uterine tissue strips. The strips excised in each direction were then randomly allocated to each of two groups: 1) SNAP (S-nitroso-N-acetylpenicillamine, an NO donor; Sigma Aldrich, St. Louis, MO, USA); or 2) NAP (N-acetyl-D-penicillamine, vehicle and time-matched control; Sigma Aldrich, St. Louis, MO, USA). These were treated at 15 min intervals with increasing concentrations (10^{-7} M to 10^{-3} M) of SNAP and NAP, respectively. The experimental protocol is illustrated in Figure 1. Contractility data was recorded throughout the treatment period and until 15 min after application of the last treatment. The data was recorded using the MP100WSW data acquisition system and AcqKnowledge 3 software (Biopac Systems Inc., Goleta, CA, USA) on a computer (ASUSTeK Computer Inc.). After each experimental run, length and weight of the tissue strips were measured.

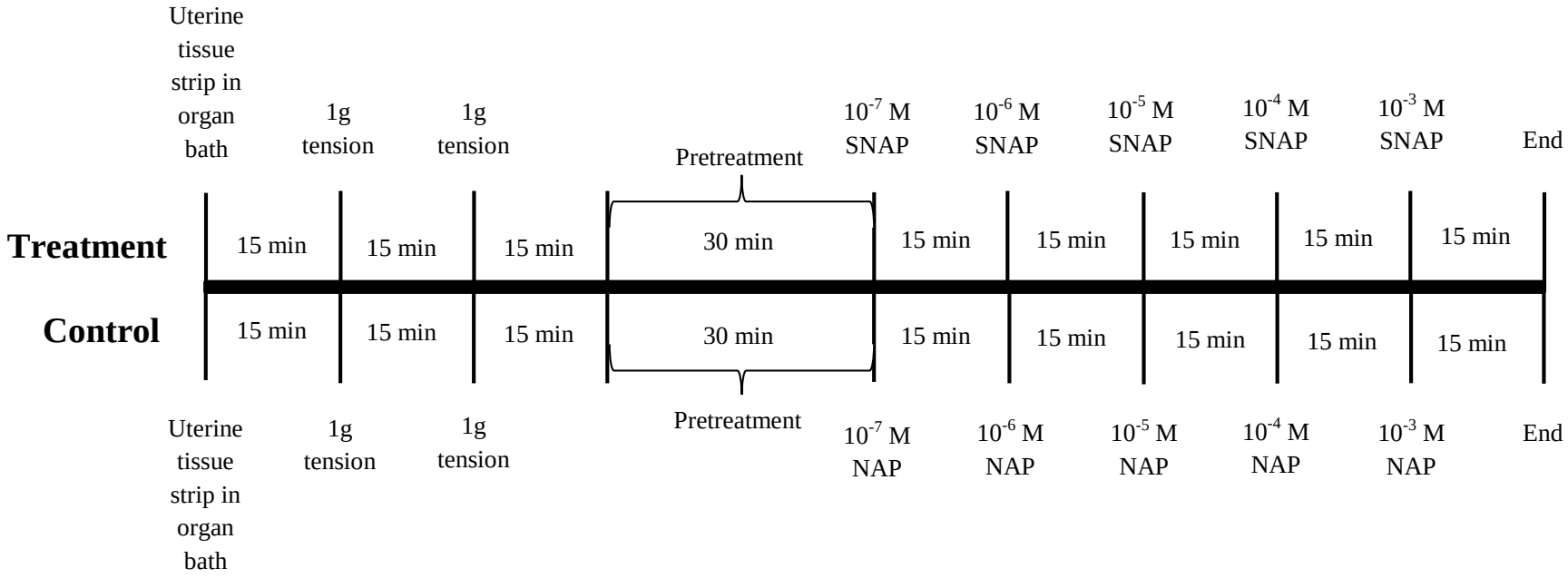


Figure 1: A schematic diagram of the experimental protocol used in this study to test the effect of nitric oxide on uterine contractility (Abbreviations: SNAP, S-nitroso-N-acetylpenicillamine; NAP, N-acetyl-D-penicillamine)

2.3.3 Contractility data analysis

From the recorded data, cumulative area under the curve (AUC) during the 30 min pretreatment period and cumulative AUC during the 15 min period following each treatment were calculated using an in-built measurement function of the AcqKnowledge 3 software. The pretreatment cumulative AUC was divided by 2 to adjust it to 15 min. Cross-sectional area of the uterine tissue strips was calculated using the formula area = mass/(density×length) and a tissue density value of 1.056 g/cm^3 (Hirsbrunner et al. 2006). The cumulative AUC values were then normalized by dividing them by the corresponding cross-sectional area. These normalized cumulative AUC values were used as a measure of contractility (dependent variable) in the statistical analyses.

2.3.4 Histological evaluation

Uterine tissue samples were fixed in 10% neutral-buffered formalin and submitted to the Animal Health Laboratory at the University of Guelph. The samples were processed by routine paraffin embedding followed by sectioning at 4 to 5 µm and staining with Hematoxylin and Eosin (H&E). Histological evaluation of the samples was performed in accordance with the grading scheme of Kenney and Doig (1986) by a reproductive and board-certified pathologist (RAF) who was blinded to the contractility data.

2.3.5 Statistical analyses

Statistical analyses were performed using SAS software (version 9.3, SAS Institute, Inc., Cary, NC, USA). A linear mixed-effects model procedure (PROC MIXED) was used. Various models that included the main effects and interactions of mare age, body weight, stage of estrous cycle, uterine histology grade, muscle layer, group and concentration were fitted and tested using the Akaike Information Criterion (AIC) method. Various correlation error structures offered by the

statistical software were applied to account for a potential autocorrelation between repeated measurements. The error structures included autoregressive 1, autoregressive heterogenous 1, toeplitz and banded toeplitz 2 through 7 as well as the heterogenous versions, and so-called unstructured and the banded versions unstructured 2 through 7. The application of unstructured correlation error structure yielded the lowest AIC (the best fit model). To assess the ANOVA assumptions, residual analyses were performed. The residuals were formally tested for normality by means of Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises, and Anderson-Darling tests. The residuals were also plotted against the predictive values and explanatory variables used in the model to identify any outliers, unequal variance, or other issues that would require further investigation. Assumptions of normality were satisfied by logarithmic transformation of the data. Post hoc analysis was performed using Least Significant Difference (LSD) test with the Bonferroni correction for multiple comparisons. Results are presented as back-transformed least squares means (LS-means) and 95% confidence intervals (CIs).

2.4 RESULTS

Of the eight mares used in this study, three were classified to be in estrus and five in diestrus. No apparent abnormalities were detected in any of the mares either on transrectal examination of the reproductive tract before euthanasia or visual examination of the reproductive tract after euthanasia. Based on histological examination, one mare was classified as having a grade I uterus (normal), five mares were classified as having grade IIA uteri (with mild inflammatory changes) and two mares as having grade IIB uteri (with moderate inflammatory changes). A total of 32 uterine tissue strips were used in the in-vitro contractility experiment. One strip excised

parallel to the circular muscle layer and allocated to the NAP (control) group lost the tie at one end during the experiment and was excluded from the data analysis.

An interaction effect of group-by-concentration on uterine contractility was observed ($P<0.0001$). The mean contractility after treatment with 10^{-4} M and 10^{-3} M SNAP were significantly lower than the mean pre-treatment contractility and the mean contractility after treatment with lower SNAP concentrations (Figure 2a). In contrast, contractility did not change significantly in the NAP treated controls (Figure 2b). The effect of treatment on uterine contractility was not influenced by the age or weight of the mare, stage of estrous cycle, uterine histology grade, or muscle layer (interaction of group with each of these factors was non-significant; $P>0.05$). The main effect of muscle layer on uterine contractility was also non-significant ($P>0.05$).

Other findings included significant main effects of age ($P<0.0001$), stage of estrous cycle ($P<0.0001$), and uterine histology grade ($P=0.0066$). The mean spontaneous contractility of uterine strips from mares greater than 8 years old (LS-mean 351.63; 95% CI 330.65, 372.61; n=114 observations; N=5 mares) was significantly lower than the corresponding value in mares aged 8 years or younger (LS-mean 975.12; 95% CI 923.76, 1026.50; n=72 observations; N=3 mares). The negative effect of age on uterine contractility was also evident on a correlation analysis between age and uterine contractility (Spearman's correlation coefficient of -0.616, $P<0.01$). The mean contractility was significantly greater in estrus (LS-mean 703.11; 95% CI 615.92, 790.30; n=72 observations; N=3 mares) than in diestrus (LS-mean 514.83; 95% CI 459.58, 570.08; n=114 observations; N=5 mares). With respect to the uterine histology, significantly greater uterine contractility was observed in grade I (LS-mean 812.34; 95% CI 543.98, 1213.09; n=24 observations; N=1 mare) compared to grade IIB (LS-mean 365.74; 95%

CI 272.97, 490.06; n=42 observations; N=2 mares). However, the mean AUC of grade IIA (LS-mean 504.63; 95% CI 422.15, 603.22; n=120 observations; N=5 mares) was not different from either of the other two grades (I and IIB).

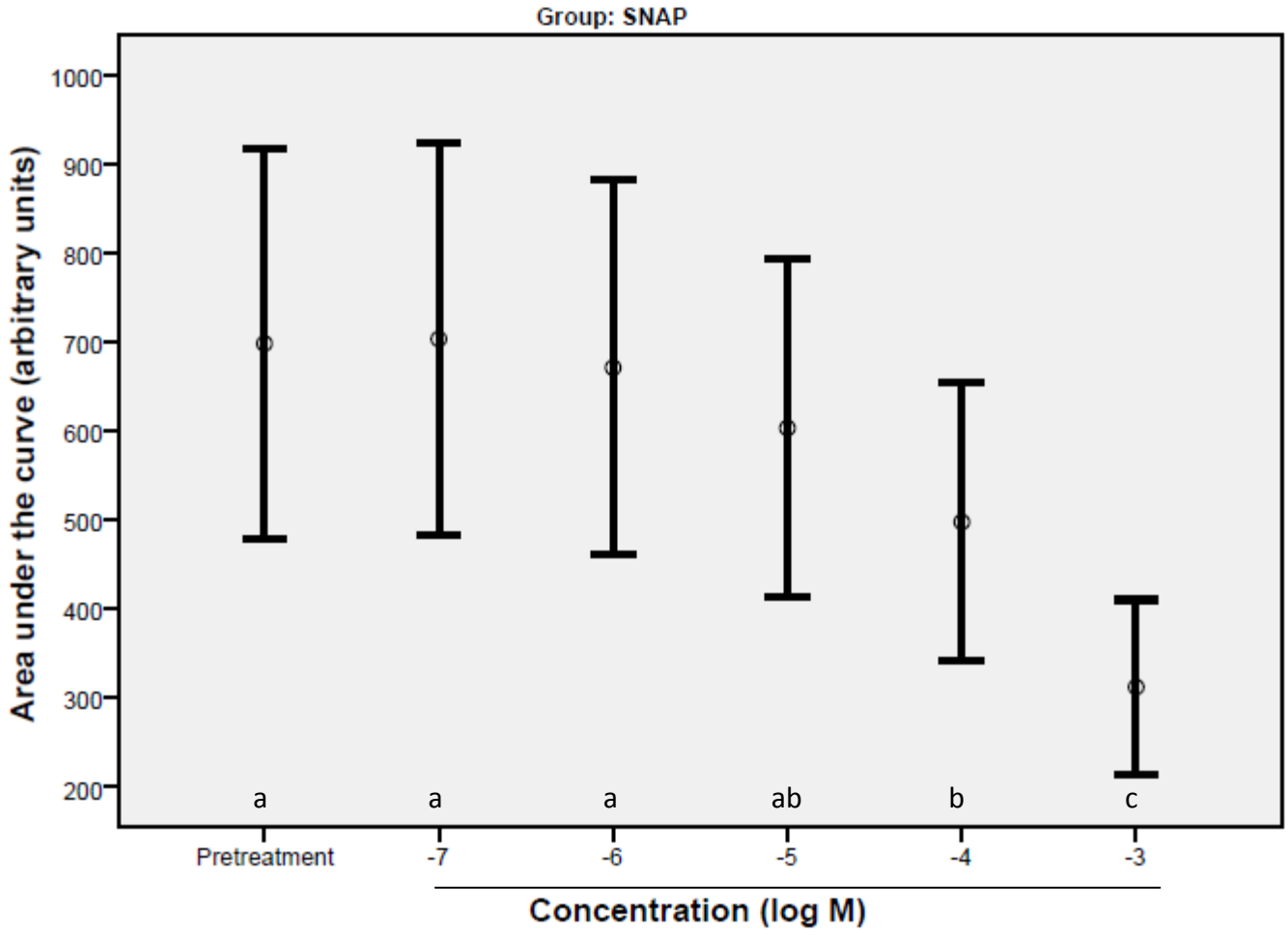


Figure 2a: Contractility (LS-means and 95% CIs of normalized cumulative area under the curve) of uterine tissue strips before (pretreatment) and after treatment with different concentrations (10^{-7} M to 10^{-3} M) of SNAP (S-nitroso-N-acetylpenicillamine). Each mean represents an average of 16 observations, (N=8 mares). Different letters indicate significant differences (P<0.05).

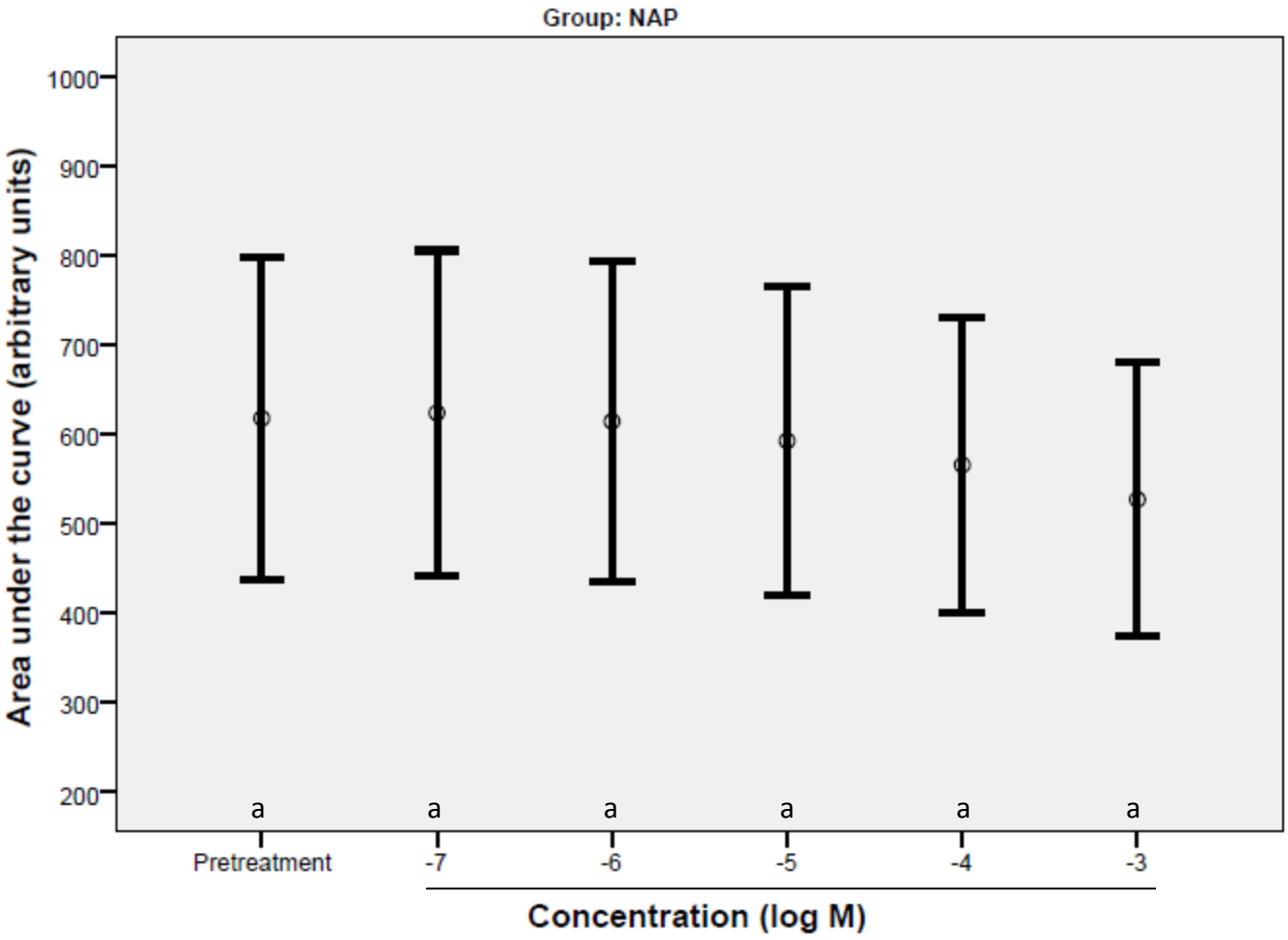


Figure 2b: Contractility (LS-means and 95% CIs of normalized cumulative area under the curve) of uterine tissue strips before (pretreatment) and after treatment with different concentrations (10^{-7} M to 10^{-3} M) of NAP (N-acetyl-D-penicillamine). Each mean represents an average of 15 observations (N=8 mares). Different letters indicate significant differences (P<0.05).

2.5 DISCUSSION

The results of this study support the hypothesis that NO has a dose-dependent inhibitory effect on spontaneous uterine contractility irrespective of the muscle layer. These findings, taken together with the earlier observations that mares susceptible to PBIE have greater amounts of uterine NO (Alghamdi et al., 2005; Woodward et al., 2013b), suggest a role of NO in the development of PBIE in mares. It is interesting that the inhibitory effect of NO on uterine contractility did not vary with age and uterine histology grade, as indicated by the non-significant interactions of group with age and uterine histology grade. It has been demonstrated previously that older mares and mares with higher uterine histology grades (IIB and III) are more susceptible to PBIE (Woodward et al., 2012). The absence of a group-by-age or a group-by-uterine biopsy grade interaction in the present study suggests that the greater susceptibility to PBIE in these mares may be due to higher uterine NO concentrations rather than increased uterine sensitivity to NO. However, further studies with mares of different ages and all four uterine histology grades are required to directly test these hypotheses. Similarly, the inhibitory effect of NO on uterine contractility was not influenced by stage of the estrous cycle based on observation of a non-significant group-by-stage interaction. While this suggests an absence of differential sensitivity of the uterine musculature to NO during different stages of the estrous cycle, it does not preclude a role of NO in regulating uterine contractility during the estrous cycle. There is evidence of a differential expression of NOS isoforms across different stages of the estrous cycle (Welter et al., 2004; Honnens et al., 2011; Gebhardt et al., 2012). However, studies on differences in the actual uterine NO concentrations between estrus and diestrus in the mare are lacking.

Studies on in-vitro uterine contractility in the mare have reported a wide range of percentages (0% to 91.2%) of uterine tissue strips that showed spontaneous contractility (Ousey et al., 2000; Rigby et al., 2001; Hirsbrunner et al., 2006; Steckler et al., 2012). Possible reasons for this great variability, such as use of anesthetized versus euthanized mares, individual variation and differences in survivability of tissue ex vivo, number of active receptors and muscle activity, have been proposed previously (Hirsbrunner et al., 2006; Steckler et al., 2012). The higher percentage (100%) of uterine tissue strips demonstrating spontaneous contractility after placement in the organ baths in the present study may be partly due to the establishment of a very consistent protocol of tissue handling. Training in the uterine tissue handling procedures was undertaken and the experimental protocol was optimized using nine ovine and two porcine uteri prior to commencement of the study (Appendix 2.1). Ovine and porcine uteri were used for training and optimization instead of equine uteri because of a greater local availability of ovine and porcine reproductive tracts.

The significant main effects of mare age and uterine histology grade on uterine contractility and the significant negative association of increasing age with uterine contractility observed in this study are consistent with the previously reported influence of age and uterine histology score on susceptibility of mares to PBIE (Woodward et al., 2012). The main effect of age on uterine contractility was tested in the present study with age as a continuous variable. The categorization of age was performed afterwards in order to compare the results with those of an earlier study (Woodward et al., 2012) in which age was used as a categorical variable in the statistical analyses. Similarly, the analysis of correlation between age and uterine contractility was performed afterwards to further evaluate the main effect. Future studies with larger sample sizes at the mare level are needed to investigate the effects of age and uterine histology grade on

uterine contractility in mares and the mechanisms underlying these effects. The significant main effect of stage of estrous cycle on uterine contractility (greater contractility during estrus as compared to diestrus) makes biological sense considering the functions of the uterus during the different stages of the estrous cycle. Greater contractility during estrus would aid spermatozoal transport to the site of fertilization and uterine clearance of dead spermatozoa and inflammatory debris after insemination (Troedsson et al., 1998). Due to the small number of true biological replicates in the present study, the secondary findings related to effects of age, stage of estrous cycle and, especially, uterine histology grade on uterine contractility should be interpreted with caution.

An apparent limitation of this study is the use of an NO donor (SNAP) instead of authentic NO. However, the use of NO donors instead of NO is a very common and accepted practice in both in-vitro and in-vivo studies investigating the effects of NO on different biological processes. It is widely known that NO gas is difficult to handle, requires complete exclusion of oxygen to prevent its oxidation to nitrogen dioxide, involves a complex process during preparation of different concentrations and is highly unstable in solution (Ignarro et al., 1987; Miller and Megson, 2007). When using NO donors, there is a possibility that the observed effect might be partly or fully due to breakdown products other than NO. To test this possibility, similar concentrations of NAP, the other breakdown product of SNAP besides NO, were applied to control uterine tissue strips in this study. The absence of a significant effect on uterine contractility at all the tested NAP concentrations rules out the possibility that the inhibitory effect observed in the SNAP treated group could have resulted from breakdown products of SNAP other than NO.

In conclusion, results of this study indicate that NO has a dose-dependent inhibitory effect on spontaneous uterine contractility irrespective of the muscle layer in the mare. The presence of increased NO concentrations in the uteri of mares susceptible to PBIE coupled with our findings that NO decreases uterine contractility constitute a potential mechanism underlying development of PBIE in the mare.

2.6 ACKNOWLEDGEMENTS

This work was supported by a research grant from Equine Guelph. The authors thank Drs. Luis Arroyo, Judith Koenig and Donald Trout from the Department of Clinical Studies, University of Guelph for their generous help with collection of reproductive tracts used in this study and William Sears from the Department of Population Medicine, University of Guelph for his help with the statistical analyses. The authors also thank the Ontario Trillium Foundation for a scholarship to the first author.

CHAPTER THREE

ENDOMETRIAL NITRIC OXIDE SYNTHASE ACTIVITY IN MARES SUSCEPTIBLE OR RESISTANT TO PERSISTENT BREEDING-INDUCED ENDOMETRITIS AND THE EFFECT OF A SPECIFIC INOS INHIBITOR

Firdous A. Khan[a], Tracey S. Chenier[a], Robert A. Foster[b], Joanne Hewson[c], Elizabeth L. Scholtz[a]

[a]Department of Population Medicine, [b]Department of Pathobiology, [c]Department of Clinical Studies, University of Guelph, Canada

3.1 ABSTRACT

Emerging research suggests that the nitric oxide system may play a role in the development of persistent breeding-induced endometritis (PBIE) in the mare. Differences in uterine nitric oxide (NO) levels between mares susceptible or resistant to PBIE and a dose-dependent inhibitory effect of NO on uterine contractility have been demonstrated. The objectives of the present study were to investigate the difference in total nitric oxide synthase (NOS) activity of the endometrium between susceptible and resistant mares and the effect of a specific inducible nitric oxide synthase (iNOS) inhibitor on the endometrial NOS activity. Six susceptible and six resistant mares were selected based on preset criteria and the results of an intrauterine challenge with killed spermatozoa during estrus. Endometrial biopsy samples were collected 24 hours post challenge and cultured at 37°C for 24 hours in L-arginine supplemented minimum essential medium with or without a specific iNOS inhibitor (1400W dihydrochloride, 1 mM). The medium and the cultured endometrial tissue were collected after 24 hours of culture and assayed for NO and total protein, respectively. Total NO content of the medium, normalized to endometrial tissue wet weight or total protein, was used as a measure of endometrial NOS activity. Non-

parametric tests were applied for statistical analysis of the data; $P<0.05$ was considered significant. Total NO production by the endometrial tissue was significantly greater in susceptible mares than in resistant mares. Within the susceptible mare group, iNOS inhibitor treatment significantly reduced NO production by the endometrial tissue, when normalized to total tissue protein or tissue wet weight. Within the resistant mare group, a significant reduction was observed when NO production was normalized to tissue wet weight but not when it was normalized to tissue protein content. Correlation analysis indicated a positive association between endometrial tissue wet weight and total protein content (Spearman's correlation coefficient of 0.632, $P<0.01$). Results of this study indicate that susceptible mares have greater total NOS activity in the endometrium than resistant mares, and that treatment with a specific iNOS inhibitor reduces total endometrial NOS activity in susceptible mares.
Keywords: Equine, Nitric oxide synthase, Breeding-induced endometritis, Inducible nitric oxide synthase inhibitor

3.2 INTRODUCTION

A mild transient endometritis after breeding is a normal, physiological response in mares (Liu and Troedsson, 2008). In some mares, the inflammation and intra-uterine fluid retention after breeding persist and have a significant negative impact on fertility (Newcombe, 1997). The incidence of persistent breeding-induced endometritis (PBIE) has been reported to range between 15% in a Thoroughbred broodmare population (Zent et al., 1998) to 43% in a mixed population of mares (Newcombe, 1997). It has also been reported that mares show variability in their susceptibility to persistent endometritis. Hughes and Loy (1969) demonstrated a difference between mares in their ability to resolve endometritis after experimental inoculation of the uterus with *Streptococcus zooepidemicus*. Similar findings have been reported in more recent studies using intrauterine infusion of live (Alghamdi et al., 2005) or killed (Woodward et al., 2013b) spermatozoa. This variability in susceptibility to persistent endometritis has been attributed mostly to impaired myometrial contractility (Troedsson et al., 1993b) and delayed uterine clearance (Troedsson and Liu, 1991; LeBlanc et al., 1994a; Troedsson, 2011) in susceptible mares.

A possible role of the nitric oxide system in the development of persistent endometritis has been suggested by Alghamdi et al. (2005) and Woodward et al. (2013b) based on a hypothesized inhibitory effect of nitric oxide (NO) on uterine contractility. The only previously documented evidence that NO has an inhibitory effect on uterine contractility in the mare was the reported inability of the myometrial tissue to respond in-vitro to electrical stimulus in the presence of NO (Liu et al., 1997). However, there is recent evidence that NO has a dose-dependent inhibitory effect on spontaneous uterine contractility in the mare (Khan et al., 2016). Nitric oxide is synthesized in the body from L-arginine by the enzyme nitric oxide synthase

(NOS) that has three isoforms: endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS), and iNOS (reviewed in Khan et al., 2015). Expression of eNOS and iNOS in the equine endometrium has been demonstrated in multiple studies (Welter et al., 2004; Alghamdi et al., 2005; Roberto Da Costa et al., 2007). The eNOS isoform is constitutively expressed and regulates vascular function, whereas iNOS expression is typically upregulated during an inflammatory process (Moncada and Higgs, 1993). In a study involving collection of uterine secretions and endometrial biopsy samples 13 hours after insemination, Alghamdi et al. (2005) observed that susceptible mares have higher amounts of NO in uterine secretions and greater expression of iNOS in the endometrium than resistant mares. The expression of eNOS and nNOS was not different between the two groups of mares. Woodward et al. (2013b) reported higher amounts of uterine NO in susceptible mares compared to resistant mares and a significantly greater increase in uterine NO production at 6 and 12 hours post-insemination in susceptible mares. There was no difference in iNOS expression between susceptible and resistant mares and the expression of the other two isoforms was not investigated. Further studies to investigate the differences in expression of the NOS isoforms and the total NOS activity between mares susceptible or resistant to PBIE, and the effect of specific iNOS inhibitors on endometrial NO production will provide valuable information on the pathogenesis of PBIE. Studies in this area may provide a basis for the use of iNOS inhibitors as prophylactic or therapeutic options for PBIE in mares.

The objectives of this study were to investigate in-vitro (i) the difference in total NOS activity of the endometrium between susceptible and resistant mares and (ii) the effect of a specific iNOS inhibitor on total NOS activity of the endometrium in susceptible and resistant mares. It was hypothesized that (i) susceptible mares would have greater total NOS activity in

the endometrium than resistant mares and (ii) treatment with a specific iNOS inhibitor would reduce the total NOS activity of the endometrium in susceptible mares.

3.3 MATERIALS AND METHODS

All animal procedures in this study were approved by the University of Guelph Animal Care Committee (Animal Utilization Protocol number 3213) and conformed to the recommendations of the Canadian Council on Animal Care.

3.3.1 Selection of mares

Thirty mares of various breeds (Standardbred, Quarter Horse, Morgan, mixed breed) and ages (3 to 17 years) housed at the Arkell Equine Research Station at the University of Guelph were screened for inclusion in this study. Criteria for the selection of mares were similar to those reported previously by Alghamdi et al. (2005) and Woodward et al. (2013b). Mares were examined by transrectal palpation and ultrasonography (Aloka SSD 500, 5.0 MHZ linear array transducer, Aloka Co. Ltd., Tokyo, Japan) to determine stage of the estrous cycle and the presence of any apparent abnormalities. The vulva and perineum were washed at least three times using warm water and gentle soap (Jergens®, Cincinnati, Ohio, USA) and dried using paper towels. Endometrial samples were collected for cytology and bacterial culture by manually guiding a guarded swab (Kalayjian Industries Inc., Signal Hill, CA, USA) through the vulva, vagina, and cervix into the uterine body using a long sterile sleeve and sterile gel (Muko, Cardinal Health Canada Inc., Mississauga, ON, Canada). The swab was rolled three times in the uterine lumen, left in place for 30 seconds, and retracted into the guarding sheath. The guarding sheath was then rolled three times to collect a sample in the cap for cytology before removal from the uterus. This was followed by collection of an endometrial biopsy sample using a

standard trans-cervical, rectally guided technique (Kenney, 1978) and a Pilling-Weck biopsy punch (Jorgensen Laboratories, Loveland, CO, USA). The biopsy sample was immediately fixed in 10% neutral-buffered formalin.

The contents of the cap of the guarded swab were pressed onto a glass slide. The slide was air dried, sprayed with a cytofixative (Cytoprep, Fisher Scientific Ltd., Nepean, ON, Canada) and stained with a modified Giemsa stain (Protocol Wright Giemsa Stain, Fisher Scientific, Ottawa, ON, Canada). The stained slide was examined under the microscope and the presence of more than two neutrophils per five high power (×400) fields was considered to be indicative of endometritis (Woodward et al., 2013b). The swab was submitted to the Animal Health Laboratory (AHL) at the University of Guelph for routine aerobic bacterial culture. The biopsy sample was submitted to the AHL and processed by routine paraffin embedding followed by sectioning at 4 to 5 µm and staining with Hematoxylin and Eosin (H&E). Histological evaluation of the sample was performed by a reproductive and board-certified pathologist (RAF) in accordance with the grading scheme of Kenney and Doig (1986). Eighteen mares that had two or fewer neutrophils per five high power fields on endometrial cytology and no bacterial growth on culture were selected for inclusion in the study. These mares were then assigned, based on results of the endometrial biopsy (Alghamdi et al., 2005; Woodward et al., 2013b), to groups potentially resistant (n=9; grade I or IIA) or susceptible (n=9; grade IIB or III) to PBIE.

3.3.2 Preparation of killed spermatozoa suspension for intrauterine challenge

Semen samples from one stallion were centrifuged at 400 × g for 10 minutes. After removing the supernatant, the spermatozoa pellet was resuspended in INRA 96 extender (IMV Technologies, L'Aigle, France). Multiple aliquots of 1×10^9 killed spermatozoa in 30 mL extender were prepared and subjected to two cycles of freezing (-20°C) and thawing (room temperature). After

the second thawing, a drop of the spermatozoa suspension from each aliquot was observed under the microscope for motility. No motility was observed, confirming that the spermatozoa were killed. The aliquots were stored at -20°C until use for intrauterine challenge.

3.3.3 Final classification of mares

The final classification of mares into susceptible and resistant groups was based on their response to challenge using an approach similar to that used previously by Woodward et al. (2013b). Mares were administered 125 µg cloprostenol (Estrumate®, Intervet Canada Corp., Kirkland, QC, Canada) intramuscularly and examined 48 to 72 hours later by transrectal palpation and ultrasonography. Mares with a relaxed cervix, uterine edema, at least one follicle with diameter greater than or equal to 35 mm and no corpus luteum (CL) were considered to be in estrus and subjected to an intrauterine challenge with killed spermatozoa. Before challenge, the vulva and perineum were washed and endometrial samples were collected for cytology and bacterial culture as previously described. Intrauterine infusion of 1×10^9 killed spermatozoa suspended in 30 mL extender was then performed using a 21 inch insemination pipette (Partnar Animal Health, Ilderton, ON, Canada) guided manually through the vulva, vagina, and cervix into the uterine body. Immediately following intrauterine infusion, mares were administered 2500 IU human chorionic gonadotropin (hCG, Chorulon®, Intervet Canada Corp., Kirkland, QC, Canada) intravenously. The mares were examined by transrectal ultrasonography every 24 hours until 96 hours post infusion. Mares that had two or fewer neutrophils per five high power fields on endometrial cytology, no bacterial growth on culture, and cleared uterine fluid within 24 hours post infusion were considered to be resistant mares (N=6). Mares that had two or fewer neutrophils per five high power fields on endometrial cytology, no bacterial growth on culture,

and did not clear uterine fluid by 96 hours post infusion were considered to be susceptible mares (N=6). Six mares did not fit either of these criteria and were excluded.

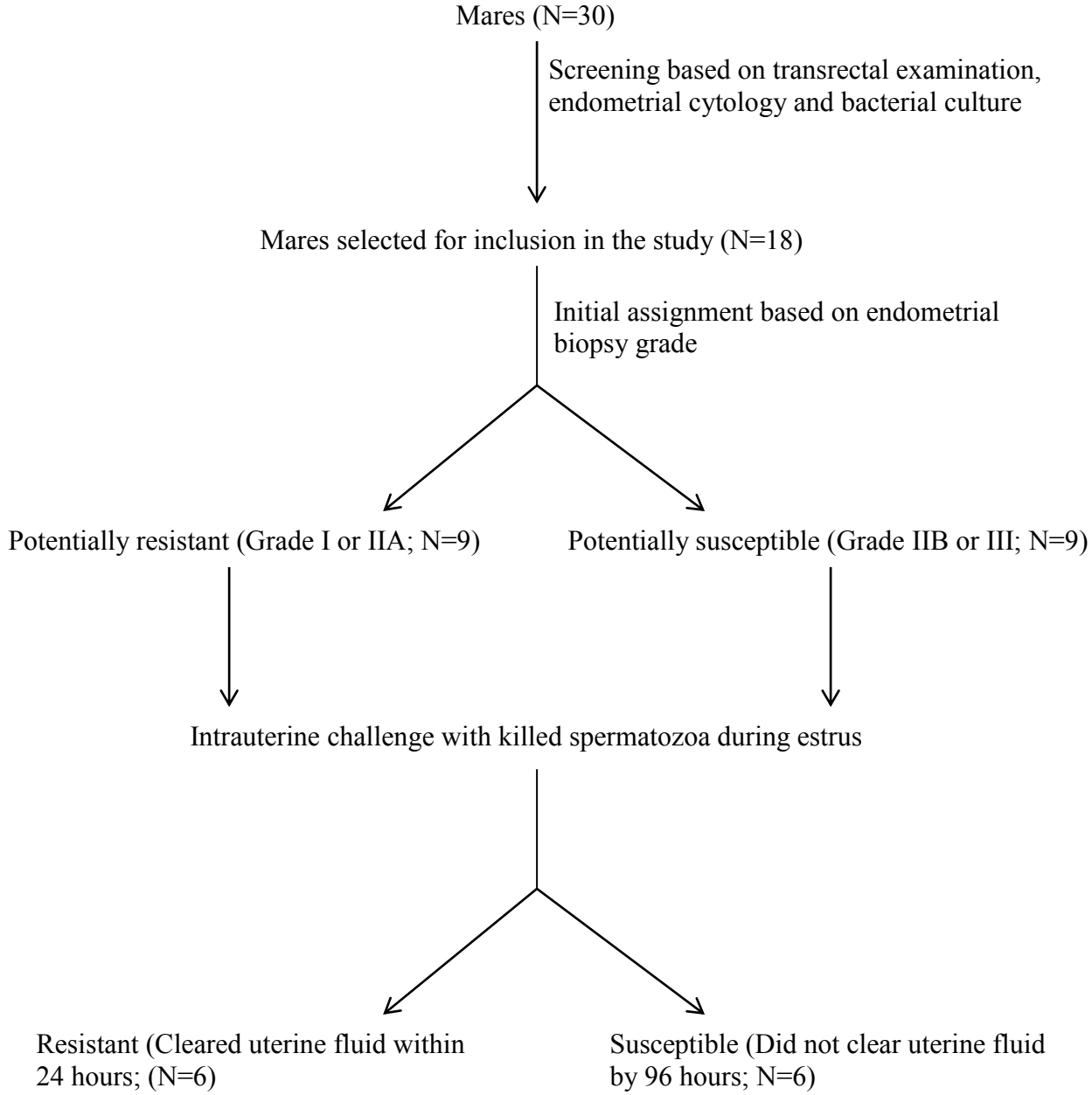


Figure 1: A schematic diagram of the selection and classification of mares

3.3.4 Determination of total NOS activity of the endometrium

An endometrial biopsy sample was collected from the base of the right uterine horn immediately following transrectal examination 24 hours post infusion with the killed spermatozoa. The sample was divided into two halves using a sterile scalpel blade and each half was transported to the laboratory in physiological saline containing 1% (v/v) penicillin-streptomycin (10,000 units/mL penicillin; 10,000 µg/mL streptomycin; Gibco^{TM}, ThermoFisher Scientific, Waltham, MA, USA).

Each of the two pieces of endometrial tissue were weighed, assigned randomly to either control or treatment groups, and cultured at 37°C for 24 hours in minimum essential medium (MEM, Gibco^{TM}, ThermoFisher Scientific, Waltham, MA, USA) containing 1% (v/v) penicillin-streptomycin and 5% fetal bovine serum (Gibco^{TM}, ThermoFisher Scientific, Waltham, MA, USA). At the beginning of the culture, the treatment group received 1 mM iNOS inhibitor (1400W dihydrochloride/N-([3-(Aminomethyl)phenyl]methyl)ethanimidamide dihydrochloride, Sigma-Aldrich, Oakville, ON, Canada) and the control group received a similar volume (250 µL) of MEM. Thirty minutes later, both groups received 1 mM L-arginine (Sigma-Aldrich, Oakville, ON, Canada). The medium and the cultured endometrial tissue were collected at 24 hours of culture and stored at -20°C until assays for NO and total protein, respectively, were performed.

Concentration of NO in the stored culture medium was determined using a commercially available assay kit (Nitrate/Nitrite Colorimetric Assay Kit, Cayman Chemical Co., Ann Harbor, MI, USA). Nitric oxide is highly unstable and reacts with several molecules present in biological fluids to form nitrates and nitrites. The assay measures the sum of both nitrate and nitrite concentrations as an index of total NO production. Samples were run in duplicate in a 96-well plate supplied with the kit. The minimum sensitivity was 2.0 µM and the intra-assay coefficient

of variation was 9.7%. Protein extraction from the tissue samples was performed using T-Per® Tissue Protein Extraction Reagent containing Halt Protease Inhibitor Cocktail (ThermoFisher Scientific, Waltham, MA, USA). Protein concentration of the extracts was determined using a Coomassie (Bradford) Protein Assay Kit (ThermoFisher Scientific, Waltham, MA, USA). The minimum sensitivity was 125 µg/mL and the intra-assay coefficient of variation was 3.7%. The protein extraction and the NO and protein assays were performed according to the manufacturers' instructions.

The total NO produced by the cultured endometrial tissue was calculated by multiplying NO concentration of the stored culture medium with the volume of the medium. Similarly, total protein content of the cultured endometrial tissue was calculated by multiplying protein concentration of the protein extract with the extract volume. The total nitric oxide production was divided by total protein or tissue weight for normalization. The normalized nitric oxide production was used as a measure of the total NOS activity.

3.3.5 Statistical analyses

Statistical analyses were performed using SPSS software (version 16.0, SPSS Inc., Chicago, IL, USA). Data were formally tested for normality by means of Shapiro-Wilk and Kolmogorov-Smirnov tests. Since assumptions of normality were not satisfied by raw or transformed data, non-parametric tests were applied for the analyses. The Mann-Whitney test was applied to evaluate differences in total NOS activity between susceptible and resistant mares and the Wilcoxon Signed-Rank test was applied to evaluate differences between control and treatment groups. Association between endometrial tissue weight and total protein content was determined using Spearman's correlation analysis. Results are presented as medians and 25^{th} and 75^{th} percentiles.

3.4 RESULTS

Within the control subgroup, the total NO production by the endometrial tissue was significantly greater in susceptible mares than in resistant mares, both when normalized to the total tissue protein (Figure 2) or the tissue wet weight (Figure 3). Similar results were obtained within the treatment subgroup when the total NO production was normalized to the tissue wet weight (Figure 3). Within the susceptible mare group, iNOS inhibitor treatment significantly reduced the NO production from the endometrial tissue. This was true both on normalization of the total NO production to the tissue protein content (Figure 2) or normalization to the tissue wet weight (Figure 3). Within the resistant mare group, a significant reduction was observed when the NO production was normalized to the tissue wet weight (Figure 3). A similar but statistically non-significant trend was observed when the NO production was normalized to the tissue protein content (Figure 2). Correlation analysis indicated a positive association between endometrial tissue wet weight and total protein content (Spearman's correlation coefficient of 0.632, $P<0.01$).

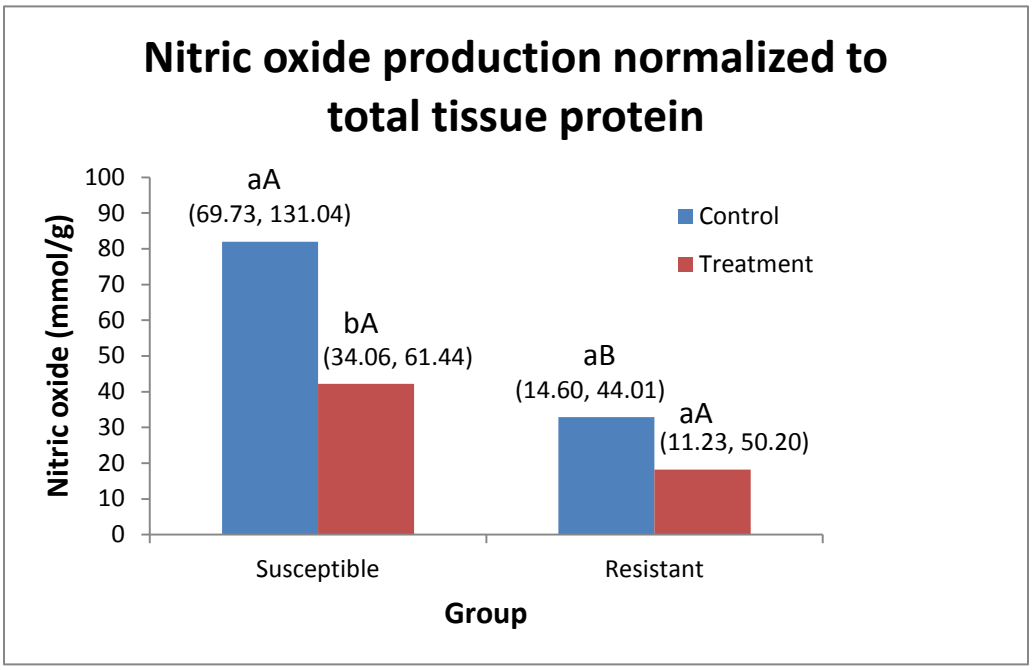


Figure 2: Nitric oxide production (normalized to total tissue protein) by the endometrial tissue of susceptible and resistant mares and the effect of a specific inducible nitric oxide synthase (iNOS) inhibitor (1400W dihydrochloride, 1 mM) in-vitro. The bars represent medians and the numbers in parentheses represent 25th and 75th percentiles. Different lowercase letters indicate significant differences (Wilcoxon Signed-Rank test, P<0.05) between control and treatment subgroups within susceptible and resistant mares. Different uppercase letters indicate significant differences (Mann-Whitney U-test, P<0.05) between susceptible and resistant mares within each of the control and treatment subgroups.

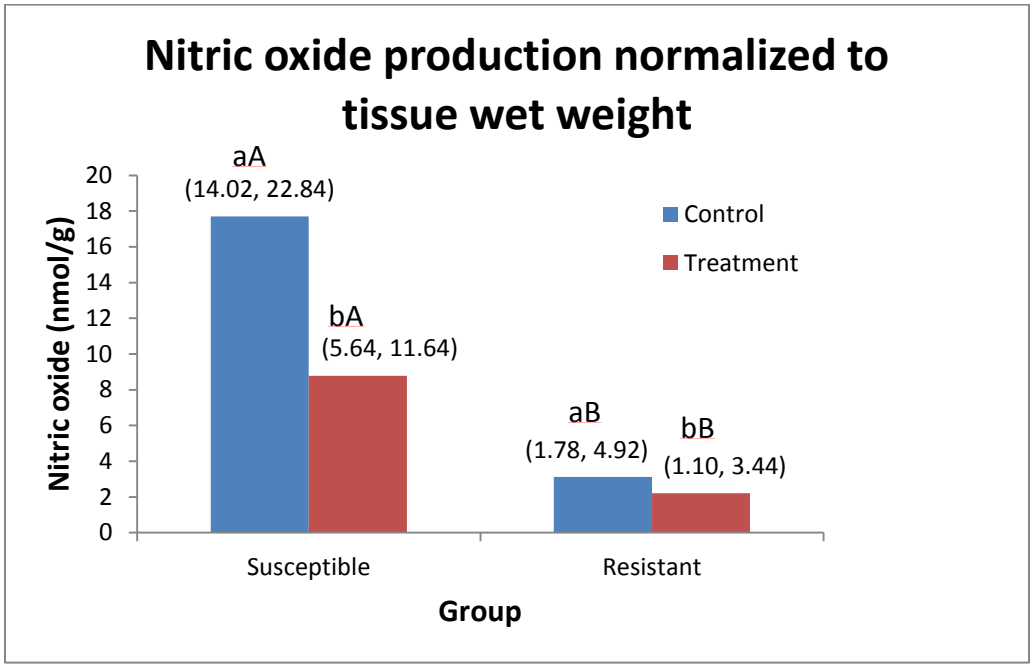


Figure 3: Nitric oxide production (normalized to tissue wet weight) by the endometrial tissue of susceptible and resistant mares and the effect of a specific inducible nitric oxide synthase (iNOS) inhibitor (1400W dihydrochloride, 1 mM) in-vitro. The bars represent medians and the numbers in parentheses represent 25th and 75th percentiles. Different lowercase letters indicate significant differences (Wilcoxon Signed-Rank test, P<0.05) between control and treatment subgroups within susceptible and resistant mares. Different uppercase letters indicate significant differences (Mann-Whitney U-test, P<0.05) between susceptible and resistant mares within each of the control and treatment subgroups.

3.5 DISCUSSION

The results of this study support the hypothesis that susceptible mares have greater total NOS activity in the endometrium than resistant mares. The greater total NOS activity in the endometrium of susceptible mares in the present study is in alignment with the previously reported greater intrauterine NO levels (Alghamdi et al., 2005; Woodward et al., 2013b) and higher iNOS expression (Alghamdi et al., 2005) in susceptible mares in comparison to resistant mares. These findings coupled with the recently reported dose-dependent inhibitory effect of NO on uterine contractility in the mare (Khan et al., 2016) indicate that the nitric oxide system may play an important role in the development of PBIE in mares. The present study also supports the hypothesis that treatment with a specific iNOS inhibitor (1400W dihydrochloride, 1 mM) reduces the total endometrial NOS activity in susceptible mares. This effect was tested in-vitro in the current study. Further in-vitro studies are required to evaluate the endometrial response to different doses of specific iNOS inhibitors. There is also a need to investigate the dose-response in-vivo followed by clinical trials on the efficacy of suitable iNOS inhibitors in prevention or treatment of PBIE in mares.

The use of total NO produced by the endometrial tissue as a measure of the total NOS activity in the present study was adopted from a previous study that used a similar approach in the rat (Yallampalli et al., 1993). In addition to the measurement of endometrial tissue weight and its use for normalization of total NO production (Yallampalli et al., 1993), the total protein content of the cultured endometrial tissue was also determined and used for normalization in the present study. This was based on the concern that the tissue wet weight may not be as accurate as the protein content when used as a measure of the total cellular mass of the endometrial tissue. However, very similar results were obtained when the total NO production was normalized to

either the tissue weight or the total protein content. Analysis of correlation between tissue wet weight and total protein content indicated a significant positive association between the two parameters. These findings suggest that either of the two parameters or both together may be used for normalization of total NO production in future studies. The slight discrepancies in results that were observed here might be due to a small sample size rather than due to the different normalization parameters.

Although the basic approach for selection and classification of mares in the present study was similar to that used previously by Alghamdi et al. (2005) and Woodward et al. (2013b), a notable difference was the use of an ovulatory agent (hCG) immediately after the intrauterine challenge with killed spermatozoa. The purpose of using an ovulatory agent in this study was to minimize variability in the interval to ovulation and to better synchronize endometrial tissue collection with respect to the time of ovulation. It was thought that extreme variation in cervical closure post-ovulation in spontaneously ovulating mares may confound the results by affecting uterine fluid clearance and endometrial status post-challenge. The intervals from intrauterine challenge to ovulation were not reported in either of the two previous studies (Alghamdi et al., 2005; Woodward et al., 2013b). Of the 18 mares that received hCG in the present study, 3 (16.7%) ovulated within 24 hours, 13 (72.2%) ovulated between 24 to 48 hours, and 2 (11.1%) ovulated between 48 to 72 hours. These results are very similar to those of a large retrospective study involving records from 559 cycling mares in which the ovulation distribution was 16% within 24 hours, 76% between 24 to 48 hours, and 9% between 48 to 72 hours after hCG administration (Barbacini et al., 2000). Further studies are needed to assess the impact of ovulation timing, relative to the time of insemination, on endometrial NOS activity and uterine NO concentration.

In conclusion, results of this study indicate that susceptible mares exhibit greater total NOS activity in the endometrium than resistant mares. This study also demonstrates that treatment with a specific iNOS inhibitor reduces the total NOS activity of the endometrium in susceptible mares. These findings provide a proof of principle for the use of iNOS inhibitors in future clinical trials as prophylactic or therapeutic options for PBIE in the mare.

3.6 ACKNOWLEDGEMENTS

This work was supported by a research grant from Equine Guelph and an in-kind contribution from the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA). The authors thank students Shawn-Elizabeth Maloney, Melanie Harness, Sarah Dyck, Evelyn Helps, Megan Ballantine, and Kate Sweetman and the staff at the Arkell Equine Research Station for their help during the selection and classification of mares and the collection of samples. The authors also thank Dr. Monica Antenos and other members of the laboratory of Dr. William Allan King in the Department of Biomedical Sciences at the University of Guelph for their help with the endometrial biopsy culture and the nitric oxide and total protein assays. A scholarship awarded to the first author by the Ontario Trillium Foundation is also greatly appreciated.

CHAPTER FOUR

GENERAL DISCUSSION

Persistent breeding-induced endometritis (PBIE) continues to be a major cause of infertility in mares, resulting in huge economic losses to horse breeders. A better understanding of the mechanisms underlying PBIE in mares is required to devise new strategies to counter this problem. It is well established that mares susceptible to persistent endometritis have delayed uterine clearance (Hughes and Loy, 1969; Troedsson and Liu, 1991; LeBlanc et al., 1994a), owing mainly to impaired myometrial contractility (Troedsson et al., 1993b; Rigby et al., 2001). However, the reasons behind the impaired myometrial contractility in susceptible mares are not clear. Previous studies demonstrating increased amounts of uterine nitric oxide (NO) in susceptible mares (Alghamdi et al., 2005; Woodward et al., 2013b), and the fact that NO is a well-known smooth muscle relaxant, suggested that NO may be one of the factors causing reduced myometrial contractility in susceptible mares. However, with the exception of one study suggesting an inhibitory effect of NO on electrically induced uterine contractility in-vitro (Liu et al., 1997), there was no information about the effect of NO on uterine contractility in the mare.

The research study reported in the second chapter of this thesis evaluated the effect of different concentrations of NO on spontaneous uterine contractility and whether this effect varied between the longitudinal and circular muscle layers of the uterus. The results provided conclusive evidence of a dose-dependent inhibitory effect of NO on spontaneous uterine contractility in-vitro, lending support to the idea that NO may be a factor affecting susceptibility of mares to PBIE. A good next step would be to test the effect of NO on uterine contractility in-vivo in mares. The effect of NO on spontaneous uterine contractility in this study did not vary

between the two (longitudinal and circular) uterine muscle layers. The effect was also not influenced by age of the mare, stage of estrous cycle, and uterine histology grade. Further studies directed specifically at testing the effects of age, stage of estrous cycle and uterine histology grade are required for confirmation of the results obtained in this study.

The application of an in-vitro model to study the effect of NO on uterine contractility in this study was based on multiple factors including widely reported use of this approach in experiments on uterine contractility in different species (Yallampalli et al., 1993; Bradley et al., 1998; Kitazawa et al., 2003; Ocal et al., 2004; Hirsbrunner et al. 2006) and relatively easier logistics and less expense compared to in-vivo methods for measurement of uterine contractility. The in-vitro model allows a greater degree of control over experimental conditions, which makes it relatively easier to maintain uniformity across different technical and biological replicates. Another advantage is that this model allows evaluation of basal contractility of individual muscle layers (longitudinal or circular) of the uterus and the effect of various test substances on each of the two uterine muscle layers. A limitation of the in-vitro muscle contractility model is that this approach does not exactly mimic the biological systems in vivo, especially with respect to the influence of neuroendocrine regulation on uterine contractility. A certain degree of caution should, therefore, be exercised in interpreting results from in-vitro muscle contractility models and extrapolating them to live animals.

The second study, reported in the third chapter, evaluated the difference in total nitric oxide synthase (NOS) activity of the endometrium between susceptible and resistant mares and the effect of a specific inducible nitric oxide synthase (iNOS) inhibitor on the total endometrial NOS activity. Susceptible mares had significantly greater endometrial NOS activity than resistant mares. The iNOS inhibitor treatment resulted in a significant reduction in endometrial

NOS activity. This provides a basis for the clinical testing of iNOS inhibitors as therapeutic or prophylactic options for PBIE. Measurement of total endometrial NOS activity reflects endogenous uterine NO production relatively more closely than measurement of mRNA or protein expression of NOS isoforms. Endometrial NOS activity was not measured directly in the present study. Instead, the total amount of NO produced by endometrial tissue samples from L-arginine in the tissue medium was measured and normalized to the endometrial tissue weight or protein content. The normalized NO production was then used as a measure of endometrial NOS activity. This approach was adopted from a previous study on endometrial NO production in the rat (Yallampalli et al., 1993). This method is actually not very different from the 'direct' methods used for measurement of tissue NOS activity, which involve incubation of tissue homogenates or protein extracts with L-arginine followed by quantitation of NO or NO-derived metabolites by radiolabeling, colorimetry, fluorometry or chemiluminescence (Schmidt and Mayer, 2001; Bryan and Grisham, 2007). The approach used for determination of NOS activity in the present study allowed evaluation of the difference in total nitric oxide synthase (NOS) activity of the endometrium between susceptible and resistant mares and the effect of a specific iNOS inhibitor on the total endometrial NOS activity using the same set of endometrial tissue samples. Application of the other approaches would have necessitated collection of at least one additional endometrial biopsy sample from each mare.

Selection of mares and their classification into susceptible and resistant mares in this study was based on inclusion and classification criteria applied previously by Alghamdi et al. (2005) and Woodward et al. (2013b). The criteria are fairly stringent, which increases the herd size requirement for selection and classification into susceptible and resistant mares. However, this approach greatly reduces confounding effects by excluding mares with preexisting

endometritis. The initial number of mares from which selection and classification into susceptible and resistant mares was performed in the previous studies (Alghamdi et al., 2005; Woodward et al., 2013b) was not reported, which precludes a comparison with the current study regarding the total number of mares. However, the number of mares (thirty) required to arrive at the sample size of six mares in each group in this study is likely higher than what would be expected in an average herd. This may be attributed to the fact that most of the mares in this research herd were previously used in embryo collection studies resulting in a higher prevalence of endometritis. Twelve of thirty mares were excluded during the initial screening process due to preexisting endometritis. Of the remaining eighteen mares, six mares did not meet the final classification criteria and were excluded.

The initial assignment of mares to potentially susceptible and resistant groups was in agreement with their final classification into susceptible and resistant mares in all but three mares. One mare was assigned to the potentially susceptible group (grade IIB) but was confirmed to be resistant to PBIE after intrauterine challenge with killed spermatozoa. Two mares were assigned to the potentially resistant group (grade IIA) but were later confirmed to be susceptible to PBIE after the intrauterine challenge. The assignment of mares to potentially susceptible and resistant groups, and the final classification based on intrauterine challenge with killed spermatozoa were performed in two different estrous cycles. Therefore, the minor discrepancy between the initial assignment and the final classification could be attributed to an improvement or worsening of uterine health over time. Bidirectional changes in susceptibility status of mares (from susceptible to resistant or from resistant to susceptible) have been reported previously by Woodward et al. (2012).

The timing of administration of iNOS inhibitors to mares in in-vivo studies should be determined with special consideration to the time of ovulation. Although the effects of selective iNOS inhibitors on ovulation in the mare are not known, administration of N^{ω}-nitro-l-arginine methyl ester, a non-specific NOS inhibitor, and aminoguanidine, a relatively more specific iNOS inhibitor, have been shown to cause delayed ovulation in mares (Pinto et al., 2002a). A safer initial approach might be to begin administration of the iNOS inhibitor after confirmation of ovulation. Alternatively, a pilot study investigating the effect of different times of administration on ovulation should be conducted before the actual clinical trial.

The research reported in this thesis provides evidence that the nitric oxide system may constitute an important part of the mechanism underlying PBIE in mares. The results of this research, indicating a significant inhibition of endometrial NOS activity by the iNOS inhibitor treatment, can serve as a foundation for future clinical applications of iNOS inhibitors in prevention or treatment of PBIE in mares.

REFERENCES

Acosta TJ, Beg MA, Ginther OJ. Aberrant blood flow area and plasma gonadotropin concentrations during the development of dominant-sized transitional anovulatory follicles in mares. Biol Reprod 2004;71:637–42.

Aguilar HN, Mitchell BF. Physiological pathways and molecular mechanisms regulating uterine contractility. Hum Reprod Update 2010;16:725–44.

Alghamdi AS, Foster DN, Carlson CS, Troedsson MH. Nitric oxide levels and nitric oxide synthase expression in uterine samples from mares susceptible and resistant to persistent breeding-induced endometritis. Am J Reprod Immunol 2005;53:230–7.

Anteby EY, Hurwitz A, Korach O, Revel A, Simon A, Finci-Yeheskel Z, Mayer M, Laufer N. Human follicular nitric oxide pathway: relationship to follicular size, oestradiol concentrations and ovarian blood flow. Hum Reprod 1996;11:1947–51.

Asbury AC, Halliwell RE, Foster GW, Longino SJ. Immunoglobulins in uterine secretions of mares with differing resistance to endometritis. Theriogenology 1980;14:299–308.

Asbury AC, Schultz KT, Klesius PH, Foster GW, Washburn SM. Factors affecting phagocytosis of bacteria by neutrophils in the mare's uterus. J Reprod Fertil Suppl 1982;32:151–9.

Bader H. An investigation of sperm migration into the oviducts of the mare. J Reprod Fertil Suppl 1982;32:59–64.

Barbacini S, Zavaglia G, Gulden P, Marchi V, Necchi D. Retrospective study on the efficacy of hCG in an equine artificial insemination programme using frozen semen. Equine Vet Educ 2000; 12:312–17.

Bradley KK, Buxton IL, Barber JE, McGaw T, Bradley ME. Nitric oxide relaxes human myometrium by a cGMP-independent mechanism. Am J Physiol 1998;275:C1668–73.

Bredt DS, Snyder SH. Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. Proc Natl Acad Sci USA 1989;86:9030–3.

Brinsko SP, Rigby SL, Varner DD, Blanchard TL. A practical method for recognizing mares susceptible to post-breeding endometritis. Proc Am Assoc Equine Pract 2003;49:363–5.

Bryan NS, Grisham MB. Methods to detect nitric oxide and its metabolites in biological samples. Free Radic Biol Med. 2007;43: 645–57.

Buxton IL. Regulation of uterine function: a biochemical conundrum in the regulation of smooth muscle relaxation. Mol Pharmacol 2004;65:1051–9.

Canisso IF, Stewart J, Coutinho de Silva MA. Endometritis: Managing persistent post-breeding endometritis. Vet Clin North Am Equine Pract 2016;32:465–80.

Carmo MT, Losinno L, Aguilar J, Rose J, Araujo GHM, Alvarenga MA. Levels of hormones and nitric oxide present in follicular fluid under or not under superovulation in mares. Reprod Fertil Dev 2010;22:359 (Abstract).

Costa AS, Mateus L, Redmer DA, Skarzynski DJ, Ferreira-Dias G. Effects of TNFa and a nitric oxide donor on equine luteal production of factor(s) that stimulate endothelial cell proliferation. Reprod Dom Anim 2006;41:361 (Abstract).

Dascanio JJ. External reproductive anatomy. In: McKinnon AO, Squires EL, Vaala WE, Warner DD, editors. Equine Reproduction, New Jersey: Wiley-Blackwell; 2011, p. 1577–81.

Dixit VD, Parvizi N. Nitric oxide and the control of reproduction. Anim Reprod Sci 2001;65:1–16.

El-Sherry TM, Derar R, Bakry R. Changes in blood flow in ovine follicles and serum concentration of estradiol 17 beta (E2) and nitric oxide (NO) around the time of ovulation in Ossimi ewes. Anim Reprod Sci 2013;138:188–93.

Evans MJ, Hamer JM, Gason LM, Graham CS, Asbury AC, Irvine CH. Clearance of bacteria and non-antigenic markers following intra-uterine inoculation into maiden mares: Effect of steroid hormone environment. Theriogenology 1986;26:37–50.

Evans MJ, Hamer JM, Gason LM, Irvine CH. Factors affecting uterine clearance of inoculated materials in mares. J Reprod Fertil Suppl 1987;35:327–34.

Evans V. Horse Racing in Canada-The economics of Horse Racing in 2010. Equine Canada 2012, p. 1–43.

Ferreira-Dias G, Costa AS, Mateus L, Korzekwa AJ, Galvão A, Redmer DA, Lukasik K, Szóstek AZ, Woclawek-Potocka I, Skarzynski DJ. Nitric oxide stimulates progesterone and prostaglandin E2 secretion as well as angiogenic activity in the equine corpus luteum. Domest Anim Endocrinol 2011;40:1–9.

Fioratti EG, Villaverde AISB, Avanzi BR, Leal ACMS, Caldas-Bussiere MC, Alvarenga MA. Nitric oxide concentration in free uterine fluid after dexamethasone treatment of mares resistant and susceptible to endometritis. Anim Reprod Sci 2010;121S:S113–4.

Förstermann U, Sessa WC. Nitric oxide synthases: regulation and function. Eur Heart J 2012;33:829–37.

Fumuso EA, Aguilar J, Giguère S, Rivulgo M, Wade J, Rogan D. Immune parameters in mares resistant and susceptible to persistent post-breeding endometritis: effects of immunomodulation. Vet Immunol Immunopathol 2007;118:30–9.

Fumuso E, Giguère S, Wade J, Rogan D, Videla-Dorna I, Bowden RA. Endometrial IL-1beta, IL-6 and TNF-alpha, mRNA expression in mares resistant or susceptible to post-breeding endometritis. Effects of estrous cycle, artificial insemination and immunomodulation. Vet Immunol Immunopathol 2003;96:31–41.

Galvão A, Skarzynski D, Lukasik K, Valente L, Ramilo D, Mateus L, Ferreira-Dias G. The effect of cytokines on equine luteal production of nitric oxide and angiogenic activity and luteal cells viability. Reprod Dom Anim 2008a;43(Suppl 5):84 (Abstract).

Galvão A, Skarzynski D, Valente L, Tramontano A, Mollo A, Mateus L, Ferreira-Dias G. Influence of TNFa and IFNc on equine luteal production of nitric oxide and mitogenic factors. Reprod Dom Anim 2008b;43(Suppl 5):86 (Abstract).

Galvão A, Valente L, Skarzynski DJ, Szóstek A, Piotrowska-Tomala K, Rebordão MR, et al. Effect of cytokines and ovarian steroids on equine endometrial function: an in vitro study. Reprod Fertil Dev 2013b;25:985–97.

Galvão A, Tramontano A, Rebordão MR, Amaral A, Bravo PP, Szóstek A, Skarzynski D, Mollo A, Ferreira-Dias G. Opposing roles of leptin and ghrelin in the equine corpus luteum regulation: an in vitro study. Mediators Inflamm 2014; Article ID 682193:1–13.

Galvão AM, Szóstek AZ, Skarzynski DJ, Ferreira-Dias GM. Role of tumor necrosis factor-α, interferon-γ and Fas-ligand on in vitro nitric oxide activity in the corpus luteum. Cytokine 2013a;64:18–21.

Gebhardt S, Merkl M, Herbach N, Wanke R, Handler J, Bauersachs S. Exploration of global gene expression changes during the estrous cycle in equine endometrium. Biol Reprod 2012;87:136.

Ginther OJ, Gastal EL, Gastal MO, Beg MA. Conversion of a viable preovulatory follicle into a hemorrhagic anovulatory follicle in mares. Anim Reprod 2006;3:29–40.

Ginther OJ, Wolf CA, Baldrighi JM, Greene JM. Relationships among nitric oxide metabolites and pulses of a PGF2α metabolite during and after luteolysis in mares. Theriogenology 2015;84:193–9.

Grüninger B, Schoon HA, Schoon D, Menger S, Klug E. Incidence and morphology of endometrial angiopathies in mares in relationship to age and parity. J Comp Pathol 1998;119:293–309.

Hanukoglu I. Steroidogenic enzymes: structure, function, and role in regulation of steroid hormone biosynthesis. J Steroid Biochem Mol Biol 1992;43:779–804.

Hemberg E, Lundeheim N, Einarsson S. Retrospective study on vulvar conformation in relation to endometrial cytology and fertility in Thoroughbred mares. J Vet Med A Physiol Pathol Clin Med 2005;52:474–7.

Hesla JS, Preutthipan S, Maguire MP, Chang TS, Wallach EE, Dharmarajan AM. Nitric oxide modulates human chorionic gonadotropin-induced ovulation in the rabbit. Fertil Steril 1997;67:548–52.

Hirsbrunner G, Reist M, Couto SS, Steiner A, Snyder J, Vanleeuwen E, Liu I. An in vitro study on spontaneous myometrial contractility in the mare during estrus and diestrus. Theriogenology 2006;65:517–27.

Honnens A, Weisser S, Welter H, Einspanier R, Bollwein H. Relationships between uterine blood flow, peripheral sex steroids, expression of endometrial estrogen receptors and nitric oxide synthases during the estrous cycle in mares. J Reprod Dev 2011;57:43–8.

Hughes JP, Loy RG. Investigations on the effect on intrauterine inoculation of Streptococcus zooepidemicus in the mare. Proc Am Assoc Equine Pract 1969;15:289–92.

Ignarro LJ, Bush PH, Buga GM, Wood KS, Fukuto JM, Rajfer J. Nitric oxide and cyclic GMP formation can cause electrica1 field stimulation that can cause relaxation of corpus cavernosum smooth muscle. Biochem Biophys Res Comm 1990;170:843–50.

Ignarro LJ, Byrns RE, Buga GM, Wood KS. Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical. Circ Res 1987;61:866–79.

Jablonka-Shariff A, Olson LM. The role of nitric oxide in oocyte meiotic maturation and ovulation: meiotic abnormalities of endothelial nitric oxide synthase knock out mouse oocytes. Endocrinology 1998;139:2944–54.

Jacobs RD, Warren LK, Mortensen CJ. Effect of oral L-arginine supplementation on uterine blood flow and fluid clearance in mares during estrus. J Equine Vet Sci 2013;33:373–4 (Abstract).

Katila T. Onset and duration of uterine inflammatory response of mares after insemination with fresh semen. Biol Reprod Mono 1995;1:515–7.

Katila T. Post-mating inflammatory response of the uterus. Reprod Domest Anim 2012;47:31–41.

Katila T. What do we know about susceptibility of mares to endometritis? Pferdeheilkunde 2008;24:61–5.

Kelley DE, Warren LK, Mortensen CJ. L-Arginine supplementation reduces uterine fluid accumulation post foaling in the mare. J Equine Vet Sci 2011;31:315 (Abstract).

Kelley DE, Warren LK, Mortensen CJ. Oral L-arginine supplementation impacts several reproductive parameters during the postpartum period in mares. Anim Reprod Sci 2013;138:233–40.

Kenney RM, Doig PA. Equine endometrial biopsy. In: Morrow DA, editor. Current Therapy in Theriogenology, Volume 2, Philadelphia: Saunders Co; 1986, p. 723–9.

Kenney RM. Cyclic and pathologic changes of the mare endometrium as detected by biopsy, with a note on early embryonic death. J Am Vet Med Assoc 1978; 172:241–62.

Khan FA, Chenier TS, Murrant CL, Foster RA, Hewson J, Scholtz EL. Nitric oxide's dose-dependent inhibition of uterine contractility: a potential mechanism underlying persistent breeding-induced endometritis in the mare. Clin Theriogenol 2016;8:307.

Khan FA, Das GK, Pande M, Pathak MK, Sarkar M. Biochemical and hormonal composition of follicular cysts in water buffalo (*Bubalus bubalis*). Anim Reprod Sci 2011;124:61–4.

Khan FA, Scholtz EL, Chenier TS. The nitric oxide system in equine reproduction: current status and future directions. J Equine Vet Sci 2015;35:481–7.

Kitazawa T, Hatakeyama H, Cao J, Taneike T. Pregnancy-associated changes in responsiveness of the porcine myometrium to bioactive substances. Eur J Pharmacol 2003;469:135–44.

Kotilainen T, Huhtinen M, Katila T. Sperm induced leukocytosis in the equine uterus. Theriogenology 1994;41:629–36.

Kuenzli KA, Buxton IL, Bradley ME. Nitric oxide regulation of monkey myometrial contractility. Br J Pharmacol 1998;124:63–8.

LeBlanc MM, Johnson RD, Calderwood Mays MB, Valderrama C. Lymphatic clearance of india ink in reproductively normal mares and mares susceptible to endometritis. Biol Reprod Mono 1995;1:501–6.

LeBlanc MM, Neuwirth L, Asbury AC, Tran T, Mauragis D, Klapstein E. Scintigraphic measurement of uterine clearance in normal mares and mares with recurrent endometritis. Equine Vet J 1994a;26:109–13.

LeBlanc MM, Neuwirth L, Mauragis D, Klapstein E, Tran T. Oxytocin enhances clearance of radiocolloid from the uterine lumen of reproductively normal mares and mares susceptible to endometritis. Equine Vet J 1994b;26:279–82.

LeBlanc MM, Neuwirth L, Jones L, Cage C, Mauragis D. Differences in uterine position of reproductively normal mares and those with delayed uterine clearance detected by scintigraphy. Theriogenology 1998;50:49–54.

LeBlanc MM. Persistent Mating induced Endometritis in the Mare In: Robinson NE, editor. Current Therapy on Equine Medicine 5. Philadelphia: W.B. Saunders; 2003, p. 234–7.

Liu I, Rakestraw PA, Coit C, Harmon F, Snyder J. An in vitro investigation of the mechanism of neuromuscular regulation in myometrial contractility. Pferdeheilkunde 1997;13:557.

Liu IKM, Troedsson MHT. The diagnosis and treatment of endometritis in the mare: Yesterday and today. Theriogenology 2008;70:415–20.

Metcalf ES, Scoggin K, and Troedsson MHT. The effect of platelet-rich plasma on endometrial proinflammatory cytokines in susceptible mares following semen deposition. J Equine Vet Sci 2012;32:498 (Abstract).

Mitchell G, Liu IK, Perryman LE, Stabenfeldt GH, Hughes JP. Preferential production and secretion of immunoglobulins by the equine endometrium–a mucosal immune system. J Reprod Fertil Suppl 1982;32:161–8.

Miller MR, Megson IL. Recent developments in nitric oxide donor drugs. Br J Pharmacol 2007;151:305–21.

Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med 1993;329:2002–12.

Mortensen CJ, Kelley DE, Warren LK. Supplemental L-arginine shortens gestation length and increases mare uterine blood flow before and after parturition. J Equine Vet Sci 2011;31:514–20.

Nemade VR, Carrette O, Larsen WJ, Markoff E. Involvement of nitric oxide and the ovarian blood follicle barrier in murine follicular cyst development. Fertil Steril 2002;78:1301–8.

Newcombe JR. The effect of the incidence and depth of intra-uterine fluid in early dioestrus on pregnancy rate in mares. Pferdeheilkunde 1997;13:545.

Ocal H, Yuksel M, Ayar A. Effects of gentamicin sulfate on the contractility of myometrium isolated from non-pregnant cows. Anim Reprod Sci 2004;84:269–77.

Ousey JC, Freestone N, Fowden AL, Mason WT, Rossdale PD. The effects of oxytocin and progestagens on myometrial contractility in vitro during equine pregnancy. J Reprod Fertil Suppl 2000;56:681–91.

Palmer RMJ, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature 1988;33:664–8.

Palmer RMJ, Ferige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 1987;327:524–6.

Pancarci SM, Güngör O, Atakişi O, Ciğremiş Y, Ari UÇ, Bollwein H. Changes in follicular blood flow and nitric oxide levels in follicular fluid during follicular deviation in cows. Anim Reprod Sci 2011;123:149–56.

Pande M, Das GK, Khan FA, Sarkar M, Prasad JK, Pathak MC, et al. Uterine infection influences size and follicular fluid composition of the largest follicle in buffalo (*Bubalus bubalis*). Reprod Domest Anim 2013;48:79–84.

Pinto CR, Paccamonti DL, Eilts BE, Short CR, Gentry LR, Thompson DL Jr, Godke RA. Evidence for a nitric oxide-mediated modulation of equine granulosa cell steroidogenesis. Theriogenology 2002b;58:579–83.

Pinto CR, Paccamonti DL, Eilts BE, Short CR, Godke RA. Effect of nitric oxide synthase inhabitors on ovulation in hCG-stimulated mares. Theriogenology 2002a;58:1017–26.

Pinto CR, Paccamonti DL, Eilts BE, Venugopal CS, Short CR, Gentry LR, Thompson DL Jr, Godke RA. Concentrations of nitric oxide in equine preovulatory follicles before and after administration of human chorionic gonadotropin. Theriogenology 2003;60:819–27.

Pycock JF. Cervical function and uterine fluid accumulation in mares. Equine Vet J 1993;25:191.

Pycock JF. How to maximize the chances of breeding successfully from the older maiden mare. Proc Am Assoc Equine Pract 2006;52:245–9.

Radomski MW, Palmer RMJ, Moncada S. Modulation of platelet aggregation by an L-arginine-nitric oxide pathway. Trends Pharmacol Sci 1991;12:87–8.

Reilas T, Rivera Del Alamo MM, Liepina E, Yeste M, Katila T. Effects on the equine endometrium of cervical occlusion after insemination. Theriogenology 2016;85:617–24.

Ricketts SW, Alonso S. The effect of age and parity on the development of equine chronic endometrial disease. Equine Vet J 1991;23:189–92.

Riddle WT, LeBlanc MM, Stromberg AJ. Relationships between uterine culture, cytology and pregnancy rates in a Thoroughbred practice. Theriogenology 2007;68:395-402.

Rigby SL, Barhoumi R, Burghardt RC, Colleran P, Thompson JA, Varner DD, Blanchard TL, Brinsko SP, Taylor T, Wilkerson MK, Delp MD. Mares with delayed uterine clearance have an intrinsic defect in myometrial function. Biol Reprod 2001;65:740–7.

Roberto da Costa RP, Costa AS, Korzekwa AJ, Platek R, Siemieniuch M, Galvão A, Redmer DA, Silva JR, Skarzynski DJ, Ferreira-Dias G. Actions of a nitric oxide donor on prostaglandin production and angiogenic activity in the equine endometrium. Reprod Fertil Dev 2008;20:674–83.

Roberto da Costa RP, Ferreira-Dias G, Mateus L, Korzekwa A, Andronowska A, Platek R, Skarzynski DJ. Endometrial nitric oxide production and nitric oxide synthases in the equine endometrium: relationship with microvascular density during the estrous cycle. Domest Anim Endocrinol 2007;32:287–302.

Roberto da Costa RP, Ferreira-Dias G, Mateus L, Korzekwa A, Andronowska A, Skarzynski DJ. Endothelial and inducible nitric oxide synthases in the equine endometrium. Reprod Dom Anim 2005;40:344 (Abstract).

Rosselli M, Keller PJ, Dubey RK. Role of nitric oxide in the biology, physiology and pathophysiology of reproduction. Hum Reprod Update 1998;4:3–24.

Schmidt HH, Lohmann SM, Walter U. The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. Biochim Biophys Acta 1993;1178:153–75.

Schmidt K, Mayer B. Assay of tissue activity of nitric oxide synthase. Curr Protoc Toxicol. 2001;10:10.2.1–10.2.13.Scott MA. A glimpse at sperm function in vivo: sperm transport and epithelial interaction in the female reproductive tract. Anim Reprod Sci 2000;60:337–48.

Sheldon IM, Noakes DE, Rycroft AN, Pfeiffer DU, Dobson H. Influence of uterine bacterial contamination after parturition on ovarian dominant follicle selection and follicle growth and function in cattle. Reproduction 2002;123:837–45.

Shirasuna K. Nitric oxide and luteal blood flow in the luteolytic cascade in the cow. J Reprod Dev 2010;56:9–14.

Shukovski L, Tsafriri A. Involvement of nitric oxide in the ovulatory process in the rat. Endocrinology 1994;135:2287–90.

Steckler D, Naidoo V, Gerber D, Kähn W. Ex vivo influence of carbetocin on equine myometrial muscles and comparison with oxytocin. Theriogenology 2012;78:502–9.

Stuehr DJ. Structure-function aspects in the nitric oxide synthases. Annu Rev Pharmacol Toxicol 1997;37:339–59.

Troedsson MH, Desvousges A, Alghamdi AS, Dahms B, Dow CA, Hayna J, Valesco R, Collahan PT, Macpherson ML, Pozor M, Buhi WC. Components in seminal plasma regulating sperm transport and elimination. Anim Reprod Sci 2005;89:171–86.

Troedsson MHT, Liu IKM, Crabo BG. Sperm transport and survival in the mare: a review. Theriogenology 1998;50:807–18.

Troedsson MHT, Liu IKM, Ing M, Pascoe J, Thurmond M. Multiple site electromyography recordings of uterine activity following an intrauterine bacterial challenge in mares susceptible and resistant to chronic uterine infection. J Reprod Fertil 1993b;99:307–13.

Troedsson MHT, Liu IKM, Thurmond M. Immunoglobulins (IgG and IgA) and complement (C3) concentrations in uterine secretion following an intrauterine challenge of Streptococcus

zooepidemicus in mares susceptible to versus resistant to chronic uterine infection. Biol Reprod 1993a;49:502–6.

Troedsson MHT, Liu IKM. Uterine clearance of non-antigenic markers (51Cr) in response to a bacterial challenge in mares potentially susceptible and resistant to chronic uterine infections. J Reprod Fertil Suppl 1991;44:283–8.

Troedsson MHT, Loset K, Alghamdi AM, Dahms B, Crabo BG. Interaction between equine semen and the endometrium: the inflammatory response to semen. Anim Reprod Sci 2001;68:273–8.

Troedsson MHT, Steiger BN, Ibrahim NM, Foster DN, Crabo BG. Biol Reprod Suppl 1995;52:307.

Troedsson MHT. Endometritis. In: McKinnon AO, Squires EL, Vaala WE, Warner DD, editors. Equine Reproduction, New Jersey: Wiley-Blackwell; 2011, p. 2608–19.

Troedsson MHT. Uterine clearance and resistance to persistent endometritis in the mare. Theriogenology 1999;52:461–71.

Troedsson MHT. Breeding-induced endometritis in mares. Vet Clin North Am Equine Pract 2006;22:705–12.

Troedsson MH, deMoraes MJ, Liu IK. Correlations between histologic endometrial lesions in mares and clinical response to intrauterine exposure with Streptococcus zooepidemicus. Am J Vet Res 1993c;54:570–2.

Troedsson MH, Woodward EM. Our current understanding of the pathophysiology of equine endometritis with an emphasis on breeding-induced endometritis. Reprod Biol 2016;16:8–12.

Tunón A-M, Katila T, Magnusson U, Nummijärvi A, Rodriguez-Martinez H. T-cell distribution in two different segments of the equine endometrium 6 and 48 hours after insemination. Theriogenology 2000;54:835–41.

Van Voorhis BJ, Dunn MS, Snyder GD, Weiner CP. Nitric oxide: an autocrine regulator of human granulosa luteal cell steroidogenesis. Endocrinology 1994;135:1799–806.

Walker MW, Kinter MT, Roberts RJ, Spitz DR. Nitric oxide-induced cytotoxicity: involvement of cellular resistance to oxidative stress and the role of glutathione in protection. Pediatr Res 1995;37:41–9.

Watson ED, Stokes CR, Bourne FJ. Cellular and humoral mechanisms in mares susceptible and resistant to persistent endometritis. Vet Immunol Immunopathol 1987;16:107–21.

Watson ED. Post-breeding endometritis in the mare. Anim Reprod Sci 2000;60–61:221–32.

Welter H, Bollwein H, Weber F, Rohr S, Einspanier R. Expression of endothelial and inducible nitric oxide synthases is modulated in the endometrium of cyclic and early pregnant mares. Reprod Fertil Dev 2004;16:689–98.

Woodward EM, Christoffersen M, Campos J, Betancourt A, Horohov DW, Scoggin KE, Squires E, Troedsson MHT. Endometrial inflammatory markers of the early immune response in mares susceptible or resistant to persistent breeding induced endometritis (PBIE). Reproduction 2013a;145:289–96.

Woodward EM, Christoffersen M, Campos J, Horohov DW, Scoggin KE, Squires E, Troedsson MHT. An investigation of uterine nitric oxide production in mares susceptible and resistant to

persistent breeding-induced endometritis and the effects of immunomodulation. Reprod Dom Anim 2013b;48:554–61.

Woodward EM, Troedsson MH. Equine breeding-induced endometritis. J Equine Vet Sci 2013;33:673–82.

Woodward EM, Troedsson MHT. Inflammatory mechanisms of endometritis. Equine Vet J 2015;47:384–9.

Woodward EM, Christoffersen M, Campos J, Squires EL, Troedsson MH. Susceptibility to persistent breeding-induced endometritis in the mare: relationship to endometrial biopsy score and age, and variations between seasons. Theriogenology 2012;78:495–501.

Yallampalli C, Garfield RE, Byam-Smith M. Nitric oxide inhibits uterine contractility during pregnancy but not during delivery. Endocrinology 1993;133:1899–902.

Zent WW, Troedsson MHT, Xue JL. Postbreeding uterine fluid accumulation in a normal population of thoroughbred mares: a field study. Proc Am Assoc Equine Pract 1998;44:64–5.

APPENDICES

Appendix 2.1 Optimization of the in-vitro muscle contractility protocol

The in-vitro muscle contractility model described in the second chapter of this thesis was optimized using nine ovine uteri and two porcine uteri collected post-mortem at the Meat Science Laboratory in the Department of Animal Biosciences at the University of Guelph. Three of the nine ovine uteri and the two porcine uteri were used for gaining hands-on experience in general handling of uterine tissue after collection, perfection of fine dissection skills required for preparation of uterine tissue strips, and selection of a suitable range of doses to be used later in a formal study involving six ovine uteri. The study was designed to test whether NO has dose-dependent inhibitory effects on uterine contractility in sheep, and whether the magnitude of the inhibitory effect is influenced by incremental dosing.

Reproductive tracts were collected from non-pregnant ewes (n=6) 10 to 15 min post-slaughter and transported within 5 minutes to the muscle contractility laboratory in a flask containing Krebs-Henseleit solution (KHS) consisting of (in mM): NaCl, 118; KCl, 4.75; $CaCl_2$, 2.54; $MgSO_4$, 1.18; $NaHCO_3$, 24.8; KH_2PO_4, 1.18; Glucose, 10. All chemicals used in the preparation of KHS were purchased from Fisher Scientific, Waltham, MA, USA. The solution was kept at room temperature and pre-aerated with a 95% O_2–5% CO_2 mixture to reach a pH of 7.3–7.4. From each uterus, three full-thickness uterine tissue strips (10–12mm x 2mm) were excised parallel to the longitudinal muscle fibers. Each strip was tied at the ends with 5.0 gauge suture silk and suspended in an individual organ bath containing 10 mL of warm (37°C) Krebs-Henseleit solution continuously aerated with 95% O_2–5% CO_2. The strips were attached to a fixed point at one end and an isometric force transducer (model FT03, Grass Medical

Instruments, Quincy, MA, USA) at the other. After a 15 min equilibration period, 1g tension was applied, followed 15 min later by another 1g tension. Fifteen min after application of the second 1g tension, spontaneous contractility data was recorded for 30 min in order to determine the baseline contractility of the uterine tissue strips. The strips were then randomly allocated to each of three groups: 1) SNP (sodium nitroprusside, an NO donor) incremental; or 2) NAP (N-acetyl-D-penicillamine, time- and vehicle-matched control), treated at 15 min intervals with increasing concentrations (10^{-6} M to 10^{-3} M) of SNP and NAP, respectively; or 3) SNP isolated, 10^{-4} M time-matched single treatment. The experimental protocol is illustrated in Figure 1.

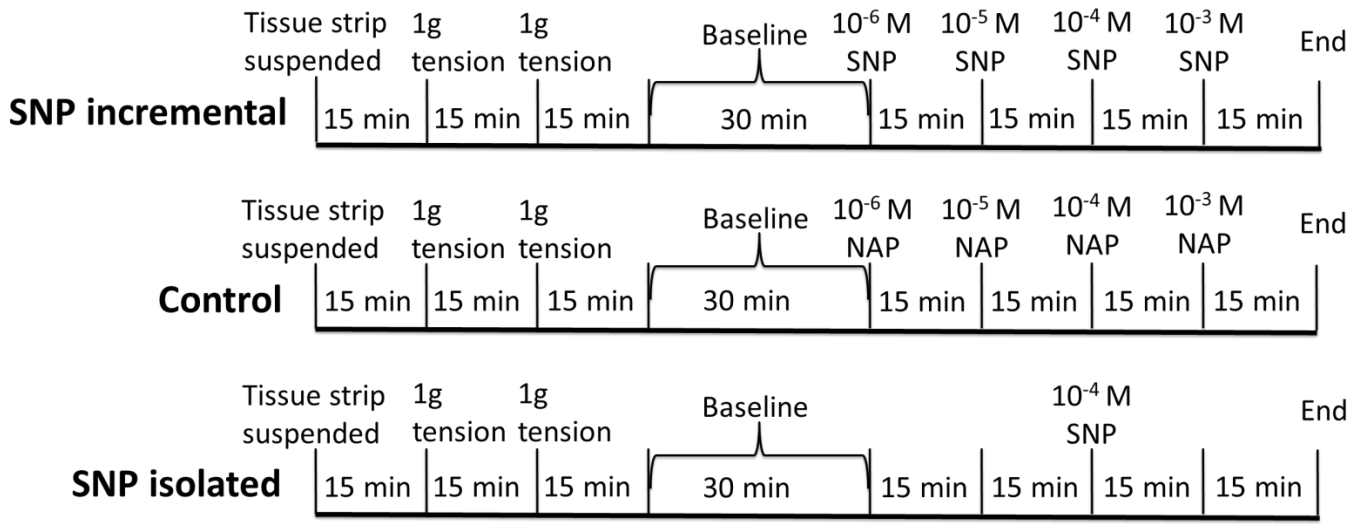


Figure 1: A schematic diagram of the experimental protocol used in this study to test the effect of nitric oxide on uterine contractility (Abbreviations: SNP, Sodium nitroprusside; NAP, N-acetyl-D-penicillamine)

Contractility data was recorded throughout the treatment period and until 15 min after application of the last treatment. The data was recorded using the MP100WSW data acquisition system and AcqKnowledge 3 software (Biopac Systems Inc., Goleta, CA, USA) on a computer (ASUSTeK Computer Inc.).

Statistical analyses were performed using SPSS software (version 16.0, SPSS Inc., Chicago, IL, USA). Formal testing of the data for normality by means of Shapiro-Wilk and Kolmogorov-Smirnov tests indicated that the data was normally distributed ($P>0.05$). The percent change in contractility relative to baseline, measured as the total area under the curve during the 15 min period post treatment, demonstrated a dose-dependent inhibitory effect ($P<0.05$, repeated measures ANOVA) of SNP at 10^{-5} M to 10^{-3} M concentrations (Figure 1). There was no difference in the percent change in contractility relative to baseline between 10^{-4} M SNP incremental and 10^{-4} M SNP isolated doses (Figure 2).

Results of this study suggested that NO has a dose-dependent inhibitory effect on spontaneous uterine contractility in sheep. This study also provided evidence that the magnitude of response of the uterine tissue to a particular dose of NO is not significantly affected by prior incremental treatments with lower NO concentrations.

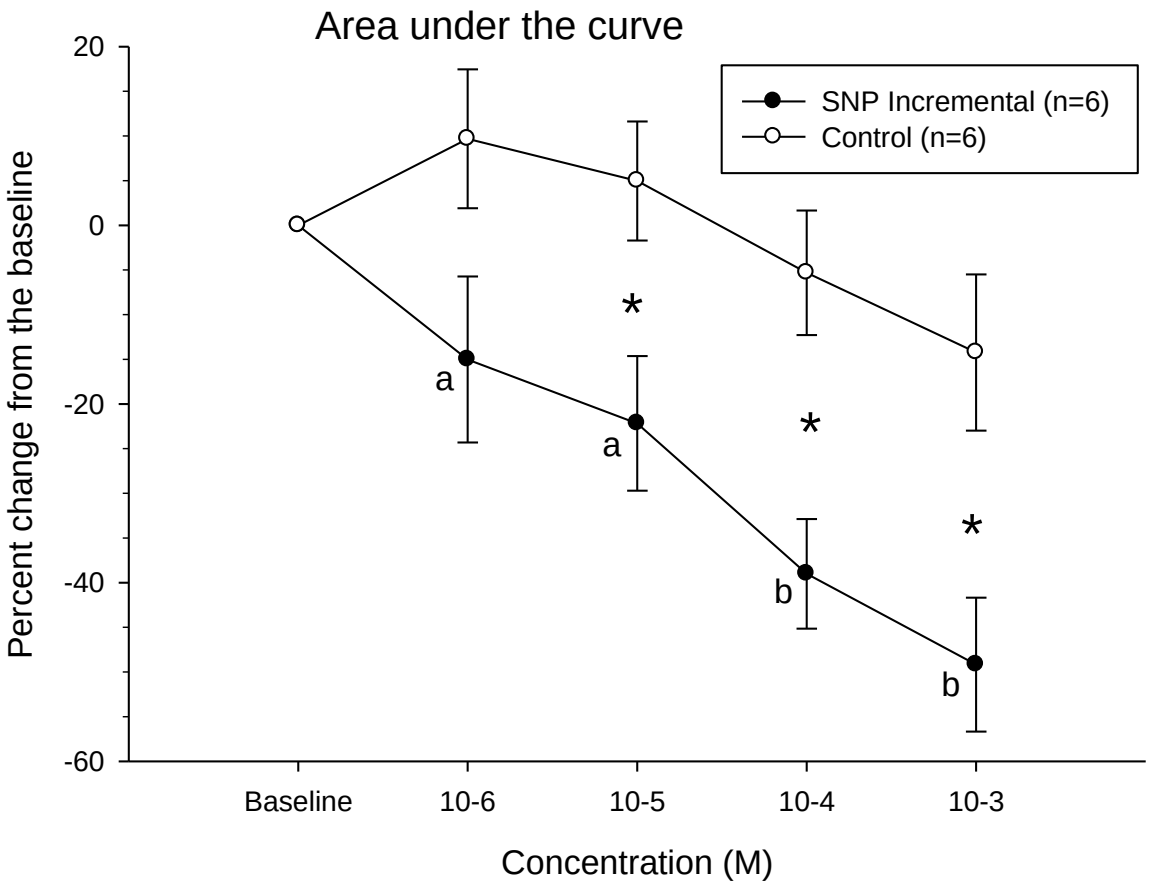


Figure 2: Percentage change in uterine contractility from the baseline after treatment with different concentrations (10^{-6} M to 10^{-3} M) of sodium nitroprusside (SNP Incremental) and N-acetyl-D-penicillamine (Control). Asterisks indicate significant differences between the two groups (P<0.05); Different letters (a,b) indicate significant differences between doses within the SNP incremental group (P<0.05)

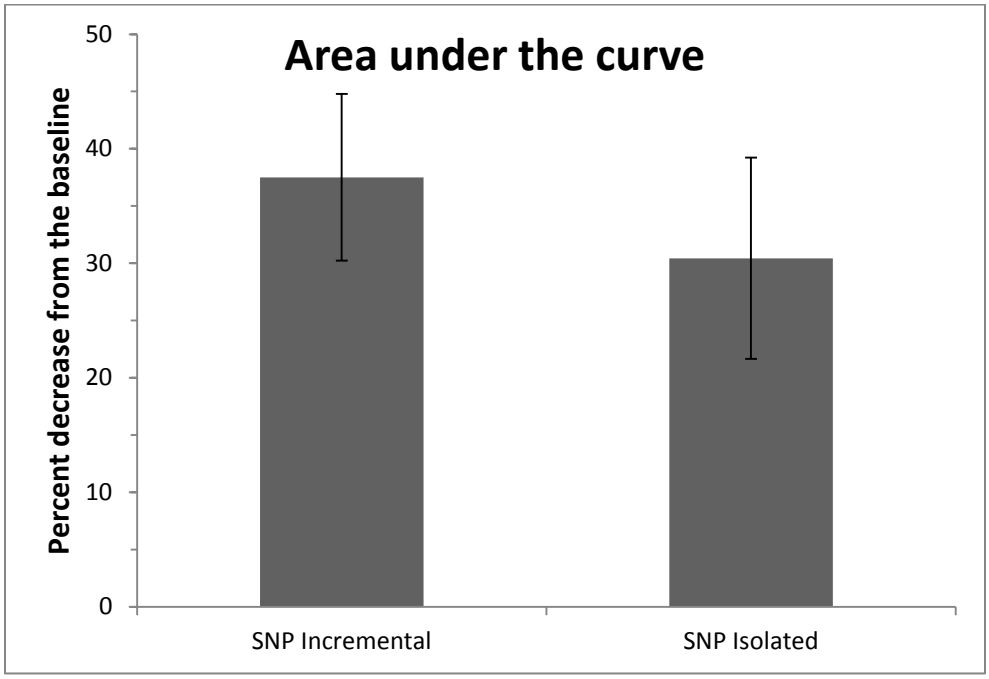
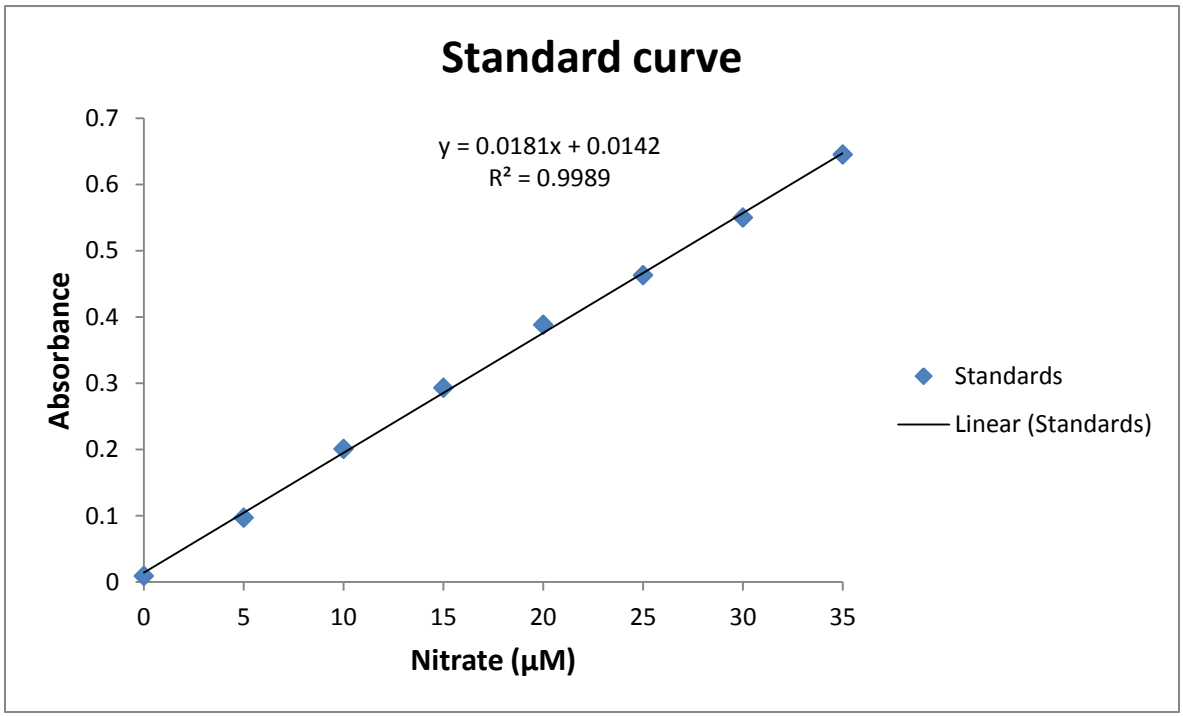


Figure 3: Percentage decrease in uterine contractility from the baseline after treatment with 10^{-4}M sodium nitroprusside in SNP incremental and SNP Isolated groups (6 observations in each group). Difference between the two groups was not statistically significant (P>0.05)

Appendix 3.1 Standard curve of the Nitrate/Nitrite Colorimetric Assay



Appendix 3.2 Standard curve of the Coomassie (Bradford) Protein Assay