EFFECTS OF ENDOPHYTE-INFECTED TALL FESCUE SEED AND BROMOCRIPTINE ON ENDOCRINE AND IMMUNE FUNCTION IN HORSES

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EFFECTS OF ENDOPHYTE-INFECTED TALL FESCUE SEED AND BROMOCRIPTINE ON ENDOCRINE AND IMMUNE FUNCTION IN HORSES

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the College of Agriculture, Food and Environment at the University of Kentucky

By

Jessica Marie Hanneman

Lexington, Kentucky

Director: Dr. Amanda A. Adams, Associate Professor of Equine Immunology

2018

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ABSTRACT OF THESIS

EFFECTS OF ENDOPHYTE-INFECTED TALL FESCUE SEED AND BROMOCRIPTINE ON ENDOCRINE AND IMMUNE FUNCTION IN HORSES

Consumption of endophyte-infected (E+) grasses has long been associated with health problems in animals. In cattle E+ tall fescue consumption leads to fescue toxicosis, and in horses it leads reproductive problems. The health-related issues associated with endophyte consumption have been attributed to the effects caused by the ergot alkaloids produced by the fungus. These ergot alkaloids are considered D2-like receptor agonists, and 5-HT2 serotonin and α-adrenergic receptor partial agonists. Many studies in humans, swine, cattle, and horses have identified that ergopeptines cause a decrease in prolactin production due to their dopaminergic activities. Additionally, these molecules have been found to cause vasoconstriction in cattle and horses through their other agonistic activities. Furthermore, dopamine agonists are currently being used to treat pituitary pars intermedia dysfunction (PPID) in horses, a condition in which the horse lacks sufficient dopamine. However, the ergot alkaloids found in E+ tall fescue had not previously been investigated for their potential benefits in treating PPID horses. Moreover, little research has investigated the effects of ergot alkaloids and dopamine agonists on the immune system of horses, even though many health problems associated with E+ tall fescue consumption suggest there to be an elicited inflammatory response. Thus, the primary objective of this study was to establish an understanding of immune and hormone responses to ergot alkaloids and bromocriptine in the horse. The hypothesis of this body of research was that ergot alkaloids and bromocriptine both would elicit inflammatory and hormone responses in the horse. Specifically, this research was conducted to determine the effects of E+ tall fescue seed consumption on immune, hormone, and vasoconstrictive responses, in both non-PPID and PPID horses. In addition, both the in vitro and in vivo effects of bromocriptine on cytokine production from equine peripheral blood mononuclear cells (PBMCs) were investigated. In the first study, there were no significant changes in body morphometrics, vasoconstriction, hormone responses or cytokine expression due to the consumption of ergot alkaloids in non-PPID and PPID horses. The second study was an in vitro study in which PBMCs were exposed to varying concentrations of either bromocriptine, a D2-like receptor agonist that is used as a model for ergot alkaloid consumption, or dopamine. This experiment demonstrated that exposure to dopamine or a dopamine agonist at a concentration greater than 10⁻⁵M is toxic to PBMCs, and that bromocriptine elicits an anti-inflammatory effect at concentrations less than 10⁻⁵M. Concentrations of dopamine less than 10⁻²M, on the other hand, did not cause any significant changes in cytokine expression. A third study was conducted that evaluated the effects of an intravenous injection of bromocriptine on hormone and immune responses in the aged mare. This study identified that bromocriptine maximally reduced prolactin levels 12 hours post-injection and prolactin returned to baseline levels approximately 56 hours post-injection. Additionally, only a significant increase in IL-1β was detected 12 hours post-injection, which suggests
bromocriptine was activating an innate immune response. Overall, the body weights and rectal temperatures of horses did not significantly change in any of the experiments, which indicated that aged non-pregnant horses are able to tolerate E+ tall fescue. In addition, this body of research identified that intravenous delivery of a semi-synthetic dopamine agonist, bromocriptine, and not an oral delivery of an E+ tall fescue seed derived dopamine agonist, caused a decrease in prolactin concentrations, but revealed conflicting results regarding inflammatory responses. In summary, further research is warranted to determine the mechanism of action that dopamine agonists have on the immune system of horses.

Keywords: Horse, Inflammation, Ergot Alkaloid, Bromocriptine, Dopamine

Jessica Marie Hanneman

July 24, 2018
Date
EFFECTS OF ENDOPHYTE-INFECTED TALL FESCUE SEED AND BROMOCRIPTINE ON ENDOCRINE AND IMMUNE FUNCTION IN HORSES

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DEDICATION

This thesis is dedicated to Keira Hanneman.
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CHAPTER 1
LITERATURE REVIEW

Endophyte-Infected Tall Fescue

1.1 History of Tall Fescue

Hundreds of species of fescues exist throughout the world with the majority falling under the genus, Festuca (Craven et al., 2009). However, a select few have been separated into the genus Lolium, known as the ryegrasses (Terrell, 1966). In particular, many grasses that fell under Festuca subgenus Schedonorus were placed into the genus Lolium as new molecular information about the plants had been discovered in recent years that made it apparent these species were evolutionarily different (Darbyshire, 1993). Within this genus is the species of tall fescue 6x Lolium arundinaceum, which is a broad-leaved cool season grass (Craven et al., 2009). The hexaploidy Lolium arundinaceum has become a popular grass on the North American continent and is closely related to 2x Lolium pratense, a European meadow fescue (Craven et al., 2009).

Lolium arundinaceum, a tall fescue, originated in Europe, but the exact time in which it was brought to North America is unknown (Hoveland, 2009). However, some fescues were reported to have been distributed in the United States during the 1800s (Kennedy, 1900). Two types of tall fescue, Alta and Kentucky 31 (KY-31), became popular in the United States in the mid-1900s (Hoveland, 2009) due to their persistence and high yield. The Alta fescue was developed particularly for its hardiness in the winter and ability to persist in dry climates (Hoveland, 2009) and was developed by the Oregon Agricultural Experiment Station and USDA (Cowan, 1956). Like the Alta fescue, KY-31 was developed based on its ability to withstand the winter months, but also drought
conditions, insects, and heavy foot traffic of grazing animals (Hoveland, 2009). It was discovered in 1931 in the mountains of eastern Kentucky and was further developed at the University of Kentucky, until it began being sold in 1943 (Fergus, 1952; Fergus and Buckner, 1972). Its popularity as a cultivar grew throughout the mid-1900s, until reports of decreased animal performance began to surface (Hoveland, 2009). This decrease in animal performance was later attributed to the endophyte that was found to by living in symbiosis with the grass.

1.2 Endophyte

The endophyte, *Epichloë coenophiala*, is a type of fungus that grows in the intercellular spaces of tall fescues, like KY-31. *E. coenophiala*, like tall fescue, originated in Europe and was brought to the United States in the seeds of grasses (Siegel et al., 1984). The endophyte is non-infectious and can only be transmitted via the seeds of the plant. Additionally, the endophyte and tall fescue have a mutualistic relationship (Christensen and Voisey, 2009) in which both the grass and the fungus benefit from the relationship. The hyphae of the endophyte have the ability to grow throughout all parts of the plant; however, KY-31 typically has few hyphae found in the blade of the grass (Hinton and Bacon, 1985). Also, cold weather has a negative impact on the growth of the hyphae, which is further impacted by the fact that the hyphae grow in conjunction with the grass tissue. Though the hyphae of the endophyte can grow throughout the grass, they are restricted to the intercellular spaces of tall fescue, which is beneficial for the fungus as the grass is able to provide the fungus with a space that lacks competition and plentiful nutrients to survive (Christensen and Voisey, 2009). The presence of hyphae is beneficial
for the grass as they allow for the improved use of nitrogen by the plant (Arachevaleta et al., 1989), ability to withstand drought conditions (Bouton, 1993; West et al. 1993), and resistance to insect consumption (Latch, 1997). Some of these benefits, like insect resistance have been attributed to the presence of ergot alkaloids, which are produced by the hyphae of the endophyte (Christensen and Voisey, 2009). The benefits provided to tall fescue by the mutualistic relationship with the endophyte give endophyte-infected (E+) tall fescue a competitive advantage over other grasses in its environment.

Furthermore, research has been conducted investigating the effects endophyte removal from tall fescue has on the survival of tall fescue (Hoveland et al., 1982). Researchers have found in many cases that removal of the endophyte results in a decrease in plant competitiveness and a decrease in persistence (Hoveland, 2009). Despite this, current research has involved the development of novel forms of endophyte, like AR542 (Bouton et al. 2002), that are still able to provide the host with competitive advantages, while reducing the negative impact on livestock by not producing particular ergot alkaloids (Bouton, 2009).

### 1.3 Ergot alkaloids

Many alkaloids have been used for medical purposes for years. However, many alkaloids have also been found to have negative effects on humans, especially if not used in moderation. Some common alkaloids include morphine, cocaine, caffeine, and ergotamine (Bush and Fannin, 2009). The ergot alkaloids produced by the endophyte, *Epichlöe coenophiala*, are similar in structure to these other alkaloid molecules, due to the organic, nitrogenous base structure (Bush and Fannin, 2009; Tudzynki et al., 2001).
Additionally, many ergot alkaloids found in E+ tall fescue, like ergovaline and ergovalanine, are derived from lysergic acid, but each differ in the placement of amino acids. Despite their differences in amino acid structure, at position three, all of the ergopeptines contain proline (Bush and Fannin, 2009). In addition, ergot alkaloids share highly similar chemical structures to the man-made hallucinogenic drug lysergic acid diethylamide (LSD) (Hofmann, 1978).

Like LSD, ergot alkaloids have been found to cause deleterious effects in humans. Since the 1500s, infected rye grasses have been reported to cause ergotism, which can occur in both the gangrenous and convulsive forms (Hofmann, 1978; Caporael, 1976). Due to this known association between ergotism and rye, historians and scientists have proposed that the strange symptoms of women during the Salem witch trials were potentially caused by the consumption of ergot alkaloids from ergot infecting the rye being consumed (Caporael, 1976). However, ergot alkaloids have also been found to have medicinal properties; and were used first in the 1500s to accelerate childbirth and later in the 1800s to reduce hemorrhaging postpartum (Hofmann, 1978).

Similar to in humans, ergot alkaloids have been hypothesized to be the culprit of fescue-related animal health problems. Even though no study has definitively determined that ergot alkaloids are the causative agents (Bush and Fannin, 2009), these molecules have been found to induce vasoconstriction through their α-adrenergic receptor binding capacity (Klotz et al., 2007; Klotz et al., 2008), as well as through their serotonergic receptor binding capacity (Klotz et al., 2012; Dyer, 1993; Schöning et al., 2001). Additionally, ergot alkaloids are considered D2-like receptor agonists (Larson et al., 1995; Larson et al., 1999), meaning that they have the potential to bind to various
dopamine receptors throughout the body (Levite, 2016), and thus can elicit the changes in hormone responses exhibited with E+ tall fescue consumption.

1.4 Animal Health and Fescue Consumption

As previously stated, animals grazing on pastures of tall fescue have been found to suffer from health problems, which have been referred to as fescue toxicosis. In cattle, fescue toxicosis has been found to manifest in three major syndromes. The first of these syndromes has been referred to as fescue foot. This is a gangrene-like condition that affects the foot, tail, and ears of the animal (Hoveland, 2009). Animals exhibiting signs of fescue foot have also been found to have an increased respiration rate. A second major syndrome associated with fescue toxicosis has been termed fat necrosis and involves the build-up of fat along the digestive tract, as well as digestive and birthing problems (Bush et al., 1979). In particular, cattle have been found to be more prone to fat necrosis when grazing on pastures high in soil nitrogen (Bush et al., 1979). The third major syndrome is known as summer syndrome, which overall results in a decreased tolerance for heat. More specifically, symptoms include an increased respiration rate, retention of the winter hair coat, decreased feed consumption and lower rate of gain, and decreased production of milk, (Stuedemann and Hoveland, 1988).

Horses have also been found to suffer from fescue toxicosis, which primarily manifests in the form of reproductive difficulties that often result in the loss of a fetus, foal, and/or mare. Several studies involving the comparative length of gestation between E+ and E- fescue consumption have demonstrated that horses consuming E+ tall fescue will generally have a longer gestation period (Garrett et al., 1980). Additionally, horses
consuming E+ tall fescue have been found to have an increased risk of abortion, dystocia, and a thickened placenta and chorioallantois, which increases the risk of foal death (Cross et al., 1995; Cross, 2009). Additionally, these thickened placentas have the potential to separate from the uterus prior to parturition resulting in a condition known as a red-bag (Cross, 2009).

Like cattle, horses consuming E+ tall fescue have also been found to suffer from agalactia, or a decrease in milk production (Cross et al., 1995; Cross, 2009). For both species this has been found to be caused by a decrease in circulating prolactin concentrations. It has been hypothesized that prolactin concentrations in these animals decrease when consuming E+ tall fescue due to the dopaminergic activity of ergot alkaloids acting on the D2-like receptors found on lactotrophs in the pituitary (Cross et al., 1995; Strickland et al., 1992; Freeman et al., 2000). Also, E+ tall fescue consumption has been associated with vasoconstriction in both cattle and horses. A study by McDowell et al. (2013) demonstrated that horses consuming E+ tall fescue had a decreased diameter of the distal palmar artery, and several studies in cattle have demonstrated vasoconstriction as well (Oliver et al., 1998; Egert et al., 2014, Klotz et al., 2007) Additionally, studies have found that this vasoconstriction has led to an increase in body temperature in cattle (Aldrich et al., 1993; Solomons et al., 1989). However, horses have not been found to have an increase in body temperature when consuming E+ tall fescue, but some studies have reported an increase in laminitic symptoms (Douthit et al., 2012).

Though symptoms of fescue toxicosis demonstrate inflammatory-like conditions, like increased temperature and necrosis, little research has investigated the inflammatory
effects of E+ tall fescue consumption. Studies have demonstrated that there might be a decrease in passive immunity for foals whose mothers were grazing E+ tall fescue due to inadequate amounts of IgG antibodies (Cross, 2009), meaning that foals were not receiving the appropriate number of antibodies from the mare. Additionally, these foals were found to be more susceptible to infections as they were not able to mount an adequate immune defense (Cross, 2009). However, a study by McDowell et al. (2013) did not detect any significant changes in cytokine gene expression in mares consuming E+ tall fescue, while a study by Filipov et al. (2000) found E+ tall fescue to have an anti-inflammatory effect in steers.

**Dopamine Agonists**

2.1 Dopamine Receptors

One of the mechanisms in which E+ tall fescue may be having an effect on immune function is through the ergot alkaloids acting as dopamine agonists. Dopamine is a major signaling molecule and as such, dopamine receptors exist on many cells throughout the body, including immune cells (Levite et al., 2001; McKenna et al., 2002; Besser et al., 2005; Watanabe et al., 2006; Cosentino et al., 2007; Nakano et al., 2008; Kustrimovic et al., 2014). These receptors are all G-protein-coupled receptors and have differing binding affinities for dopamine (Beaulieu and Gainetdinov, 2011). There are five known types of dopamine receptors that fall into two families, D1-like receptors and D2-like receptors. D1-like receptors, include DAR1 and DAR5, are $G_{ai}$ coupled protein receptors and result in a decrease in cellular cyclic adenylyl monophosphate (cAMP) (Levite, 2016). On the other hand, D2-like receptors are $G_{ai}$ coupled protein receptors,
which activate adenylyl cyclase and lead to an increase in cellular cAMP. DAR2, DAR3, or DAR4 activation of this pathway can then lead to activation of cytokine gene expression (Levite, 2016).

*In vitro* studies have demonstrated that T-effector cells exposed to an optimal concentration of dopamine (10^{-8}M) produce TNF-α, IL-10, and IL-6 (Levite, 2016). This has been hypothesized to be primarily due to the activation by D2-like receptors, particularly DAR2 which has the highest affinity for dopamine. Other *in vitro* studies have demonstrated that concentrations of dopamine greater than 10^{-5}M have apoptotic and toxic effects on lymphocytes (Levite, 2016). Additionally, the inflammatory effects of dopamine on immune cells can vary between different immune cell types. Varying concentrations of dopamine as well as the quantity and type of receptors on the surface of cells also play a role in the inflammatory effects due to the differing affinities dopamine receptors have for dopamine.

### 2.2 Ergot alkaloids

As mentioned previously, it has been hypothesized that the cause of animal health problems related to tall fescue consumption stems from the ergot alkaloids produced by the endophyte. Ergot alkaloids produced by E+ tall fescue stem from the precursor molecule lysergic acid, and as such lysergic acid can be found in the plant as well. This molecule has significant similarities to the man-made psychedelic drug LSD but varies slightly in the chemical structure (Hofmann, 1978). Also, like the ergot alkaloids found in E+ tall fescue have been found to be a D2-like receptor agonists, and 5-HT2 serotonin receptor and α-adrenergic receptor partial agonists (Schiff, 2006). Through their
dopaminergic activity, ergot alkaloids consumed with tall fescue have been found to cause decreases in prolactin in horses and cattle (Thomson et al., 1996; Baldwin et al., 2016). This proposed mechanism of this involves the binding of the dopamine agonist to dopamine receptors found on the lactotrophs in the pituitary (Freeman et al., 2000). However, the exact mechanism is unknown due to the location of the lactotrophs, which are found on the inside of the pituitary gland (Freeman et al., 2000).

Also, the ergot alkaloids, such as ergovaline, have been found to cause vasoconstriction (Klotz et al., 2007; Klotz et al., 2008). These molecules have been hypothesized to cause this physiological reaction by binding to the 5-HT2 serotonin and α-adrenergic receptors found in the veins. A study by Klotz et al. (2008) demonstrated that ergovaline and lysergic acid both had vasoconstrictive effects on the veins of cattle; however, ergovaline was found to be more vasoconstrictive. Though it is more vasoconstrictive, currently, a consistent and simple method to measure the amount of ergot alkaloids absorbed into the blood after E+ tall fescue consumption has not been developed. However, even though ergot alkaloids have not been quantified in the blood, a study by Schultz et al. (2006) has demonstrated that when comparing the quantities of ergovaline and lysergic acid consumed, horses eliminate a greater quantity of lysergic acid than was consumed. This has suggested that ergot alkaloids, like ergovaline, are being catabolized prior to entering the blood stream. In humans, ergopeptines have been found to be metabolized by cytochrome P4503A4 (CYP3A4) in the small intestine and liver prior to entering the blood, which results in the oxidation of the ergopeptine to recreate a molecule similar to the precursor lysergic acid (Sevrioukova and Poulos,
In a study by Tydén et al. (2004), the same enzyme, CYP3A, was found in the intestine of the horse.

2.3 Bromocriptine

Bromocriptine is a semi-synthetic ergopeptine sold under the commercial name, Parlodel, and is commonly used to treat hyperprolactinemia and symptoms of Parkinson’s disease in humans (Serioukova and Poulos, 2012). As an ergopeptine, bromocriptine shares many structural similarities with the ergot alkaloids produced by E+ tall fescue. Thus, bromocriptine is capable of binding to the same receptors as ergot alkaloids and studies have demonstrated that it can induce the same decrease in prolactin (Baldwin et al., 2016) and as such, bromocriptine has been used as a model for E+ fescue consumption. In human studies, bromocriptine has been administered orally and found to be catabolized by CYP3A4 in the liver to bromo-lysergic acid (Serioukova and Poulos, 2012). Therefore, the active agent in human studies has been bromo-lysergic acid, rather than bromocriptine. In most studies involving bromocriptine for agricultural research, the drug has been administered via intravenous, intramuscular, or subcutaneous injection (Thomson et al., 1996; Baldwin et al., 2016; Auchtung et al., 2003; Bennet-Wimbush et al., 1998; Thompson et al., 2017). However, a study involving sows (Farmer et al., 1998) and a case study involving a horse (Meirelles et al., 2012) both administered bromocriptine orally and exhibited similar effects on prolactin compared to those studies in which bromocriptine was injected into cattle and horses. Though studies involving bromocriptine had examined the effects on hormone production in the horse, there has
been a lack of research in regards to effects on immune function, especially in the old horse population.

**Old Horses**

3.1 Old Horse and Inflammation

In a study by Ireland et al. (2010), horses were defined as geriatric, or old, once they reach the age of fifteen. In that same study, approximately 29% of the horses in the United Kingdom were found to be old. In addition, a recent study conducted in 2016 by NAHMS showed that approximately 12 percent of the American equine population are considered geriatric (>20 years old). With an increasing elderly equine population, an increasing amount of research has been invested in the health of this aging population. As horses age, like humans, they exhibit signs of inflamm-aging (Adams et al., 2008), which is defined as a chronic low-grade systemic inflammation (Franceschi et al., 2000). In the study by Adams et al. (2008), it was demonstrated that old horses had an increased production of IL-1β, IL-15, IL-18, and TNF-α when compared to younger horses. Additionally, old horses have been found to be more susceptible to disease and infection (Paradis, 2002; Adams et al., 2008). With the increased susceptibility to disease, increased baseline inflammation, and increasing population numbers, research regarding the health of the old horse has been an important focus for the equine industry. Moreover, it is important to understand the effects of E+ tall fescue consumption may have on the immune function, in particular, inflamm-aging, of the aged horse.
3.2 Pituitary Pars Intermedia Dysfunction

As previously stated, as horses age they become more susceptible to disease. One disease that particularly affects the old horse population is pituitary pars intermedia dysfunction (PPID) (Siard et al., 2015; Pease et al. 2011; Diez de Castro et al., 2014). Horses suffering from PPID tend to retain their winter coat, have loss of muscle, laminitis, and abnormally high levels of ACTH (Siard et al. 2015; Diez de Castro et al., 2014), with a normal level of ACTH to being defined as less than 35 pg/mL, according to the Equine Endocrinology Group 2015 recommendations (Frank et al. 2015). These increased levels of ACTH have been associated with a lack of dopamine reaching the pituitary gland.

Two common methods used for determining if a horse has PPID are measuring baseline ACTH levels or performing a thyrotropin releasing hormone (TRH) stimulation test (Diez de Castro et al., 2014). TRH is a hormone that stimulates the pars intermedia, thereby causing an increased secretion of ACTH (McFarlane et al., 2006) Both tests involve measuring ACTH found in the plasma; however, the TRH stimulation test creates an exaggerated response (Diez de Castro et al., 2014), which is beneficial for assessing horses in the early stages of PPID, when using the Equine Endocrinology Group’s reference range. Additionally, a study by Diez de Castro et al. (2008), emphasized that feed consumption and season can affect the results of baseline ACTH and ACTH after a TRH stimulation test. In that study, they found that food consumption increased the ACTH concentration in the horse, and that ACTH concentrations increased when seasons changed from summer to fall (Diez de Castro et al., 2008).
After identifying if a horse has PPID, veterinarians often prescribe pergolide as a treatment (Herbert et al., 2013). As stated in the next section, pergolide is a dopamine agonist and therefore simulates the presence of dopamine in the body. The administration of pergolide does not cure the disease, but it has been found to decrease the presence of symptoms. With the effectiveness of administration of a dopamine agonist, in recent years researchers have been investigating alternative drugs to pergolide, like cabergoline (Hebert et al. 2013), as well as the potential of nutritional sources.

3.3 Pergolide

Pergolide, a drug initially developed for the treatment of Parkinson’s disease, is a dopamine agonist that is currently used in treatment of horses suffering from pituitary pars intermedia dysfunction (PPID), a condition in which horses lack dopamine (Pease et al., 2011; Love, 1993). The lack of dopamine in the pituitary pars intermedia allows for an overproduction of pro-opiomelanocortin (POMC) peptides, and horses suffering from PPID also have increased levels of adrenocorticotropic hormone (ACTH), which leads to an increase in cortisol levels (Love, 1993). This drug is administered orally and acts on the dopamine receptors in the pituitary gland to inhibit POMC peptide production (Love, 1993), and decrease ACTH secretion (McFarlane et al. 2016). Given the dopamine agonist properties of both E+ tall fescue and pergolide, it is of interest to understand the effects that ergot alkaloids may have on hormone and immune responses in aged horses.
Based on the literature, it is evident that symbiotic relationship between the endophyte, *Epichlöe coenophiala*, and the tall fescue, *Lolium arundinaceum*, lends a competitive advantage for the grass in its environment. Throughout history though, the ergot alkaloids produced by the endophyte have primarily been found to be harmful to humans and animals. Significant evidence has made it clear that consumption of the molecules similar to those produced by the fungus act in a dopaminergic manner in humans and animals causing a decrease in prolactin concentrations (Serioukova and Poulos, 2012; Thompson et al., 2017; Baldwin et al., 2016). Though the prolactin response to ergot alkaloids had been assessed, the response to other hormones involved in dopaminergic pathways, like adrenocorticotropic hormone (ACTH), had not been thoroughly evaluated in the horse. Additionally, the inflammatory effects of dopamine and dopamine agonists on human immune cells have been well documented (Levite 2016). However, the inflammatory effects of ergot alkaloids found in E+ tall fescue have only been investigated in a few animal studies (McDowell et al., 2013; Filipov et al. 2000), even though symptoms of fescue toxicosis are reflective of increased inflammation. Therefore, further understanding of the effects of dopamine agonists on the immune function in horses is important to develop a better understanding of the effects of E+ tall fescue consumption in the horse.
Hypothesis and Specific Aims

The overall hypothesis for this compilation of research is that ergot alkaloids and bromocriptine elicit pro-inflammatory, prolactin, and adrenocorticotropic hormone responses in horses, due to their dopaminergic activities. This body of research had the following main specific aims:

1. To determine the effects ground endophyte-infected (E+) tall fescue seed consumption on immune, prolactin, and vasoconstrictive responses in old and young horses
2. To determine any change in adrenocorticotropic hormone (ACTH) levels in horses with pituitary pars intermedia dysfunction (PPID) after consumption of E+ tall fescue
3. To determine, at a cellular level, the effects of bromocriptine and dopamine on cytokine production by equine peripheral blood mononuclear cells (PBMCs)
4. To determine the effects of an intravenous bromocriptine injection on immune and hormone responses in old mares
CHAPTER 2

EFFECT OF ENDOPHYTE-INFECTED TALL FESCUE SEED CONSUMPTION ON THE HORMONE AND IMMUNE RESPONSES IN OLD AND YOUNG HORSES

Abstract

Most research investigating the effects of endophyte-infected (E+) tall fescue has involved the study of fescue toxicosis in cattle and the mare. To date, little research has been conducted to study the immunological effects E+ tall fescue consumption could have on the non-pregnant mare or gelding, or potential effects on horses suffering from pituitary pars intermedia dysfunction (PPID). The equine population in this study consisted of old (n=14; age: 22.5±2.9) and young horses (n=16; age: 11.5±2.0). Immune and hormone responses to ground E+ tall fescue seed consumption (Group 1) were compared to certified endophyte-free (E-) ground tall fescue seed consumption (Group 2). Horses were randomly assigned to Group 1 or Group 2. Those in Group 1 were fed 1.8 μg ergovaline and ergovalanine (E)/kg BW from Day 1-Day 28 and 3.7 μg E/kg BW from Day 29 – Day 56, while those in Group 2 were fed comparable amounts of E-ground tall fescue seed. Body weights, body condition scores, and rectal temperatures were measured on Day 1, Day 28, and Day 56. Additionally, we assessed the horses’ immune response every two weeks by isolating peripheral blood mononuclear cells (PBMC) and intracellularly staining them for TNF-α and IFN-γ, as well as determining their relative cytokine gene expressions for TNF-α, IFN-γ IL-6, IL-1β, and IL-10. Hormone responses were also assessed every two weeks for changes in basal
adrenocorticotropic hormone (ACTH) levels in plasma and prolactin levels in serum. All data was analyzed using two-way repeated measures ANOVAs on SigmaPlot v.13 software. Overall, no significant treatment by time effects were determined by intracellular staining, cytokine gene expression, basal ACTH levels, or prolactin levels. However, significant time effects were identified for ACTH and prolactin, suggesting that season has an effect on these hormone responses, which has been previously shown. Additionally, lack of change in inflammatory cytokine production has been supported by a previous study in the horse and suggests the possibility that E+ tall fescue consumption in the horse does not cause any negative inflammatory side-effects.

**Introduction**

Tall fescue (*Lolium arundinaceum*) is a common cool-season grass in the United States that is often infected by a type of fungi, known as an endophyte (*Epichloë coenophiala*). This endophyte-infected (E+) tall fescue originated in Europe and is thought to have been brought to the United States in the 1800s. Kentucky 31, a type of E+ tall fescue was discovered in 1931 in the mountain pastures of Eastern Kentucky. This grass became popularly planted in the United States beginning in the 1940s, due to its ability to adapt to a variety of soil types and it allowed for year-long grazing (Hoveland, 2009). Additionally, the mutualistic relationship between the endophyte and the tall fescue allows the fungus to grow in between the cells of the grass and receive all nutrients necessary for survival, while the tall fescue has a greater utilization of nitrogen from the soil (Arachevaleta et al., 1989), resistance to stress during times of drought.
(Arachevaleta et al., 1989; Bouton et al., 1993; West et al., 1993), resistance to insects and disease, and increased vigor of seedlings (Latch, 1997).

*Epichlöe coenophiala* lives in symbiosis with the tall fescue and produces ergot alkaloids, like ergovaline, and other molecules, such as lysergic acid (Fleetwood et al., 2007; Schardl et al., 2006). These molecules are thought to benefit tall fescue by contributing to the grass’s resistance to insects, disease, and ability to compete in its environment (Christensen and Voisey, 2009). The molecules produced are considered to be D2 receptor agonists (Larson BT et al., 1999; Cross et al., 1995), as well as 5-HT2 serotonin receptor and α-adrenergic receptor partial agonists (Schiff, 2006), which when consumed by grazing livestock, such as cattle and horses, can lead to a decrease in livestock performance.

Decreased performance in livestock grazing on E+ tall fescue became evident throughout the years (Hoveland et al., 1983; Stuedemann and Hoveland, 1988) with many animals exhibiting signs of fescue toxicosis. Cattle exhibiting signs of fescue toxicosis often suffer from three associated syndromes: fescue foot, similar to gangrene, fat necrosis, and/or summer syndrome. Fat necrosis can result in the accumulation of hard fat along the intestinal tract, which can then lead to digestive difficulties as well as birthing complications (Bush et al., 1979). Summer syndrome occurs in cattle consuming E+ tall fescue and is identified by the inability to shed their haircoat, heat intolerance, and decreased milk production, conception rates, and feed intake (Stuedemann and Hoveland, 1988).

Much of the work regarding E+ tall fescue in the horse has been in relation to its negative effects on the pregnant mare and fetus, like abortion, dystocia, thickened
placenta, agalactia, and prolonged gestation (Cross et al., 1995). Studies by Klotz et al. in 2007 and 2008 have identified that the ergot alkaloids produced by the endophyte 

*(Epichlöe coenophiala)* growing in symbiosis with the tall fescue are capable of causing vasoconstriction in the veins of bovine *in vitro*. Furthermore, an *in vivo* study by McDowell et al. (2013) has demonstrated that consumption of E+ tall caused vasoconstriction in horses. It has also been consistently documented that E+ tall fescue supplementation in horses causes changes in hormone production, like a decrease in prolactin (Cross et al., 1995; Thompson and Oberhaus, 2015). Additional research in the horse has shown that other dopamine agonists, like bromocriptine, can cause changes in prolactin levels as well (Thomson et al., 1996), suggesting that ergot alkaloids with their dopamine agonist activity are capable of causing hormone changes.

Though E+ tall fescue research in the horse has primarily focused on reproductive aspects, little research has investigated the potential effects of E+ tall fescue consumption on immune function. In 2000, Filipov et al. examined the effects of ergotamine treatment on immune function in cattle and found it to be anti-inflammatory in nature, while work by McDowell et al., in 2013, showed no change in inflammation in mares.

Additionally, prior to this study, no research had been conducted in the horse to investigate the potential beneficial impact that the consumption of ergot alkaloids produced by E+ tall fescue could have on old horses suffering from pituitary pars intermedia dysfunction (PPID). This condition results from a lack of dopamine production and can be assessed by measuring increased levels of adrenocorticotropic hormone (ACTH) (Diez de Castro et al., 2013). In a recent study by McFarlane et al. (2016), administration of pergolide, another dopamine agonist, in horses was shown to
cause a reduction in plasma ACTH levels and other PPID symptoms. Furthermore, a study by Browning et al. (1998), demonstrated that consumption of ergotamine tartrate, similar to the ergot alkaloids found in E+ tall fescue, by cattle caused a significant ($P<0.001$) reduction in plasma ACTH levels.

Overall, the purpose of this study was to assess the effects E+ tall fescue consumption on inflammatory and hormone regulation in the horse. Additionally, it was of interest to investigate potential beneficial effects E+ tall fescue consumption could have on the PPID horse due to the dopamine agonist activity of ergot alkaloids.

**Methods**

*Animals*

For this study, 14 old horses (age: 22.5±2.9) and 16 young horses (age: 11.5±2.0) from the University of Kentucky, Department of Veterinary Science herd were kept on pastures containing an average of 7% tall fescue (Figure 2.1). The presence of ergovaline and ergovalanine (total E: 0.53ppm±0.01ppm) in pasture samples were measured by HPLC at the University of Kentucky, Department of Plant and Soil Sciences (Blankenship et al. 2001; Yates and Powell, 1988). This study occurred from mid-October to mid-December, and all of the horses appeared healthy and exhibited no clinical signs of infectious disease. Of the old horses, eight were considered to have PPID prior to the beginning of the study, based on levels of basal ACTH that were found to be greater than 35 pg/mL in plasma, per the Equine Endocrinology Group 2016 recommendations [all basal ACTH concentrations were measured by Cornell Animal Health Diagnostic Center, Endocrinology Laboratory]. All of the research in this study
was approved by the University of Kentucky Institutional Animal Care and Use Committee, protocol number 2014-1225.

_Fescue Seed Supplementation_

All horses were allowed water and pasture ad libitum throughout the day and placed into individual pens during daily morning feedings. Their basal diet consisted of 50% oats and 50% vitamin and mineral fortified alfalfa pellets (approximately 2.25kg fed twice a day). Their diets were supplemented with either E+ ground KY31 tall fescue seed (4.06 µg E/g seed) or certified endophyte-free (E-) ground KY32 tall fescue seed, and top-dressed with a mixture of water, molasses, and oil for palatability. The total amount of ergovaline and ergovalinine (E) in KY31 and KY32 tall fescue seeds were measured using HPLC (Blankenship et al., 2001; Yates and Powell, 1988) by the laboratory of Dr. Lowell P. Bush at the University of Kentucky, Department of Plant and Soil Sciences. Animals were sex and age matched, and randomly assigned to either the supplementation group (Group 1) or control group (Group 2) (Figure 2.1). Every morning, Group 1 received a low dose of E (1.8 µg E/kg BW) from Day 1 – Day 28 and a higher dose of ergot alkaloids (3.7 µg E/kg BW) from Day 29 – Day 56, while the controls received equal amounts of the KY32 ground fescue seed. The doses of ergot alkaloids were based on a previous study by McDowell et al. (2013), in which vasoconstriction of the distal palmar artery was identified at the higher dose fed in this study.
Body Weight, Body Condition Score, and Rectal Temperature

Body weights, body condition scores (BCS), and rectal temperatures were taken from all horses on Day 1, Day 28, and Day 56 prior to feedings. Body weights were measured using a scale. Body condition scores were taken using the Henneke system (Henneke et al., 1983) by two trained individuals and the scores were averaged.

Doppler Ultrasonography

A subgroup of 15 horses from Group 1 and Group 2 (n=7, age: 17.1±5.6; n=8, age: 18.0±6.2) were selected at random to be assessed for vasoconstriction. The same 15 horses were assessed on Day 0 and Day 56 of the study to determine vasoconstriction induced by E+ tall fescue consumption. The general procedure was outlined in a previous study (McDowell et al., 2013). In short, an ultrasound machine (Sonosite Titan with an 11 mm convex array 5/8 MHz transducer; Bothell, WA) was used to scan the distal palmar artery of the left front limb using Doppler ultrasonography. Twice, an image of the cross-section of the distal palmar artery was frozen for each horse and the transverse and longitudinal diameters were averaged to obtain the average diameter of the distal palmar artery at each time point (McDowell et al., 2013).

Sample Collection

Every two weeks, EDTA plasma and heparinized blood samples were collected via venipuncture from the jugular vein. After collection, plasma samples were centrifuged at 800g for 10 minutes. Samples were then stored at -20°C. The EDTA plasma was sent to Cornell Animal Health Diagnostic Center, Endocrinology Laboratory.
for basal ACTH analysis, which was performed an Immulite® 1000 (Siemens, Berlin, Germany) chemiluminescence immunoassay (Place et al. 2010). Plasma was also sent to Louisiana State University for prolactin analysis by a radioimmunoassay (RIA) method validated by Colborn et al. (1991).

Peripheral Blood Mononuclear Cell Culture

As previously described by Adams et al. (2008), peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized blood samples. This was performed using a density gradient of Ficoll-Paque Plus™ (Amersham Biosciences, Piscataway, NJ) and phosphate buffered saline (PBS) (Adams et al., 2008). Cell concentration was determined using the VICELL™ Counter-XR (Beckman Coulter, Miami, FL) and cells were plated at a concentration of 4 x 10^6 cells/mL in RPMI [a mixture of RPMI 1640 (Gibco, Grand Island, NY), 2.5% fetal equine serum (Sigma-Aldrich, St. Louis, MO), 55μM 2-mercaptoethanol (Gibco, Grand Island, NY), 2mM L-glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin (HyClone Pen/Strep/Glutamine solution; Thermo Scientific)] (Adams et al. 2008). Two wells of PBMCs were plated for each horse with 10 μL of phorbol 12-myristate 13-acetate (PMA; 25 ng/mL; Sigma Aldrich) and ionomycin (1 mM; Sigma) added to each well, and incubated for 24 hours at 37 °C, 5% CO². At 20 hours, cells were stimulated with 2 μL Brefeldin A (10 μg/mL; Sigma Aldrich) (Adams et al. 2008).
Intracellular Staining and Flow Cytometry

After all stimulations from each horse completed the incubation period, 200 µL of PBMCs in eRPMI were aliquoted into two 96-well V-bottom plates and 500 µL were saved for RNA isolation. The plated cells were centrifuged and resuspended in 2% paraformaldehyde (Sigma Aldrich, St. Louis, MO). Once resuspended, cells were incubated at 4 ºC overnight. Following the incubation, the samples were washed with saponin buffer [a mixture containing PBS, 1% fetal bovine serum, 0.1% saponin, and 0.1% sodium azide (Sigma)] and stained for either IFN-γ or TNF-α using a 1:100 dilution of IFN-γ FITC mouse anti-bovine antibody (AbD Serotec, Raleigh, NC) in saponin buffer or 1:10 dilution of TNF-α anti-equine monoclonal antibody (HL801), respectively (Adams et al., 2008). The stained cells were then placed in the dark on ice to be incubated for 30 minutes before another wash with saponin buffer. The PBMCs being stained for TNF-α were then resuspended in a 1:1000 dilution of FITC-conjugated goat F(ab')2 anti-mouse IgG (H + L) (Invitrogen; 2mg/mL) in saponin buffer. While the PBMC being stained for TNF-α were incubating in the dark on ice for another 30 minutes, the cells being stained for IFN-γ were resuspended in FACS Flow (Becton Dickinson, San Jose, CA) (Adams et al. 2008). Post-incubation, the TNF-α plate was washed with saponin buffer and resuspended in FACS Flow. All PBMCs from both plates were then transferred into round-bottom tubes and remained out of the light until flow cytometric analysis could be conducted using the BD Accuri™ C6 Plus and BD Accuri™ C6 Software v.1.0.264.21 (BD Biosciences, San Jose, CA). PBMC samples were then gated based on granularity and cell size to determine the percent of lymphocytes producing IFN-γ and TNF-α (Adams et al., 2008).
RNA Isolation and Cytokine Gene Expression

PBMCs set aside for RNA isolation from all stimulations of each horse were centrifuged and resuspended in Trizol (Ambion). Later, RNA was extracted from those samples using a Trizol method that had been adjusted (Elzinga et al., 2017). Next 0.5 μg of RNA from each sample was reverse transcribed into cDNA by adding reverse transcription master mix (Promega, Madison, WI) and incubating for 15 minutes at 42 °C followed by 5 minutes at 95 °C. Then, 4.5 μL of the cDNA from each sample was mixed with 5 μL of Master Mix (SensiMix HI-ROX 2x, Bioline). A primer-probe of interest (TaqMan, Equine-specific, intron spanning primers and probes; Applied Biosystems) was then added to the cDNA and Master Mix combination. The Applied Biosystems Real-Time PCR (ABI Viia7) was used to run all samples against the genes: beta-glucuronidase (B-gus; Ec03470630_m1), interferon gamma (IFN-g; Ec03468606_m1), IL-6 (Ec03468678_m1), IL-1B (Ec04260298_s1), TNF-a (Ec03467871_m1), and IL-10 (Ec03468647_m1) (Adams et al. 2015). Duplicates of each sample were performed and all underwent an incubation period of 10 minutes at 95°C, which was followed by ten consecutive cycles of 15 seconds at 95°C and 60 seconds at 60°C (Elzinga et al. 2017). Applied Biosystems (ABI) system software was used to analyze the data and any changes in gene expression were calculated using the equation RQ=2^-ΔΔCT (Livak and Schmittgen, 2001). Our calibrator was generated by averaging the values of all media samples from Day 0 for the β-gus housekeeping gene.
Statistical Analysis

All statistical analysis was conducted using SigmaPlot version 13.0 (Systat Software, Inc., Richmond, CA). If the data were not normally distributed, they were log transformed to achieve normality. Within each age group (old and young), all data were analyzed using two-way repeated measures ANOVAs (treatment by time). Additional t-tests were run to directly compare old PPID vs old non-PPID horse responses. Statistically significant data was achieved if the data had a p-value less than 0.05, while data were considered to be trending if there was a p-value less than 0.10.

Results

Body Condition Morphometrics

Previous work by Adams et al. (2009), demonstrated that changes in body morphometrics in the horse can affect changes in immune function. Therefore, body weight, BCS, and rectal temperatures were measured to determine if supplementation with ground E+ tall fescue seed had an effect on those parameters. Additionally, it was important to determine that those parameters did not confound the results, as studies have determined that fat composition (Vick et al., 2007) and body weight (Adams et al., 2009) can have effects on inflammation in horses. Results for this study indicated that supplementation with ground E+ tall fescue seed did not have significant effects on body morphometrics in the horse. The weights (Figure 2.2A) and body condition scores (Figure 2.3A) of the old horses did not yield any significant changes, but a significant treatment versus control and treatment by time interactions ($P<0.05$) were exhibited for the decreases in rectal temperature at Day 56 (Figure 2.4A). Additionally, the young
horses did not exhibit any significant weight (Figure 2B) or rectal temperature changes (Figure 2.4B). However, the body condition scores (BCS) of the young horses did exhibit significant \((P<0.05)\) time effects at Day 28 and Day 56 (Figure 2.3B). On Day 28, the young horses in Group 1 had an average BCS of 6.6±0.6, while Group 2 had an average BCS of 6.5±1.0. By the end of the study, the average BCS of Group 1 had increased to 6.8±0.8, while that of Group 2 increased to 6.6±1.0. However, these changes in BCS lacked significance \((P>0.05)\).

*Vasoconstriction*

Vasoconstriction has been previously identified in horses consuming E+ tall fescue by McDowell et al. 2013. Therefore, vasoconstriction was measured in the distal palmar artery of a subset \((n=15)\) from Group 1 and Group 2 containing both old and young horses from each group. The subset of Group 1 \((n=7, \text{ age: } 17.1±5.6)\) and Group 2 \((n=8, \text{ age: } 18.0±6.2)\) assessed for vasoconstriction had a distal palmar artery with an average diameter of 0.27±0.15 cm and 0.31±0.11 cm, respectively (Figure 2.5). By Day 56, Group 1 had an average diameter of 0.19±0.09 cm and Group 2 had an average diameter of 0.22±0.08 cm. Though both groups exhibited changes, neither of the changes were deemed significant \((P>0.05)\).

*Hormone Concentrations*

A decrease in prolactin concentrations is a common effect of fescue toxicosis in both cattle and horses. The prolactin levels of horses were necessary to measure to ensure an appropriate dose of E+ tall fescue was being consumed by the supplementation group.
The prolactin concentrations in the old and young horses were generally higher for Group 1 throughout the study, which consumed the ground E+ tall fescue seed, but these differences were not significant, \( P>0.05 \) (Figure 2.6). However, both the old and young horses had significant time effects \( P<0.05 \), as prolactin levels decreased at Day 28 and Day 56 for the old horses and Day 56 for the young horses. Additionally, a two-way repeated measures ANOVA did not indicate a significant treatment by time effect on prolactin levels between old and young horses \( P>0.05 \).

As previously mentioned, the ergot alkaloids and other molecules produced by the endophyte are dopamine agonists. Other dopamine agonists, like pergolide, are being used to treat PPID in old horses (McFarlane et al. 2016). PPID is a disease that only affects the old horse (>15y.o.), and due to this, only old horses were assessed for baseline adrenocorticotropic hormone (ACTH) levels. These levels decreased in both Group 1 and Group 2 from Day 0 to Day 56, with significant time effects at Day 14, Day 28, Day 42, and Day 56 (Figure 2.7A). No significant differences were found between Group 1 and Group 2 at any of the time points. Furthermore, in the subset of old horses with PPID, the same results were found (Figure 2.7B).

Inflammatory and Anti-inflammatory Cytokine Production

In the old horse population, there were no significant treatment or time effects in the percent of lymphocytes staining positive for TNF-\( \alpha \) throughout this study (Figure 2.8A). However, a significant \( P<0.05 \) time effect was detected for the percent of lymphocytes staining positive for IFN-\( \gamma \) at Day 56 (Figure 2.8C). In contrast, the young horses exhibited significant \( P<0.05 \) time effects for the percent of lymphocytes staining
positive for TNF-α at Day 14 and Day 56 (Figure 2.8B), as well as for IFN-γ at Day 14, Day 28, Day 42, and Day 56 (Figure 2.8D). Additionally, t-tests indicated the percent of lymphocytes staining positive for TNF-α was significantly higher (P<0.05) in the old horse than the young horse at the start of the study, and the same was indicated for IFN-γ, which reflects inflamm-aging that occurs in horses, as previously demonstrated (Adams et al., 2008).

Cytokine gene expression by stimulated PBMCs from old horses showed similar results to the TNF-α and IFN-γ flow cytometry results with no significant treatment effects occurring in either cytokine, when comparing time points to the baseline, Day 0. However, significant (P<0.05) time effects from the baseline were exhibited in cytokine gene expression for IL-6 at Day 42 and Day 56, as well as for IL-1β at Day 14, in the old horse group (Figure 2.9). Like the old horse group, the young horse group exhibited similar time effects for TNF-α and IFN-γ cytokine gene expression when compared to the flow cytometry results. Significant time effects (P<0.05) were found at Day 14 and Day 42 for cytokine gene expression of TNF-α, as well as Day 28 and Day 42 for IFN-γ in the young horse group (Figure 2.10). Additionally, in the young horse group, significant time effects were found at Day 28 and Day 42 for IL-6, Day 14 for IL-1β, and Day 28 and Day 42 for IL-10. Though significant time effects were detected, no significant treatment by time effects were detected for either the old or young horse groups in regards to cytokine gene expression.
Discussion

One of the negative side effects of E+ fescue consumption in cattle is the decreased rate of gain (Hoveland et al., 1983); however, in this study there were no significant weight changes in the horse. Furthermore, the changes in body condition scores found in the young horses were most likely attributed to the fact this is a subjective scoring system. Though these scores are subjective, they were important to measure as a study by Vick et al. (2007) indicated that changes in fat composition of horses can affect inflammation. Also, cattle consuming E+ tall fescue have been reported to have an increase in body temperature (Hoveland et al., 1983). Unlike studies in cattle, but similar to other studies in the horse (McDowell et al., 2013), our study did not indicate that E+ tall fescue consumption had a significant effect on rectal temperature.

Previous work by McDowell et al. (2013) indicated that our higher dose of 3.7 μg E/kg BW should have resulted in significant changes in vasoconstriction of the distal palmar artery. However, in this study, there was no significant treatment effect on vasoconstriction. This was supported by the study by Douthit et al. (2012), in which significant changes in vasoconstriction in horses being fed E+ tall fescue were also not identified.

Research in cattle (Baldwin et al., 2016) and the horse (Thompson & Oberhaus 2015) have frequently observed a decrease in circulating prolactin levels caused by E+ tall fescue consumption. Therefore, it would have been expected to identify significant decreases in prolactin levels after consumption of E+ tall fescue. However, even at higher doses of E+ tall fescue consumption, McDowell et al. (2013) did not see any significant differences in prolactin concentrations. Similar to research conducted by McDowell et al.
(2013), results in this study revealed no significant changes in prolactin. Instead, a significant decrease in prolactin over time was identified in this study. These results more than likely indicate a seasonal change, as prolactin levels would be expected to decrease moving from fall to winter months (Johnson 1986). Therefore, the decrease in prolactin was likely due to the seasonal changes occurring, as this study occurred during the fall and winter.

Contradictory to the study by Browning et al. (1998) that found significant decreases in ACTH levels in cattle, no significant treatment effects were exhibited in the old horse group consuming E+ tall fescue. Though significant treatment effects were not found, there were significant time effects. These decreases in basal ACTH levels in both Group 1 and Group 2 were likely caused by seasonal changes, which was expected as ACTH levels tend to decrease when the seasons change from fall to winter (Diaz de Castro et al 2014).

This study was the first to investigate changes in TNF-α or IFN-γ caused by E+ fescue consumption in equine lymphocyte populations. The results generated by this study did not indicate any significant treatment effects caused by the consumption of endophyte in either the old or the young horse. However, it should be noted that the old horses began the study with significantly (P<0.05) higher percentages of lymphocytes staining positive for TNF-α and IFN-γ than the young horses, which supported previous work regarding inflammation in the old horse (Adams et al. 2008).

By assessing the cytokine gene expression, it was apparent that the results for TNF-α and IFN-γ closely mimicked those found using flow cytometry. This consistency suggests that it is not likely that a dose of 3.7 μg E/kg BW/day would cause an
inflammatory response in a horse involving production of TNF-α and/or IFN-γ, which supports the work by McDowell et al. (2013). Additionally, in both the old and young horses only significant time effects were found for the inflammatory cytokines IL-6 and IL-1β, as well as the anti-inflammatory cytokine IL-10. These time effects therefore do not suggest that the endophyte consumption was causing an inflammatory nor an anti-inflammatory response in non-pregnant mares or geldings.

Little research had been conducted to measure the effects of ground E+ tall fescue seed consumption on inflammatory responses in the horse, in particular, the non-pregnant old and young horse. This study did not indicate any significant changes to inflammatory or anti-inflammatory cytokines produced by PBMC’s. Furthermore, the endocrine and vasoconstrictive results of this study were inconsistent with previous studies performed by Thomson et al. (1996) and McDowell et al. (2013), which revealed that E+ fescue consumption can cause decreased production of prolactin and vasoconstriction of the distal palmar artery, respectively. In this study, E+ tall fescue consumption did not have significant effects on prolactin and ACTH levels, and any changes in the two hormones were likely due to normal seasonal decreases. Additionally, even though research by McDowell et al. (2013) showed significant changes in vasoconstriction after consumption of 3.7 μg E/kg BW, this was not the maximal dose in that study. Therefore, based on the study by McDowell et al. (2013) it might have been necessary to give a higher dose of ergot alkaloids, such as 18 μg E/kg BW, in order to see comparable changes.

Further research could be conducted to determine if feeding a higher dose of ergot alkaloids would have an impact on immune function in older horses. However, this would need to be conducted by pelleting the seed, as the dusty nature of the ground seed
and the large volume of seed needed to meet the dose requirements made it difficult to get horses to consume the diet, which would only intensify by increasing the ergot alkaloid dose requirements. Finally, investigating other dopamine agonists, like bromocriptine, could ensure that the desired amount of dopamine agonist was achieved, as this compound is capable of being injected directly into the animal.

In conclusion, no significant changes to body morphometrics, vasoconstriction, prolactin, or ACTH were identified in horses consuming ground E+ tall fescue seed. Furthermore, this study found that consumption of E+ tall fescue at a dose of 3.7 μg E/kg BW/day did not cause any significant changes in inflammatory or anti-inflammatory cytokine production in the old or young non-pregnant mare or gelding.
Figures

Figure 2.1. Schematic of sample size and average age of both Group 1 and Group 2. Group 1 horses received E+ tall fescue seed supplementation and Group 2 received E- tall fescue seed supplementation, serving as a control group. Both groups contained the same number of old and young horses, as well as the same number of PPID and non-PPID horses. Additionally, these paired subsets between groups had comparable average ages.
Figure 2.2. Effect of ground E+ (KY31) seed versus ground E- (KY32) seed consumption on weights in the (A) old horse and (B) young horse. Group 1 receiving KY31, is indicated by the black bars. Group 2 receiving the E- tall fescue, KY32, is indicated by the open bars. Neither treatment effects nor time effects were measured for body weight changes throughout the study ($P>0.05$).
Figure 2.3. Effect of ground E+ (KY31) seed versus ground E- (KY32) seed consumption on body condition score in the (A) old horse and (B) young horse. Group 1 receiving the E+ tall fescue, KY31, is indicated by the black bars. Group 2 receiving the E- tall fescue, KY32, is indicated by the white bars. Neither treatment effects nor time effects were identified for the BCS of old horses. However, the BCS of the young horses exhibited significant ($P<0.05$) time effects at Day 28 and Day 56 compared to Day 0, indicated by an asterisk (*).
Figure 2.4. Effect of ground E+ (KY31) versus ground E- (KY32) seed consumption on rectal temperature in the (A) old horse and (B) young horse. Group 1 receiving the E+ tall fescue, KY31, is indicated by the black bars. Group 2 receiving the E- tall fescue, KY32, is indicated by the white bars. Significant treatment vs. control and treatment by time interactions ($P<0.05$) were observed for the decrease in rectal temperature at Day 56 for the old horse group, indicated by an asterisk (*). However, neither significant treatment vs. control nor significant treatment by time interactions were found in regards to any changes in young horse rectal temperatures.
Figure 2.5. Effect of ground E+ (KY31) seed versus ground E- (KY32) seed consumption on vasoconstriction of the distal palmar artery in mixture of old (n=7) and young horses (n=8) from Group 1 (n=7) and Group 2 (n=8). Group 1 received the E+ tall fescue, KY31, and is indicated by the black bars. Group 2 received the E- tall fescue, KY32, and is indicated by the open bars. Diameters of both groups decreased over time; however, it was not significant.
Figure 2.6. Effect of ground E+ (KY31) seed versus ground E- (KY32) seed consumption on prolactin levels in the (A) old horse and (B) young horse. Group 1 received KY31 and is indicated by the black bars. Group 2 received KY32 and is indicated by the white bars. Additionally, the old horses had a significant time effect at Day 28 compared to Day 0, while both old and young horses had significant time effects at Day 56 compared to Day 0. Significant time effects are indicated by an asterisk (*). However, no treatment by time interactions were identified.

Figure 2.7. Effect of ground E+ (KY31) seed versus ground E- (KY32) seed consumption on basal ACTH levels in (A) old horses and (B) PPID old horses. Group 1 receiving the E+ tall fescue, KY31, is indicated by the black bars. Group 2 receiving the E- tall fescue, KY32, is indicated by the white bars. Basal ACTH levels decreased from Day 0 to Day 56 for both Group 1 and Group 2 (A and B). Significant time effects from Day 0 are indicated by an asterisk (*).
Figure 2.8. Effect of ground endophyte-infected tall fescue versus ground endophyte-free tall fescue seed consumption on the percent of lymphocytes staining positive for TNF-α in the (A) old horse and (B) young horse, and the percent of lymphocytes staining positive for IFN-γ in the (C) old horse and (D) young horse. Group 1 received the E+ tall fescue, KY31, and is indicated by the black bars. Group 2, the control group, received E- tall fescue, KY32, and is indicated by the white bars. Significant treatment effects were not observed in the old or young horse groups. However, the young horse samples stained for TNF-α experienced significant ($P<0.05$) time effects at Day 14 and Day 56, as depicted by asterisks (*), but the old horse group did not exhibit any significant time effects for that cytokine. A significant ($P<0.05$) time effect was detected for the percent of lymphocytes staining positive for IFN-γ in the old horse at Day 56, as well as at Day 14, Day 28, Day 42, and Day 56 in the young horse.
Figure 2.9. Effect of ground endophyte-infected tall fescue seed (KY31) versus ground endophyte-free tall fescue seed (KY32) consumption on cytokine gene expression in the old horse. Group 1 received KY31 (black bars), while Group 2 received KY32 (white bars). Five cytokines were assessed: (A) TNF-α, (B) IFN-γ, (C) IL-6, which had a significant ($P<0.05$) time effect from the baseline at Day 42 and Day 56, (D) IL-1β, which had a significant ($P<0.05$) time effect from the baseline at Day 14, and (E) IL-10. Time effects are indicated with an asterisk (*).
Figure 2.10. Effect of ground endophyte-infected tall fescue seed (KY31) versus ground endophyte-free tall fescue seed (KY32) consumption on cytokine gene expression in the young horse. Group 1 received KY31 (black bars), while Group 2 received KY32 (white bars). Five cytokines were assessed: (A) TNF-α, which expressed significant time effects ($P<0.05$) at Day 14 and Day 42, (B) IFN-γ, which expressed significant time effects ($P<0.05$) at Day 28 and Day 42, (C) IL-6, which expressed significant time effects ($P<0.05$) at Day 28 and Day 42, (D) IL-1β, which expressed significant time effects ($P<0.05$) at Day 14, and (E) IL-10 which expressed significant time effects ($P<0.05$) at Day 28 and Day 42. No significant treatment by time interactions were detected. Time effects are indicated with an asterisk (*).
CHAPTER 3

EFFECTS OF BROMOCRIPTINE AND DOPAMINE ON EQUINE CYTOKINE PRODUCTION IN VITRO

Abstract

Today, the majority of research investigating the effects of ergot alkaloids found in endophyte-infected (E+) tall fescue have focused on understanding the effects on equine reproduction or fescue toxicosis in cattle. However, these studies often involve the consumption of tall fescue seeds or leaves, which can have varying concentrations of ergot alkaloids and often spill from the mouth of the animal, leaving exact consumption concentrations unknown. Bromocriptine, a semi-synthetic ergopeptine, on the other hand, is a compound capable of being injected directly into an animal and has been found to exhibit similar effects to E+ tall fescue consumption in cattle. Additionally, very little research has been conducted to understand the effects of bromocriptine or ergot alkaloids in vitro, more specifically on immune function, in order to further understand the mechanisms of action of each on the animal. Both bromocriptine and ergot alkaloids are D2-like receptor agonists, meaning that they are capable of binding to dopamine receptors. In vitro research in humans and mice have demonstrated that dopamine can act directly on immune cells and elicit a variety of effects. Thus, the purpose of this research was to investigate the effects of bromocriptine and dopamine on equine cytokine production by peripheral blood mononuclear cells (PBMCs) in vitro. Two experiments were conducted, the first evaluated the effects of bromocriptine in vitro on PBMCs from old (n=8; age: 24.75±2.31) horses, and the second evaluated the effects of dopamine in
\textit{vitro} on PBMCs from old horses (n=8; age: 25.25±2.55). Isolated PBMCs were exposed to either bromocriptine or dopamine concentrations ranging from $10^{-2}$M or $10^{-11}$M. Cell viability was determined, and all samples were stained intracellularly for TNF-\(\alpha\) and IFN-\(\gamma\), as well as assessed for relative levels of cytokine gene expressions for TNF-\(\alpha\), IFN-\(\gamma\) IL-6, IL-1\(\beta\), and IL-10. After analyzing the data using one-way ANOVAs, it was found that concentrations of bromocriptine and dopamine were toxic to equine PBMCs at concentrations greater than $10^{-5}$M. Additionally, bromocriptine was found to downregulate the expression of TNF-\(\alpha\) and IL-1\(\beta\), while dopamine did not cause any significant changes in cytokine production. Therefore, this study found that bromocriptine caused a decrease in inflammatory cytokine production when acting on equine PBMCs \textit{in vitro}.

\textbf{Introduction}

Currently, there is little research characterizing effects that ergot alkaloids have on the immune function of horses. Most research to-date has investigated the effects of ergot alkaloids on whole animal health, including reproduction in horses and fescue toxicosis in cattle. A study in cattle by Filipov et al. (2000) found a decrease in circulating TNF-\(\alpha\) after administration of ergotamine tartrate, which suggested an anti-inflammatory effect was caused by the ergopeptine. Research by McDowell et al. (2013), on the other hand, identified that horses consuming ground E+ tall fescue seed had significantly increased vasoconstriction in the distal palmar artery, and no change to inflammatory cytokine production or prolactin levels. Due to the conflicting results, the study discussed in Chapter 2 of this publication was conducted to assess the effect of E+
tall fescue consumption on immune function in the horse. This study identified no significant differences in immune function of horses consuming 3.7 μg E/kg body weight in ground E+ tall fescue seed and those consuming an equivalent amount of ground endophyte free (E-) tall fescue seed. In addition, though the horses had been consuming 3.7 μg E/kg body weight, it was unknown how much, if any, of the ergot alkaloids were being absorbed into the blood stream. Thus, the exact mechanism or effect of ergot alkaloids on immune cells remains to be understood.

However, it is known that ergot alkaloids are a type of D2-like receptor agonist (Cross et al. 1995; Larson et al. 1999). In addition to being a D2-like receptor agonist, ergot alkaloids have also been found to be α-adrenergic and 5-HT2 serotonin receptor partial agonists (Schiff 2006). Therefore, ergot alkaloids, like ergovaline, should bind to dopamine receptors found on a variety of cells throughout the body (Levite 2016).

In fact, dopamine has been found to have effects on inflammatory cytokine production by lymphocytes depending on the concentration of dopamine that the cells are exposed to in vitro (Levite 2016). Levite (2016) describes high doses (>10⁻⁵M) as being toxic to immune cells, and depending on the immune cell type low doses (<10⁻⁷M) could have inflammatory or anti-inflammatory effects. This is important because immune cells have been found to have a variety of dopamine receptors on their surfaces (Levite et al. 2001, McKenna et al 2002, Besser et al. 2005, Watanabe et al 2006, Cosentino et al. 2007, Nakano et al. 2008, Kustrimovic et al. 2014), with each receptor having a different affinity for dopamine (Beaulieu & Gainetdinov 2011). For example, DAR3, DAR2, and DAR4 have all been referred to as D2-like receptors (Levite 2016), but DAR3 has the greatest affinity for dopamine out of all dopamine receptors, while DAR1, a D1-like
receptor, has the least affinity for dopamine. Additionally, all dopamine receptors are types of G-protein-coupled receptors, with D1-like receptors activating the G-protein, G_{\text{as}}, and D2-like receptors activating the G-protein, G_{\text{ai}} (Levite 2016). These two pathways within the cell either activate adenylyl cyclase or inhibit it, respectively. Activation of adenylyl cyclase leads to increased levels of cyclic adenosine monophosphate (cAMP), which is an important intracellular signaling molecule and plays a role in the activation of multiple cellular pathways (Levite 2016). Therefore, the response to the presence of dopamine at a low concentration likely activates DAR3 and thereby inhibits adenylyl cyclase and cAMP production, while a high concentration of dopamine has the potential to increase levels of cAMP within immune cells.

Furthermore, studies in humans have demonstrated that bromocriptine exhibits immunosuppressive effects by acting through dopamine receptors on immune cells in vitro, and has been shown to affect prolactin responses (Morikawa et al., 1994). Moreover, research in horses by Thomson et al. (1996) has shown that injections of bromocriptine, a semisynthetic ergopeptine and D2-receptor agonist, cause changes in hormone responses, in particular a decrease in prolactin. Bromocriptine in cattle has been used to simulate the effects of E+ tall fescue consumption (Baldwin et al., 2016). However, no research has been conducted in horses or cattle to determine the effects of bromocriptine on immune responses in vitro.

Therefore, the purpose of this study was to determine if bromocriptine or dopamine affect equine peripheral blood mononuclear cells (PBMCs) production of inflammatory (IL-1β, IL-6, TNF-α, and IFN-γ) and anti-inflammatory (IL-10) cytokine responses, similar to what has been shown in human and murine studies. Hence, this research will
provide insight into mechanisms of action that ergot alkaloids found in E+ tall fescue may have on lymphocyte production of cytokines.

**Methods**

*Bromocriptine Preparation*

A working stock of bromocriptine was prepared by dissolving 2-bromo-α-ergocryptine methanesulfonate salt (bromocriptine; Sigma-Aldrich, St. Louis, MO) in dimethyl sulfoxide (DMSO; Sigma-Aldrich) to reach a concentration of 9.407 x 10⁻¹M. Serial dilutions of bromocriptine were then prepared using cRPMI [a mixture of RPMI 1640 (Gibco, Grand Island, NY), 2.5% fetal equine serum (Sigma-Aldrich), 55μM 2-mercaptoethanol (Gibco), 2mM L-glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin (Hyclone Pen/Strep/Glutamine solution; Thermo Scientific)] to obtain concentrations ranging from 9.407 x 10⁻¹M bromocriptine to 9.407 x 10⁻¹¹M bromocriptine. Dilutions were prepared using sterile technique in a laminar flow hood.

*Dopamine Preparation*

A working stock of dopamine was prepared by dissolving dopamine hydrochloride (dopamine; Sigma-Aldrich) in DMSO to reach a concentration of 8.391 x 10⁻¹M. Serial dilutions of dopamine were prepared using DMSO to obtain concentrations ranging from 8.391 x 10⁻¹M dopamine to 8.391 x 10⁻¹¹M dopamine. Dilutions were prepared using sterile technique in a laminar flow hood.
Animals and Sample Collection

For the first experiment, heparinized blood was collected via venipuncture from the jugular vein of randomly selected old horses (n=8, age: 24.75±2.31) from University of Kentucky, Department of Veterinary Science herd. Old horses (age: ≥15) were used as it has been shown that inflamm-aging occurs at the lymphocyte level, which provides a natural model to study the immunomodulatory effects of bromocriptine and dopamine. Samples were collected in mid-January from horses that appeared healthy with no clinical signs of infectious disease.

The second experiment involved the use of a different group of randomly selected old horses (n=8, age: 25.25±2.55). Heparinized blood was collected via venipuncture from the jugular vein in mid-April, and all horses were a part of the University of Kentucky, Department of Veterinary Science herd. All horses appeared to be in good health and did not exhibit signs of infectious disease. These experiments were approved by the University of Kentucky Institutional Animal Care and Use Committee, protocol number 2014-1225.

Peripheral Blood Mononuclear Cell Isolation

For both experiments, peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized blood samples following a procedure previously used by Adams et al., in 2008. PBMCs were isolated using Ficoll-Paque Plus™ (Amersham Biosciences, Piscataway, NJ) gradient centrifugation. Once isolated, PBMCs were washed twice with 1X phosphate buffered saline (PBS) (Adams et al. 2008). The cell concentration and
percent viability of each sample was identified using the VICELL™ Counter-XR (Beckman Coulter, Miami, FL), which uses a trypan blue to stain dead cells.

**PBMC Stimulation and Viability**

For the first experiment, PBMCs for each horse were plated at a volume of 989 μL and a concentration of $4 \times 10^6$ cells/mL cRPMI. Each well received 11 μL of the appropriate bromocriptine dilution to reach a final volume of 1mL. Therefore, the final concentrations of bromocriptine in each well ranged from $10^{-2}$M to $10^{-12}$M. For each horse, there were two negative controls, one media well containing cRPMI and one well containing media and 11 μL of DMSO. Additionally, the positive control (DMSO/PHA) contained cRPMI and DMSO. All wells in the plate, except for the negative controls, were stimulated with 10 μL of phytohemagglutinin (PHA; 50 μg/mL; Sigma-Aldrich). The plates were incubated for 48 hours at 37 °C, 5% CO₂. After 44 hours had passed, 2 μL Brefeldin A (10 μg/mL; Sigma Aldrich) was added to every well. Following the incubation period, percent viability of each control and dilution was determined using the VICELL™ Counter-XR.

The second experiment differed in that PBMCs for each horse were plated at a volume of 988 μL and each well received 12 μL of the appropriate dopamine dilution to reach a volume of 1mL. Final concentrations of dopamine in each well ranged from $10^{-2}$M to $10^{-12}$M. Additionally, the negative controls (Media; and DMSO) and positive control (DMSO/PHA) were constructed the same as those from the first experiment, with the addition of a second positive control only containing media and PHA. The cells were
incubated and viability was determined in the same manner as that of the first experiment.

*Intracellular Staining and Flow Cytometry*

After stimulations, 200 µL of PBMCs in cRPMI were aliquoted into two 96-well V-bottom plates to be intracellularly stained and 500 µL were aliquoted for RNA isolation. The V-bottom plated cells were pelleted by centrifugation and then resuspended in 2% paraformaldehyde (Sigma-Aldrich). PBMCs were incubated overnight at 4 °C. The following day, cells were washed with saponin buffer [a mixture containing PBS, 1% fetal bovine serum, 0.1% saponin, and 0.1% sodium azide (Sigma-Aldrich)] and stained for either IFN-γ or TNF-α. Either 1:100 dilution of IFN-γ FITC mouse anti-bovine antibody (AbD Serotec, Raleigh, NC) in saponin buffer or 1:10 dilution of TNF-α anti-equine monoclonal antibody (HL801) was used following a protocol by Adams et al. (2008). Once stained, PBMCs were incubated for 30 minutes in the dark on ice, and then washed with saponin buffer. The cells being stained for TNF-α were resuspended in a 1:1000 dilution of FITC-conjugated goat F(ab’)2 anti-mouse IgG (H + L) (Invitrogen; 2mg/mL) in saponin buffer and incubated in the dark on ice for another 30 minutes. Cells stained for IFN-γ were resuspended in 1X PBS, and the same was done for those stained for TNF-α after the second incubation. Flow cytometric analysis could be conducted using the Attune® Nxt™ Flow Cytometer and Attune® Nxt software version 2.5 (ThermoFisher Scientific). Each sample was gated based on cell size and granularity to identify the percent of lymphocytes producing IFN-γ and TNF-α (Adams et al. 2008).
RNA Isolation and Cytokine Gene Expression

The PBMC aliquots for RNA isolation from each experiment were centrifuged and resuspended in Trizol (Ambion). These samples were placed in a -80°C freezer. Once all samples were ready for RNA extraction, samples were thawed and processed using a previously published method (Elzinga et al. 2017). Once isolated, 0.5 μg of RNA from each sample was reverse transcribed into cDNA. This was performed by adding a reverse transcription master mix to each sample (Promega, Madison, WI) and incubating for a 15-minute period at 42°C followed by 5 minutes at 95°C. Then, 4.5 μL of the cDNA from each sample was mixed with 5 μL of Master Mix (SensiMix HI-ROX 2x, Bioline). A primer-probe of interest (TaqMan, Equine-specific, intron spanning primers and probes; Applied Biosystems) was then added to the cDNA and Master Mix combination. The Applied Biosystems Real-Time PCR (ABI Viia7) was used to run all samples against the genes: beta-glucuronidase (β-gus; Ec03470630_m1), interferon gamma (IFN-g; Ec03468606_m1), IL-6 (Ec03468678_m1), IL-1B (Ec04260298_s1), TNF-a (Ec03467871_m1), and IL-10 (Ec03468647_m1) (Adams et al. 2015). Each sample was run in duplicate. All samples were incubated for 10 minutes at 95°C, which was followed by ten consecutive incubation cycles of 15 seconds at 95°C and 60 seconds at 60°C (Elzinga et al. 2017). The Applied Biosystems (ABI) system software was used to analyze the data and changes in expression of genes were calculated using the equation, RQ=2^{ΔΔCT} (Livak & Schmittgen 2001). The calibrator for each experiment was generated by averaging the ΔCT values for all media samples from the appropriate experiment. Beta-glucuronidase (β-gus) was used as the housekeeping gene.
Statistical Analysis

Statistical analysis was conducted using SigmaPlot version 13.0 (Systat Software, Inc., Richmond, CA). All data were analyzed using one-way ANOVAs and displayed as line graphs (mean±SE). If the data were not normally distributed, they were log transformed to achieve normality and if not achieved ANOVA on ranks were performed. Additionally, t-tests were run when investigating differences in cell viability to determine the point at which cell viability was no longer affected by bromocriptine and dopamine. Only dilutions that were not negatively affected by viability were considered for the cytokine statistical analysis. The data were considered significant at p-values less than 0.05, and trending, if the p-value was less than 0.10.

Results

Cell Viability

Levite (2016) stated that high concentrations of dopamine (>10⁻⁵M) have been found to be toxic to some immune cells and even cause cell death. Therefore, it was important to identify if similar results were exhibited in the equine immune cells being exposed to bromocriptine and dopamine. Results for both experiments, as depicted in Figure 3.1, indicated that equine PBMCs exposed to concentrations of bromocriptine between 10⁻²M and 10⁻⁴M had a significant (P<0.05) decrease in cell viability, when compared to the positive control (DMSO/PHA). Additionally, t-tests indicated that there were no significant differences (P>0.05) in cell viability between the positive control, DMSO/PHA, and PBMCs exposed to bromocriptine or dopamine concentrations between 10⁻⁵M and 10⁻¹¹M. Additionally, no significant changes were identified in the negative
controls (Media; and DMSO). Therefore, only doses that did not negatively impact cell viability were used for the cytokine statistical analysis.

**Intracellular Staining of Inflammatory Cytokines**

In a study by Besser et al. (2005), it had been shown that the presence of dopamine caused an increase in TNF-α production in human T lymphocytes *in vitro*. Therefore, this study investigated whether similar effects were exhibited with equine lymphocytes by exposing cells to various concentrations of bromocriptine and dopamine.

In this study, the percent of lymphocytes staining positive for TNF-α significantly decreased after incubation with either 10⁻⁷M bromocriptine or 10⁻⁹M bromocriptine (Figure 3.2A), when compared to the positive control (DMSO/PHA). However, as shown in Figure 3.2B, the percent of lymphocytes staining positive for TNF-α did not significantly change in the presence of decreasing concentrations of dopamine.

Also, research involving human and murine lymphocytes demonstrated that dopamine had little effect on the presence of IFN-γ (Besser et al. 2005), and therefore it was important to determine if those same effects were present in the horse. Like previous studies, there were no significant changes between in the percent of lymphocytes staining positive for IFN-γ when cells were incubated with concentrations of bromocriptine or dopamine less than or equal to 10⁻⁵M (Figure 3.3).

Additionally, significant changes in the negative controls (Media; and DMSO) for both cytokines were not identified.
Cytokine Gene Expression

Changes in lymphocyte cytokine production after exposure to dopamine or dopamine agonist in human and murine studies have been shown by Josefsson et al. (1996) and Bergquist et al. (2000). In the first experiment of this study, bromocriptine had significant effects on cytokine gene expression (Figure 3.4). As indicated in the methods section, doses at which cell viability was not affected by bromocriptine and dopamine were analyzed for any effects on cytokine production, starting with the $10^{-5}$M dose. Results from the first experiment indicated that, when compared to the positive control (DMSO/PHA), bromocriptine induced a significant ($P<0.05$) decrease in TNF-$\alpha$ at all doses ($10^{-5}$M – $10^{-11}$M), in IFN-$\gamma$ at $10^{-5}$M, and in IL-1$\beta$ from $10^{-7}$M – $10^{-11}$M. No significant changes ($P>0.05$) in the IL-6 or IL-10 gene expression in PBMCs for any concentration of bromocriptine were measured. Also, as shown in Figure 3.5, there were no significant changes ($P>0.05$) in TNF-$\alpha$, IFN-$\gamma$, IL-6, IL-1$\beta$, or IL-10 gene expression after equine PBMC’s were exposed to varying concentrations of dopamine; and no significant changes in the negative controls (Media; and DMSO) were identified.

Discussion

The effects of dopamine and dopamine agonists on human and murine immune cells have been extensively studied. However, no research had investigated the effects of dopamine or bromocriptine on immune cell function of the horse in vitro. Research in humans and mice have shown that depending on the concentration of dopamine present, inflammatory or anti-inflammatory effects may be measured (Levite 2016). This has been attributed to the varying affinity that dopamine and its agonists have for dopamine
receptors (Beaulieu & Gainetdinov 2011). Additionally, though research in cattle and horses have compared the effects of two types of dopamine agonists, bromocriptine and ergot alkaloids (Baldwin et al. 2016; Thomson et al. 1996), these studies did not investigate effects on immune function, particularly at the cellular level. Therefore, this study was important for understanding any direct effects that bromocriptine could have on equine PBMCs, determining if the equine PBMC response to dopamine is similar to mice and humans, and if equine PBMCs respond similarly to dopamine and bromocriptine.

In previous studies, it has been shown that high concentrations of dopamine (>10^{-5}\text{M}) can be toxic to immune cells (Levite 2016). Those findings were supported by this study, which found significant decreases in cell viability when PBMCs were exposed to either bromocriptine or dopamine concentrations that were greater than 10^{-5}\text{M}. This indicated that the response to dopamine and bromocriptine were similar in regards to cell viability and that concentrations of dopamine and its agonists greater than 10^{-5}\text{M} were toxic to equine PBMCs.

In a study by Besser et al. (2005), it was found that dopamine at a concentration of 10^{-8}\text{M} was capable of increasing TNF-\alpha production by lymphocytes. However, those results were not supported by the findings in this study. Intracellular staining of lymphocytes exposed to bromocriptine at intermediate concentrations, from 10^{-7}\text{M} to 10^{-9}\text{M}, had a decrease in the percent of lymphocytes staining positive for TNF-\alpha. Additionally, there was no significant change in the percent of lymphocytes staining positive for TNF-\alpha after exposure to dopamine. Also, these results were similar for TNF-\alpha gene expression of PBMCs exposed to bromocriptine or dopamine, where all
concentrations of bromocriptine induced a significant decrease in TNF-α expression when compared to the positive control (DMSO/PHA) and those exposed to dopamine did not induce a change. Therefore, the findings in this study did not indicate that equine lymphocytes have the same TNF-α response to dopamine or dopamine agonists as lymphocytes from mice or humans. This suggests that the binding of dopamine and its agonists to dopamine receptors on equine lymphocytes might induce the activation or inactivation of different pathways compared to mice and humans. Though there did not seem to be any significant change in cells exposed to non-toxic amounts of dopamine, there were some anti-inflammatory effects on PBMCs exposed to concentrations of bromocriptine less than 10^{-4}M, since there was a significant decrease in TNF-α gene expression compared to the positive control (DMSO/PHA).

While the TNF-α results did not support previous research, the results for IFN-γ supported research conducted with human and murine lymphocytes. Research by Besser et al. (2005) indicated lymphocytes in the presence of dopamine did not have any change in the production of IFN-γ. In this study, there were no significant differences in the percent of lymphocytes staining positive for IFN-γ when exposed to concentrations of bromocriptine or dopamine equal to or less than 10^{-5}M. Similarly, the gene expression of IFN-γ in PBMCs incubated with varying concentrations of bromocriptine only showed a significant decrease in gene expression at a concentration of 10^{-5}M bromocriptine, while cells exposed to the same dopamine concentrations did not have any significant changes in gene expression. Therefore, the results from this study support the research performed in humans and mice, in regards to changes in IFN-γ production in response to dopamine or its agonists.
Depending on the specific immune cell type and concentration of dopamine or agonist, different effects have been noted in relation to the production of other cytokines. In one study by Josefsson et al. (1996), it was found that high concentrations of dopamine inhibited the production of IL-6 and IFN-γ, while Levite (2016) has shown that dopamine at a concentration of 10^{-8}M caused an increase in TNF-α, IL-10, and IL-6. In this study, no significant differences were found in the IL-6 or IL-10 gene expression of PBMCs incubated with bromocriptine. However, there were significant decreases in IL-1β gene expression at 10^{-7}M bromocriptine and lower when compared to the control, which indicates that low concentrations have the potential to induce anti-inflammatory effects on equine PBMCs.

Overall, the purpose of this study was to determine the direct effects of dopamine and a dopamine agonist, bromocriptine, on cytokine production of PBMCs, as well as to determine if equine PBMCs and human or murine lymphocytes have similar immune responses to dopamine and its agonists. This study demonstrated that concentrations of dopamine or bromocriptine greater than 10^{-5}M are toxic to equine PBMCs by causing significant decreases in cell viability, which was similar to human and murine lymphocytes. Also similar to other research was the lack of a response in IFN-γ to dopamine or bromocriptine. However, the results of this study directly opposed those reported in in vitro studies involving human and murine lymphocyte TNF-α production, as a decrease was found after immune cells were exposed to bromocriptine. Also, there was very little response in other cytokines after exposure to dopamine or bromocriptine, except for IL-1β which had an anti-inflammatory response to bromocriptine. Therefore, the differing responses by equine PBMCs to bromocriptine and dopamine suggest that
bromocriptine was not acting on a dopamine receptor on the immune cell surface. Overall, these results suggest that bromocriptine may affect the innate immune responses (TNF-α and IL-1β) and not adaptive, cell-mediated responses (IFN-γ). Additionally, this study has shown that unlike in humans and mice, it appears that in the horse bromocriptine, a semi-synthetic ergopeptine, did not act as a dopamine agonist and instead activated a different receptor to stimulate anti-inflammatory responses.
Figures

A. Bromocriptine

B. Dopamine

**Figure 3.1.** Effect of (A) bromocriptine and (B) dopamine on peripheral blood mononuclear cell viability after a 48-hour incubation period. Both bromocriptine and dopamine exhibited significant ($P<0.05$) decreases in cell viability between concentrations of $10^{-2}$M and $10^{-4}$M when compared to the positive control (DMSO/PHA) as designated by an asterisk (*). For both, cell viability was not significantly affected at or below $10^{-5}$M.
Figure 3.2. Effect of (A) bromocriptine and (B) dopamine on the percent of TNF-$\alpha^+$ lymphocytes. When comparing the percent of TNF-$\alpha^+$ lymphocytes to the positive control (DMSO/PHA), significant ($P<0.05$) decreases were found at concentrations of $10^{-7}$M and $10^{-9}$M bromocriptine as designated by an asterisk (*). However, no significant differences ($P>0.05$) were found for any of the concentrations of dopamine.
Figure 3.3. Effect of (A) bromocriptine and (B) dopamine on the percent of IFN-γ⁺ lymphocytes. When comparing the percent of IFN-γ⁺ lymphocytes to the positive control (DMSO/PHA), no significant ($P>0.05$) differences were found at any concentrations of bromocriptine or dopamine.
Figure 3.4. Effect of bromocriptine on PBMC gene expression of (A) TNF-α, (B) IFN-γ, (C) IL-6, (D) IL-1β, and (E) IL-10. When comparing the gene expression of TNF-α to the positive control (DMSO/PHA), significant ($P<0.05$) decreases were determined at all concentrations of bromocriptine as designated by an asterisk (*). Significant ($P<0.05$) decreases in gene expression were measured for IFN-γ at $10^{-5}$M and for IL-1β at concentrations less than $10^{-5}$M as designated by an asterisk (*). However, no significant differences ($P>0.05$) were found for IL-6 or IL-10.
Figure 3.5. Effect of dopamine on PBMC gene expression of (A) TNF-α, (B) IFN-γ, (C) IL-6, (D) IL-1β, and (E) IL-10. No significant differences ($P>0.05$) were found when comparing the gene expression of any of the cytokines to the positive control (DMSO/PHA).
CHAPTER 4

EFFECTS OF AN INTRAVENOUS INJECTION OF BROMOCRIPTINE ON PROLACTIN AND IMMUNE FUNCTION IN THE HORSE

Abstract

Bromocriptine, a D2-like receptor agonist and semi-synthetic ergopeptine, has been used for a number of years in research to simulate the effects of fescue toxicosis in cattle and horses. Bromocriptine causes a decrease in prolactin, similar to other D2-like receptor agonists. Furthermore, research using human cells has established that dopamine agonists acting on D2-like receptors can cause an increase in inflammation. However, the in vivo effects of bromocriptine on equine immune function have not been previously measured. Aged mares (n=19, age: 17.5±2.6) were evaluated in this study by comparing the immune and prolactin responses between the treatment group (n=9; age: 17.6±3.0) that received an intravenous injection of bromocriptine to the control group (n=10, age: 17.4±2.3), which received a vehicle injection of 7.6% ethanol. Heparinized blood and plasma samples were collected over a period of 56 hours at T0, T12, T24, T48, and T56. Body weights and rectal temperatures were measured at T0 and T56. Peripheral blood mononuclear cells were isolated from the heparinized blood and intracellularly stained for TNF-α and IFN-γ. Also, PBMC gene expression of TNF-α, IFN-γ IL-6, IL-1β, and IL-10 were measured at each time point. Prolactin levels were measured in the plasma samples at all time points. Statistical analysis was performed using two-way repeated measures ANOVAs on SigmaPlot v.13 software with significance set for \( P<0.05 \). Body weights and rectal temperatures did not significantly change throughout the study.
Prolactin levels were significantly lower in the bromocriptine treatment group from T12 to T48 hours in comparison to the vehicle controls, with prolactin maximally decreasing at T12 hours. Additionally, an overall significant treatment by time effect was measured in the gene expression of IL-1β with a significant treatment effect occurring at T12. However, all other cytokines and intracellular staining exhibited significant time effects. Therefore, this study supported previous studies indicating that prolactin maximally decreased twelve hours post-injection of bromocriptine and returned to baseline approximately 56 hours post-injection. Additionally, the results from this study suggested that bromocriptine only had a significant effect on inflammation through IL-1β.

Introduction

Bromocriptine, a semi-synthetic ergopeptine, originally sold under the name Parlodel, has been used for humans suffering from hyperprolactinemia, prolactinomas, and other conditions (Freeman et al., 2000). It has been used to treat those disorders, primarily due to its dopaminergic activity and its ability to reduce prolactin levels. Bromocriptine is a D2-like receptor agonist, meaning that it has the capability of binding to DAR2, DAR3, and DAR4 throughout the body. In particular, when binding to dopamine receptors found in the pituitary gland, bromocriptine has been found to inhibit the production of prolactin by lactotrophs (Freeman et al., 2000).

Dopamine receptors have been found throughout the body, including on immune cells. Studies involving dopamine and immune cells have indicated that high concentrations of dopamine (>10^{-5}M) can be toxic; and depending on the type of immune cells and concentration, dopamine can cause either an inflammatory or anti-inflammatory
response (Levite, 2016). In humans, primary binding of dopamine occurs on the DAR3, which has been found to elicit an increase in TNF-α production (Levite, 2016). However, in the presence of higher concentrations of dopamine, DAR5 and DAR1 have the capability of binding dopamine, which has been found to have a general anti-inflammatory effect (Levite, 2016). Therefore, due to its dopaminergic activity in human studies, it was possible that bromocriptine could induce effects on equine immune cells.

Though bromocriptine has been used historically to treat diseases in humans, in recent years, bromocriptine has been used to simulate endophyte-infected (E+) fescue consumption in animals (Evans et al., 1999; Baldwin et al., 2016). Research in cattle has demonstrated that animals receiving either bromocriptine or a diet of E+ tall fescue exhibited similar decreases in prolactin concentrations (Baldwin et al., 2016). It has been hypothesized that the decreases in prolactin caused by ergot alkaloids found in E+ fescue are due to these molecules having dopaminergic activity like bromocriptine (Thompson and Oberhaus, 2015; Cross et al., 1995; Larson et al., 1999). As such, research investigating the effects of bromocriptine in the horse often examine changes in prolactin (Thomson et al., 1996; Thompson et al., 2017).

While research has demonstrated that dopamine and dopamine agonists can have effects on immune function in vitro, little research has investigated the effects of dopamine agonists on immune function in livestock. Some studies in cattle and horses have evaluated inflammatory parameters after E+ fescue consumption, but the results are conflicting. In cattle, ergot alkaloids have been shown to elicit an anti-inflammatory response (Filipov et al., 2000), while in horses no change in inflammation has been measured (McDowell et al., 2013). The effects of bromocriptine on inflammation in
cattle and in horses have not been investigated. Therefore, the purpose of this study was to determine the effects of bromocriptine on immune function, in particular, inflammatory cytokine production in horses.

Methods

Animals

For this study, a total of 19 aged mares (17.5±2.6) from the University of Kentucky, Department of Veterinary Science herd were randomly assigned to either the bromocriptine treatment (n=9, age: 17.6±3.0) or vehicle control (n=10, age: 17.4±2.3) group. This study occurred over the course of 56 hours in mid-May. At the time of this study, all horses appeared healthy and exhibited no clinical signs of infectious disease, nor had above normal rectal temperatures. This study protocol was approved by the University of Kentucky Institutional Animal Care and Use Committee, protocol number 2018-2922.

Preparation of Injections

Each horse in the vehicle control group (n=10) was administered a single intravenous injection of 3 mL of 7.6% ethanol. The 7.6% ethanol solution was prepared using aseptic techniques in a laminar flow hood by mixing ethyl alcohol (Sigma-Aldrich, St. Louis, MO) with sterile saline (Henry Schein).

The bromocriptine treatment group horses were each administered a single intravenous injection of 100 mg of bromocriptine. The bromocriptine injections were prepared using aseptic techniques in a laminar flow hood. The bromocriptine mixtures
were prepared by mixing 2-bromo-α-ergocryptine methanesulfonate salt (bromocriptine; Sigma-Aldrich, St. Louis, MO) in 3 mL of 7.6% ethanol solution, as prepared above for the vehicle control group.

Sample Collection

Body weights were taken using a scale and rectal temperatures were collected using a digital thermometer at T0 and T56. Samples of heparinized blood and EDTA plasma were collected from the jugular vein via venipuncture at T0 (pre-injection), which occurred between 0800 and 1000 hours; and all other collections occurred 12, 24, 48, and 56 hours afterwards. The intravenous injection of either the bromocriptine treatment or vehicle control solution was administered to each horse in the jugular vein immediately after the first blood collection. After plasma samples were collected, they were centrifuged at 800g for 10 minutes and stored at -20 °C, until they were sent to Louisiana State University for prolactin analysis by a radioimmunoassay (RIA) method validated by Colborn et al. (1991).

Peripheral Blood Mononuclear Cell Culture

Peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized blood samples by using a density gradient of Ficoll-Paque Plus™ (Amersham Biosciences, Piscataway, NJ) and phosphate buffered saline (PBS) (Adams et al., 2008). VICELL™ Counter-XR (Beckman Coulter, Miami, FL) was used to determine PBMC cell concentration. Each sample was plated into two wells at a concentration of 4 x 10⁶ cells/mL cRPMI [a mixture of RPMI 1640 (Gibco, Grand Island, NY), 2.5% fetal equine
serum (Sigma-Aldrich, St. Louis, MO), 55μM 2-mercaptoethanol (Gibco, Grand Island, NY), 2mM L-glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin (Hyclone Pen/Strep/Glutamine solution; Thermo Scientific)] (Adams et al. 2008). Ten μL of phorbol 12-myristate 13-acetate (PMA; 25 ng/mL; Sigma Aldrich) and ionomycin (1 mM; Sigma) were added to each well, and the plates were incubated for 24 hours at 37 °C, 5% CO₂. After 20 hours had passed, cells were stimulated with 2 μL Brefeldin A (10 μg/mL; Sigma Aldrich) (Adams et al. 2008).

Intracellular Staining and Flow Cytometry

After incubation, 200 μL from each sample were aliquoted into two 96-well V-bottom plates and 500 μL were aliquoted for RNA isolation. Once plated, the cells were centrifuged and resuspended in 2% paraformaldehyde (Sigma Aldrich). The plates were then incubated at 4 °C for 51 hours. Afterwards, samples were washed with saponin buffer [a mixture containing PBS, 1% fetal bovine serum, 0.1% saponin, and 0.1% sodium azide (Sigma)] and stained for either IFN-γ or TNF-α using a 1:100 dilution of IFN-γ FITC mouse anti-bovine antibody (AbD Serotec, Raleigh, NC) in saponin buffer or 1:10 dilution of TNF-α anti-equine monoclonal antibody (HL801), respectively (Adams et al. 2008). Next, stained cells were removed from the light and placed on ice for 30 minutes before washing with saponin buffer. Cells stained for TNF-α were then resuspended in a 1:1000 dilution of FITC-conjugated goat F(ab’)2 anti-mouse IgG (H + L) (Invitrogen; 2mg/mL) in saponin buffer and placed in the dark on ice for another 30 minutes. Meanwhile, the cells being stained for IFN-γ were resuspended in 1X PBS and the same was done for the TNF-α plate after being washed with saponin buffer. After
resuspending the cells, flow cytometric analysis was conducted using the Attune® Nxt™ Flow Cytometer and Attune® Nxt software version 2.5 (ThermoFisher Scientific).

Samples were then gated based on cell size and granularity to identify the percent of lymphocytes producing IFN-γ and TNF-α (Adams et al. 2008).

**RNA Isolation and Cytokine Gene Expression**

PBMCs aliquoted for RNA isolation were centrifuged and resuspended in Trizol (Ambion) and placed in a -80 °C freezer for later use. A modified RNA extraction method was used to isolate RNA from the PBMCs (Elzinga et al., 2017). After isolation, 0.5 μg of RNA from each sample was reverse transcribed into cDNA using a reverse transcription master mix (Promega, Madison, WI) by incubating the samples for 15 minutes at 42 °C, which was followed by 5 minutes at 95 °C. Afterwards, 4.5 μL of cDNA was mixed with 5 μL of Master Mix (SensiMix HI-ROX 2x, Bioline) for each sample. Next, a primer-probe (TaqMan, Equine-specific, intron spanning primers and probes; Applied Biosystems) was added to the cDNA and Master Mix combination. Samples were run against the genes: beta-glucuronidase (B-gus; Ec03470630_m1), interferon gamma (IFN-g; Ec03468606_m1), IL-6 (Ec03468678_m1), IL-1E (Ec04260298_s1), TNF-α (Ec03467871_m1), and IL-10 (Ec03468647_m1), using the Applied Biosystems Real-Time PCR (ABI Viia7) (Adams et al., 2015). Each sample was run in duplicate and all were incubated for 10 minutes at 95°C. This was followed by ten consecutive cycles of 15 seconds at 95°C and 60 seconds at 60°C (Elzinga et al., 2017). The generated data was analyzed using Applied Biosystems (ABI) system software and changes in gene expression were calculated using the equation $RQ = 2^{-\Delta\Delta CT}$ (Livak &
Schmittgen, 2001). The calibrator was calculated by averaging the values of all media samples from the beta-glucuronidase (β-gus) housekeeping gene at T0.

Statistical Analysis

The statistical analysis was performed using SigmaPlot version 13.0 (Systat Software, Inc., Richmond, CA). Data were log transformed if not normally distributed. Two-way repeated measures ANOVAs were used to analyze the data and depicted using line graphs (mean±SE). The data were considered to be statistically significant at a p-value less than 0.05, and trending at a p-value less than 0.10.

Results

Body Weight and Rectal Temperature

Previous research in cattle suggested that consumption of E+ tall fescue can cause a decrease in appetite resulting in a decreased rate of gain (Hoveland et al., 1983; Stuedemann & Hoveland, 1988), along with an increase in body temperature (Stuedemann and Hoveland, 1988). However, research in horses consuming E+ tall fescue revealed no changes in body temperature and weight (McDowell et al., 2013). Additionally, work by Adams et al. (2009) indicated that immune function can be affected by changes in body morphometrics. Therefore, it was important to investigate if the horses in this study had any changes in body morphometrics caused by the administration of bromocriptine, a semi-synthetic ergopeptide. As shown in Table 4.1, no significant changes in body weight or rectal temperature were found in this study.
Hormone Concentrations

Both horses and cattle consuming ergopeptines have been found to have decreased levels of circulating prolactin (Cross et al., 1995; Baldwin et al., 2016). Similarly, cattle and horses being administered bromocriptine have also exhibited decreased prolactin levels (Baldwin et al., 2016, Thomson et al., 1996, Thompson et al., 2017, Evans et al., 1999). It was necessary, then, to determine any changes in prolactin levels to ensure the effectiveness of the administered bromocriptine. As depicted in Figure 4.1, there were significant treatment versus control by time effects ($P<0.05$). The significant differences ($P<0.05$) between bromocriptine treatment and vehicle control group prolactin responses were measured at T12, T24, and T48 hours post-injection.

Intracellular Staining of Inflammatory Cytokines

Samples were assessed for the presence of intracellular inflammatory cytokines, TNF-α and IFN-γ. There were no significant differences ($P>0.05$) between treatment or control groups when evaluating the percent of lymphocytes staining positive for TNF-α (Figure 4.2) or IFN-γ (Figure 4.3). However, overall significant time effects were found for both TNF-α and IFN-γ. When assessing the percent of lymphocytes staining positive for TNF-α, both the bromocriptine treatment and the vehicle control groups had significant decreases from T0 to T48, and the bromocriptine treatment group had an additional time effect from T0 to T56. Additionally, the percent of lymphocytes staining positive for IFN-γ significantly decreased in both the bromocriptine treatment group and vehicle control group from T0 to T48, with the bromocriptine treatment group having an additional decrease from T0 to T12 and T24.
Cytokine Gene Expression

PBMCs were assessed for changes in the gene expression of TNF-α, IFN-γ, IL-1β, IL-6, and IL-10. The results from this study (Figure 4.4) closely mimicked the flow cytometry results with all cytokines exhibiting significant time effects. Additionally, there were no treatment by time effects, except for IL-1β. As seen in Figure 4C, there was a significant ($P<0.05$) difference between treatment and control groups at T12, an increase in IL-1β production by the bromocriptine treatment group and a decrease by the vehicle control group.

Discussion

Bromocriptine has been used to treat symptoms from a multitude of diseases in humans, including Parkinson’s disease, hyperprolactinemia, and prolactinomas. Primarily, bromocriptine has been used clinically to decrease levels of prolactin by acting on dopamine receptors found on lactotrophs in the pituitary gland (Freeman et al., 2000). Many in vitro studies have also demonstrated that dopamine receptors exist on immune cells and the binding of dopamine or dopamine agonists to those receptors can cause inflammatory or anti-inflammatory responses. Recently, bromocriptine has been used as a model for fescue toxicosis in cattle by simulating the effects of E+ fescue consumption (Baldwin et al., 2016). Additionally, research in cattle and horses have demonstrated that both bromocriptine and E+ fescue consumption leads to decreases in prolactin. However, prior to this study, no research had investigated the effects of bromocriptine on the immune system of the horse.
Symptoms of fescue toxicosis can include an increase in body temperature (Stuedemann & Hoveland, 1988) and suppression of appetite resulting in a decrease in animal performance (Hoveland et al., 1983; Stuedemann & Hoveland, 1988). Due to the significant chemical similarities between ergot alkaloids and bromocriptine, it was important to determine if an injection of bromocriptine caused any changes to body weight and rectal temperature in horses. However, in this study no significant changes to body weight or rectal temperature were identified.

As previously stated, bromocriptine acting on dopamine receptors has the ability to decrease circulating prolactin levels, and this has been documented in humans, swine, cattle, and horses (Serioukova and Poulos, 2012; Farmer et al., 1998; Baldwin et al., 2016; Thompson et al., 2017; Thomson et al., 1996). In fact, a recent study in the horse by Thompson et al. (2017) indicated that 12 hours after an intravenous injection of bromocriptine, prolactin levels maximally decreased and then continued to increase for 24 hours afterwards. Similar results were found in this study, as bromocriptine treatment induced a significant maximal response by decreasing prolactin at 12 hours post-injection. Additionally, prolactin levels remained significantly lower in the bromocriptine treatment group compared to the vehicle control group until 56 hours post-injection. Thus, it took approximately 56 hours after an injection of bromocriptine for prolactin levels to return to baseline.

Research at the cellular level in humans identified dopamine receptors on immune cells (Levite et al., 2001; McKenna et al., 2002; Besser et al., 2005; Watanabe et al., 2006; Cosentino et al., 2007; Nakano et al., 2008; Kustrimovic et al., 2014), and have also demonstrated that the concentration of dopamine, or agonist, and the specific
receptor (DAR1, DAR2, DAR3, DAR4, DAR5) being activated can result in inflammatory, anti-inflammatory, or toxic effects (Levite, 2016). Though the inflammatory effects of dopamine and dopamine agonists have been investigated in vitro, no research has investigated the in vivo effects of bromocriptine on inflammation in the horse. In this study, there were no significant treatment by time effects for the percent of cells staining positive for either TNF-α or IFN-γ.

Additionally, all cytokines analyzed for gene expression (TNF-α, IFN-γ, IL-1β, IL-6, and IL-10) exhibited overall significant time effects (P<0.05). However, unlike the decrease in percent of lymphocytes staining positive for TNF-α, the gene expression of TNF-α increased overall. This may indicate that treatment may take longer to have an impact on cytokine production, as cytokines are transcriptionally regulated. Also, a significant treatment by time interaction (P<0.05) was identified for the gene expression of IL-1β. Expression of IL-1β was significantly higher in the bromocriptine treatment group compared to the vehicle control group at T12 hours post-injection.

Overall, this study supported the results of previous studies in humans, cattle, and horses that demonstrated a decrease in prolactin levels following the administration of bromocriptine. Furthermore, this study supported the work by Thompson et al. (2017), which found prolactin to maximally decrease 12 hours after intravenous administration of bromocriptine. In addition to Thompson et al. (2017), this study further characterized the effects of intravenous administration of bromocriptine beyond 12 hours, by demonstrating that it takes approximately 56 hours for prolactin levels to return to baseline after the injection. Also, this was the first study to investigate the effects of bromocriptine on inflammatory responses in the horse. In this study significant changes
to inflammatory cytokine production caused by bromocriptine were not identified, except for IL-1β particularly at T12. This study supported previous research involving the decrease in prolactin caused by bromocriptine and suggested that a single 100 mg intravenous dose of bromocriptine was not high enough to cause many significant changes to inflammation in the horse. Further research should examine the effects of repeated bromocriptine injections on immune function. Moreover, it would be interesting to determine the effects of oral administration of bromocriptine. In summary, acute changes in prolactin levels induced by bromocriptine, a D2-like receptor agonist, did not have a significant impact on immune responses in horses.
Tables and Figures

Table 4.1. Body morphometrics for bromocriptine treated (n=9) and vehicle control (n=10) horses pre (T0) and post injection (T56).

<table>
<thead>
<tr>
<th>Group</th>
<th>T0 Weight (kg; mean±SD)</th>
<th>T56 Weight (kg; mean±SD)</th>
<th>T0 Rectal Temp (°C; mean±SD)</th>
<th>T56 Rectal Temp (°C; mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromocriptine</td>
<td>552.6±32.9</td>
<td>539.4±31.9</td>
<td>37.4±0.2</td>
<td>37.8±0.6</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle Control</td>
<td>541.9±78.6</td>
<td>534.2±79.7</td>
<td>37.5±0.2</td>
<td>37.7±0.2</td>
</tr>
</tbody>
</table>

Figure 4.1. Circulating prolactin concentrations (ng/mL) in control (n=10) and bromocriptine treated (n=9) horses pre (T0) and post (T12, T24, T48, and T56) injection. There were significant (P<0.05) treatment by time effects at T12, T24, and T48 as indicated by an asterisk (*).
Figure 4.2. Percent of positive TNF-α lymphocytes in control (n=10) and bromocriptine treated (n=9) horses pre (T0) and post (T12, T24, T48, and T56) injection. There was an overall time effect ($P<0.05$) for both groups observed. No significant treatment effects were identified.
Figure 4.3. Percent of positive IFN-γ lymphocytes in control (n=10) and bromocriptine treated (n=9) horses pre (T0) and post (T12, T24, T48, and T56) injection. There was an overall time effect ($P<0.05$) for both groups observed. No significant treatment effects were identified.
Figure 4.4. Cytokine gene expression (A) TNF-α, (B) IFN-γ, (C) IL-1β, (D) IL-6, and (E) IL-10 from stimulated PBMCs in control (n=10) and bromocriptine treated (n=9) horses pre (T0) and post (T12, T24, T48, and T56) injection. An overall significant treatment by time effect \( (P<0.05) \) was measured for IL-1β, with a significant treatment effect occurring at T12 as indicated by an asterisk (*).
CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

The relationship between the endophyte *Epichlöe coenophiala* and the tall fescue *Lolium arundinaceum* has been found to be mutualistic in nature, meaning that both organisms benefit from the other. The grass provides nutrients for the fungus to survive (Christensen and Voisey, 2009), while the endophyte produces ergot alkaloids that aid in the prevention of insect attack and disease (Latch, 1997). The ergot alkaloids produced by the endophyte have also been found to be beneficial for medicinal purposes. For example, beginning in the 1800s, physicians used ergot alkaloids to help treat medical conditions, like postpartum hemorrhaging (Hofmann, 1978); and in recent years, clinicians have used ergot alkaloid derivatives, like bromocriptine, to treat hyperprolactinemia in women and Parkinson’s disease (Serioukova and Poulos, 2012). However, within the past seventy-five years, scientists found that consumption of ergot alkaloids produced by E+ tall fescue could cause fescue toxicosis in cattle and horses, which results in decreased levels of prolactin, decreased rate of gain (Stuedemann and Hoveland, 1988), lack of hair loss, increased respiration rate, gangrene (Hoveland, 2009), and vasoconstriction in cattle (Oliver et al., 1998; Egert et al., 2014, Klotz et al., 2007) along with reproductive difficulties, agalactia (Cross et al. 1995), and vasoconstriction in horses (McDowell et al., 2013).

Even though many of the symptoms of fescue toxicosis in cattle and horses suggest there could be inflammatory effects, very little research has investigated this and the few studies that have been conducted have yielded conflicting results (McDowell et
al., 2013; Filipov et al., 2000). Therefore, the primary objective of this body of research was to investigate the effects of dopamine agonists, ergot alkaloids and bromocriptine, on immune function in the horse. This study hypothesized that exposure to ergot alkaloids or bromocriptine would elicit an inflammatory response and a decrease in prolactin levels. Additionally, this study was interested in determining the effect of E+ tall fescue on horses with pituitary pars intermedia dysfunction (PPID), a condition in which horses lack dopamine. Due to the dopaminergic activity of ergot alkaloids, it was proposed that consumption of those molecules could have the potential to benefit PPID horses by lowering their levels of ACTH. Also, this study intended to use bromocriptine as a model for E+ fescue consumption both in vitro and in vivo.

Overall, the results from the first study involving the consumption of ground E+ tall fescue seed did not support the hypothesis as significant treatment effects were not identified for the immune or hormone responses. Even though this study did not identify any significant treatment effects on hormone responses, significant time effects caused by seasonal changes were identified. It is known that with changes in season from fall to winter there is a decrease in prolactin (Johnson, 1986). Therefore, season could have confounded any treatment effects that may have been measured in this study. However, it is also possible that if a higher dose of E+ tall fescue had been consumed, then the seasonal effects could have been overcome. Similar to the response in prolactin concentrations, the same seasonal effects were exhibited for the baseline adrenocorticotropic hormone (ACTH) levels (Diez de Castro et al., 2014), which likely mitigated any potential effects the E+ tall fescue might have exerted on the production of hormones. And a higher dose of E+ tall fescue seed could have overcome those seasonal
effects. Hence, the effects of E+ tall fescue consumption on ACTH levels of PPID horses remains to be determined.

In regards to the *in vitro* study, the results did not support the hypothesis as decreases in the expression of inflammatory cytokines, TNF-α and IL-1β, were found for peripheral blood mononuclear cells (PBMCs) exposed to bromocriptine and no change in inflammatory cytokine production was measured for PBMCs exposed to dopamine. These results contradicted previous studies using human lymphocytes, which reported increases in TNF-α after exposure to dopamine (Levite 2016).

However, in the *in vivo* bromocriptine study, prolactin levels significantly decreased after administering bromocriptine intravenously to nine horses. The maximal decrease in prolactin occurred around twelve hours post-injection, and returned to baseline levels around 56 hours post-injection, which supported the research by Thompson et al. (2017), Thomson et al. (1996), Baldwin et al. (2016), and many others in equine and bovine fields. Additionally, this study supported the inflammatory response aspect of the hypothesis by demonstrating that an intravenous injection of bromocriptine resulted in an increase in the gene expression of IL-1β, a pro-inflammatory cytokine. Even though some inflammation was exhibited, the results remained contradictory to previous research in humans that suggested bromocriptine, a dopamine agonist, should have cause an increase in TNF-α production.

Therefore, the results from this body of research demonstrated that equine lymphocytes may respond differently to dopamine agonists than human immune cells. Additionally, it is possible that systemic exposure to a dopamine agonist might result in
differing effects from an *in vitro* experiment due to the contributing effects of hormones and other physiological processes on the immune system.

In furthering this research, it would be important to investigate the effects of E+ fescue consumption on immune function in either the summer or winter months as these are the seasons in which prolactin has been found to be at its highest and lowest, respectively, in the horse. By evaluating the effects on prolactin during a season in which the concentrations remain more constant, it would allow for a more accurate determination of ergot alkaloid consumption on inflammatory responses in the horse. Additionally, pelleting the E+ tall fescue seed could potentially improve the consumption of the seed as the volume and amount of dust from grinding the seeds caused a lack of palatability and did not allow for the possibility of feeding a higher quantity of ergot alkaloids to the animals. By pelleting the seed, a higher dose of ergot alkaloids could be more easily consumed by the horse. Additionally, it would be interesting to perform a study involving the oral administration of bromocriptine. This route of administering the bromocriptine would better mimic the consumption of E+ tall fescue compared to intravenous, intramuscular, or subcutaneous injection routes. It would also allow for potential analysis of the metabolism of bromocriptine by cytochrome P4503A (CYP3A), an enzyme in the intestine of the horse (Tydén et al, 2004) that has been found to metabolize bromocriptine in humans to its metabolite bromo-lysergic acid (Serioukova and Poulos, 2012). Consequently, this could help clarify which molecules are actually found in the blood stream of animals consuming E+ tall fescue, ergovaline or the precursor molecule lysergic acid, as both ergovaline and bromocriptine have lysergic acid as a common metabolite.
Overall, this body of research demonstrated that bromocriptine, a semi-synthetic ergopeptine, has an effect on both the immune and hormone response of old horses. This research supported previous research regarding the effects of dopamine agonists on prolactin in the horse, and contradicted research with human immune cells in response to dopamine agonists. The data collected identified that E+ tall fescue consumption was not found to have an effect on immune function, prolactin, vasoconstriction, body temperature, or body weight, indicating that older non-pregnant horses can tolerate E+ tall fescue. Bromocriptine data revealed conflicting results when comparing *in vitro* to *in vivo*. Additional research investigating the use of different forms of E+ tall fescue seed, such as pellets formulated with a higher concentration of ergot alkaloids, as well as an oral bromocriptine challenge, would provide further understanding of how dopamine agonists affect immune responses in horses.
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