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ASSESSMENT OF BOVINE VASCULAR SEROTONIN RECEPTOR POPULATIONS AND TRANSPORT OF ERGOT ALKALOIDS IN THE SMALL INTESTINE

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ASSESSMENT OF BOVINE VASCULAR SEROTONIN RECEPTOR POPULATIONS
AND TRANSPORT OF ERGOT ALKALOIDS IN THE SMALL INTESTINE

THESIS

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

By

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Lexington, Kentucky

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2017

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ABSTRACT OF THESIS

ASSESSMENT OF BOVINE VASCULAR SEROTONIN RECEPTOR POPULATIONS AND TRANSPORT OF ERGOT ALKALOIDS IN THE SMALL INTESTINE

Prior work using a contractility bioassay determined that the serotonin (5-HT) receptor subtype 5-HT_{2A} is present in bovine lateral saphenous veins and plays a role in ergot alkaloid-induced vascular contraction in steers grazing endophyte-infected (*Epichloë coenophiala*) tall fescue (*Lolium arundinaceum*). A study was conducted to determine what 5-HT receptors are involved in vasoconstriction of bovine gut vasculature. The findings of this study indicate that 5-HT_{2A} is present and may play a role in ergot alkaloid induced vasoconstriction. A second study was conducted to determine if ergot alkaloids were transported in the small intestine. The active transporter, peptide transporter 1 (PepT1), was evaluated for its role in the transport of various concentrations of ergot alkaloids across Caco-2 cell monolayers. Results indicate that CEPH, ERT, EXT, and LSA do move across Caco-2 cell monolayers, but appear to utilize PepT1 at larger concentrations. Overall, the demonstrated presence of 5-HT_{2A} receptors in the bovine gut vasculature established a potential for vascular interference by ergot alkaloids entering the bloodstream through transepithelial absorption.

KEYWORDS: fescue toxicosis, serotonin receptors, mesenteric and ruminal vasculature, Caco-2 cells, ergot alkaloid transport, peptide transporter 1

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December 5th, 2017
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FREQUENTLY USED ABBREVIATIONS

5-HT- Serotonin

AP+ - Apical chamber with inhibitor

AP- - Apical chamber without inhibitor

BA+ - Basolateral chamber with inhibitor

BA- - Basolateral chamber without inhibitor

CEPH – Cephalexin

CNS – Central nervous system

CV - Cardiovascular

d – Day

E+ - Endophyte infected

E- - Endophyte free

EA – Ergot alkaloid

ERT – Ergotamine

EP - Epinephrine

EXT - Ergovaline seed extract

FBS – Fetal bovine serum

GI – Gastrointestinal

h – Hour

HBSS – Hank's balanced salt solution

KCl – Potassium chloride

LSA – Lysergic acid

MA – Mesenteric artery

MV – Mesenteric vein

min – Minute

MYS – Methysergide

NATE - Nateglinide

NCC – No cell control

NE – Norepinephrine

PBS – Phosphate buffered saline

PepT1 – Peptide transporter 1

RA – Ruminal artery

RV – Ruminal vein

SI – Small intestine

CHAPTER ONE: INTRODUCTION

Grasses that are important to animal agriculture can become infected with endophytic fungi some of which produce ergot alkaloids in the tissues of their plant hosts. Ergot alkaloids are secondary metabolites and mycotoxins that are produced by numerous species of fungi belonging to the genera *Claviceps* and *Epichloë* (Strickland et al., 2011). One host grass that is beneficial as a pasture-based feed source is tall fescue (*Lolium arundinaceum*) (Strickland et al., 2011). Tall fescue has numerous desirable traits as a forage for cattle and other domestic herbivores, but the ergot alkaloids that are produced lead to the livestock disorder called fescue toxicosis. Some symptoms include rough hair coat, excessive salivation, elevated body temperature, and dry gangrene in the extremities (Strickland et al., 2011). Such symptoms are vague and could also point to other maladies.

At present, the site(s) and mechanism(s) of ergot alkaloid absorption have not been fully described. Some studies have indicated that some absorption and metabolism of alkaloids occurs before reaching the duodenum (Westendorf et al., 1993; Schumann et al., 2009) and before excretion of feces (Schumann et al., 2009). While there are studies linking the absorption of ergot alkaloids across ruminant gastric tissues (Hill et al., 2001; De Lorme et al., 2007) there is not much information available concerning the absorption and transport of ergot alkaloids across the small intestine.

CHAPTER TWO: LITERATURE REVIEW

Introduction

Tall fescue (*Lolium arundinaceum*) is a perennial grass and is one of the most abundant forage crops in the United States (Jackson et al., 1984; Buckner et al., 1979). It is the one of the most widely used cool-season grasses in the southeastern region and predominates in the transition zone between the northern and southern regions of the eastern United States (Williams et al., 1984; Paterson et al., 1995), also referred to as the fescue suitability zone (Hannaway et al., 2009). It was discovered on a Kentucky farm in the 1930s and marketed as 'Kentucky 31' in 1942 (Fergus & Buckner, 1972; Bacon, 1995). Tall fescue became prevalent in an area that needed a quality forage as it exhibited numerous desirable traits such as increased hardiness, resistance to pests, high adaptability, and extended grazing season (Stuedemann & Hoveland, 1988; Bacon, 1995). Despite all of the positive traits tall fescue exhibited, it became evident that livestock were not thriving when grazing tall fescue, developing unthrifty appearances and general reduced performance (Stuedemann & Hoveland, 1988). These symptoms were termed fescue toxicosis and have been associated with the endophytic fungus *Neotyphodium coenophialum* (Bacon et al., 1977) later reclassified as *Epichloë coenophiala* (Leuchtman et al., 2014).

Symbiosis with the Endophyte

The distribution of tall fescue has been attributed to its symbiotic relationship with the endophytic fungus *E. coenophiala* (Glenn et al., 1996; Strickland et al., 2011). *E. coenophiala* is located throughout the plant but is of highest concentration around the crown meristem during vegetative periods (Scharidl & Phillips, 1997) and in the side

branches of flower heads during the reproductive stage (Bacon & Siegel, 1988). Bacon et al. (1977) first reported the presence of the endophyte from tall fescue pastures in Georgia. Cattle grazing on E+ (endophyte positive) pastures displayed fescue toxicosis symptoms while cattle that were housed and fed E+ showed no symptoms (Bacon et al., 1977). This phenomenon was also observed in a study conducted by Schmidt et al. (1982). Cattle that were fed E- (endophyte negative) hay and seed showed no symptoms of fescue toxicosis while cattle fed E+ hay and seed exhibited symptoms (Schmidt et al., 1982). Both studies imply that there is a correlation between fescue toxicosis and endophyte presence.

The endophyte cannot survive without a host and contributes toxic secondary metabolites to its host that allows protection against ingestion by herbivores and other invertebrate agricultural pests (Scharl & Phillips, 1997; Rowan et al., 1986) amongst other benefits. The host plant benefits by gaining increased drought tolerance, root growth, nematode tolerance, and improved germination (Arachavaleta et al., 1992; Norman, 2008). Arachavaleta et al. (1989) conducted a study in which 75% of E- tall fescue plants survived during extreme drought conditions, but 100% of E+ tall fescue plants survived and displayed greater regrowth once harvested. This implies that the endophyte does indeed improve plant hardiness.

Tall Fescue Toxicosis

Signs and Symptoms

Once considered a superior forage, tall fescue developed a reputation as an inferior forage based on production issues and poor animal performance (Stuedemann & Hoveland, 1988). Animals grazing on tall fescue may exhibit three conditions: fat

necrosis, fescue foot, and tall fescue toxicosis (Paterson et al., 1995). Fescue foot is recognized by symptoms such as swelling around the fetlock and hoof, dry gangrene at the tips of the tail and ears, and lameness (Cunningham, 1948). In advanced cases, animals that are affected by fescue foot may lose their hooves to sloughing which is an effect of ergot alkaloids on blood vessels (Strickland et al., 2009). Ergot alkaloids damage the cells that line blood vessels, enhance blood clotting, and lead to vasoconstriction of the lumens of blood vessels (Tor-Agbidye et al., 2001). Fat necrosis is not as obvious as fescue foot besides poor performance and an unthrifty appearance. This is due to necrotic fat constricting internal organs (Strickland et al., 2009).

Fat necrosis and fescue foot are acute issues of concern to producers but fescue toxicosis is a more common and chronic problem that results in higher economic losses (Schmidt & Osborn, 1993). Tall fescue toxicosis is also known as fescue toxicosis, summer slump, or summer syndrome due to the unthrifty appearance and poor performance of the animal during the summer months (Schmidt & Osborn, 1993). Fescue toxicosis is problematic within the fescue suitability zone (Hannaway et al., 2009) and is related to high temperatures rather than an increase of toxic compounds during warmer months (Hemken et al., 1984). Signs and symptoms are usually not overly severe and are typically not recognized by producers until damage is already done. Issues from grazing on tall fescue pastures, such as reduced conception rates and decreased calf weight gains, have resulted in economic losses estimated around \$609 million annually (Paterson et al., 1995; Hoveland, 1993). When taking into account the effects on the small ruminant and equine industries, combined losses attributed to fescue toxicosis exceed \$1 billion (Strickland et al., 2011).

Causative Agent: Secondary Metabolites

The symbiotic relationship between tall fescue and endophyte bestows a competitive edge to the plant by fungal production of biologically active secondary metabolites. Numerous alkaloids were isolated from tall fescue and some proved to be biologically active (Bacon, 1995). Alkaloids in E+ tall fescue include the pyrrolopyrazine, peramine, the pyrrolizidines (lolines), and the ergot alkaloids (ergoline derivatives such as clavines, lysergic acid amides, and peptide alkaloids) (Figure 2.1).

Pyrrolopyrazines

Perennial ryegrass (*Lolium perenne*) that is infected with the endophytic fungus *Epichloë festucae* var. *lolii* is resistant to a diverse number of insect species, including the Argentine stem weevil (*Listronotus bonariensis*), due to the pyrrolopyrazine peramine (Rowan et al., 1986). Biosynthetically, it appears that peramine is derived from the cyclization of the amino acids arginine and proline (Rowan et al., 1986). Rowan et al. (1986) isolated peramine through ethanol extraction, solvent partitioning, repeated chromatography, and analysis by high resolution mass spectroscopy. It appears that peramine is produced continuously by the endophyte, but does not accumulate in older plant tissues (Koulman et al., 2006). Using a linear ion trap mass spectrometer, Koulman et al. (2006) developed a highly sensitive method to analyze peramine and located peramine in the cut leaf fluid of all grass-endophyte associations analyzed, including tall fescue. However, peramine was not found in the guttation fluid of all grass-endophyte associations. Lolines and ergot alkaloids were found in the guttation fluid in some grass-endophyte associations. This indicates that peramine and other fungal alkaloids were mobilized into plant fluids by the host plant. Siegel et al. (1990) showed that peramine

and the ergopeptine, ergovaline, were detected in 66% and 60% of endophyte-infected grasses. While peramine and ergovaline are produced in similar quantities, they are produced independently of one another meaning that breeding tall fescue with reduced ergovaline and toxicity to livestock does not affect insect resistance from peramine (Roynance et al., 1994).

Pyrrolizidines (Lolines)

Pyrrolizidines, or lolines (saturated 1-aminopyrrolizidines with an oxygen bridge), are the most abundant alkaloids present in tall fescue and act as insecticides when produced in symbioses with *Epichloë* species and grasses, particularly *Lolium* and *Festuca* (Blankenship et al., 2001). Prior to 2001, it was unknown if lolines were of plant origin or of fungal origin. Porter (1994) reported that lolines were not observed in fungal cultures. Using GC-MS and ¹³C NMR spectroscopic analyses, Blankenship et al. (2001) demonstrated that three of the loline alkaloids in plant tissues (loline, *N*-acetylnorloline, and *N*-formylloline were produced by the fungal endophyte *N. uncinatum* under certain minimal medium conditions. The structure of lolines consists of a saturated necine ring with –NRR' substituent at C1 and an oxygen bridge between carbon 2 and carbon 7 (Blankenship et al., 1991; Yates et al., 1990; Powell & Petroski, 1992). Bush et al. (1993) suggested that lolines are biosynthetically derived from spermine or spermidine. Loline alkaloids are distributed throughout the plant and offer protection to the plant in the form of broad-spectrum insecticides (Dahlman et al., 1997). However, lolines do not appear to be as toxic to mammalian species although activity has been noted in mammalian tissues and small mammals (Bush et al., 1993). Immunosuppressive effects have been observed in mouse feeding trials (Dew et al., 1990). It had been suggested that lolines may be

involved in vasoconstriction and may utilize α_2 -adrenergic, serotonin, or D₂ dopamine receptors (Larson et al., 1995; Strickland et al., 1996). However, Klotz et al. (2008) demonstrated that bovine lateral saphenous veins did not show a contractile response when exposed to *N*-acetyl loline.

Ergot Alkaloids

Although concentrations of ergot alkaloids are lower than that of lolines, ergot alkaloids appear to impact mammals much more significantly. Ergot alkaloids are compounds that contain an ergoline ring (Bush et al., 1997) that is a component of lysergic acid, an intermediate compound produced when ergot alkaloids are degraded (Merill et al., 2007, De Lorme et al, 2007; Norman, 2008). The major classes of ergot alkaloids are the clavines, lysergic acid amides, and ergopeptines (Porter, 1995; Norman, 2008). The most prevalent member of the ergopeptine class is ergovaline (Yates et al., 1985; Yates & Powell, 1988) and is suspected to be the causative agent behind fescue toxicosis. Low concentrations of ergovaline are enough to cause fescue toxicosis in heat stressed animals (Cornell, 1990). However, a single alkaloid or group of alkaloids has not been definitively identified as the primary cause of fescue toxicosis.

Endocrine Changes

Hormones that are associated with metabolic rate regulation include triiodothyronine (T₃), cortisol, and thyroxine (T₄). Studies have shown that E+ does not alter hormone profiles (Fiorite et al., 1991; Hurley et al., 1980; Aldrich et al., 1993). One consistent indicator of fescue toxicosis is decreased serum prolactin (Paterson et al., 1995; Elsasser and Bolt, 1987; Schillo et al., 1988). Ergot alkaloids have been identified as potential agents in alteration of the production of numerous pituitary hormones (Porter

et al., 1990; Browning et al., 1997) and are influential in prolactin secretion. Prolactin is a protein hormone that is secreted by the adenohypophysis (anterior pituitary gland) with secretion and inhibition being mediated by dopamine. Ergot alkaloids may be responsible for decreased levels of serum prolactin by binding to D₂ dopamine receptors and producing a second messenger response that depresses levels of prolactin (Larson et al., 1995; Hurley et al., 1980; Elsasser and Bolt, 1987).

It has been found that phenothiazine may enhance prolactin secretion as it acts as a dopamine antagonist (Boling et al., 1989). Boling et al. (1989) conducted two experiments to determine the effects of phenothiazine and temperature on prolactin concentrations and growth of calves that consumed high endophyte or low endophyte tall fescue. It was found that while phenothiazine may reduce decreases in prolactin from a high endophyte tall fescue diet, reductions might also depend on ambient temperatures (Boling et al., 1989). Other dopamine antagonists such as haloperidol and domperidone (Paterson et al., 1995; Bolt et al., 1983; Boling et al., 1989; Cross et al., 1995) have been tested as possible pharmaceuticals to treat fescue toxicosis, but the actual mechanism behind fescue toxicity has not been identified conclusively.

Alkaloid Mode of Action

Catecholamines

Ergot alkaloids found in E+ act similarly to dopamine and other catecholamines such as serotonin (5-HT), epinephrine (EP), and norepinephrine (NE) and can cause vasoconstriction by binding biogenic amine receptors (Figure 2.2). Catecholamines have major roles in metabolic pathways such as lipolysis, glycogenolysis, and contraction-relaxation of smooth muscles (Norman, 2008). Specifically, catecholamines interact with

receptors such as α -2 adrenergic receptor, serotonergic-2 receptor, and dopaminergic-2 receptors (Norman, 2008). These receptors exist throughout the central nervous system, blood vessels, and in the gastrointestinal tract (Oliver, 1997). Sibley and Creese (1983) investigated the interactions of ergot alkaloids with bovine anterior pituitary D-2 dopamine receptors utilizing radioligand binding techniques and computer modeling technologies. Ergolines interacted with receptors in an agonist-specific way and exhibited competition curves which were divided into high and low affinity components (Sibley & Creese, 1983). Only the low-affinity compounds were observed when in the presence of guanine nucleotides, suggesting that guanine nucleotides convert high affinity compounds to low affinity compounds as guanine nucleotides regulate binding at specific dopamine receptors (Creese et al., 1979). In contrast, ergot alkaloids that contained a cyclic peptide side chain (ergopeptines) exhibited competition curves that were monophasic and unaffected by guanine nucleotides (Sibley & Creese, 1983). This implies that ergopeptines have similar binding properties of antagonist ligands. Ergot alkaloid action at the site of these receptors can cause metabolic changes by alteration of homeostatic mechanisms (Strickland et al., 1993).

Blood Flow and Ergot Alkaloid Vasoactivity

Epinephrine and NE are involved in the control of blood flow in the body and play a role in the regulation of vasoconstriction. Changes in peripheral blood flow are related to the ability to dissipate body heat. Plasma EP and NE were not altered after E+ was consumed (Henson et al., 1987; Elsasser & Bolt, 1987). Henson et al. (1987) showed that hypothalamic and pituitary levels of EP, NE, and dopamine levels were unchanged. This indicates that an alternate mechanism is involved at the receptor level.

Body heat regulation can occur through skin vaporization and increased respiration rates. The inability to dissipate body heat may be a result of decreased blood flow. Rhodes et al. (1991) showed that cattle that consumed an E+ diet displayed a 50% reduction in blood flow to the skin which is characteristic of heat stress symptoms that accompany fescue toxicosis. Aiken et al. (2009) also observed vasoconstriction as luminal areas of caudal arteries were restricted 27 and 51 hours after heifers consumed a diet of 0.39 μg ergovaline/kg body weight or 0.79 μg ergovaline/kg body weight.

Using radiolabeled microspheres, Rhodes et al. (1991) showed that cattle on an E+ diet at high ambient temperatures showed reduced blood flow to the gastrointestinal tract. However, Harmon et al. (1991) conducted a study in which the results contradicted that of Rhodes et al. (1991). Harmon et al. (1991) found that there was no effect of an E+ diet on the flow of blood through the portal vein. Discrepancies could be explained by amounts of E+ fescue fed to animals, by differences in ambient temperatures, species differences, and diet differences.

Egert et al. (2014) conducted an experiment in which branches of mesenteric artery (MA) and mesenteric vein (MV) were collected from steers dosed with either E+ or E- fescue seed in order to determine if prior exposure to E+ tall fescue seed affected vasoactivity. Tissues were exposed to ergocryptine, ergotamine, ergocristine, ergocornine, ergonovine, lysergic acid, ergovaline-containing tall fescue seed extract (EXT), and 5-hydroxytryptamine (5-HT). Steers that were previously exposed to E+ seed had a smaller MA contractile response than E- steers to ergocryptine, ergotamine, ergocristine, ergocornine, ergonovine, EXT, and 5-HT. Steers with prior exposure to E+ seed had a smaller MV contractile response than E- steers to ergocryptine, ergotamine, ergocristine,

ergonovine, EXT, and 5-HT. There was no contractile response to lysergic acid in MV or MA. The EXT produced the most potent response in both MV and MA. This indicates that ergot alkaloids were vasoactive in the midgut and that steers previously exposed to E+ had diminished contractility. This suggests that prior dietary exposure to ergot alkaloids may alter nutrient absorption by decreasing blood flow.

Footo et al. (2013) investigated the effects of ergot alkaloids on reticuloruminal epithelial blood flow and volatile fatty acid (VFA) absorption by washing the reticulorumen of steers on an E+ diet or E- diet in the presence of accumulating levels of ergot alkaloids in steers housed in thermoneutral and heat stressed conditions. Steers were exposed to a VFA-containing buffer incubated in the sequence: control, 1xEXT, and 3xEXT. There were two 30 min sampling periods: a 30 min incubation of a treatment buffer with no sampling and a 30 min incubation of the same treatment buffer with the addition of chromium-EDTA and deuterium oxide. Steers on the E+ diet exposed to heat stressed conditions decreased their feed intake. At heat stressed conditions, serum prolactin decreased in both groups with no difference in 1xEXT and 3xEXT treatment groups. An interaction between the seed treatment and buffer treatment was present, indicating that the E+ seed treatment decreased reticuloruminal epithelial blood flow at thermoneutral conditions during the control incubation. Presence of EXT in the buffer decreased epithelial blood flow by at least 50% although there was no difference between steers exposed to 1xEXT and 3xEXT. At heat stressed conditions, there was a seed and buffer treatment interaction, indicating that reduced blood flow induced by incubating the EXT was larger for steers on the E- treatment. Time either increased or did not affect blood flow or VFA flux when an additional experiment was conducted to determine the

time effect on blood flow and VFA flux. This indicated that differences were due to ergot alkaloid presence in the rumen.

Footo et al. (2014) conducted a study using Holstein steers to determine the effects of EXT on total, passive, and facilitated acetate and butyrate flux across ruminal tissue and barrier function. Isolated tissues were mounted on Ussing chambers and exposed to a vehicle control, low EXT, and high EXT. Results indicated that there was no effect of acute exposure on total, passive, and facilitated flux of acetate and butyrate across rumen epithelium. Barrier function was measured by inulin flux, which was not affected by EXT treatment, indicating no change in barrier function due to ergot alkaloid exposure. Ergovaline was found in serosal buffer of the high EXT treatment indicating that ergovaline has the potential to be absorbed via the rumen, contributing to reduced blood flow. It is possible that ergot alkaloids have vasoconstrictive potential over vasculature in the gastrointestinal tract. As the barrier function of the gastrointestinal tract may become compromised, absorption of nutrients may become altered. As well as nutrient absorption becoming altered, the protective barrier the gastrointestinal tract provides may begin to break down, which could have a profound effect on the health of livestock.

Gastrointestinal Tract Anatomy, Histology, and Physiology

The gastrointestinal (GI) tract is a multilayered barrier that plays roles in absorption, digestion, and protection. It is responsible for the absorption of fluids and nutrients, secretion of drugs and drug metabolites, and acts as a barrier against harmful bacteria and toxins from the intestinal lumen (Nunes et al., 2015; Barreau & Hugot, 2014; Lennernas, 2013). These processes occur due to the infolding structure of the GI tract.

Around 90% of all absorption in the GI tract occurs in the small intestine region (Nunes et al., 2015). In ruminants, the small intestine extends from the abomasum to the large intestine and is broken up into three regions: the duodenum, jejunum, and the ileum.

The small intestine is composed of four connected layers: the mucosa, submucosa, muscularis propria, and the serosa (Pereira et al., 2015; Rao & Wang, 2010) (Figure 2.3). The mucosa is the innermost layer and also the most complex as it is composed of three layers. The epithelial monolayer is the first layer and is in contact with the intestinal lumen (Pereira et al., 2015). This monolayer separates the intestinal lumen from the lamina propria. The monolayer of cells is in contact with immunological agents, commensal bacteria, mucus, and nutrients (Pereira et al., 2015). The second layer is the lamina propria and it is composed of connective tissue, capillaries, and lymph nodes. It absorbs digestive products. The third and final layer of the mucosa is the muscularis mucosae which is a thin layer of smooth muscle (Pereira et al., 2015; Abreu, 2012; Rao & Wang, 2010). It is at the base of crypts and contains vascular and lymphatic capillaries that also aid in the digestive process.

Enterocytes and Secretory Cells

According to Crosnier et al. (2006), unique mechanisms in the intestinal epithelium, such as cell proliferation, differentiation, and apoptosis, occurs in that particular order along the crypt-villus axis. Proliferative crypts are invaginations that contain intestinal epithelial stem cells and transit-amplifying cells (Pereira et al., 2015). Transit-amplifying cells are partially differentiated cells that divide four to five times before terminal differentiation (Pereira et al., 2015). Two types of cells are generated from this process: absorptive enterocytes and secretory cells. Enterocytes make up more

than 80% of all intestinal cells. The monolayer of enterocytes is highly absorptive and has a large absorptive area due to being composed of a large number of villi (Lennernas, 2013). Enterocytes are columnar, highly polarized and have distinct apical and basolateral membranes (Nunes et al., 2015; DeSesso & Jacobson, 2001; Reitsma et al., 2014). Secretory cells are further subdivided into goblet cells, enteroendocrine cells, and Paneth cells (Pereira et al., 2015). Secretory cells have roles in innate immunity through protein secretion, hormone secretion, and mucin secretion while enterocytes act as a barrier and absorb nutrients.

Intestinal Absorption and Transport

Membrane transporters act as gatekeepers for cells and control influx and efflux of sugars, nucleotides, amino acids, organic ions, and drugs (Estudante et al., 2012; Hediger et al., 2004). The two major sites that affect the amount of a drug that passes into the systemic circulatory system after an oral dose are the liver and the intestines. There has been extensive research focused on the role of drug transporters in the liver (Shitara et al., 2006; DeGorter et al., 2009; Li et al., 2009; Faber et al., 2003) and kidneys (Shitara et al., 2006; van Montfoort et al., 2003; Kikuchi et al., 2010) but not as much on the role of influx and efflux transporters in the small and large intestines (Oswald et al., 2007).

For orally administered drugs and nutrients to reach capillaries that lead to the portal vein, they must first pass through the gut wall mucosa. The closely-linked polarized enterocytes (Estudante et al., 2012) that make up the mucosa are sealed by tight junctions. Intestinal drug transporters are of increasing interest as several drug transporters have been identified in enterocytes that make up the gut wall mucosa.

Molecules may pass through gut membranes by passive diffusion or active mechanisms (Estudante et al., 2012).

Factors Governing Permeability Across Intestinal Epithelium

The extent and rate to which a drug or nutrient is absorbed across the intestinal wall depends on numerous factors. They are physiochemical properties, physiological properties, anatomical properties, biochemical factors, and factors related to dosage form (Nunes et al., 2015; Agoram et al., 2001; Ungell et al., 1998). According to a study by Dahan et al. (2009) the fundamental events that control absorption are the permeability of the drug or nutrient through the GI membrane and the solubility of a drug or nutrient in the GI environment. Nutrients also face physical barriers, such as luminal barriers and gut wall barriers, which control the amount that is absorbed across the intestine.

The intestinal epithelium is covered with a mixture of water, mucus, and glycocalyx (Pereira et al., 2015). This mixture acts as a protective barrier but it is also permeable to nutrients and small molecules. The mucus component is a hydrogel-like structure that is responsible for particle clearance due to its rapid turnover time (Atuma et al., 2001). The structure of intestinal mucus can also increase transcellular transport of a compound (Ensign et al., 2012).

Not only does the mixture of water, mucus, and glycocalyx influence the absorption of nutrients but the epithelium itself does as well. The epithelium is reinforced by tight junctions and efflux transporters. Tight junctions keep bacteria and molecules larger than 200 Da out via the paracellular transport pathway (Chen et al., 2011). Tight junctions also work together with efflux transporters, particularly P-glycoprotein 1 (P-gp;

MDR1; ABCB1) and breast cancer resistant protein (BCRP; ABCG2). Efflux transporters push unwanted substances back into the intestinal lumen.

Transport Pathways Across Intestinal Epithelium

Transport across the intestinal membrane is a complex process and involves several pathways in parallel and along the small intestine (Nunes et al., 2015; Balimane et al., 2000; Lennernäs, 2014). Transport of drugs varies from one drug to the next depending on the properties of that particular drug. There are two general types of transport: passive transport and active transport. Passive transport and active transport can be further subdivided based on properties and functions (Figure 2.4).

Passive Transport

Passive transport involves the movement of molecules across a concentration gradient without the use of energy. It is not saturable and shows a low specificity for structure of the molecule (Shargel & Yu, 1999). The only form of passive transport that requires a carrier protein or ionophore and that is a saturable process is facilitated diffusion. It does not need energy as it is dependent on the concentration gradient (Dobson & Kell, 2008). Passage of molecules from the intestinal lumen through the mucosa occurs mainly by two passive mechanisms: transcellular diffusion (through cells) and paracellular diffusion (between adjacent cells) (Nunes et al., 2015; Antunes et al., 2013).

Transcellular Diffusion

Passive transcellular transport allows compounds to cross from one side of the cellular membrane to the other through the lipid bilayer. Large particles and macromolecules typically utilize the transcellular route (Barreau & Hugot, 2014) as this

type of transport is not saturable and therefore not subject to inhibition. This form of transport is also much less sensitive to the structure of drugs (Nunes et al., 2015; Sugano et al., 2010). Rapidly and completely absorbed drugs are typically lipophilic and will distribute readily into cell membranes of the intestinal epithelium (Artursson et al., 2001). As the lipid bilayer is highly hydrophobic, diffusion of compounds across the cell membranes is limited to uncharged and desolvated compounds and depends on molecular size and affinity for the lipid bilayer (Nunes et al., 2015). It can be assumed that drugs that meet this criterion are transported almost exclusively by passive transcellular diffusion (Artursson et al., 2001).

Paracellular Diffusion

Drugs that are slowly or incompletely passively absorbed distribute poorly into cell membranes and it is assumed that these drugs are transported paracellularly (Artursson et al., 2001). Drugs that typically follow the paracellular transport pathway are peptides and hydrophilic drugs, but it is not known if these drugs use paracellular routes exclusively (Artursson et al., 2001; Nellans, 1991). Paracellular transport allows water, solutes, and ions to pass through and is restricted by tight junction proteins (Antunes et al., 2013; Nunes et al., 2015). The surface area of tight junction proteins is 0.1% of the total surface area and tight junctions restrict the paracellular transport of drugs (Artursson et al., 2001; Furuse et al., 1993). Drug transport across the intestinal epithelium by paracellular transport is thought to be minimal and dependent on the size of the molecule attempting to utilize this pathway. Tight junction permeability is further restricted due to the fact that tight junctions are charge selective (Nunes et al., 2015). Compounds and

molecules transported by the paracellular mechanism are not exposed to lysosomes within the enterocyte and are not broken down (Nunes et al., 2015; Reitsma et al., 2014).

Active Transport

Active transport is a form of transport that requires energy and is saturable. Saturation is limited by the number of protein transporters that are present (Cabrera-Pérez et al., 2015). Active transport requires energy as molecules are pumped against a concentration gradient or going “up-hill.” Various hydrophilic drugs have chemical structures that are similar to various nutrients that capable of being transported across the intestinal epithelium by active, carrier-mediated transport (Artursson et al., 2001). Active transport is selective and can be interfered by the presence of inhibitors (Cabrera-Pérez et al., 2015).

There are two types of active transport: primary active transport and secondary active transport. Primary active transporters utilize energy coming from the hydrolysis of ATP while secondary active transporters utilize the energy that is generated from an electrochemical gradient from a primary transporter (Cabrera-Pérez et al., 2015). There is no direct ATP coupling occurring in secondary active transport. Secondary active transport uses cotransporters such as symporters or antiporters. Symporters transport molecules in the same direction while antiporters transport molecules in opposite directions.

Endocytosis

Endocytosis (also known as vesicle-mediated transport, is a form of active transport involving the cell membrane in which materials are taken into the cell via cell membrane invaginations. The cell membrane forms a fold around the desired material,

enclosing the material, then fuses again, forming a vesicle (Cabrera-Pérez et al., 2015). This vesicle transports the engulfed material across the membrane to the inside of the cell (Cabrera-Pérez et al., 2015). Endocytosis can further be divided into two subtypes: phagocytosis and pinocytosis. Phagocytosis allows for an entire cell to be engulfed whereas pinocytosis engulfs external fluids. It is possible that endocytosis plays a minor role in drug and nutrient absorption, sans protein based molecules (Cabrera-Pérez et al., 2015).

Influx and Efflux Facilitated Transport

There are studies linking the absorption of ergot alkaloids across pre-gastric tissues, but there is a paucity of information concerning the absorption and transport of ergot alkaloids across the small intestine. Influx and efflux transport systems belong to either the ATP-binding cassette (ABC) transporter family or the solute carrier (SLC) transporter family (Maubon et al., 2007; Petzinger & Geyer, 2006; Ware, 2006) (Figure 2.5). Both transporter families play essential roles concerning transport of compounds across cell monolayers. Therefore, transporter proteins may play an important role in the drug pharmacokinetic profiles by controlling drug disposition.

ATP- Binding Cassette Transporters

ATP-binding cassette (ABC) proteins are present in almost all living organisms ranging from prokaryotes to mammalian species (Szakács et al., 2008). They are active transporters and require energy in the form of ATP. ABC transporters restrict the fraction of a drug absorbed by pumping the compound out of the cells into the lumen (Chan et al., 2004; Varma et al., 2010). There are three main functional categories of ABC transporters: importers, exporters (effluxers), and those involved in DNA repair and

translation. In prokaryotes, they exist as importers. In eukaryotes, ABC transporters function as exporters or effluxers, pumping out toxins, drugs, or possibly nutrients. The final subgroup does not act as a transporter, but is involved in DNA repair and translation processes (Davidson et al., 2008).

ABC proteins involved in transport are bound to the membrane and built from a combination of membrane-spanning regions and cytoplasmic ATP binding domains (Szakács et al., 2008). All ABC transporters contain four core domains: two trans-membrane (T) domains and two cytosolic (A) domains (Dean et al., 2001). The T domains switch between inward and outward facing positions which are powered by ATP hydrolysis (Dean et al., 2001). ATP binds to the A subunits and is hydrolyzed. Transport is linked to the ATPase activity of ABC proteins (Senior et al., 2004). ABC subfamilies involved in intestinal transport are the ABCB subfamily and the ABCC subfamily, with P-glycoprotein and breast cancer resistance protein being two of the primary members.

P-glycoprotein

P-glycoprotein, also known as ABCB1 and P-gp, was first isolated from cancer cells and plays an important role in expelling drugs out of the cell which has led to multidrug resistance (Liu & Liu, 2013). P-gp is ATP-dependent and has a broad substrate specificity. In humans, P-gp is encoded by two genes, MDR1 and MDR3, and in rodents it is encoded by MDR1a, MDR1b, and MDR2 (Kunta & Sinko, 2004). It is located at brush border membranes of enterocytes and functions as an efflux pump before drugs and toxins can access the portal circulation. Various anticancer drugs, such as vincristine, immunosuppressants, steroids, verapamil, and digoxin act as substrates for P-gp (Adachi et al., 2001). It has been suggested that P-gp and cytochrome P450 3A4 (CYP3A4)

cooperate in the intestinal absorption of drugs. CYP3A4 is an enzyme found in intestines and liver and oxidizes small foreign organic molecules such as drugs and toxins for removal from the body.

Breast Cancer Resistance Protein

Breast cancer resistant protein, also known as BCRP or ABCG2, is a half-transporter that is expressed in breast tissue, the ovaries, colon, liver, and small intestine (Colabufo et al., 2009; Allikmets et al., 1998; Maliepaard et al., 2001). BCRP is located at the apical part of the plasma membrane in epithelia (Liu & Liu, 2013). It is thought that BCRP plays a protective role in the GI tract as it prevents the absorption of toxic substances. BCRP effluxes similar substrates as P-gp which suggests that BCRP acts as a half-transporter compared to P-gp regarding drug and nutrient absorption (Taguchi et al., 1997). This could be explained by the fact that BCRP only has six transmembrane domains while P-gp has twelve.

Solute Carrier Transporters

The solute carrier (SLC) group of transporters includes over 300 members of transporters that are organized in 52 families (Hediger et al., 2004). Most SLC transporters are located within the cell membrane and can transport a diverse array molecules and ions. Transport proteins within the SLC group are either facilitative transporters or secondary active transporters. For instance, oligopeptide transporter 1 [PepT1] and organic ion transporter polypeptide A [OATPA] rely on ion gradients (e.g. H⁺ and Na⁺ gradients) that are created by primary active carriers (Varma et al., 2010). The SLC family does not contain transport proteins that are classified as primary active transporters, ion channels, or aquaporins. SLC transporters enhance the absorption of

drugs and nutrients into cells that can result in increased plasma levels of drugs and nutrients (Liang et al., 1995).

Peptide Transporter 1

Peptide transporter 1, also known as PepT1 or SLC15A1, is a proton-coupled transport protein that is encoded by the SLC15A1 gene in humans (Liang et al., 1995). PepT1 acts as a cotransporter (Adibi, 1997) and is functional in the intestines. In the intestines, PepT1 is located in the brush border membrane of the epithelium. It plays an important role in uptake of nutrients, specifically dipeptides and tripeptides, across the apical membrane (Adibi, 1997). PepT1 is of pharmacological interest because it accepts numerous drugs and prodrugs as substrates (Brandsch, 2013) and due to overexpression in malignant cancer cells (Nakanishi et al., 2000; Gonzalez et al., 1998) it is an important target for drug design to improve bioavailability. Prodrugs are drugs that are metabolized to a metabolically active form once administered. Prodrugs are grouped into two categories: those that are designed to resemble peptides for uptake via di and tri-peptide transporters and those that are developed to improve solubility or lipophilicity (Brodin et al., 2002). Certain β -lactam antibiotics fall into the second category of prodrugs (Bretschneider et al., 1999). In recent years, it has been established that PepT1 is largely involved in intestinal absorption of β -lactam antibiotics (Bretschneider et al., 1999; Sugawara et al., 2003; Terada et al., 1997). β -lactam antibiotics possess similar features common with dipeptides, including a terminal carboxylic acid group, an α -amino group, and a peptide bond (Dantzig et al., 1988).

Organic Anion-Transporting Proteins

Organic anion-transporting proteins (OATPs) are transport proteins that allow the transport of organic anions across the cell membrane. They act as gatekeepers and are present in the lipid bilayer of the cellular membrane of the brain, eyes, smooth muscles, and basolateral membrane of proximal tubular cells of the kidneys. They belong to the SLC family and are more specifically members of the solute carrier organic anion (SLCO) gene subfamily (Kullak-Ublick et al., 1997). In humans, OATP A is encoded by the *SLCO1A2* gene (Kullak-Ublick et al., 1997). This transporter is found in almost every tissue and plays a role in the transport of drugs across the cell membrane. However, a study by Meier et al. (2007) found no detectable levels of *SLCO1A2* mRNA in the duodenum of humans. Substrates of OATP-A include bile acids, thyroid hormones, and steroid hormones (Kullak-Ublick et al., 1995; Bossuyt et al., 1996; Fujiwara et al., 2001).

Caco-2 Cells as an Intestinal Model

Importance of Cell Models

It is important to evaluate molecule permeability in the early stages of drug development and nutrient profiling because it allows for selection of the best molecule for the targeted transporter (Cabrera-Pérez et al., 2015). Cell-based models of different barriers have been useful as they are relatively simple, have a high-throughput, are predictable in behavior and abilities, and are cost effective (Cabrera-Pérez et al., 2015). The main limitation of using a cell model is the variability in results (Cabrera-Pérez et al., 2015). Results vary based on temperature, protocol, cell line, passage number, culture medium, and the manipulator, making it very difficult to compare published data (Cabrera-Pérez et al., 2015). However, suitable permeability assays can be developed if

the protocol is confirmed by a standardized procedure and if high-permeability internal standards, markers, and reference compounds are used (Volpe, 2010).

Caco-2 Cell Background

Because the oral route is how most nutrients are obtained, the intestinal barrier has received extensive attention. The Caco-2 cell line is a heterogeneous human epithelial cell line that was derived from a colorectal carcinoma by the Sloan-Kettering Institute for Cancer Research (Fogh & Trempe, 1975). Caco-2 cells are considered to be the “gold standard” cell model for predicting intestinal absorption (Pereira et al., 2015; Hidalgo, 2001). Caco-2 cells were found to simulate the intestinal barrier and are used as an in vitro model (Artursson et al., 2001). In a study conducted by Hilgers et al. (1990) it was found that after 21 days, Caco-2 cells seeded into 3- μm -pore membrane inserts at 60,000 cells/cm² showed confluent monolayers with well-defined tight junctions, transporters, and enzymes. While Caco-2 cells are derived from a large intestinal carcinoma, when cultured under certain conditions, the cells differentiate and become polarized, resembling enterocytes that line the small intestine (Pinto et al., 1983; Hidalgo et al., 1989).

Caco-2 cells are not used as individual cells but rather are typically grown as monolayers on a membrane-containing insert (Figure 2.6). When cultured this way, cells will differentiate and form a polarized epithelial monolayer, providing a physical and biochemical barrier towards small molecules and ions (Hidalgo et al., 1989; Artursson, 1990). The insert system is composed of 10 μM thick membrane with different pore sizes. When epithelial cells are seeded onto the membrane, this creates apical and basolateral compartments which are similar to the epithelia lining the intestinal lumen (Pereira et al.,

2015). The Caco-2 monolayer on a membrane insert is widely used in the pharmaceutical industry as an in vitro model to predict the absorption of nutrients and drugs and has its advantages and limitations (Shapell and Smith, 2005; Mulac et al., 2012; Watanabe et al., 2003; Bravo et al., 2003; Artursson et al., 2001).

Validation of the Caco-2 Cell Line

The primary goal of establishing a Caco-2 monolayer is to create an in vitro model that mimics the intestinal barrier to study and profile drugs, toxins, and nutrients and how they are absorbed through said barrier. Three characteristics allow the model to be validated: these characteristics are monolayer integrity, differentiation, and functionality. Model integrity can be studied by measuring transepithelial electrical resistance (TEER). TEER measurements measure absolute values of resistance per unit of area and are measured in units of ohms (Pereira et al., 2015). Tight junctions modulate the transport of molecules by paracellular transport and the tighter the tight junctions, the higher the TEER measurements (Pereira et al., 2015). In addition to TEER, small molecules such as mannitol, Lucifer yellow, and dextran can also be used as evidence of membrane integrity (Pereira et al., 2015).

Transport and Possible Metabolic Routes of Alkaloids in Ruminants

As mentioned previously, of the ergopeptine alkaloids produced by tall fescue, ergovaline is produced in the largest proportions by the endophyte and has been implicated as the primary toxin associated with tall fescue toxicosis (Bush et al., 1997; Paterson et al., 1995; Strickland et al., 1993). Yates et al. (1985) determined that ergovaline was the most prominent ergopeptine alkaloid in samples from KY-31 pastures. Based on its concentration in the plant, ergovaline was presumed to be the toxin

responsible for causing fescue toxicosis. However, it has been suggested that lysergic acid is involved in disease progression as well. Stuedemann et al. (1998) used urinary and biliary alkaloid excretion patterns and ELISA quantification to suggest that lysergic acid amides and biotransformed ergopeptine alkaloids were rapidly absorbed. This suggests that another ergot alkaloid may be responsible for fescue toxicity. Hill et al. (2001) supported these findings with studies that were conducted to compare alkaloid transport in ovine omasal, reticular, and ruminal tissues and to determine whether transport was active or passive. The aforementioned tissues were removed and placed in parabolic chambers with equimolar concentrations of lysergic acid, lysergol, ergonovine, ergotamine, and ergocryptine with Krebs's Ringer phosphate (KRP) solution on the mucosal side of the tissues. Samples were taken from KRP on the serosal side during allotted times during the 240 min incubation period. Ruminal tissue had greater ergot alkaloid transport potential than omasal tissue and reticular tissue due to a larger surface area. Transport of alkaloids seemed to be an active process, with lysergic acid and lysergol having the greatest transport potential. Ergopeptine alkaloids appeared to pass across omasal tissues in larger quantities than in ruminal tissues but this transport was minimal when compared to lysergic acid and lysergol transport.

Hill et al. (2001) reported that lysergic acid crossed ruminal epithelium at greater rates than other alkaloids tested and suggested that lysergic acid may be responsible for fescue toxicosis. However, not all alkaloids present in E+ tall fescue, including ergovaline, were tested for in determination of transport across ruminal epithelium. Durix et al. (1999) used 4 lactating goats to develop an HPLC assay to quantify ergovaline in milk after intravenous injection of ergovaline into the right jugular vein. It was suggested

that the physiological response to ergovaline did not require metabolism by the gastrointestinal tract after administration of purified ergovaline resulted in physiological effects that were similar to ergovaline toxicity. This implies that metabolism by gastrointestinal microbes or the liver may not be required for ergovaline's immediate physiological effects.

At present, the site(s) and mechanism(s) of ergot alkaloid absorption have not been fully described. In two experiments conducted by Westendorf et al. (1993), different combinations of the alkaloids *N*-formyl and *N*-acetyl loline were incubated in ruminal fluid for three timepoints (0, 24, and 48 hr). Disappearance of *N*-formyl and *N*-acetyl loline increased over time. Significant amounts of both alkaloids were metabolized and converted to loline. The second experiment was comprised of abomasally cannulated sheep that were given doses of 945 mg and 2346 mg of ergovaline and ergovalinine per day. Up to 50-60% of the alkaloids in the abomasal contents were recovered as compared to the 5% recovered in fecal material. From the *in vitro* experiment, it can be concluded that alkaloids in general may be degraded by ruminal microbes. However, in the *in vivo* study, recoveries of pyrrolisidine alkaloids in abomasal fluids and feces were lower than those of the ergot alkaloids. In support of these findings, Schumann et al. (2009) conducted a study in order to examine the effects of ergot contaminated concentrate at differing levels of feed intake on ergot alkaloid metabolism and carry over into milk. The findings of this study were similar to the findings of Westendorf et al. (1993). It was determined that 67% of ergot alkaloids examined reached the duodenum. It was also found that only ~24% of alkaloids were excreted with fecal material. In both studies,

there is a sharp drop-off of alkaloid recovery from abomasal/ duodenal contents and fecal material, suggesting that some alkaloid absorption could occur in the small intestine.

Ergot Alkaloid Transport Across the Small Intestine

Eckert et al. (1978) found that many toxicants are absorbed via passive transport across the gastrointestinal epithelia and that absorption of ergot alkaloid compounds appears to be limited to the small intestine in nonruminants. Savary et al. (1991) published one of the few studies linking ergovaline absorption across the digestive tract in ruminant species. Ergovaline and its diastereoisomer ergovalinine were found in serum from steers that were grazing on endophyte-infected fescue. However, quantitative assessment was not possible as serum levels of ergovaline and ergovalinine were close to the analytical limit of detection when using high-pressure liquid chromatography (HPLC). Shapell and Smith (2005) used Caco-2 cells as a model of human intestinal cells to demonstrate the movement of a 60:40 ratio of ergovaline/ergovalinine isomers. Isomer movement was assessed using HPLC. It was found that ergovaline and ergovalinine crossed intestinal cells at similar rates. It was suggested that either isomer, or a combination, could be involved in the pathogenesis of fescue toxicosis. However, no other ergot alkaloids were tested in this study.

Summary and Statement of Objectives

The objectives of this thesis are to determine if ergotamine, ergovaline, and lysergic acid utilize peptide transporter 1 in a Caco-2 cell monolayer small intestine model, and to identify serotonin receptors in ruminal and mesenteric vasculature that could be affected by ergot alkaloid absorption.

Figure 2.1 Alkaloids present in endophyte positive tall fescue; A: pyrrolopyrazine, peramine, B: pyrrolizidine, general loline structure, C: ergoline ring structure, D: clavine structure, E: lysergic acid amide structure, F: peptide alkaloid structure

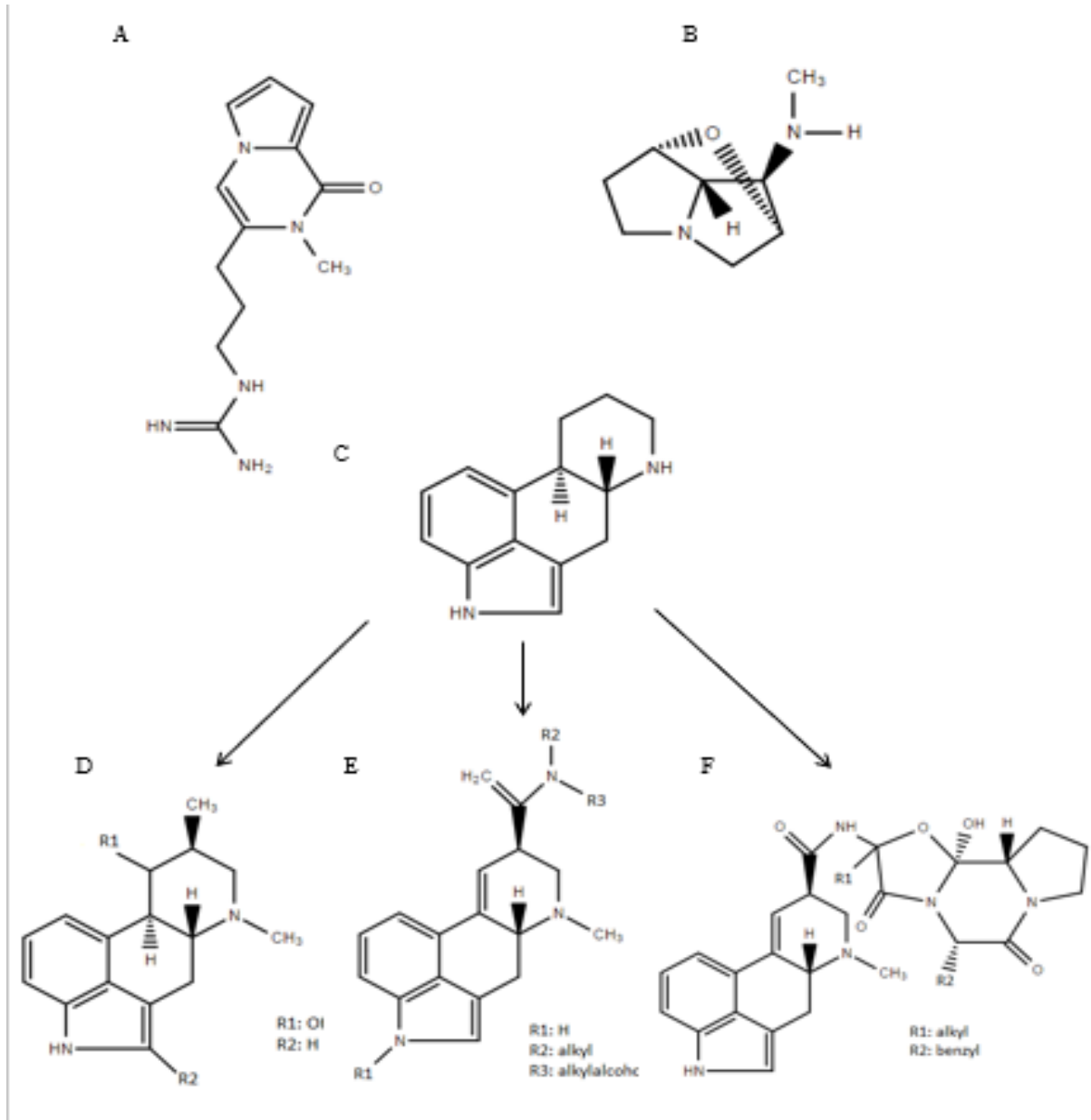


Figure 2.2 Vasoconstriction causing catecholamines: A: serotonin (5-HT), B: epinephrine (EP), C: norepinephrine (NE)

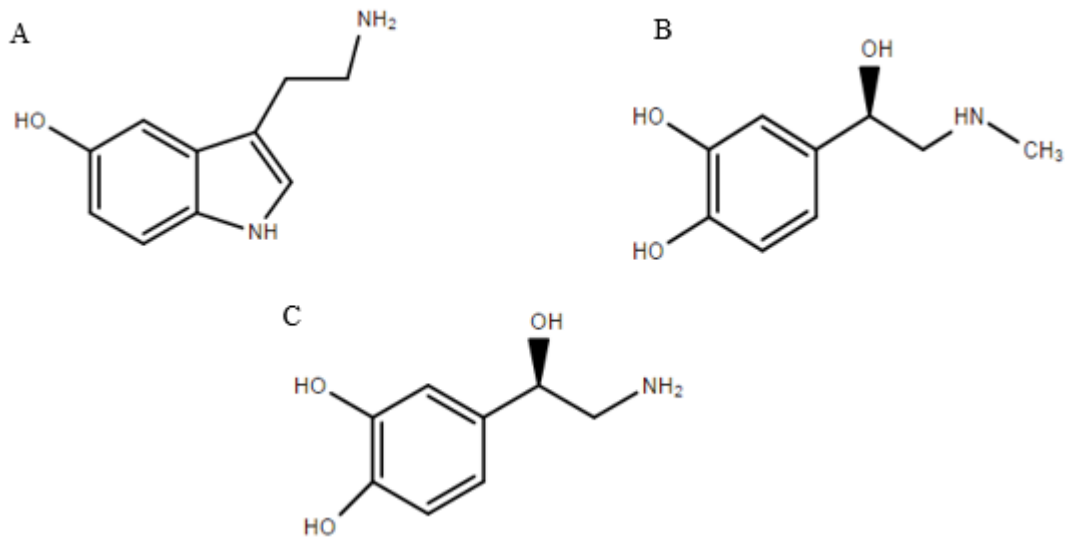


Figure 2.3 Histology of the small intestine. The small intestine is composed of four layers: A: serosa (outermost layer), B: muscularis propria, C. submucosa, and D. mucosa (innermost layer).

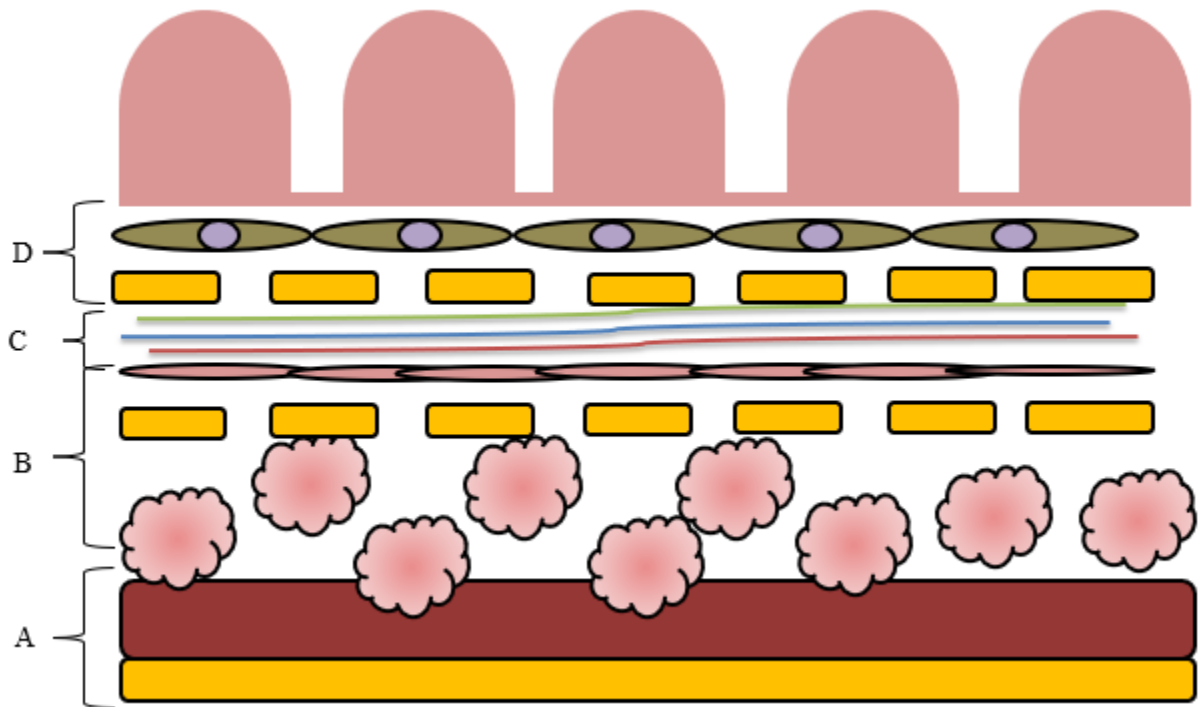


Figure 2.4 General forms of A: passive transport and B: active transport

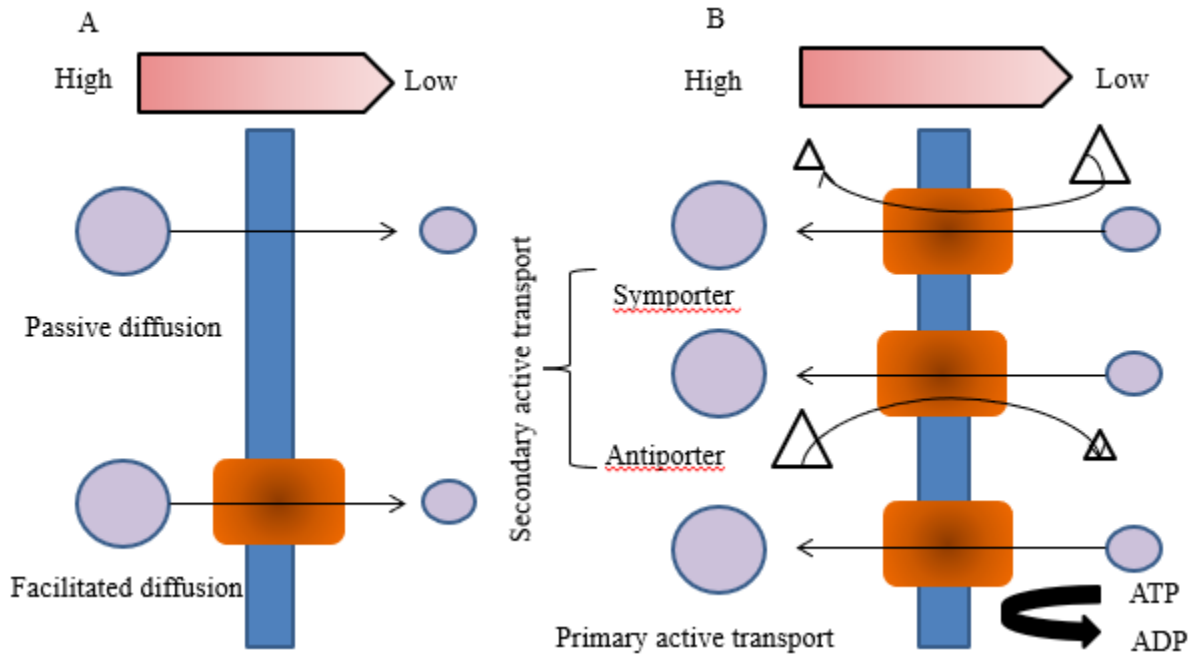


Figure 2.5 ATP-binding cassette (PepT1 and OATP A) and solute carrier transporter (P-gp and BCRP) cellular locations in intestinal epithelial cells.

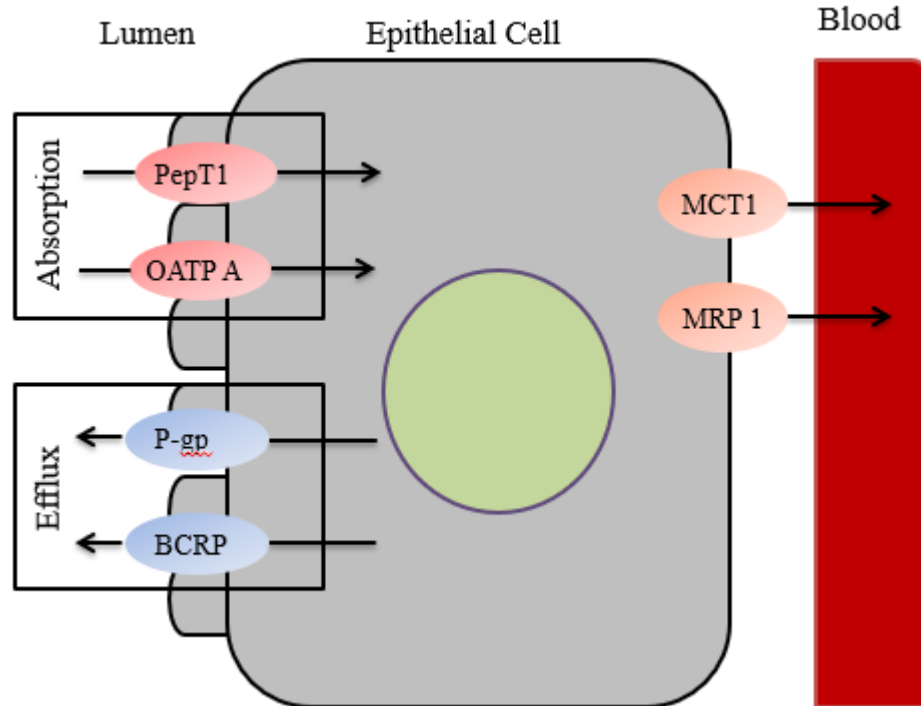
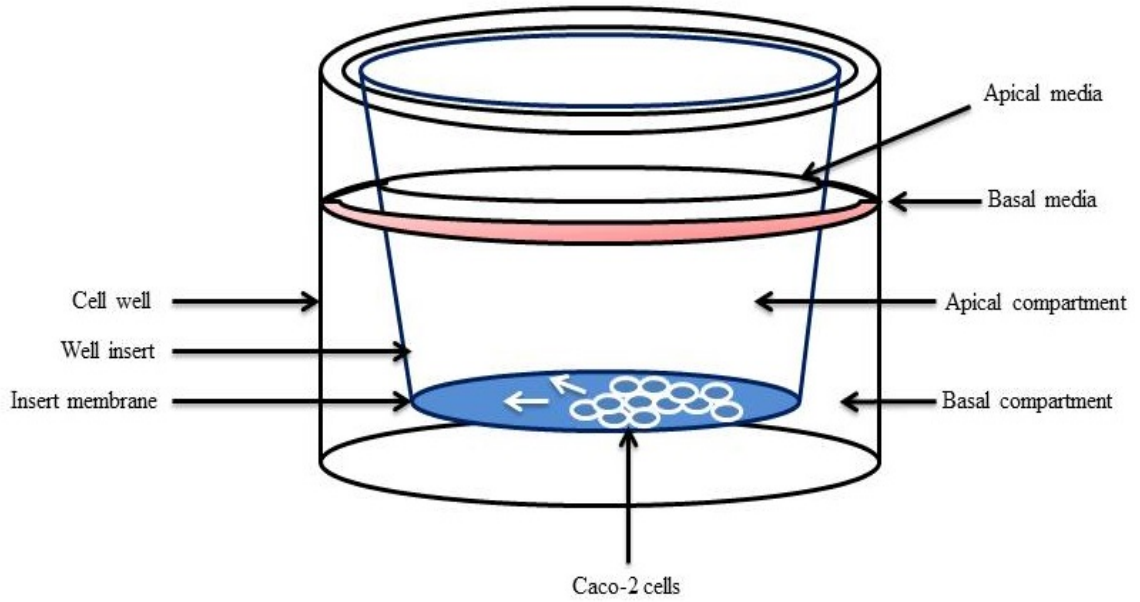


Figure 2.6 Cell membrane insert dividing growth wells into apical and basal compartments.
White arrows represent potential cellular growth on a membrane insert.



CHAPTER THREE: PHARMACOLOGIC ASSESSMENT OF BOVINE RUMINAL AND MESENTERIC VASCULAR SEROTONIN RECEPTOR POPULATIONS

Introduction

Epichl e coenophiala is an endophyte symbiotically related with tall fescue grass (*Lolium arundinaceum*). *E. coenophiala* produces numerous ergot alkaloids (EA) which have been identified as causative agents of vasoconstriction associated with fescue toxicosis. Various studies have reported that EA induce vasoconstriction in bovine uterine and umbilical arteries (Dyer, 1993), right ruminal artery and vein (Foote et al., 2011), mesenteric artery and vein (Egert et al., 2014), and lateral saphenous vein (Klotz et al., 2008; Klotz et al., 2010). Ergot alkaloids have structural similarities with biogenic amines, such as serotonin (5-hydroxytryptamine, 5-HT), and cause vasoconstriction by binding to receptors (Dyer, 1993; Berde, 1980), possibly causing decreased blood flow observed in gastrointestinal vasculature (Rhodes et al., 1991; Foote et al., 2011).

Foote et al. (2011) found that ergot alkaloids could play a role in vasoconstriction of the bovine right ruminal artery and vein, possibly reducing blood flow to or from the rumen and resulting in decreased absorption rates of nutrients and fermentation products. Ergot alkaloid-induced vasoconstriction was not only seen in ruminal vasculature but also in mesenteric vasculature. Egert et al. (2014) demonstrated that various ergot alkaloids were vasoactive in bovine mesenteric vasculature. Further, steers with prior exposure to endophyte-infected tall fescue had a diminished response to many ergot alkaloids and 5-HT in the mesenteric vasculature. Because there are a number of different serotonin receptor subtypes (Hoyer et al., 1994), it is necessary to assess the receptor populations available for possible ergot alkaloid interaction. Therefore, the objective of this study was

to pharmacologically determine which 5-HT receptors are involved in contractility of bovine gut vasculature using agonists selective for 5-HT_{1D}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}, 5-HT₄, and 5-HT₇ receptors.

Materials and Methods

No live animals were involved in this study, thus approval from the University of Kentucky Animal Care and Use Committee was not required.

Animals and Tissue Collection

Blood vessels were collected from the carcasses of twenty-four mixed breed and gender cattle between ten and thirty months of age (542 ± 23 kg) that were slaughtered at the University of Kentucky Meats Lab or Hi-View Meats (Sadieville, KY). As previously described by Klotz and Barnes (2014), the gastrointestinal tract was removed and the ileocecal fold and ileal flange were identified as landmarks in order to remove multiple branches of common mesenteric artery (MA) and vein (MV) segments. The ventral coronary and caudal grooves were identified as landmarks to remove segments of the right ruminal artery (RA) and vein (RV) as previously described (Klotz et al., 2011). Vessel segments of 2 – 3 cm were immersed and transported in oxygenated Krebs-Henseleit buffer (95% O₂/ 5% CO₂; pH = 7.4; 11.1 mM D-glucose; 1.2 mM MgSO₄; 1.2 mM KH₂PO₄; 4.7 mM KCl; 118.1 mM NaCl; 3.4 mM CaCl₂; 24.9 mM NaHCO₃; Sigma Chemical Co., St. Louis, MO, USA). The MA, MV, RA, and RV were stored on ice until they were separated and cleaned of surrounding fat and fascia. Vessels were sliced into approximately 2-mm segments and viewed under a dissection microscope (Stemi 2000-C, Carl Zeiss Inc., Oberkochen, Germany) at 12.5x magnification to check for vessel abnormalities (structural damage and branches) and to assess vascular dimensions. Cross-

sections with structural damage were replaced with undamaged segments. Because MV and RV are pliant, vascular dimensions were only recorded for MA and RA cross sections using Axiovision (version 20, Carl Zeiss Inc.).

Standard Preparations

All receptor agonists were obtained from Tocris Bioscience (Minneapolis, MN, USA). Stock solutions of the agonists TCB-2 (TCB-2; Cat. No. 2592), CP 93129 dihydrochloride (CP 93129; Cat. No. 1032), LP 44 (LP 44; Cat. No. 2534), BIMU-8 (BIMU-8; Cat. No. 4374), BW 723C86 hydrochloride (BW 723C86; Cat. No. 1059), and L-694, 247 (L-694, 247; Cat. No. 0781) (Table 3.1) were diluted to corresponding concentrations for final working concentrations in tissue wells of 1×10^{-11} to 1×10^{-4} M for LP 44 and 5×10^{-9} to 1×10^{-4} M for all other agonists. LP 44, BW, L-694, TCB-2, and BIMU-8 were prepared in dimethylsulfoxide (DMSO) (472301; Sigma Chemical Co.). CP 93129 was prepared in H₂O. Eight standard additions of LP 44 were prepared based on available stock of LP 44 (1×10^{-11} , 1×10^{-10} , 1×10^{-9} , 1×10^{-8} , 1×10^{-7} , 1×10^{-6} , 1×10^{-5} , and 1×10^{-4} M). For all other agonists, there were a total of ten standard additions (5×10^{-9} , 1×10^{-8} , 5×10^{-8} , 1×10^{-7} , 5×10^{-7} , 1×10^{-6} , 5×10^{-6} , 1×10^{-5} , 5×10^{-4} , and 1×10^{-4} M). All additions were added to tissue chambers in increasing order of concentration in volumes that did not exceed 0.5% of the total buffer.

Myograph Protocol

Mesenteric and ruminal artery and vein cross-sections were mounted onto luminal supports in individual wells of a multimyograph (DMT 610M, Danish Myo Technology, Atlanta, GA) containing 5 mL Krebs-Henseleit buffer subject to constant gassing (95% O₂/ 5% CO₂; pH =7.4; 37°C). The incubation buffer was the same composition as the

transport buffer plus 3×10^{-5} M desipramine (D3900; Sigma Chemical Co.) to inactivate neuronal catecholamine uptake and 1×10^{-6} M propranolol (P0844; Sigma Chemical Co.) to block β -adrenergic receptors. Vessels were allowed to equilibrate under the above conditions for 90 min with buffer changes occurring every 15 min to allow a vessel to equilibrate at a resting tension of approximately 1 g. Experimental tissues were exposed to a reference addition of 120 mM KCl (Sigma Chemical, Co.) for 15 min to confirm tissue responsiveness and viability. Vessels were rinsed with new incubation buffer every 15 min until approximately 1 g of tension was reached. Once vessels had returned to resting tension, standard additions were added for contractile response experiments. Additions were added in 15-min cycles. Each cycle consisted of a 9-min incubation period, 2 2.5-min buffer washes, a final buffer replacement, and 1-min recovery period before the next standard addition. Vessels were again exposed to 120 mM KCl once the experiment was completed to verify vessel viability for the entire experiment.

Isometric contractions in mesenteric and ruminal vessels to KCl, LP 44, CP 93129, BW 723C86, L-694, 247, TCB-2, and BIMU-8 were digitized and recorded in grams of tension using PowerLab16/35 and Chart software (version 7.3, ADInstruments, Colorado Springs, CO). Baseline tension was recorded before addition of 120 mM KCl. For all contractile response data, the maximum tension (measured in g) during the 9-min incubation was recorded as the contractile response and then corrected for baseline tension. Due to variation between animals and tissues, contractile response data was normalized as a percentage of the maximum grams of tension induced by the reference addition of KCl to compensate for differences in vessel responsiveness. Vessel contractile response data are reported as the percentage mean contractile response \pm SEM of the

maximum contractile response produced by the 120 mM KCl reference addition.

Sigmoidal concentration response curves of ruminal and mesenteric vasculature to 5-HT agonists were calculated and plotted using nonlinear regression with fixed slope (GraphPad Prism 7, GraphPad Software Inc., La Jolla, CA). Data were plotted and calculated using a 3-parameter equation:

$$y = \text{bottom} + \frac{(\text{top} - \text{bottom})}{[1 + 10^{(\log EC_{50} - x)}]}$$

where y represents contractile response, x represents agonist concentration, top and bottom are the percentage of 120 mM KCl maximum contractile response at the plateaus, and EC_{50} is the molar concentration of the agonist producing 50% of the maximum response of KCl.

Statistical Analysis

All data were analyzed using the MIXED model of SAS (SAS 9.3, SAS Inst. Inc., Cary, NC). Contractile response and EC_{50} data for each agonist were analyzed as a completely randomized design for effect of agonist concentration. Mesenteric and ruminal arteries and veins were the experimental units. A vessel x agonist concentration interaction was analyzed with vessel and agonist concentration being the main effects. Mean separation was conducted for data if the probability of a greater F -statistic in the ANOVA was significant for the effect of agonist concentration. Individual mean differences were evaluated by using the LSD feature of SAS. Data were analyzed for deviations from normality and homogeneity. Results are considered significant if probabilities are $P < 0.05$, unless reported otherwise.

Results and Discussion

Exploration of the Presence of 5-HT_{2A} Receptors: Responses to TCB-2

To our knowledge, this is the first study to profile the 5-HT receptor subtypes present in bovine ruminal and mesenteric vasculature. Because there are numerous 5-HT receptor subtypes located throughout the body, the biological responses to 5-HT are complex and not fully characterized. Serotonin can act as a vasodilator or vasoconstrictor depending on the animal species, blood vessel type, or status of the endothelial cell layer (Ni and Watts, 2006). The 5-HT_{2A} subtype is the primary receptor involved in vasoconstrictive responses. The current study found that there was a significant contractile response for mesenteric and ruminal vasculature to the 5-HT_{2A} agonist TCB-2 ($P < 0.05$) (Figure 3.1). Klotz et al. (2013) had previously identified the presence of 5-HT_{2A} receptors in bovine lateral saphenous veins using the antagonist ketanserin. The onset of the contractile response was observed at the addition of $5 \times 10^{-5} M$ TCB-2 for the MA, MV, and RV ($P < 0.05$) whereas the RA reached a maximal contractile response when exposed to a lower concentration, $5 \times 10^{-6} M$ TCB-2 ($P < 0.05$).

For EC₅₀ values, only data regarding 5-HT_{2A} was considered as all other experimental agonists failed to produce a positive sigmoidal contractile response. Because the units of expression for EC₅₀ values are logarithmic, the larger the EC₅₀ value, the lower the TCB-2 concentration needed to achieve 50% of total response and the more sensitive a vessel is to TCB-2. Thus, when comparing EC₅₀ values of vessels exposed to TCB-2 (Table 3.2), the EC₅₀ value of MA was higher than that of MV ($P = 0.0047$) and RV ($P = 0.0025$) but did not differ from RA ($P = 0.266$). Ruminal arteries also had higher EC₅₀ values than MV ($P = 0.0004$) and RV ($P = 0.0002$). While MV and RV were

different from MA and RA, they did not differ from each other ($P = 0.7018$). As RV and MV had lower EC_{50} values than MA and RA, it could be suggested that TCB-2 reached 50% of the maximum contractile response at a lower concentration, suggesting that RV and MV are more sensitive to TCB-2 than MA and RA. Egert et al. (2014) reported similar findings when exposing mesenteric vasculature to 5-HT as MA was found to be less sensitive to 5-HT exposure than MV.

A significant vessel type x concentration interaction was observed for vessels exposed to TCB-2 ($P = 0.0005$; Figure 3.1). This indicates that the contractile response seen with TCB-2 depends on both the concentration of the agonist the vessel is exposed to and the type of blood vessel being used. Mesenteric arteries and RA exposed to TCB-2 differ from RV and MV, exhibiting a larger contractile response, which is supported by the lower EC_{50} values associated with the MA and RA responses. This evidence supports the idea that MA and RA may have a larger amount of functional 5-HT_{2A} receptors present than RV and MA. This understanding is similar to the findings of Egert et al. (2014), where maximal response to 5-HT appeared greater in the MA than the MV (no direct comparison across vessel type was conducted in this study).

Previous myograph experiments using bovine vasculature have also shown varied sensitivity in response to agonist treatments. Larger contractile responses in arteries may be explained by the differential expression of the 5-HT_{2A} receptor. Using rats, Kato et al. (1999) demonstrated that the expression of 5-HT_{2A} receptors is significantly increased in the aorta compared to the vena cava. Watts (2002) also found that 5-HT_{2A} receptors are the primary contractile receptor in mesenteric arteries of hypertensive rats. These findings indicate that there may be a larger quantity of 5-HT_{2A} receptors in arteries than in veins.

Regarding the larger responses seen in ruminal vasculature as compared to mesenteric vasculature, differences could be due to the type of blood vessel (e.g. ruminal vessels v. mesenteric vessels) and a corresponding difference in 5-HT_{2A} receptor population numbers.

Vascular Responses to CP 93129, LP 44, and BIMU-8

Ruminal and mesenteric vessels exposed to CP 93129, LP 44, and BIMU-8 did not exhibit contractile responses ($P > 0.05$) (Figure 3.2, 3.3, and 3.4). These results indicate that the 5-HT receptor subtypes 5-HT_{1B}, 1D, and 5-HT₄ are not present or do not play a role in EA-induced vasoconstriction in bovine mesenteric or ruminal vessels. It is possible that these receptors are present but not in concentrations high enough to be detected by the bioassays used in the current, or they may be present, but not functional. For example, Banes and Watts (2003) showed that the 5-HT_{1B} agonist CP 93129 failed to cause a contraction in the aorta of rats. However, molecular expression of 5-HT_{1B} receptors was demonstrated in rats, indicating that although these receptors were present, they were not functional or involved in aortic contractions.

There was not a vessel x concentration interaction for vessels exposed to CP 93129 ($P = 1.0$; Figure 3.2), LP 44 ($P = 1.0$; Figure 3.3), or BIMU-8 ($P = 0.99$; Figure 3.4). However, these vessels did exhibit significant responses based on both vessel type and concentration of agonist. Vessels exposed to LP 44 exhibited a vessel effect ($P < 0.0001$) and an agonist concentration effect ($P = 0.014$). Mesenteric artery and RA responses were not different, but relaxed less than both MV and RV. Vessels also responded differentially to BIMU-8 exposure ($P < 0.0001$) as well as responding to agonist concentration ($P < 0.0001$). Similar to vessels exposed to LP 44, MA and RA did

not exhibit differing responses from each other but relaxed less than both RV and MV. Vessels exposed to CP 93129 exhibited a vessel effect ($P < 0.0001$) and an agonist concentration main effect ($P = 0.0002$). MA, MV, RA, and RV are all significantly different from one another ($P < 0.0001$). For both CP 93129 and BIMU-8, MA relaxed less than MV and RA relaxed less than RV. In vessels exposed to CP 93129, MA had a stronger response than RV and RA had a stronger response than MV. This finding and the fact that MA and RA relaxed less in the presence of an agonist than MV and RV, indicate that the location from which the vessel came from (mesenteric v. ruminal) may not be as important as the type of vessel (artery v. vein).

The 5-hydroxytryptamine 4 (5-HT₄) receptor is coupled to adenylate cyclase and is distributed throughout the central nervous system and peripheral tissues, specifically in those tissues and organs that contain smooth muscle, such as the bladder and the alimentary tract. As mentioned previously, it was found in this experiment that 5-HT₄ did not elicit a constrictive or relaxation response to the agonist BIMU-8 in ruminal or mesenteric vessels. 5-HT₄ receptors have previously been found to induce endothelium-independent relaxation of bovine mesenteric lymphatics (Miyhara et al., 1994). However, little information is available about the location of 5-HT₄ receptors in core body vasculature. Ullmer et al. (1995) demonstrated the diversity of receptor subtypes expressed in various blood vessels. Reverse transcription-PCR was used to distinguish mRNAs for G protein-coupled 5-HT receptors expressed in rat and pig blood vessels. Most blood vessels expressed mRNA receptors for 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2B}, 5-HT₄, and 5-HT₇ mRNA although they were expressed at differing levels. Similar analogous RT-PCR assays were performed using smooth muscle and endothelial cells from human

pulmonary artery, aorta, coronary artery, and umbilical vein. Of the known G protein-coupled 5-HT receptors, only five were found to be expressed in the various blood vessels tested. Endothelial cells were found to express receptor mRNA for 5-HT_{1D}, 5-HT_{2B}, and 5-HT₄ whereas smooth muscle cells expressed 5-HT_{1D}, 5-HT_{2A}, and 5-HT₇. This could explain why no response was seen in vessels exposed to BIMU-8 in our study. 5-HT₄ may be present in endothelial cells but absent in smooth muscle cells, meaning that contraction of the vessel cannot occur via direct acting vasoconstrictors. Ontsouka et al. (2014) compared the levels of mRNA and 5-HT₄ receptor binding sites in smooth muscle layers from the GI tract of dairy cows using real-time PCR to verify if mRNA and protein expression were correlated. It was found that mRNA levels of the 5-HT₄ receptor were affected by location along the GI tract. Levels in the external loop of the spiral colon and ileum were greater than those in the proximal loop of the ascending colon (mesenteric blood vessels utilized in the current study were collected from mesentery supporting the ileum). However, these levels of mRNA were similar to levels of receptors in the fundus abomasa, pylorus, and cecum. It could be concluded from this study that mRNA and 5-HT₄ receptor binding sites were present in the smooth muscle layer of the entire GI tract but that they were not correlated with each other. Although Ontsouka et al. (2014) studied receptors in smooth muscle rather than blood vessels, the findings were similar to that of Ullmer et al. (1995) and suggest that 5-HT receptors may be present within a tissue but may not play an active role or a role that has yet to be identified.

Vascular Responses to BW 723C86 and L-694, 247

When exposed to the agonists BW 723C86 or L-694, 247, ruminal and mesenteric vasculature did not show a contractile response ($P > 0.05$). However, MV relaxed ($P <$

0.05) (Figures 3.5 and 3.6). This indicates that 5-HT_{1D} and 5-HT_{2B} receptors may be present in MV and contribute to vasorelaxation. A significant vessel x concentration interaction was observed ($P = 0.0012$) in vessels exposed to L-694, 247, indicating that the vasorelaxation response seen depends on the concentration of the agonist the vessel is exposed to and the type of vessel being used. Ruminal arteries and across all concentrations of agonist additions. The response of MV differs from RV for the 5×10^{-8} M addition onward, and is different from the RA from 1×10^{-8} M onwards.

There was not a vessel x concentration interaction for vessels exposed to BW 723C86 ($P = 0.15$; Figure 3.5). Vessels did, however, exhibit significant responses based on both vessel type ($P < 0.0001$) and concentration of agonist ($P = 0.0013$). Similar to vessels exposed to CP 93129, MA had a stronger response than RV and RA had a stronger response than MV. This indicates that the type of vessel does have an effect on the type and intensity of the response seen.

The existence of 5-HT_{2B} receptors has been reported to cause endothelium-dependent vasorelaxation in rabbit and rat jugular vein and the porcine vena cava and pulmonary artery (Watts and Cohen, 1999). The existence of 5-HT₇ has also been demonstrated in the femoral vein and pulmonary arteries of rabbits (Martin and Wilson, 1995; Morecroft and MacLean, 1998) and in the coronary and cerebral arteries of dogs (Cushing et al., 1996; Terrón, 1996; Terrón and Falcón-Neri, 1999). Although Ullmer et al. (1995) and Bhalla et al. (2002) used RT-PCR to identify mRNA for 5-HT_{2B} and 5-HT₇ in porcine pulmonary arteries, the presence of mRNA is not indicative of whether the receptor is functional or active in producing a response, as noted previously. Jähnichen et al. (2005) further characterized the vasorelaxative responses in pulmonary arteries

precontracted to prostaglandin $F_{2\alpha}$ in weaned pigs. By using the 5-HT_{2B} receptor agonist BW 723C86, the selective 5-HT_{2C} receptor antagonist SB 242084, and the 5-HT_{2B/2C} agonist SB 206553, it was demonstrated that 5-HT in weaned pigs induced an endothelium-dependent relaxation response mediated by 5-HT_{2B} receptors and a direct relaxation response by 5-HT₇ receptors. However, these findings are in direct contrast to the response seen in slaughter-age pigs. In slaughter-age pigs, it was found that pulmonary artery vasorelaxation only is mediated by 5-HT_{2B} receptors. These results indicate that as the pig ages, it acquires a greater population of 5-HT_{2B} receptors that contribute to vasorelaxation more so than 5-HT₇. While our current study only found a vasorelaxation response in mesenteric veins in response to L-694, 247 and BW 723C86, the findings of Jähnichen et al. (2005) provide insight into the responses observed. It is possible that as ruminants age, they too, rely more heavily on 5-HT_{2B} than 5-HT₇.

Conclusions

The findings of this study indicate that 5-HT_{2A} is present in ruminal and mesenteric vasculature, plays a role in vasoconstriction, and could be influenced by ergot alkaloid exposure as has been demonstrated in peripheral blood vessels. In contrast, the 5-HT_{1D} and 5-HT_{2B} receptor subtypes do not contribute to vasoconstriction in any blood vessel tested, but may be present in mesenteric veins and possibly contribute to vasorelaxation. Further research with 5-HT_{2A} should be directed towards defining the relationship this receptor has with ergot alkaloids and associated vasoconstriction. Additionally, as this study was confined to mesenteric and ruminal vasculature, it may be possible to expand this study to include blood vessels in different anatomic locations.

Table 3.1. Agonists, function, and distribution of serotonin (5-HT) receptor subtypes.

| 5-HT Receptor | Agonist | Receptor Function | Distribution | Sources |
|--------------------|--------------------------|--|---|-----------------------------|
| 5-HT _{1B} | CP 93129 dihydrochloride | Mood, Locomotion, Vasoconstriction | CNS, Blood vessels | Weinshank et al., 1992 |
| 5-HT _{1D} | L-694, 247 | Locomotion, Vasoconstriction | CNS, Blood vessels | Hamel et al., 1993 |
| 5-HT _{2A} | TCB-2 | Appetite, Thermoregulation, Smooth muscle contractions, Vasodilation, Vasoconstriction | CNS, Smooth muscles, Blood vessels, Platelets | Morecroft and MacLean, 1998 |
| 5-HT _{2B} | BW 723C86 hydrochloride | Appetite, Vasoconstriction, CV function, GI motility | CNS, Blood vessels, CV system, GI tract | Ullmer et al., 1995 |
| 5-HT ₄ | BIMU-8 | Mood, Appetite, GI motility | CNS, GI tract | Dumuis et al., 1989 |
| 5-HT ₇ | LP 44 | Behavior, Vasoconstriction | CNS, Blood vessels | Ullmer et al., 1995 |

CNS: central nervous system; CV: cardiovascular; GI: gastrointestinal.

Table 3.2. Log EC₅₀ values in response to TCB-2 for mesenteric arteries (MA), mesenteric veins (MV), ruminal arteries (RA), and ruminal veins (RV). Values are expressed as the mean ± SEM. ^{1,2}

| Myograph Treatment | Vessel | Log EC ₅₀ , <i>M</i> |
|--------------------|--------|---------------------------------|
| TCB-2, <i>M</i> | MA | -4.79 ± 0.09 (7) ^{3a} |
| | MV | -5.23 ± 0.09 (7) ^b |
| | RA | -4.63 ± 0.11 (6) ^a |
| | RV | -5.29 ± 0.11 (6) ^b |

^{a-b}Within a column for LogEC₅₀, means without a common superscript differ ($P < 0.05$).

¹-Log EC₅₀ = measure of the potency of a drug or agonist that induces a response midway (50%) between the baseline and maximum after a certain time of exposure; expressed as the molar concentration of the agonist, in this instance, 120 mM KCl.

²EC₅₀ values were only considered for vessels exposed to TCB-2 as TCB-2 was the only agonist that elicited a vasoconstrictive response.

³Values in parentheses are the number of animals used in each experiment.

Figure 3.1 Chemical structure of the 5-HT_{2A} agonist TCB-2 and mean contractile responses of mesenteric artery (MA), mesenteric Vein (MV), ruminal artery (RA), and ruminal vein (RV) to increasing concentrations of TCB-2.

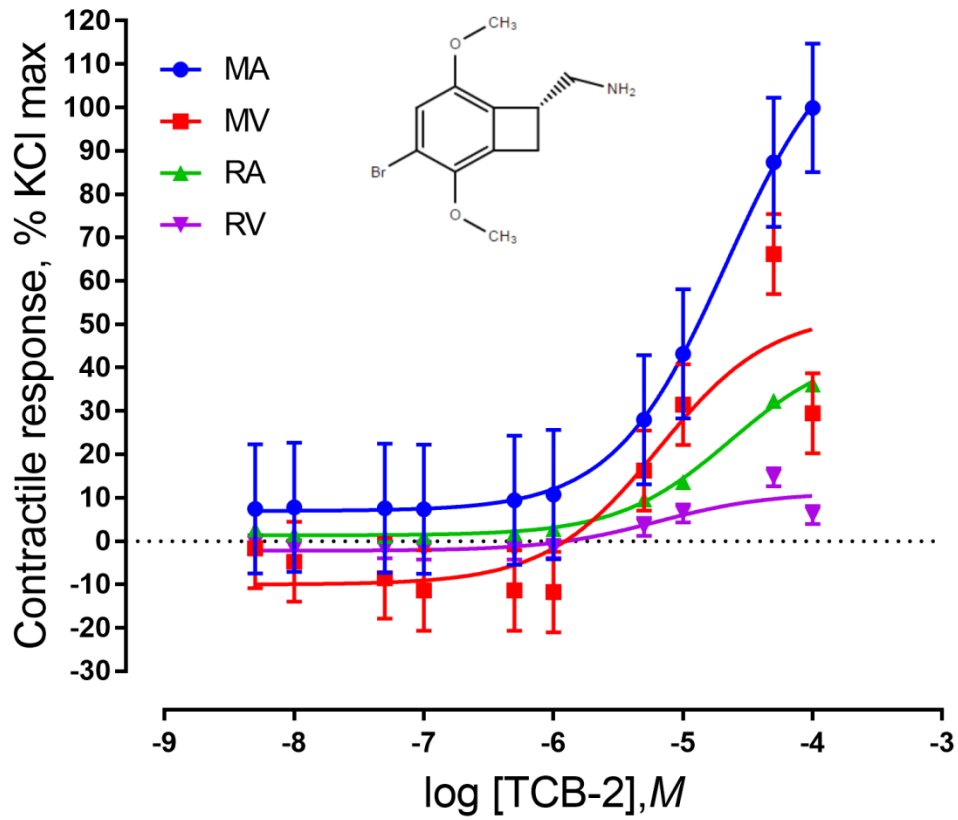


Figure 3.2 Chemical structure of the 5-HT_{1B} agonist CP 93129 dihydrochloride and mean contractile responses of mesenteric artery (MA), mesenteric Vein (MV), ruminal artery (RA), and ruminal vein (RV) to increasing concentrations of CP 93129 dihydrochloride.

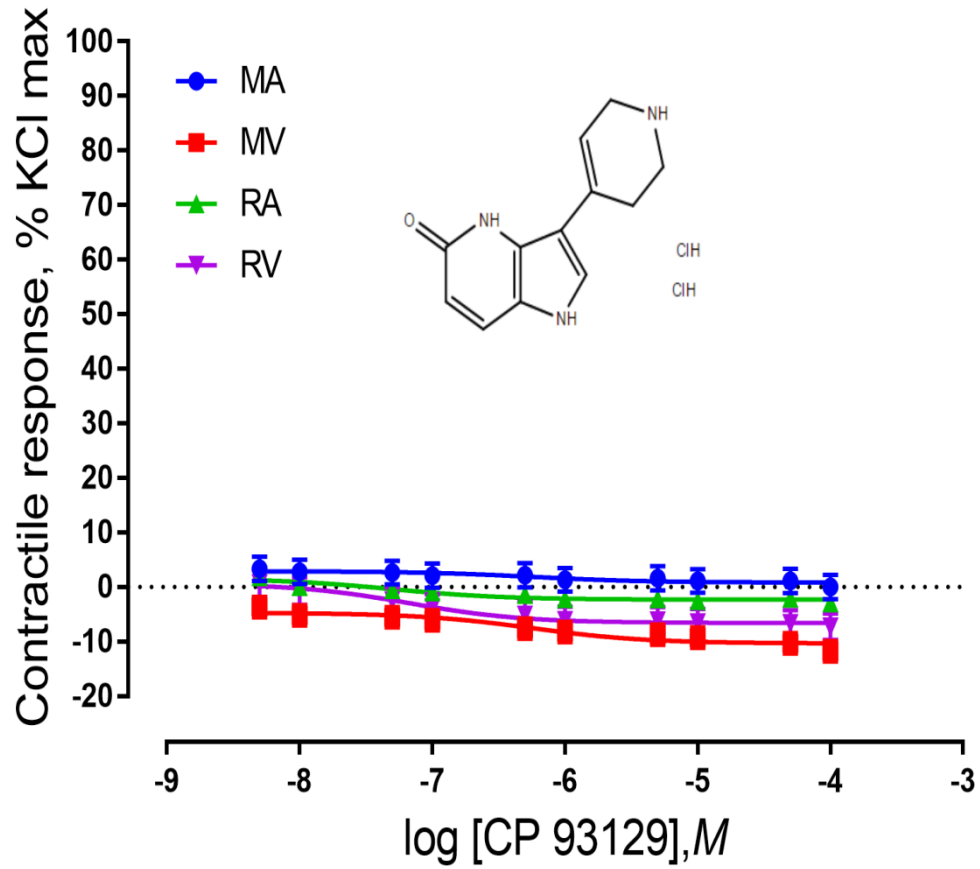


Figure 3.3 Chemical structure of the 5-HT₇ agonist LP 44 and mean contractile responses of mesenteric artery (MA), mesenteric Vein (MV), ruminal artery (RA), and ruminal vein (RV) to increasing concentrations of LP 44.

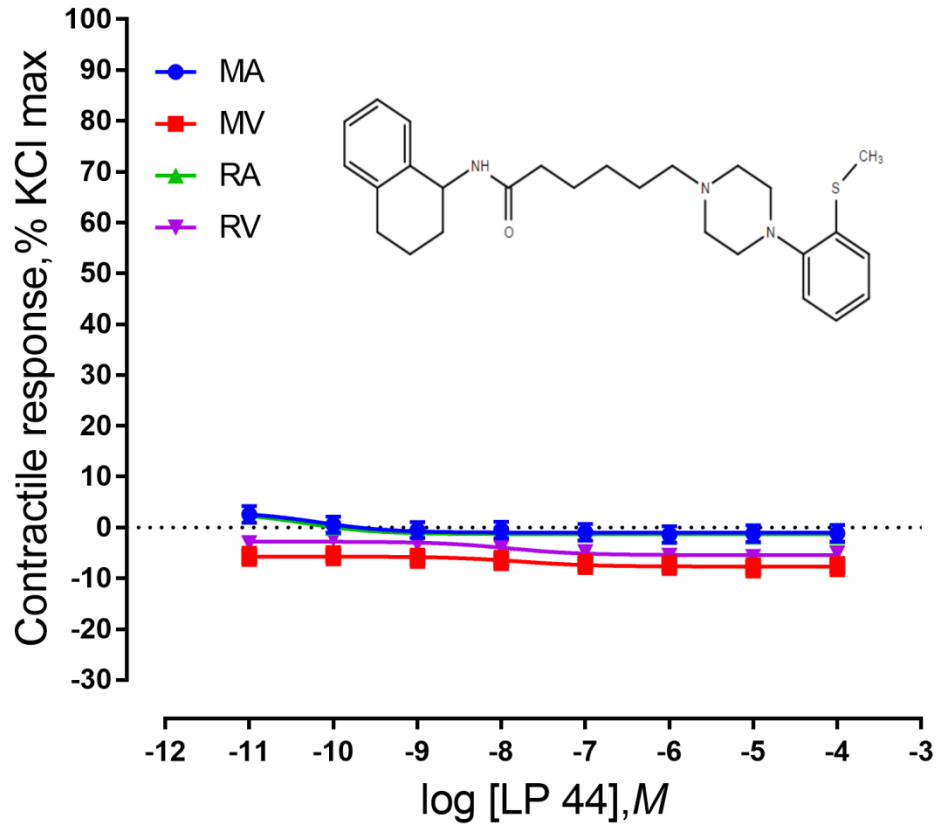


Figure 3.4 Chemical structure of the 5-HT₄ agonist BIMU-8 and mean contractile responses of mesenteric artery (MA), mesenteric Vein (MV), ruminal artery (RA), and ruminal vein (RV) to increasing concentrations of BIMU-8.

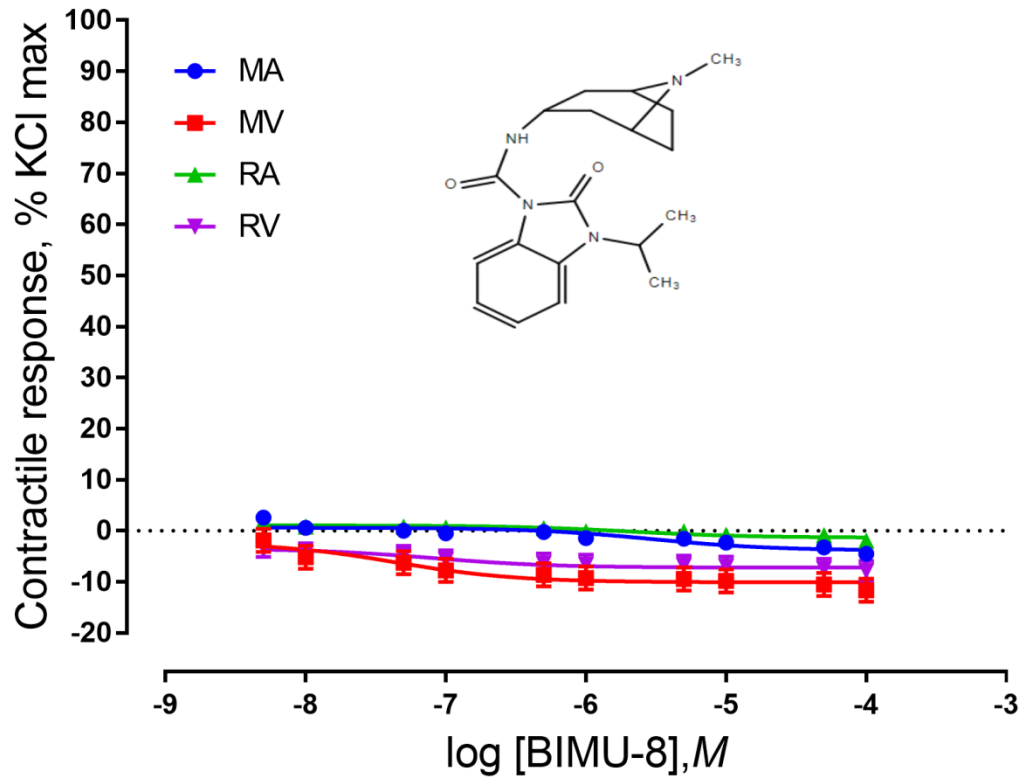


Figure 3.5 Chemical structure of the 5-HT_{2B} agonist BW 723C86 and mean contractile responses of mesenteric artery (MA), mesenteric Vein (MV), ruminal artery (RA), and ruminal vein (RV) to increasing concentrations of BW 723C86.

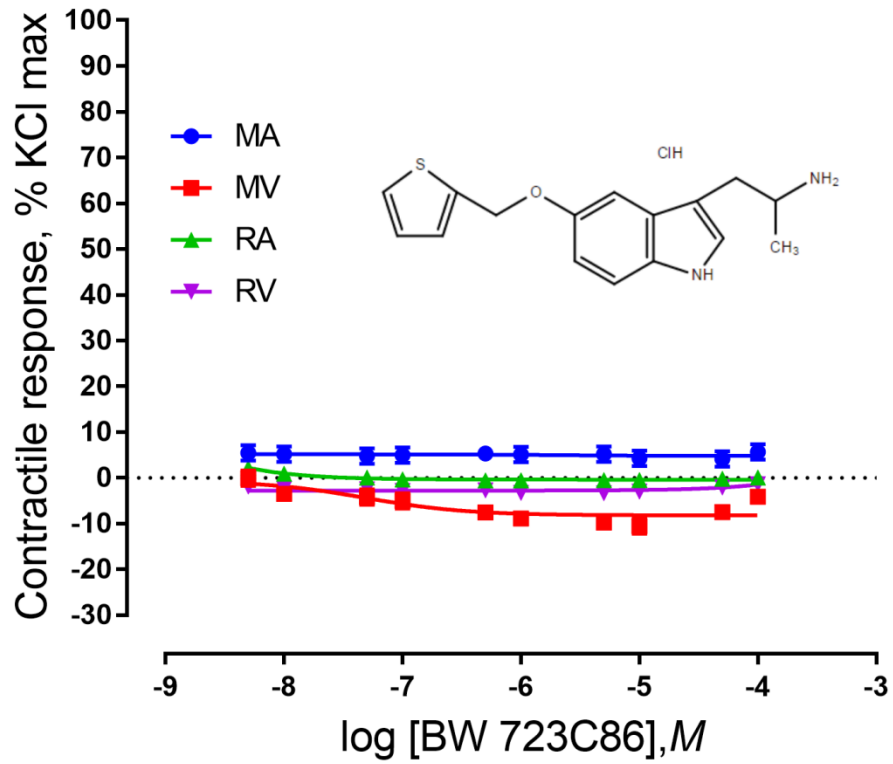
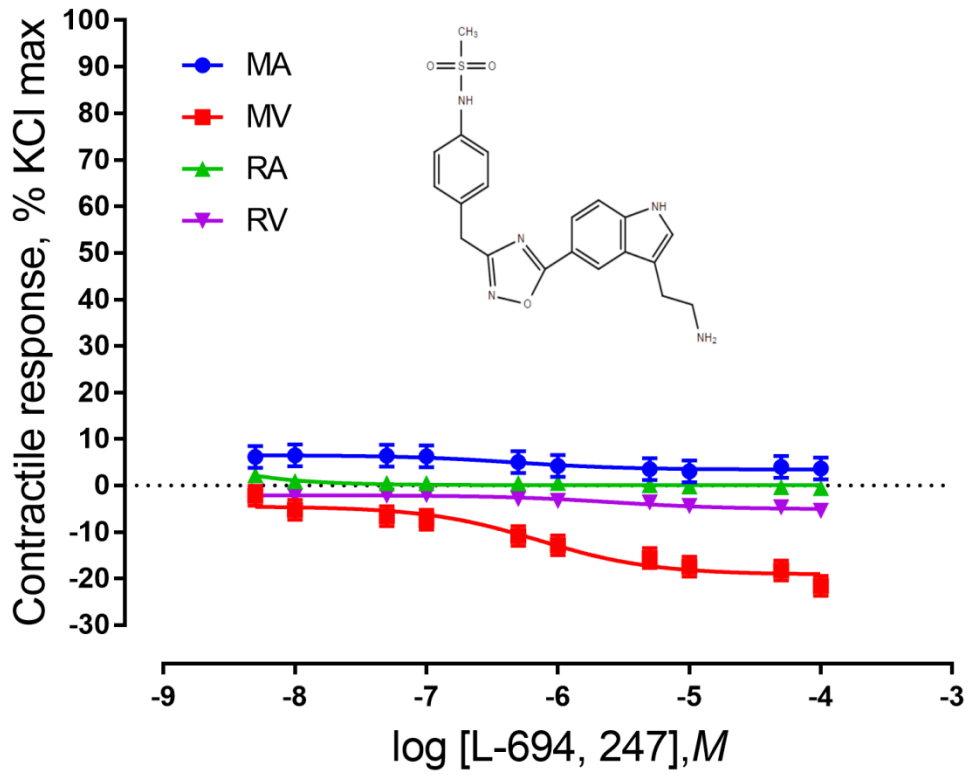


Figure 3.6 Chemical structure of the 5-HT_{1D} agonist L-694, 247 and mean contractile responses of mesenteric artery (MA), mesenteric Vein (MV), ruminal artery (RA), and ruminal vein (RV) to increasing concentrations of L-694,247.



CHAPTER FOUR: ASSESSMENT OF PEPTIDE TRANSPORTER 1 INVOLVEMENT IN TRANSPORT OF ERGOT ALKALOIDS USING A CACO-2 CELL MONOLAYER

Introduction

Tall-fescue (*Lolium arundinaceum*) is a cool-season, perennial grass that is often infected with a fungal endophyte (*Epichloë coenophiala*) that produces ergot alkaloids responsible for causing fescue toxicosis. Fescue toxicosis causes large production losses in the beef industry. The mechanisms underlying fescue toxicosis are intricate and not fully understood at this time. Little is known about the cellular mechanisms that lead to fescue toxicosis. Ergopeptines and lysergic acid amides bind to dopamine receptors and are thought to be one mechanism behind fescue toxicosis symptoms (Larson et al, 1999). α -Ergocryptine and bromocriptine are two ergopeptines that are dopamine receptor D2 (D2R) agonists (Sibley and Creese, 1983). Activation of D2R stimulates a G-protein cascade, inhibiting adenylate cyclase and reducing cAMP inside the cell. Activation of D2R is thought to lead to many of the maladies affecting livestock consuming endophyte-infected tall fescue. It has been hypothesized that ergot alkaloids are absorbed by the GI tract but there is limited evidence. Because ergopeptine alkaloids are charged in environments with a low pH, it is assumed that they are absorbed in the small intestine (Eckert et al., 1978). Shapell and Smith (2005) assessed the movement of ergovaline and its isomer ergovalinine across human intestinal cells using Caco-2 cells. It was found that ergovaline and ergovalinine readily moved across Caco-2 cells without further metabolism and at similar rates at both 6 μ M and 22 μ M concentrations. However, it was not determined whether this movement was due to passive diffusion or active transport.

Recent studies suggest that transport proteins may play a role and contribute to EA toxicity (Settivari et al., 2009; Brown et al., 2009). Intestinal absorption of numerous drugs and toxins can occur via passive diffusion (Eckert et al., 1978) but it is thought that carrier-mediated, or, active transport, may also contribute to drug absorption. Numerous transporters in the gastrointestinal tract have been identified. These include amino acid transporters, nucleotide transporters, and peptide transporters (Terada et al., 2004; Meredith et al., 2000). Peptide transporter 1 (PepT1) is an apically expressed solute carrier for peptides in the kidneys and brush border membrane of the intestines and acts in a proton dependent manner (Figure 4.1), (Adibi, 1997). PepT1 is responsible for dietary nitrogen uptake in the form of di- and tripeptides. It is pharmacologically and pharmaceutically relevant as it is hypothesized that PepT1 mediates drug transport because numerous drugs are hydrophilic in nature, limiting passive transport (Hironaka et al., 2008). PepT1 is a known transporter of ACE-inhibitors (Moore et al., 2000), thrombin inhibitors (Walter et al., 1995) and β -lactam antibiotics such as penicillins and cephalosporins (Brandsch, 2009; Hironaka et al., 2008; Terada et al., 1997; Bretschneider et al., 1999; Bailey et al., 2006). Because of the lactam ring present in ergorpeptams (Crews, 2015; Uhlig & Petersen, 2008) and peptide component of ergopeptides, it was hypothesized that ergot alkaloids are actively transported by peptide transporters.

Various studies have targeted transporters to improve bioavailability of drugs and nutrients (Cundy et al., 2004; Majumdar et al., 2004; Landowski et al., 2005). It is equally advantageous to target and identify transporters, such as PepT1, to decrease the bioavailability of toxic agents such as ergot alkaloids. To understand how and if ergot alkaloids are absorbed in the small intestine, the objective of this study was to assess the

involvement of PepT1 in the transport of the peptide alkaloids ergotamine (ERT) and ergovaline (EXT), and the ergoline alkaloid lysergic acid (LSA) across a Caco-2 cell monolayer (Figure 4.2).

Materials and Methods

Cell Maintenance. Caco-2 cells (passage 18) were obtained from American Type Culture Collection (ATCC) (HTB-37; Rockville, MD) and maintained in Falcon flasks (passages 25 -28), (75 cm², Becton-Dickinson, Bedford, MA) containing growth media consisting of Eagle's minimum essential medium (EMEM) with 2 mM L-glutamine, (ATCC, Manassas, VA), 20 % fetal bovine serum (FBS) (reduced IgG; ATCC), and 1% antibiotic/ antimycotic solution (10,000 U mL⁻¹ penicillin, 10 mg mL⁻¹ streptomycin, and 25 µg mL⁻¹ amphotericin B; Sigma Chemical Co., St. Louis, MO). Humidified incubators were maintained at 37°C and 5% CO₂. Cells were subcultured every five days (undifferentiated, subconfluent cells) using a trypsin (0.05%)-EDTA (0.02%) solution.

For transport experiments, 1×10^5 cells (passages 25 – 28) were plated in cell culture inserts (0.4 µm pore size, 1×10^8 pores/cm², 12 mm diameter, polyethylene terephthalate (PET); EMD Millipore Corporation, Billerica, MA) in 1 mL of the above growth media. Cell concentrations for seeding were determined by using a hand-held cell counter (Scepter 2.0; Millipore Sigma, Darmstadt, Germany). Inserts were placed in wells of a 12-well plate (Corning; #3513) containing 2 mL of the same media. Media in the apical side (upper chamber) and basolateral side (lower chamber) was replaced every other day.

Caco-2 Cell Monolayer Validation

Cellular differentiation and confluent monolayer establishment were determined by examining monolayers through a microscope, transepithelial electrical resistance (TEER) measurements, measuring sucrase activity, quantifying phenol red transport, and by assessing cytotoxicity of experimental compounds.

Transepithelial Electrical Resistance. Transepithelial electrical resistance (TEER) measurements were made using a TEER-meter (Millicell Electrical Resistance System; World Precision Instruments, Sarasota, FL) every other day. Electrodes were equilibrated in 0.15 mM NaCl for twenty-four hours before the initial measurements. Electrodes were sterilized for fifteen min in ethanol before measurements were taken. Measurements were performed within five min after taking culture plates out of the incubator to minimize temperature fluctuations. Electrodes were sterilized between plate measurements. To obtain a sample resistance, the unit area resistance ($\Omega \cdot \text{cm}^2$) was calculated by multiplying the sample resistance by the effective area of the membrane (1.1 cm^2 for 12-well Millicell inserts). Final TEER values were obtained after subtracting the resistance of the cell-free filter.

Sucrase Activity. Sucrase activity increases as enterocytes differentiate and is considered a good indicator of Caco-2 cell differentiation. Release of glucose in the apical compartment was measured using the fluorescent Amplex Red Glucose/ Glucose Oxidase Assay Kit (Cat. No. A22189; Molecular Probes, Eugene, OR). Sucrase activity of cells was measured on day 7 and day 18 in triplicate. Cells were washed with phosphate buffered saline (PBS; No. P4417, Sigma) twice to remove any pre-existing glucose that could possibly interfere with the reaction. Rinsed cells were pre-incubated at

37°C for 60 min with 1 mL of PBS and sucrose solution in the apical well and 2 mL of PBS in the basolateral well. After the 1 hour incubation, 200 µL of sample was collected at time intervals of 2, 4, 6, and 8 min. Samples were incubated at room temperature protected from light, for fifteen min. Amplex Red/ UltraRed Stop Reagent (Cat. No. A33855; Molecular Probes, Eugene, OR) was added to the sample to terminate the fluorescence signal-generating reaction in the enzymatic assay. Sample tubes were vortexed for approximately 3 seconds and sample fluorescence was measured using a fluorometer (Qubit 2.0; Invitrogen; Cat. No. Q32866, Carlsbad, CA).

Phenol Red Diffusion. Phenol red diffusion was used as a measure of cell monolayer integrity. Diffusion of phenol red was measured on day 7 and day 18 in triplicate. Culture media was removed from the apical and basolateral wells. Cells were washed with phenol red-free EMEM (Sigma Chemical Co.) twice, to ensure total removal of phenol red-containing growth media. One mL of phenol red containing growth media was placed in the apical insert and 2 mL of phenol red-free media was placed in the basolateral compartment. As a control, 2 mL of phenol red free and phenol red containing growth medium were incubated in wells with inserts. Cells were incubated at 37°C for 150 min. Aliquots of 200 µL were removed from the apical and basolateral chambers and plated in a 96-well plate. Absorbances were determined using a microplate reader (Synergy HT, Biotek Instruments, Inc., Winooski, VT) set to read at a wavelength of 546 nm using KC4 3.1 Rev 15 software (Biotek Instruments, Inc.). Absorbance values were corrected by subtracting the phenol red free absorbance values from the apical and basal phenol red absorbance values and percent diffusion was calculated using the formula:

$$\frac{\text{corrected basal (nm)}}{\text{corrected basal (nm)} + \text{corrected apical (nm)}} \times 100.$$

Cytotoxicity. An alamar blue assay (Thermofisher; Cat No. DAL1100) was used to assess cytotoxicity by quantifying the reducing environment of cells. The reducing environment in the alamar blue assay measures resazurin (oxidized form; blue) to resorufin (reduced form; pink). Cells for toxicity experiments (passages 25 – 27) were plated at 5×10^4 cells/well in 96-well plates (Falcon, Becton-Dickinson, Bedford, MA), using phenol red EMEM (Sigma Chemical Co.) containing 20% FBS and 1% A/A. Cells were allowed to grow for 7 days to create a semi-differentiated monolayer. Cells were exposed to 100 μ L of ERT, LSA, and EXT at concentrations of 100 μ M, 10 μ M, and 1 μ M with 10 μ L of alamar blue for 12 hours. Controls consisted of a positive growth control containing cells, HBSS media, and alamar blue and a negative control that contained HBSS media and alamar blue but no cells. All treatments were conducted in triplicate. Readings were taken at 30, 90, 360, and 720 min at absorbances of A_{570} nm and A_{600} nm in a multi-mode microplate reader (Synergy HT, Biotek Instruments, Inc.) using KC4 3.1 Rev 15 software (Biotek Instruments, Inc.). To determine the percent reduction of resazurin to resorufin, the following calculations were used:

1. % Reduced = C_{RED} test well / C_{OX} negative control well
2. % Reduced = $(\epsilon_{OX}) \lambda_2 A \lambda_1 - C_{OX}(\epsilon_{OX}) A \lambda_1 A \lambda_2 / (\epsilon_{RED}) \lambda_1 A' \lambda_2 - C_{OX}(\epsilon_{RED}) \lambda_2 A' \lambda_1) \times 100$

To calculate the percent difference in reduction between treated and control cells:

3. $(\epsilon_{OX}) \lambda_2 A \lambda_1 - (\epsilon_{OX}) \lambda_1 A \lambda_2$ of test agent / $(\epsilon_{OX}) \lambda_2 A^\circ \lambda_1 - (\epsilon_{OX}) \lambda_1 A^\circ \lambda_2$ of untreated positive growth control) $\times 100$

Where C_{RED} = is the concentration of reduced alamar blue, C_{OX} is the oxidized form of alamar blue, ϵ_{OX} is the molar extinction coefficient of oxidized alamar blue, ϵ_{RED} is the

molar extinction coefficient of reduced alamar blue, A is test well absorbance, A' is the absorbance of negative control wells, A° is the absorbance of positive growth control wells, λ_1 is A_{570} nm, and λ_2 is A_{600} nm.

Preparation of Treatment Stocks

To make stock standards for cytotoxicity and transport experiments, 100 μ M cephalixin hydrate (CEPH; C4895, Sigma Chemical Co.), a positive control for PepT1 was prepared in phenol red free Hank's balanced salt solution (HBSS, H8264, Sigma Chemical) modified with sodium bicarbonate (S5761, Sigma Chemical). D-lysergic acid dihydrate (95%; Acros Organics, Geel, Belgium) and ergotamine D-tartrate ($\geq 97\%$ purity, #45510, Fluka, Sigma Chemical Co.) were weighed out. Ergotamine was brought up in solution with methanol and 100 μ M standards of lysergic acid were prepared in 80% (vol/vol) methanol acidified with 1.2×10^{-4} M acetic acid. Ergovaline was prepared in phenol red free HBSS and vacuum filtered. Analysis showed that filtering did not alter the concentration of ergovaline present in the media. Standards only containing cephalixin, ergotamine, lysergic acid, and ergovaline were further diluted to create 10 μ M and 1 μ M stock standards. A solution of 0.5 mM nateglinide (NATE) (Cat. No. 4231, Tocris Bioscience, Minneapolis, MN), an inhibitor of PepT1, was prepared in dimethylsulfoxide (DMSO; 25-950-CQC, Mediatec Corning, Manassas, VA), and brought to volume with media containing the experimental compounds.

Transport Studies

Experimental Treatment of Cells. Apical to basolateral transport studies were conducted 21 days post-plating when cells were fully differentiated. Growth media in the apical and basolateral compartments was removed. Cells were washed with Hank's

balanced salt solution (HBSS) to remove traces of growth media. Media in the basolateral compartment was changed to 2 mL HBSS and media in the apical compartment was composed of 1 mL of HBSS containing 1 μ M, 10 μ M, or 100 μ M of cephalexin, ergotamine, lysergic acid, and ergovaline in the presence or absence of 0.5 mM of nateglinide. Samples (200 μ l) were collected from apical and basolateral compartments over a 12-hour period at 30, 60, 90, 180, 360, and 720 min. Samples that were immediately collected were stored in the dark at 4°C until analysis. Remaining media in the apical and basolateral compartments was collected and stored at -20°C in amber vials (Waters, Product Number 600000669CV, Milford, MA).

Liquid Chromatography-Mass Spectrometry. Aliquots of collected apical and basolateral media were thawed and diluted with 10% acetonitrile/water (Fisher Optima) so samples would fall within the range of standards and spiked with 20 μ L of an internal standard, methysergide (MYS; Sigma Chemical, St Louis, MO). Inserts were thawed and 200 μ L of Millipore water was added to the tubes and tubes were sonicated for 5 min. A 780 μ L-aliquot of 10% acetonitrile was added to the tubes and tubes were centrifuged for 15 seconds. Contents of the tubes were transferred to amber vials and spiked with 20 μ L of MYS. Samples were analyzed by ultra-high pressure liquid chromatography (UPLC) tandem mass spectrometry (MS/MS) (Waters Acquity triple quadrupole (TQD) mass spectrometer (product info?). Chromatographic separations were obtained using a reverse-phase ultra-performance liquid chromatography (UPLC) column (Waters BEH C18, 1.7 μ m, 2.1 mm x 150 mm). The mobile phase consisted of water containing 0.1% formic acid (solvent A; Fisher) and acetonitrile containing 0.1% formic acid (solvent B) in a linear gradient from 25% B to 75% B at a flow rate of 0.35 mL/min. The TQD

tandem mass spectrometer was operated in positive ion multiple reaction monitoring (MRM) with electrospray ionization (ESI) using nitrogen as the nebulizing gas and argon as the collision gas. Chromatograms are shown for CEPH (Figure 4.3a), ERT (Figure 4.3b), EXT (Figure 4.3c), and LYS (Figure 4.3d)

Statistical Analysis

All data were analyzed using SAS (SAS 9.3, SAS Inst. Inc., Cary, NC). Phenol red data was analyzed using PROC GLM and a multi-means comparison test with day as the independent variable and percent diffusion as the dependent variable. Sucrase activity was analyzed using a nested design with min nested in day. A one factor ANOVA was utilized with LSD measurements and a Tukey post hoc test. Transport data was analyzed using PROC GLM and an orthogonal polynomial contrast to determine the best fit of increases and decreases in the appearance of analyte in the apical and basolateral chambers. Interaction effects were analyzed with PROC GLM between apical chamber with and without inhibitor; basolateral chambers with and without inhibitor; apical chambers compared to the no cell control; and basolateral chambers compared to the no cell control. For comparisons of apical chambers and basolateral chambers, time x NATE effects, time effects, and NATE effects were analyzed. For comparisons with the no cell control, time x cell effects, time effects, and cell effects were analyzed. A Shapiro-Wilk and Bartlett's test were used to determine normality of data. All values are expressed as the mean \pm SEM. Results are considered significant if probabilities are $P < 0.05$, unless reported otherwise.

Results and Discussion

Prior work by Shapell and Smith (2005) demonstrated that ergovaline was readily absorbed across a Caco-2 cell monolayer. This study elaborated on previous work by examining the intestinal absorption of ergotamine, ergovaline, and lysergic acid, specifically by the transporter, PepT1. All significance values for time x NATE, time, and NATE effects are listed in Tables 4A – D in Appendix 1 and regression analyses for all tested compounds are listed in Table 4.1 All experimental wells (apical + inhibitor (AP+), apical without inhibitor (AP-), basolateral with inhibitor (BA+), and basolateral without inhibitor (BA-)) were compared to a no cell control (NCC) that were exposed to the same conditions that the other wells were exposed to for 21 days to determine if the cell layer or the support membrane in the insert influenced transport of CEPH, ERT, EXT, and LYS.

Cephalexin is a typical substrate for PepT1 (Dantzig and Bergin, 1988; Yang et al, 1999) and was demonstrated to have a relatively high affinity (K_m of 7.5 ± 2.8 mM) for PepT1 in intestinal segments of rats (Hironaka et al., 2008) and Caco-2 cells (Bretschneider et al., 1999). Therefore, cephalexin was used as a model in this study to demonstrate that PepT1 was functional in this system. To further show that PepT1 was functional in this study, it was inhibited using the hypoglycemic agent nateglinide. Nateglinide is a dipeptide-like drug in that it possesses a carboxyl group and peptide bond in its structure. It was demonstrated in Caco-2 cells that nateglinide inhibited the transport activity of both PepT1 and PepT2, but nateglinide itself was not transported by these transporters (Terada et al, 2000).

Intestinal absorption of numerous compounds is mainly by passive diffusion, but it is also believed that numerous membrane transporters are involved. Due to the complexity and relationship between different modes of influx and efflux, exact mechanisms and actions of compound transport are vague. Dipeptides and tripeptides are taken up by cells by the low affinity H⁺/ peptide cotransporter PepT1. Because ergotamine and ergovaline have a tri-peptide component to their structure, it was hypothesized that PepT1 may be involved in their transport from the intestinal lumen to the blood. Lysergic acid was proposed as a potential toxic agent in fescue toxicosis (Hill, 2005) and was later determined to be a product of rumen metabolism of ergopeptines (DeLorme et al., 2007). Therefore, lysergic acid is also a compound of interest regarding intestinal transport. Based on previous research that showed that lysergic acid and lysergol were transported across ruminal gastric tissues (Hill et al., 2001; Ayers et al., 2009), it was hypothesized that lysergic acid may also be transported in intestinal tissues.

Caco-2 Cell Monolayer Model Validation

Microscopy. Various studies disagree with the use of Caco-2 cells at days 18 – 21. Some believe that cells in culture as early as 6 – 9 days postseeding should be used in experimental studies (Trotter and Storch, 1991). Other studies call for using cells that are 30 days in culture (Thwaites et al., 1994). However, Briske-Anderson et al. (1997) concluded that Caco-2 cell monolayers were fully differentiated by days 15 – 18. Briske-Anderson et al. (1997) found that at days 15 - 18, sucrase and alkaline phosphatase activity are at a maximum and that cell numbers are stabilized, indicating that cell proliferation is complete. Because of these characteristics, cell models at days 18 -21

postseeding were used for homogenous cell number, density, and enzymatic purposes in the current study.

The degree of morphologic differentiation of Caco-2 cells grown in T75 cm² flasks was evaluated in Figure 4.4. Images of Caco-2 cells before seeding in wells were captured on days 1, 3, 7, 14, 18, and 21. Cells were undifferentiated on day 1 as seeding had just occurred. By day 3, cell nuclei were visible and cells were beginning to form monolayers. On days 7 and 14, cells were semi-differentiated, as full monolayers were present. The differentiation process occurs in a mosaic pattern meaning that some areas in cell culture will express fully differentiated cells as soon as days 12 to 14 while other areas will be less differentiated (Lea, 2015). By days 18 and 21, cells were fully differentiated and should, therefore, be fully polarized as indicated by TEER values. Cell layers should form a continuous barrier between the apical and basal compartment by day 21 if growth in flasks is an indicator. This is in agreement with data presented by Briske-Anderson et al. (1997).

Transepithelial Electrical Resistance (TEER) Values. Transepithelial electrical resistance (TEER) measurements were recorded every other day beginning on day 2 (undifferentiated) and ending on day 20 (fully differentiated) (Figure 4.5). Measured TEER values generally ranged from 100 to 550 Ω cm² with lower values correlating to days early in culture and higher values correlating to later days in culture. Similar values were reported in Caco-2 cells and INT 407 cells by Ismail (1999) and Yamashita et al. (2002). These values are sufficient for transport studies as determined by Bohers et al. (2001), Nigsch et al. (2007), and Balimane and Chong (2005). The TEER for cells used in experiments for CEPH, LSA, and EXT reached the maximum value at day 10 in

culture and TEER for the ERT experiment reached a maximum at day 12. The TEER values began to gradually decrease afterwards for all cells in culture before plateauing. The TEER values can be influenced by cell passage number, seeding density, temperature at the time measurements were made, and culture conditions. Usually, decreasing TEER values are indicative of cell death or poor monolayer formation. However, Derk et al. (2015) conducted a study for testing the effect of exposure nanoparticles on the lung alveolar epithelial barrier using the human lung epithelial cell line, Calu-3. They evaluated monolayer formation by measuring electrical resistance of cell monolayers and found that FBS concentration in culture medium greatly affected epithelial monolayer integrity. Each well was seeded at the same concentration (5.0×10^4 cells/well) with increasing concentrations of FBS (2%, 5%, 10%, and 15%). Lower concentrations (2% and 5%) resulted in significantly higher TEER values than those cells cultured in 10% and 15% FBS. Culture medium supplemented with 10% FBS showed consistent TEER values while medium containing 15% FBS demonstrated larger variation in TEER values. Yamashita et al. (2002) also observed variability of TEER values when cells were exposed to FBS. It was found that longer culture period did result in tighter monolayers. The maximum TEER value was reached within 5 days, but after 5 days in culture, monolayer integrity gradually decreased. It was also found that transporter activity was highest at days 5 and 6 in culture. To obtain the highest activity of the transporters P-gp and PepT1, Yamashita et al. (2002) developed a modified short-term protocol with FBS-containing medium. Their results indicated that this simple modification allowed them to obtain monolayers with better barrier properties and transport activity equivalent to the 21-day culture system. Therefore, the amount of supplemental FBS used in this study

(20%) and the length of time in culture may have led to the variation in TEER values.

The TEER data in the current study indicate that a confluent monolayer was obtained and maintained up to day 21 and the transport study.

Phenol Red Diffusion. Phenol red diffusion is a simple, non-destructive method of assessing the confluency of cell monolayers as culture media already contained phenol red. Cell monolayer integrity is verified by a lack of phenol red in the basolateral chamber. Diffusion of phenol red from the apical to the basal compartment was significantly lower ($P < 0.0006$) on day 18 with an average percent diffusion of 2.5% (Figure 4.6). Day 7 exhibited an increased amount of phenol red diffusion with an average of 21.5% diffusion. This is indicative that cell monolayers are confluent by day 18 and agrees with previously measured TEER values. Ismail (1999) also used phenol red diffusion as a measure of monolayer confluency in Caco-2 cells and the HeLa derivative cell line INT 407. Diffusion in both cell lines was measured over a period of 20 days. On day 5, diffusion of phenol red was around 15%, lower than the value reported for day 7 in this study. On day 17, phenol red diffusion values had reached a constant of around 6%, higher than the value reported in the present study. The differences in the diffusion values obtained by Ismail (1999) may be a consequence of differing of membrane inserts used, cell line used, type of medium used (DMEM), and the amount of FBS supplemented (10%) compared to the current study.

Sucrase Activity. Small intestine brush border enzymes are typically assayed using tissue homogenates, membrane preparations (Dahlqvist, 1968), or intact small intestinal preparations (Lee et al., 1998) with sucrase activity linearly increasing during cell differentiation (Galand, 1989). Therefore, it is considered a good indicator of Caco-2

cell differentiation (Van Beers et al., 1995). Sucrase is a catalyst that causes the hydrolysis of sucrose to glucose and fructose. It is generally measured from microvilli fractions or whole cell homogenates (Ferruzza et al., 2011). Figure 4.7 shows non-significant differences between concentrations at min 2, 4, 6 and 8 within days 7 and 18. However, across days sucrase activity on day 18 was greater at all measured timepoints ($P < 0.0001$) than on day 7, indicating that cell monolayers were viable and intact. This is similar to the findings of Ferruzza et al. (2011). Using a Caco-2 cell line, Ferruzza et al. used a glucose assay kit to measure sucrase activity of cells growing on cell inserts. Sucrase activity was measured (nmol/mL) between 1 and 8 min on days 8, 15, and 21 in culture. Data showed linear increases as time went on, with activity being significantly higher ($P < 0.01$) on day 21 as compared to sucrase activity on days 8 and 15, consistent with current findings that the longer in culture, the higher the sucrase activity of cells. Based on physical morphology, TEER values, phenol red exclusion data, and, sucrase activity, the Caco-2 cells in culture for 21 days were confluent, exhibited sufficient monolayer integrity, and were differentiated and ready for ergot alkaloid transport studies.

Cytotoxicity of Ergotamine, Ergovaline, and Lysergic Acid. An alamar blue assay was used to assess the toxicity of three different concentrations (1, 10, and 100 μM) of ergotamine, ergovaline, and lysergic acid on semi-differentiated Caco-2 cells. Resazurin is a blue colorimetric indicator that will reduce to a pink, fluorescent form, resorufin, in response to cellular metabolism. The intensity of the fluorescence produced is proportional to the amount of living cells and can be used to quantitatively measure toxicity and cell viability. Alamar blue is desirable as an indicator as it does not interfere

with the electron transport chain nor does it affect cell respiration and function like other redox indicators. Results were reported as percent reduction of the indicator resazurin and percent inhibition of Caco-2 cell growth.

Ergotamine. Reduction of resazurin to resorufin increased from 1 h to 3 h where it remained constant until the end of the experiment at 12 h in response to all three concentrations of ergotamine (Figure 4.8). At 6 h, there was an increase in reduction but it decreased to levels seen at 3 h by the end of the experiment at the 100 μM concentration level (Figure 4.8). Average reduction levels ranged from 45% to 56%, indicating that only half of resazurin was reduced to resorufin, which could indicate that only half of the available cells were living or functional. Ergotamine has been found to show a weak cytotoxic effect with a decline in viability to 80% in renal proximal tubule epithelial cells (Mulac and Humpf, 2011). Ergotamine cytotoxicity was assessed using a CCK-8 assay with concentrations ranging from 1 nM to 20 μM . Although effects were weak, a clear cytotoxic effect was observed in comparison to the control cells at the concentration range of 5 μM to 20 μM . A second experiment studying the impact of ergot alkaloids on the blood-brain barrier also showed that ergotamine had no impact on cell viability (Mulac et al., 2011). It is possible that reduction of resazurin was only at around 50% because of the state the cells were in. As cells were at a semi-differentiated state in the current experiment, tight junctions would not be fully formed, nor would a cell monolayer be completely intact.

Lysergic Acid. Lysergic acid was not toxic to cells at concentration. At 1 h, reduction ranged from 41% to 51% (Figure 4.9). By hour 3, more than 100% reduction had been reached at the 100 μM and 10 μM levels and reduction was at 97% at the 1 μM

level. By the sixth hour, reduction was beyond 100% at all concentrations. There are few studies assessing the toxicity of lysergic acid on cells, but there are studies assessing other derivative lysergic acid amides. Ergotmetrine (ergonovine) is one such derivative. Documented previously regarding ergotamine toxicity, the study by Mulac and Humpf (2011) assessed the toxicity of ergotmetrine using the CCK-8 assay, LDH activity, and capase-3 activation. Ergotmetrine did not have any cytotoxic effects on RPTEC and was not taken up by the cells during uptake studies. Although ergotmetrine is a derivative of lysergic acid and toxicity was assessed in a different cell line, based on this information and the results of the alamar blue assay, it can be said that lysergic acid is not toxic to Caco-2 cells.

Ergovaline. Ergovaline in a tall fescue seed extract was not toxic to cells at the 1 μM concentration. One hundred percent reduction was observed by hour 6 and cell growth was only inhibited by 7% at the first hour (Figure 4.10). Ergovaline at 10 μM and 100 μM concentrations was toxic to semi-differentiated cells. Reduction of resazurin increased from the first hour for both concentration levels but reduction was the same from 3 h to the end of the experiment (Figure 4.10). The 100- μM concentration may have been more toxic than 10 μM ergovaline as the overall average inhibition of cell growth was 44% (56% viability) at the 100 μM and 30% (70% viability) at 10 μM . In a similar study using Caco-2 cells, ergovaline toxicity was assessed using mitochondrial succinate dehydrogenase activity, alamar blue (cytochrome oxidase activity), and DNA quantitation assays (Shapell, 2003). Concentrations of 10^{-11} to 10^{-4} M to Caco-2 cells and toxicity were assessed after 24 and 72 h of exposure on days 1, 8, and 18. It was determined that undifferentiated cells were the most sensitive to 10^{-4} M ergovaline. After

72 h, undifferentiated cells in all three assays had activities reduced to less than 10% of the control means. At day 11, ergovaline toxicity had decreased in semi-differentiated cells. By day 18, alamar blue activity decreased and no change in DNA occurred until after 72 h of exposure. These findings indicated that ergovaline toxicity was dependent on differentiation state and that ergovaline was not toxic to differentiated cells. Cells tested in this study were semi-differentiated and, it can therefore be assumed that cells were more sensitive to ergovaline on day 7, but were most likely resistant by the time transport studies were conducted at day 21 when cells were fully differentiated.

Cellular Transport and Mass Spectrometry Analysis

Insert, No Cell Control, and 1 μ M Data. Cell monolayers were collected and the insert concentrations were analyzed to see if cells or the insert membrane contained accumulations of cephalexin, ergotamine, ergovaline, or lysergic acid; however, no significant concentrations were collected from inserts across any concentration. Therefore, this data was excluded from this study. Regarding the no cell control data, in Figure 4.11 the presence of cells in experimental wells appears to play a role in inhibiting transport of all compounds to the basolateral chamber as compared to wells that do not contain cells. It can also be suggested that Nateglidine exacerbates this effect. As the effects noted in Figure 4.11 were similar in wells across all compounds at every concentration, data were not included. It should also be noted that due to the fact that the results from the 1 μ M data were inconsistent and biologically irrelevant, these data were also omitted.

Cephalexin and Lysergic Acid – 10 μ M. At 10 μ M cephalexin, there was some inhibition of 10 μ M cephalexin and lysergic acid. A time effect ($P = 0.002$) and a NATE

effect ($P= 0.02$) were observed between CEPH AP+ chambers and CEPH AP- chambers. As time increased, the amount of CEPH in the both apical chambers decreased (Figure 4.12). The chambers containing nateglinide had a greater mean percent recovery of cephalixin compared to that of CEPH AP- ($P = 0.02$) meaning that the inhibitor did allow for some inhibition of cephalixin transport overall; however, CEPH AP+ and CEPH AP- did not differ from each other from timepoint to timepoint (Figure 4.12). The 10 μ M concentration of cephalixin may have been inhibited by nateglinide, but this inhibition is not time dependent.

At the 10 μ M concentration level, the transport of lysergic acid was inhibited from 180 min onwards (Figure 4.13). A time x NATE interaction effect was observed ($P = 0.01$), indicating that both time and the presence of NATE affected the percent recovery obtained from the apical wells. As time increased, the concentration of lysergic acid present in the Lysergic acid AP- wells decreased linearly ($P < 0.0001$); however, the concentration of lysergic acid present in the LSA AP+ wells remained constant and concentrations did not differ from one another. There was also a time x NATE effect ($P < 0.0001$) between the LSA BA+ and LSA BA-; as time increased the amount of lysergic acid in the basolateral compartment increased for cells not exposed to NATE; cells exposed to NATE had lower concentrations of lysergic acid present in the basolateral chamber and the amounts present did not differ from one another. A significant increase in lysergic acid in LSA BA- was not seen until the 180 min timepoint; from there the concentration increased from 2.8% recovery to 4.4% at the 360 min timepoint, and from 4.4% to 6.2% recovery at the 720 min time point (Figure 4.13). These results indicate that inhibition of PepT1 reduced the apical to basal transport of lysergic acid.

Ergotamine and Ergovaline – 10 μ M. At the 10 μ M concentration level, NATE did not inhibit transport of 10 μ M ergotamine. A time effect was observed between ERT AP+ wells and ERT AP- wells; as time progressed, the concentration of ergotamine in both AP+ and AP- decreased ($P = 0.0002$) but there was no difference in concentrations of ergotamine present in the apical chambers of ERT AP+ or ERT AP-. A time x NATE interaction effect was reported between ERT BA+ wells and ERT BA- wells ($P = 0.004$); however, this only appears to be significant due to a large flux of ergotamine that was present at the 30 min timepoint (Figure 4.14).

At the 10 μ M concentration level, a time effect ($P < 0.0001$) and a NATE effect ($P = 0.01$) are observed between EXT AP+ wells and EXT AP- wells (Figure 4.15). As time decreases, the concentration of ergovaline in both AP+ and AP- wells decreases with a corresponding increase in BA+ and BA- wells. Overall, there is a greater average percent recovery in the AP+ wells than the AP- wells ($P = 0.01$). There is an effect of NATE present, but it is not time dependent. A time effect is present between EXT BA+ and BA- wells ($P < 0.0001$) but there are no differences in concentrations. Thus, NATE does appear to inhibit transport of ergovaline, but its effects are not exacerbated by time.

Cephalexin and Lysergic Acid – 100 μ M. At the 100- μ M level, a NATE effect was observed between 100 μ M CEPH AP+ and CEPH AP- (Figure 4.16). Cephalexin AP+ was only different from CEPH AP- up to the 90 min timepoint. At 90 – 180 min, NATE appears to lose its effectiveness and the concentrations of cephalexin in the AP+ and AP- chambers are the same. In the basolateral chambers with NATE as compared to CEPH BA-, there was a time effect and a NATE effect. There was an increase in the appearance of cephalexin in the basolateral chambers of both BA+ and BA- as time

progressed ($P < 0.0001$). However, the appearance of cephalexin in the BA+ chambers was less than that of the BA- (19% v 30%; $P = 0.01$), indicating that NATE inhibited overall transport of CEPH, but was not time dependent. The only difference in concentration was the 30 min timepoint. After 30 min, concentrations in the basolateral chambers were the same (Figure 4.16). Therefore, cephalexin transport may be inhibited by nateglinide overall but IT may lose its effectiveness over time.

At the 100 μ M concentration level, NATE inhibited the transport of 100 μ M lysergic acid (Figure 4.17). A NATE effect was observed between LSA AP+ and LSA AP- ($P = 0.001$), suggesting that NATE does contribute to inhibition and retaining lysergic acid in the apical chamber. Lysergic acid AP- wells decreased in a linear fashion (Table 4.1; $P = 0.01$) while LSA AP+ does not decrease at all, staying the same across all timepoints. Wells containing NATE had a greater average percent recovery compared to LSA AP- wells ($P = 0.01$; Figure 4.17). As the concentration of lysergic acid is not different between AP+ and AP- until 180 min, it is plausible to assume that NATE needs time to take effect and that it may not reach its maximal effect until after 180 min. This is further evidenced by the findings in the basolateral chambers. A NATE effect ($P = 0.01$) was observed between LSA BA+ and LSA BA- with LSA BA- increasing quadratically (Table 4.1; $P = 0.006$). An average of 26% LSA was recovered in LSA BA- wells as compared to an average of 3% recovery in LSA BA+ chambers. LSA BA+ chambers contained lower concentrations of lysergic acid that did not differ from one another across time until the 720 min timepoint; concentrations in LSA BA+ and LSA BA- differed from the 360 min timepoint on.

Ergotamine and Ergovaline – 100 μ M. There was a time x NATE effect ($P = 0.002$) between ERT AP+ and ERT AP- wells (Figure 4.18). As time increased, the concentration of ergotamine decreased in the apical chamber. Although the concentration of ergotamine present AP+ wells decreased over time also, the presence of the inhibitor at 180 min still resulted in a greater concentration of ergotamine in the AP+ compared to AP- at 180 min. There was a time x NATE effect ($P < 0.0001$) between ERT BA+ and ERT BA-. As time increased, the appearance of ergotamine in the basolateral chamber increased in both ergotamine BA- and ERT BA+; however, NATE appears to have inhibited the transport of ergotamine as the untreated wells had larger concentrations of ergotamine in the basolateral chamber than cells apically treated with NATE. ERT BA- and ERT BA+ did not begin to differ until 180 min, indicating that perhaps, the effectiveness of NATE increased as time progressed (Figure 4.18). It can be concluded from these results that ergotamine transport is inhibited by nateglinide at the 100 μ M concentration, but it is time-dependent.

A time effect and NATE effect are present between EXT AP+ and EXT AP- (Figure 4.19). As time progresses, there is a decrease in the concentration of ergovaline in both EXT AP+ and EXT AP- wells ($P < 0.0001$). Wells that contain NATE have a greater overall percent recovery of ergovaline compared to recovery of ergovaline in AP- ($P < 0.0001$). There appears to be inhibition of ergovaline transport by NATE but only until the 180 min timepoint. A time effect is present between EXT BA+ and EXT BA-; the amount of ergovaline present in the basolateral chamber increases as time progresses ($P = 0.01$). The concentration of ergovaline present in the EXT BA+ wells and EXT BA- wells does not differ (Figure 4.19). These results indicate that NATE may inhibit the

amount of ergovalinetransported from the apical to the basolateral chamber overall, but the NATE effect appears to have limit over time.

Overall Findings

It was determined that the CEPH-NATE model was an appropriate model to use regarding PepT1 inhibition as cephalixin transport was inhibited by NATE at two concentration levels, 10 μ M and 100 μ M. In the current study, ergotamine was transported across the cell monolayer, indicating that ergotamine may primarily be transported in the small intestine. Ergotamine does not appear to utilize PepT1 until it is at the highest concentration level (100 μ M). However, inhibition is incomplete as transport is still occurring in chambers treated with nateglinide; transport by PepT1 may not be completely inhibited and could be working in tandem with another form, whether it be passive, active, or a combination. (Ganapathy et al., 1995). Evidence of such statements is provided in findings observed using porcine brain capillary endothelial cells (PBCECs) as a model of the human blood-brain barrier (Mulac et al., 2011). Mulac et al. (2011) were interested in the extent to which EA are able to cross the blood-brain barrier and their effects on barrier integrity. The alkaloids ergometrine, ergocristinine, ergotamine, ergocornine, α -ergocryptine, and ergosine were of primary interest as these were identified as the most relevant alkaloids regarding toxicity. All compounds contain an optically active carbon atom at the C-8 position and can form epimers under certain conditions including light, pH change, and temperature change. Ergometrine (ergoline alkaloid), ergocristine, and ergotamine (ergopeptine alkaloids) were chosen for permeability studies as cells exposed to these ergot alkaloids maintained barrier integrity after exposure. For permeability studies, 10 μ M of ergotamine was used. It was found

that ergotamine does have the ability to reach the brain, but no active transport properties were identified for it or ergocristine. Within hours, both ergopeptides were able to cross the blood-brain barrier. Ergometrine was also able to cross the blood-brain barrier but it was determined that it moved via active transport and is a substrate for BCRP, or breast cancer resistant protein. Although the cells used in this study were a model for the blood-brain barrier, the SI also has a high concentration of BCRP, which plays a role in intestinal absorption. As ergometrine is a derivative of lysergic acid, it may be suggested that lysergic acid is not only transported by PepT1. This aligns with the results of lysergic acid transport at 10 μ M and 100 μ M as transport to the basolateral chamber was not completely inhibited, even in the presence of nateglinide, indicating that another transporter or form of transport is involved.

Because cephalixin, ergovaline, and lysergic acid transport was reduced by nateglinide at the 10 μ M and 100 μ M concentration levels, it can be concluded that PepT1 plays some part in transport of ergot alkaloids from the lumen to the blood. Based on the results of the alamar blue assay, neither ergovaline nor lysergic acid was toxic by hour 12 to fully differentiated cells. It cannot be ruled out that passive transport or another transporter could be working in tandem with PepT1 as inhibition was not complete for any of the compounds at the 10 μ M and 100 μ M concentration levels, including the model compound cephalixin. As demonstrated in rat intestinal segments, PepT1 substantially contributed to oral absorption of cephalixin, but the function of PepT1 could be compensated by passive diffusion if cephalixin were presented at a therapeutic dose (Hironaka et al., 2008).

Ergovaline-containing tall fescue seed extract was shown to be non-toxic at the 1 μM concentration level and was considered non-toxic at higher concentrations in differentiated cells (although, in this study, it was toxic at the semi-differentiated state) based on results of the alamar blue cytotoxicity assay and previously conducted experiments. It was observed in this experiment that the concentrations 10 and 100 μM of ergovaline do move across the cell monolayer in the presence and absence of NATE, but concentrations of ergovaline are less in apical wells exposed to nateglinide. This indicates that ergovaline most likely uses PepT1, although inhibition of transport did not persist over the 12-hour time period. Similar, to ergotamine, it may also use a secondary form of transport to move across the cell monolayer. A similar experiment was conducted using a Caco-2 cell model to evaluate the movement of ergovaline and its isomer ergovalanine across the intestinal barrier to establish a route of dietary absorption (Shappell and Smith, 2005). A pre-equilibrated ergovaline and ergovalanine mixture (60:40 ratio) at two concentrations (6 μM and 22 μM) was added to the apical compartment of cell inserts. Extracted media was assessed by high performance liquid chromatography. Active transport was not evaluated in this study, but it was determined that ergovaline and ergovalanine readily crossed Caco-2 cells at similar rates. Similar to this study, cells did slow the transport of ergovaline to the basolateral chamber. In this study, 100% recovery was not achieved for most of the compounds.

Anatomic location, route of compound administration, and physiological properties play a role in how compounds are transported and absorbed. Hill et al. (2004) evaluated whether transport of alkaloids was a passive or active process depending on the location in gastric tissues. Hill et al. (2004) suggested that alkaloid transport was an

active process in ruminant gastric tissues. This study differed in that ergoline and ergopeptide alkaloid transport was studied in isolated sheep gastric tissues, specifically reticular, ruminal, and omasal tissues. Ruminal and omasal tissues were removed, placed in parabiotic chambers, and exposed to equimolar concentrations of lysergic acid, lysergol, ergonovine, ergotamine, and ergocryptine. Tissues were incubated for 240 min and analyzed by competitive ELISA and freeze-dried serosal Krebs' Ringer phosphate solution containing alkaloids was analyzed using high-pressure liquid chromatography. Ruminal tissue had greater alkaloid transport than omasal tissues due its surface area. The ruminal posterior sac had the greatest potential for alkaloid transport and the reticular tissues displayed the smallest amount of alkaloid transport. Similar to the findings of this study, lysergic acid was transported actively. Not only was lysergic acid and lysergol transported across omasal tissues, they were transported in larger quantities than the ergopeptide alkaloids, especially in omasal tissues. Of the alkaloids tested, lysergic acid had the greatest concentration in serosal chamber regardless of tissue type. These results indicated that simple ergoline alkaloids, such as lysergic acid and lysergol, were more likely to cross digestive barriers than ergopeptide alkaloids, such as ergotamine and ergovaline. Ayers et al. (2009) confirmed these findings by observing that lysergic acid was transported in greater quantities than ergovaline in both ruminal and omasal tissues. It was demonstrated by Hill et al. (2004) that ergotamine and ergocryptine had the least affinity for alkaloid transport among all tissues, with reticular tissues transporting the least amount of tissues

In the current study, it appears as though the ergoline, lysergic acid, and the ergopeptides, ergovaline and ergotamine, move readily across a cell layer representing

the small intestine. They may use the transporter PepT1, but it is highly plausible that this is not the only form of transport available to ergot alkaloids. Although both ergovaline and ergotamine were readily absorbed, ergotamine requires a greater concentration (100 μM) to be transported when compared to ergovaline, at which transport begins at 10 μM . This indicates that ergovaline may be more readily absorbed and be available in the blood sooner than ergotamine, possibly playing a larger part in symptoms of fescue toxicosis than ergotamine. Lysergic acid was also readily transported at the same concentrations as ergovaline, indicating that, it too, may play a larger role in fescue toxicosis symptoms than ergotamine, as previously suggested.

Conclusions

Ergotamine and lysergic acid were not toxic to semi-differentiated cells. High concentrations of ergovaline in a seed extract was toxic to semi-differentiated cells. Ergotamine transport was not inhibited by nateglinide until the 100 μM concentration level. This indicates that apical transport of ergotamine across an epithelial barrier may use alternative protein transporters, passive diffusion, or a combination of transport modes. It is also plausible that it does not play as large a role in toxicosis symptoms as lysergic acid and ergovaline. Cephalixin, lysergic acid, and ergovaline transport was reduced by the presence of nateglinide at the 10 μM and 100 μM concentration levels. This provides further evidence that lysergic acid and ergovaline are involved in some facet of fescue toxicosis. Further work should focus on identifying whether other forms of transport in the small intestine play a role in transport of ergot alkaloids to areas outside of the GI tract. It would also be ideal to conduct similar studies with these

compounds using harvested tissues or an animal model. Finally, an inhibition study needs to be conducted to assess the affinity of these compounds for PepT1.

Table 4.1. Orthogonal polynomial contrast regressions of cephalixin (CEPH), ergotamine (ERT), ergovaline (EXT), and lysergic acid (LSA).

| Compound | Concentration (μM) | AP+ ¹ | AP- | BA+ | BA- |
|----------|--------------------|------------------------|---------------------|---------------------|----------------------|
| CEPH | 1 | Linear; < 0.0001 | Linear; 0.0005 | Quadratic; 0.02 | Quadratic; 0.04 |
| | 10 | Linear; 0.01 | Linear; 0.003 | Linear; 0.009 | Linear; 0.0006 |
| | 100 | Linear; 0.008 | - ² | Linear; 0.0001 | Quadratic; 0.03 |
| ERT | 1 | Quadratic; 0.0001 | - | Quartic; 0.002 | - |
| | 10 | Quadratic; < 0.0001 | Linear; 0.001 | Cubic; 0.004 | Linear; 0.005 |
| | 100 | Linear; 0.0012 | Linear; < 0.0001 | Quartic; 0.003 | - |
| EXT | 1 | - | Quadratic; 0.02 | - | - |
| | 10 | Linear; 0.004 | - | Quadratic; 0.03 | Linear; < 0.0001 |
| | 100 | Linear; < 0.0001 | Linear; 0.0006 | - | Quadratic; 0.0007 |
| LSA | 1 | Quadratic; 0.0006 | Linear; < 0.0001 | - | - |
| | 10 | Linear; < 0.0001 | Linear; < 0.0001 | Linear; < 0.0001 | Quadratic; 0.02 |
| | 100 | - | Linear; 0.01 | - | Quadratic; 0.006 |

¹AP+: apical chamber with inhibitor; AP-: apical chamber without inhibitor; BA+ basolateral chamber with inhibitor; BA-: basolateral chamber without inhibitor

²Dashes show that there was no regression pattern present.

Figure 4.1 Peptide transporter 1 cellular mechanism in intestinal enterocytes

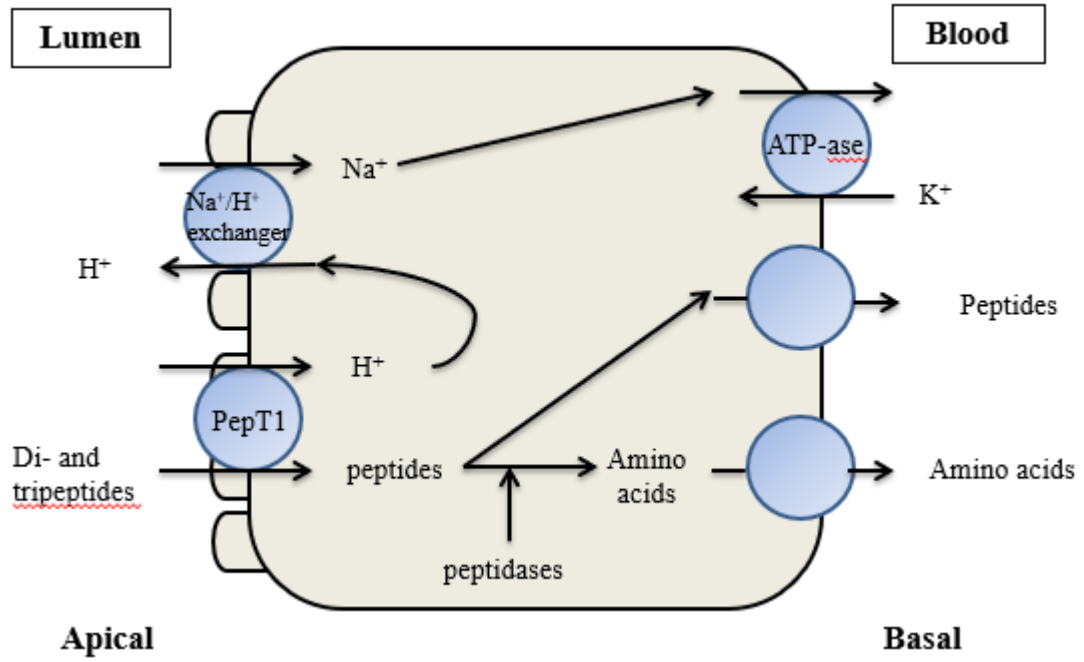


Figure 4.2 Structures of A. cephalixin (CEPH), B. ergotamine (ERT), C. lysergic acid (LSA), D. ergovaline extract (EXT), and E. nateglinide (NATE).

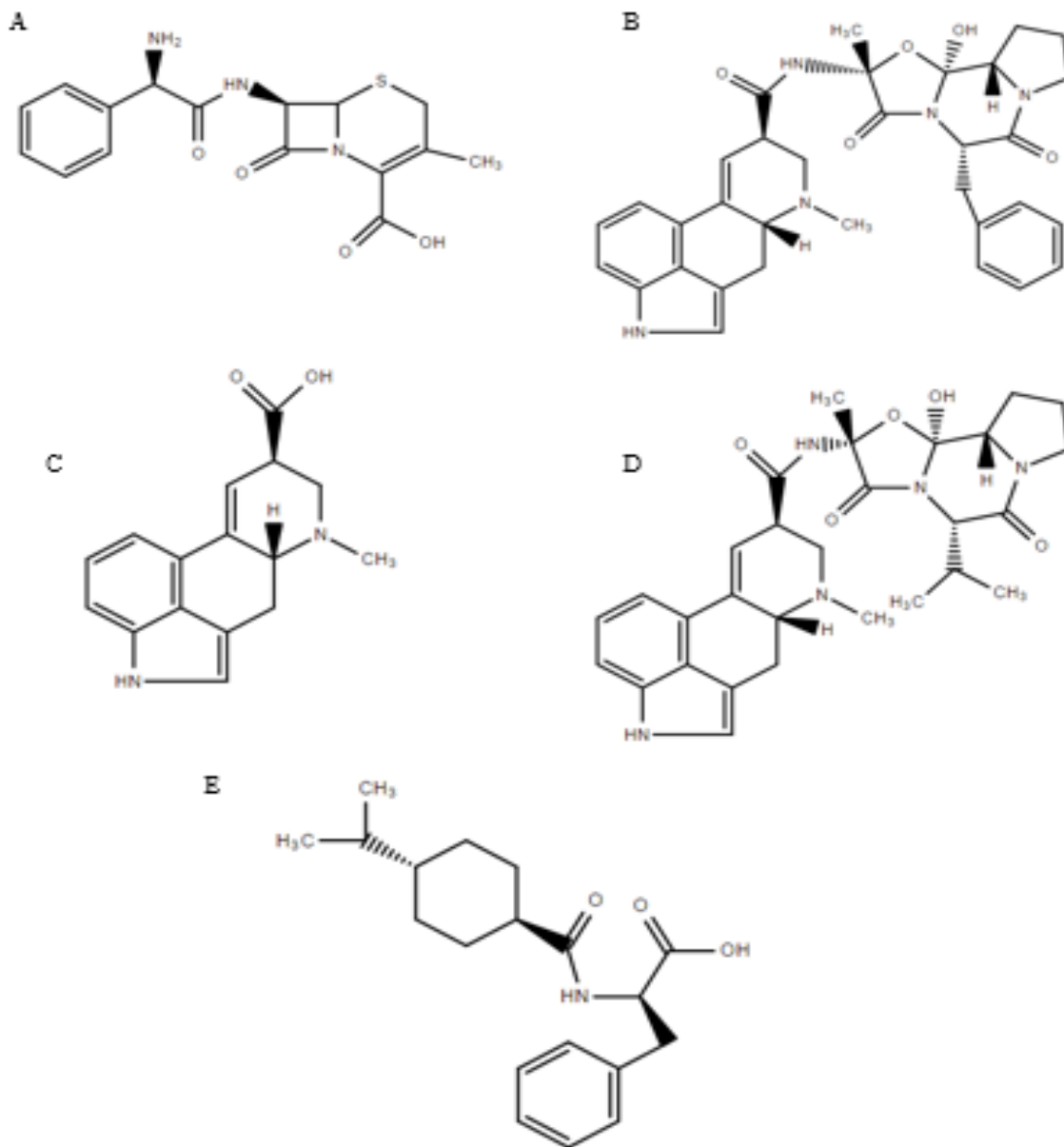


Figure 4.3a Chromatogram of Cephalexin (CEPH)

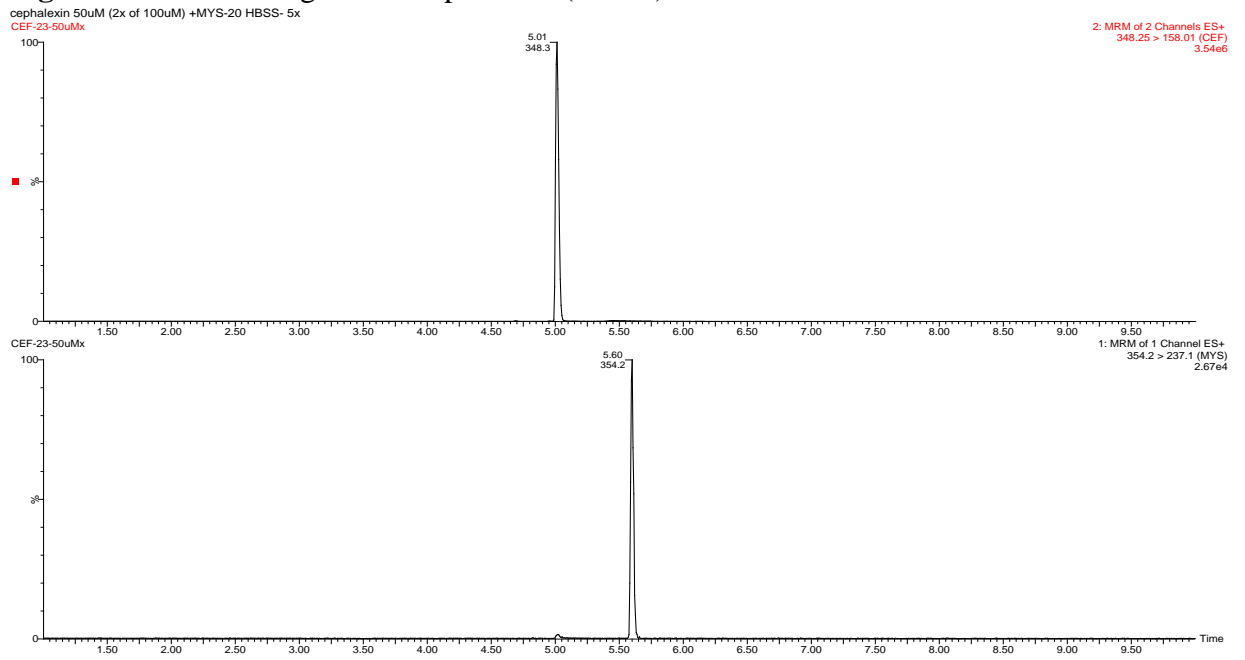


Figure 4.3b Chromatogram of ergotamine (ERT)

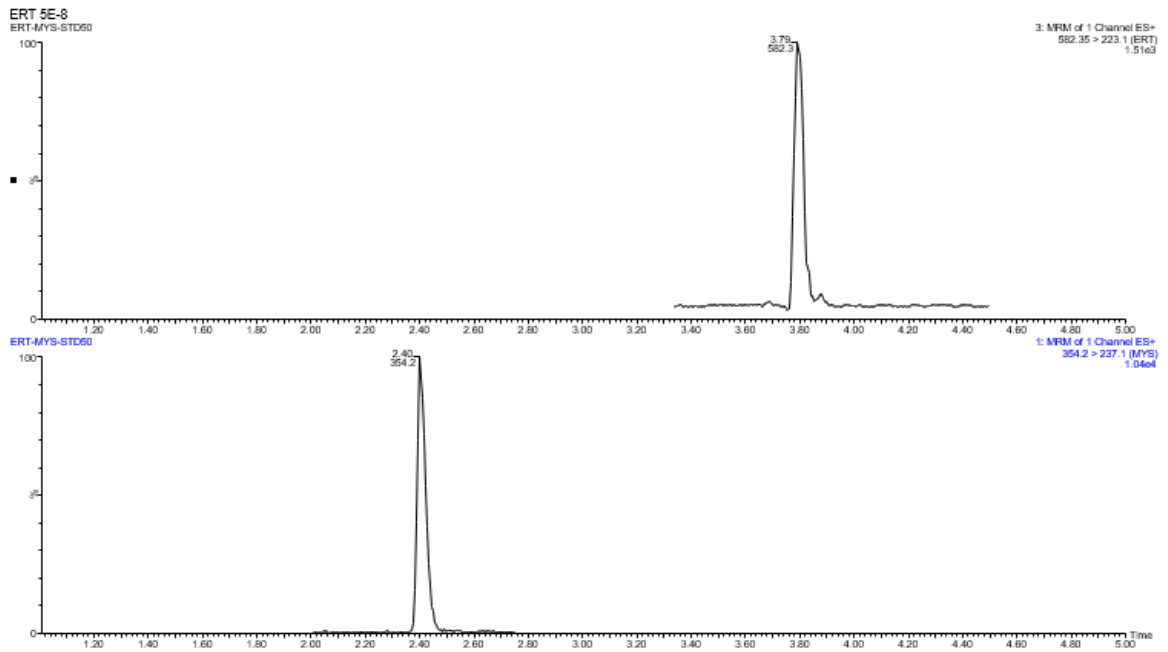


Figure 4.3c Chromatogram of ergovaline seed extract (EXT)

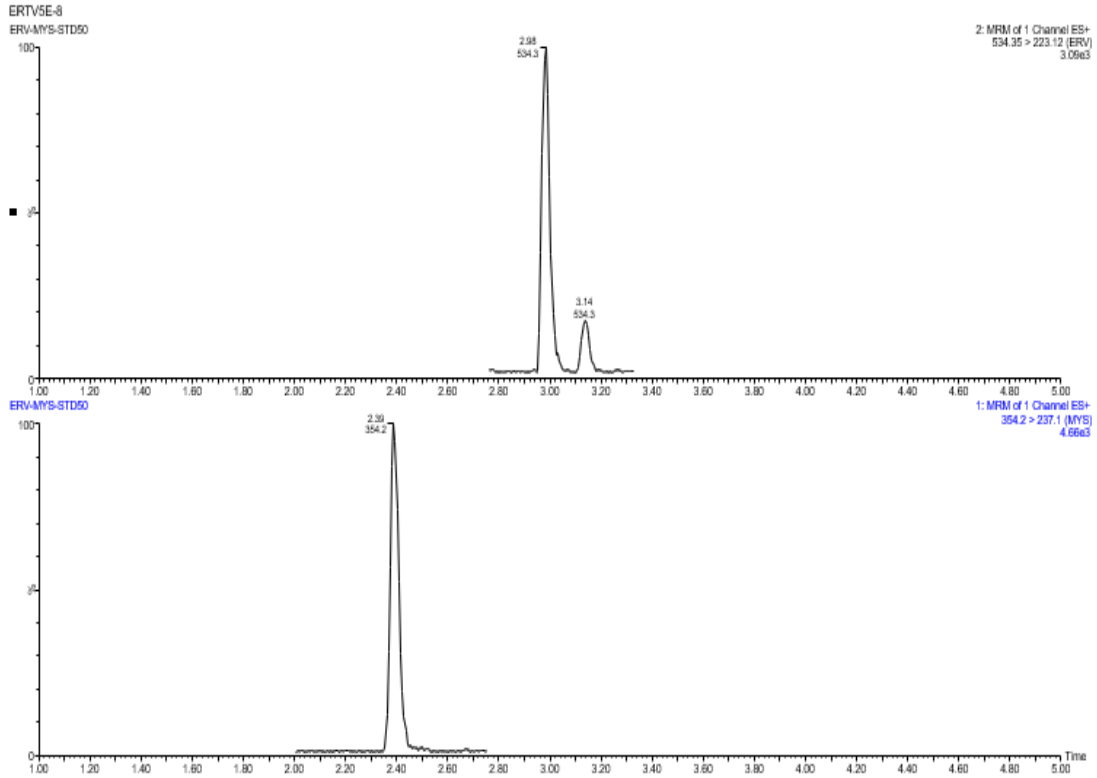
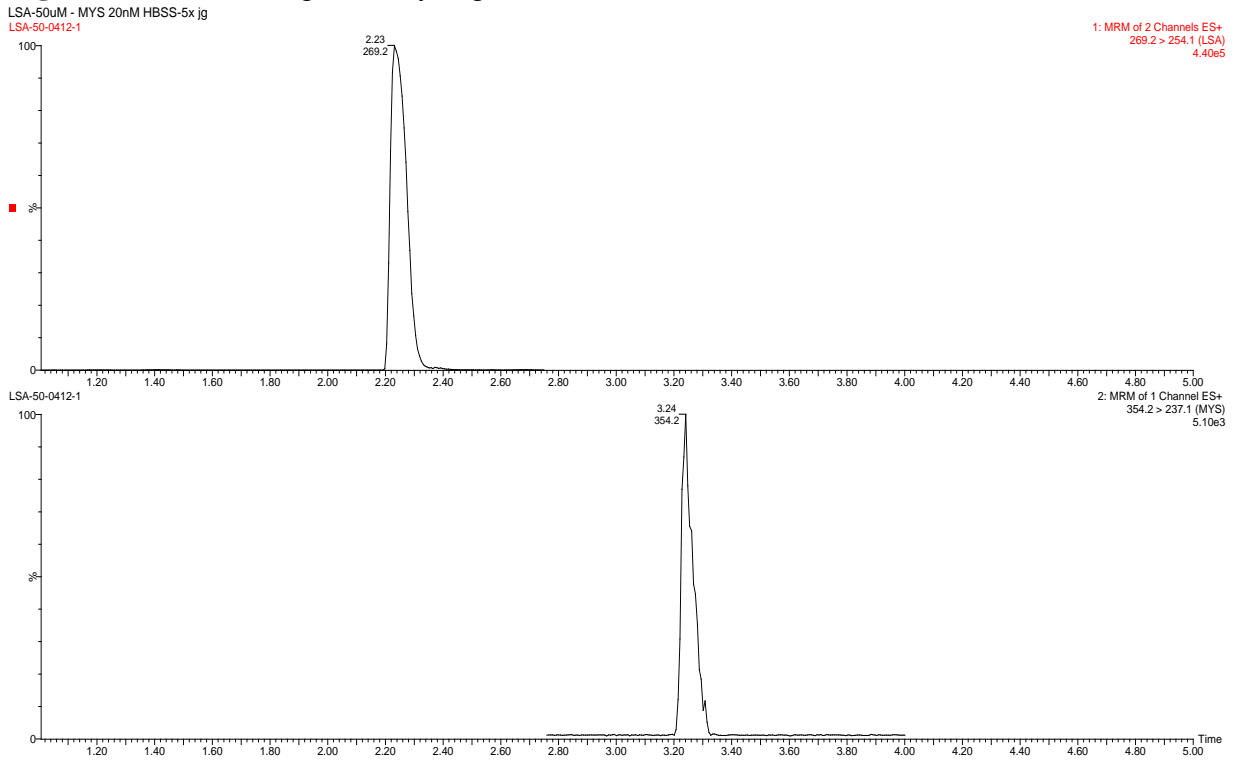


Figure 4.3d Chromatogram of lysergic acid (LSA)



Figures 4.4a -f Images of Caco-2 cells at: A: day 1, B: day 3, C: day 7, D: day 14, E. day 18, and day 21 at 100X magnification

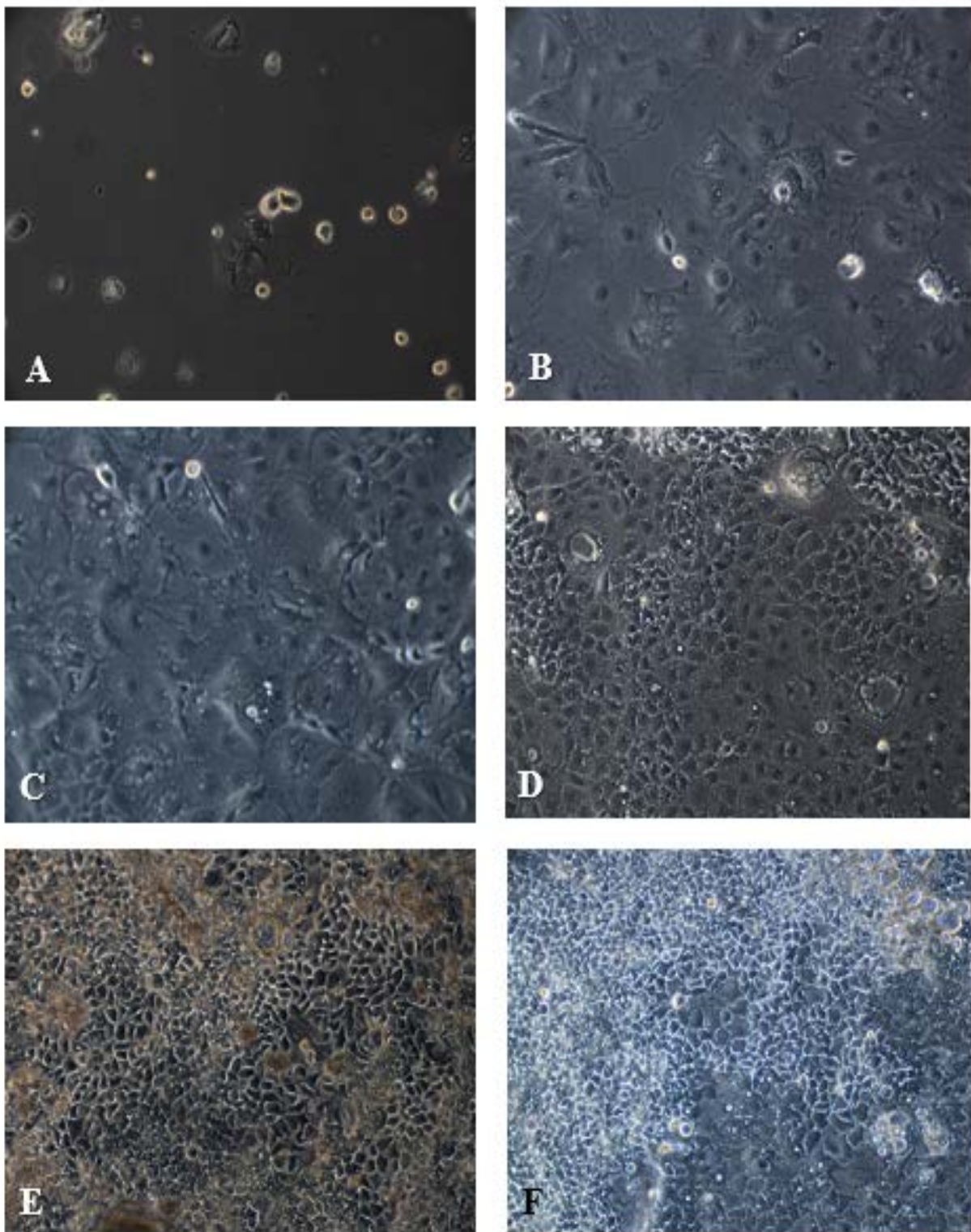


Figure 4.5 Changes in TEER values across Caco-2 cell monolayers before treatment with cephalalexin, ergotamine, ergovaline, and lysergic acid.

**Changes in transepithelial electrical resistance (TEER)
across Caco-2 cell monolayers**

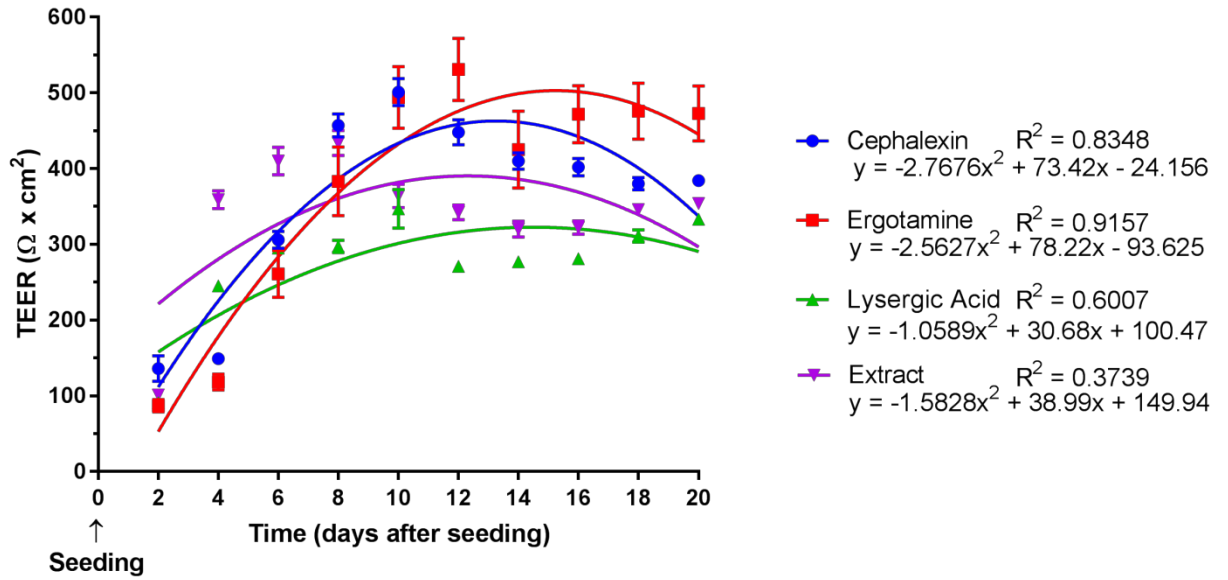


Figure 4.6 Phenol red exclusion: day 7 v. day 18 diffusion; Data are expressed as the means \pm standard error from four different experiments and were analyzed using a multiple mean comparisons test. Means with different letters are significantly different ($P < 0.05$).

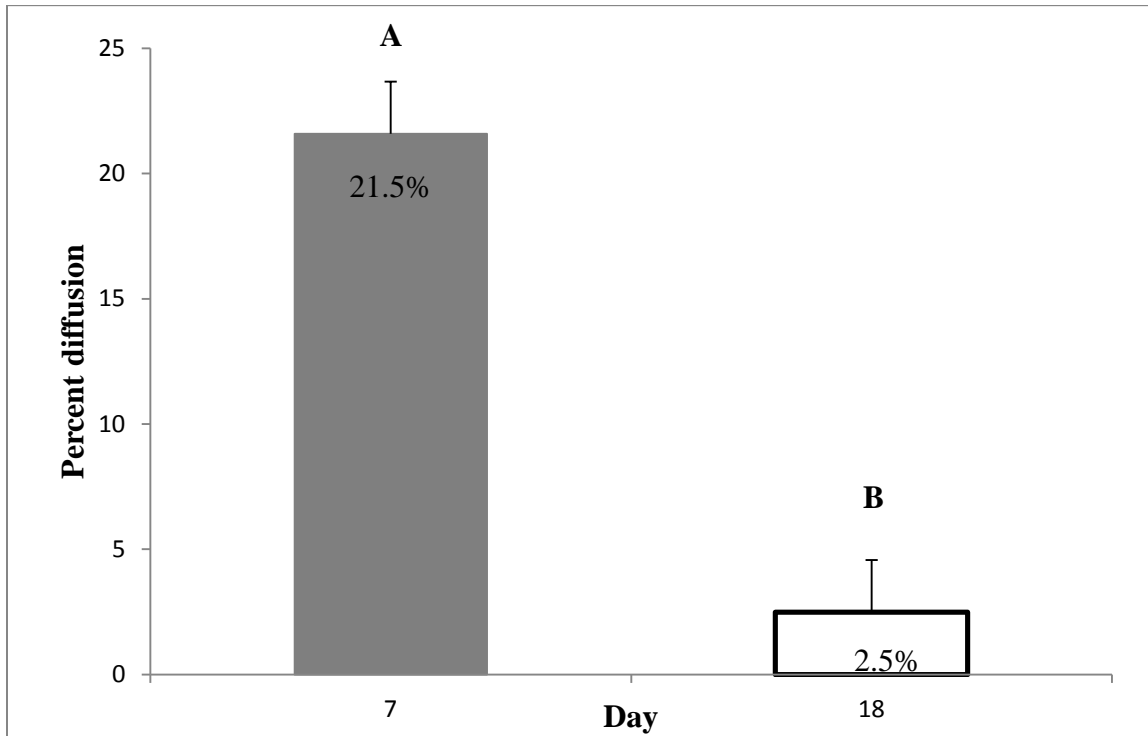


Figure 4.7 Sucrase activity of Caco-2 cells on day 7 and day 18. Data are expressed as the means \pm standard error from four different experiments and were analyzed by one-way ANOVA followed by pairwise comparison test, and Tukey post hoc test ($P < 0.05$).

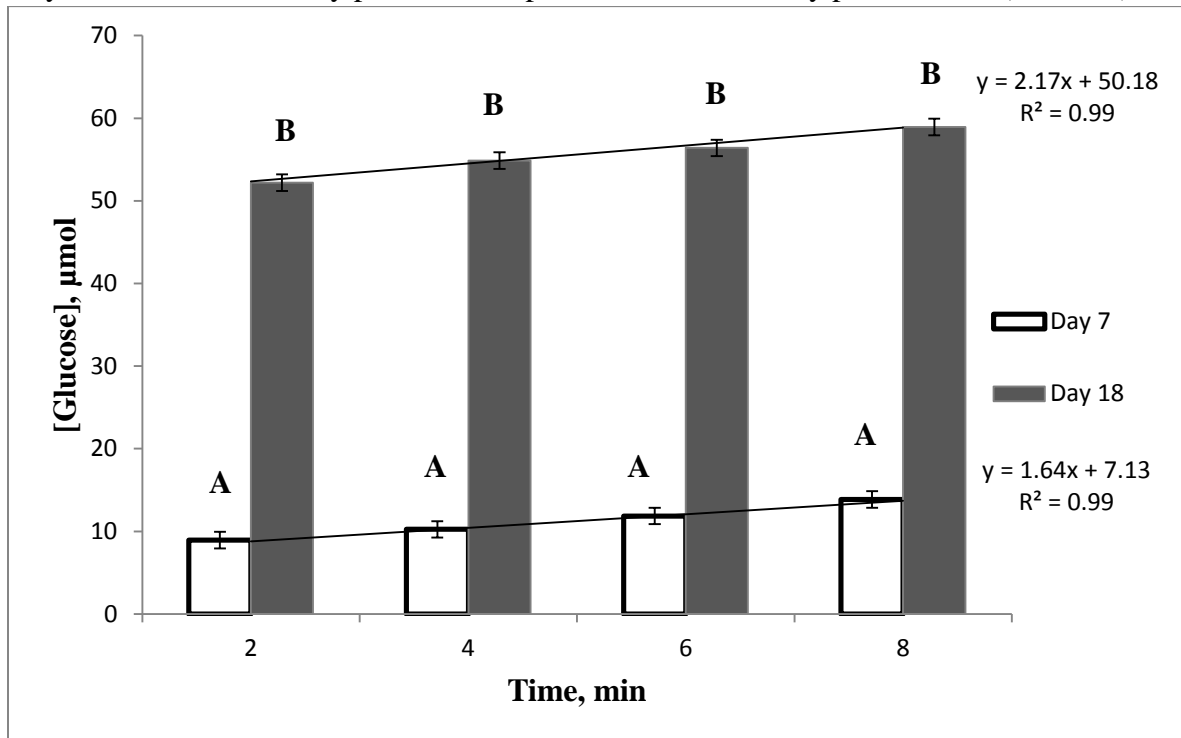


Figure 4.8 Percent reduction of resazurin to resorufin in the presence of different concentrations of ergotamine (ERT) over time. Concentrations are expressed as molar (M) and represent 10^{-6} M, 10^{-5} M, and 10^{-4} M.

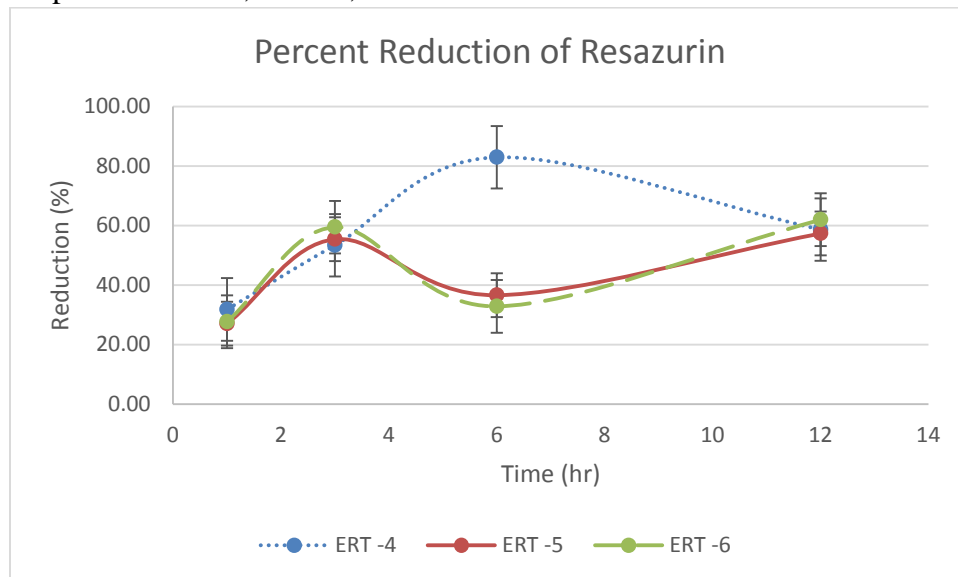


Figure 4.9 Reduction of resazurin to resorufin in the presence of different concentrations of lysergic acid (LSA) over time. Concentrations are expressed as molar (M) and represent 10^{-6} M, 10^{-5} M, and 10^{-4} M.

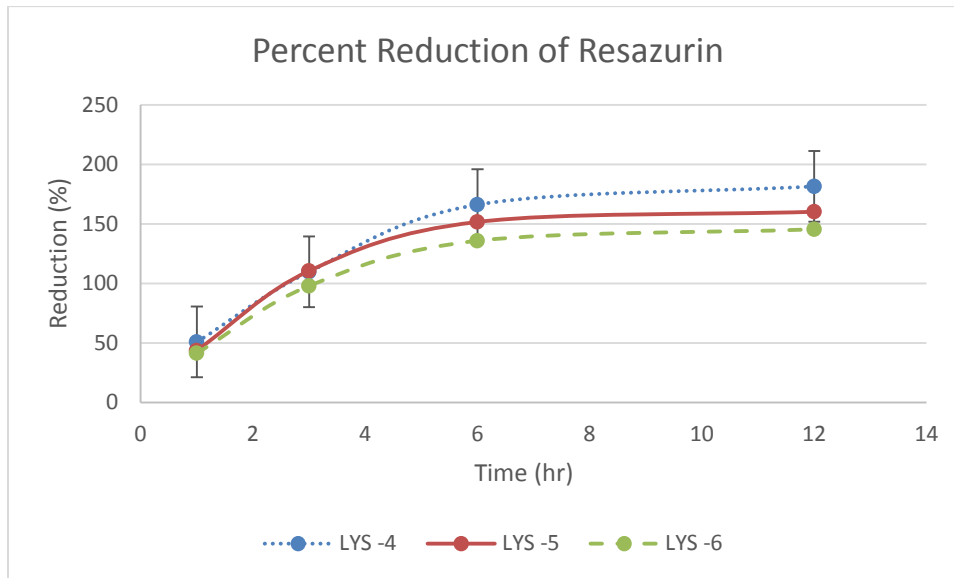


Figure 4.10 Reduction of resazurin to resorufin in the presence of different concentrations of ergovaline (EXT) over time. Concentrations are expressed as molar (M) and represent 10^{-6} M, 10^{-5} M, and 10^{-4} M.

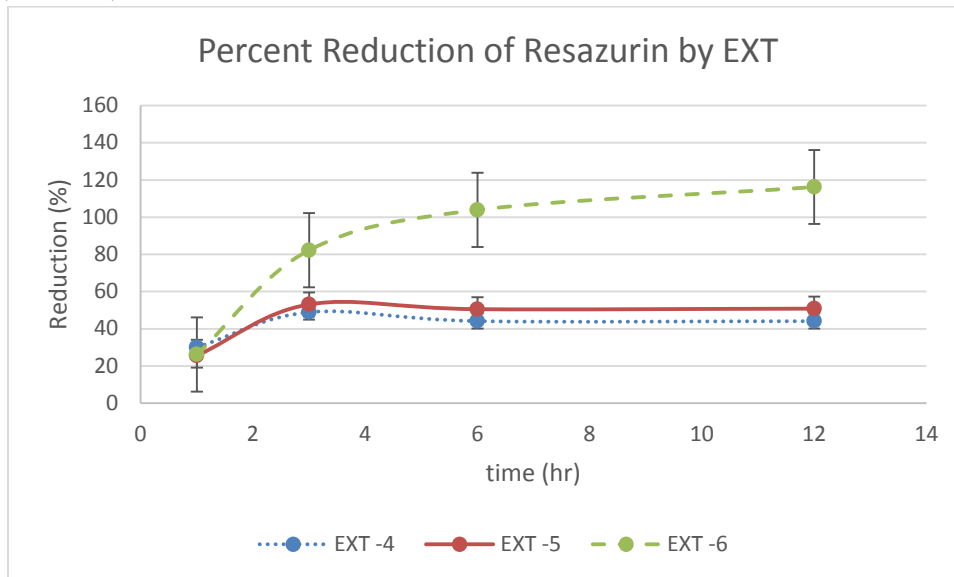


Figure 4.11 Percent recovery of 100 μM of lysergic acid (LSA) in wells with and without 0.5 mM nateglinide (NATE) compared to the no cell control (NCC). AP- (Apical chamber without NATE) and AP+ (apical chamber treated with NATE). Values are expressed as the mean \pm SEM.

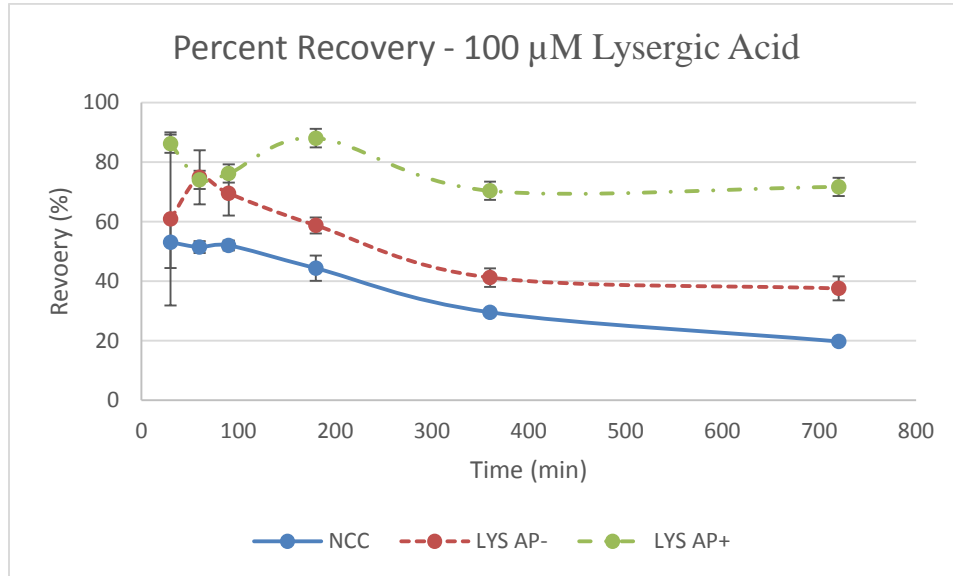


Figure 4.12 Transport of 10 μM of cephalixin (CEPH) in wells with and without 0.5 mM nateglinide (NATE). AP- (Apical chamber without NATE), BA- (Basolateral chamber without NATE), AP+ (apical chamber treated with NATE), and BA+ (Basolateral chamber treated with NATE). Values are expressed as the mean \pm SEM.

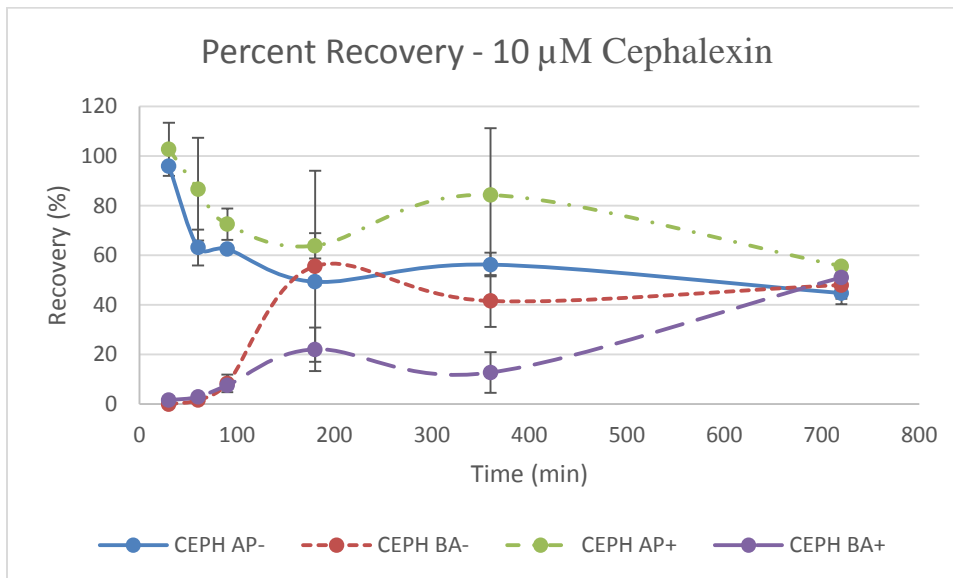


Figure 4.13 Transport of 10 μM of lysergic acid (LSA) in wells with and without 0.5 mM nateglinide (NATE). AP- (Apical chamber without NATE), BA- (Basolateral chamber without NATE), AP+ (apical chamber treated with NATE), and BA+ (Basolateral chamber treated with NATE). Values are expressed as the mean \pm SEM.

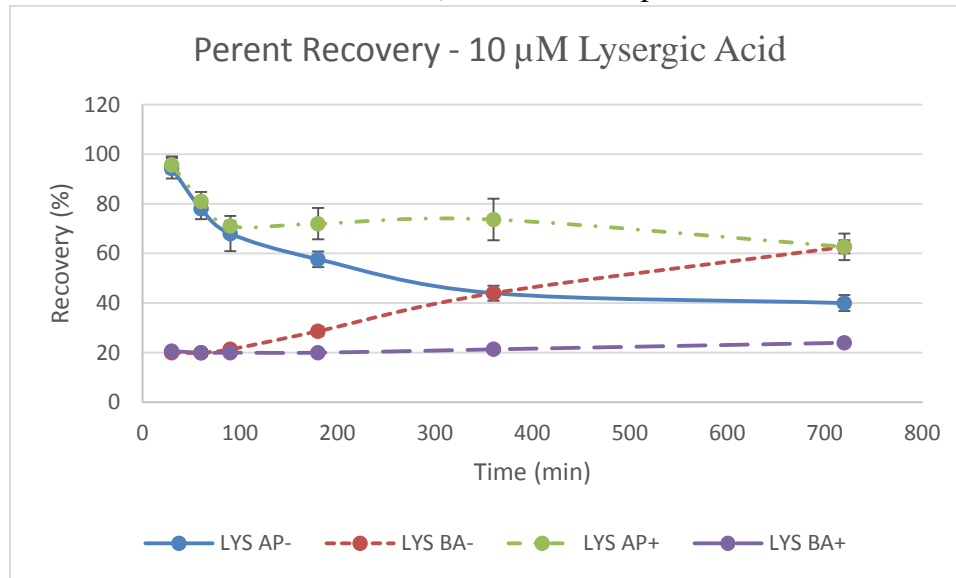


Figure 4.14 Transport of 10 μM of ergotamine (ERT) in wells with and without 0.5 mM nateglinide (NATE). AP- (Apical chamber without NATE), BA- (Basolateral chamber without NATE), AP+ (apical chamber treated with NATE), and BA+ (Basolateral chamber treated with NATE). Values are expressed as the mean \pm SEM.

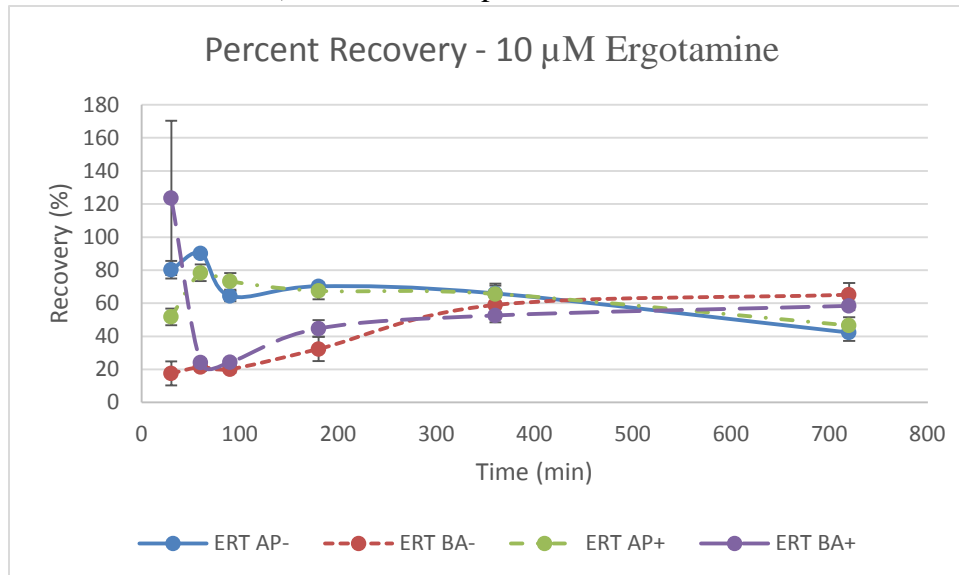


Figure 4.15 Transport of 10 μM of ergovaline (EXT) in wells with and without 0.5 mM nateglinide (NATE). AP- (Apical chamber without NATE), BA- (Basolateral chamber without NATE), AP+ (apical chamber treated with NATE), and BA+ (Basolateral chamber treated with NATE). Values are expressed as the mean \pm SEM.

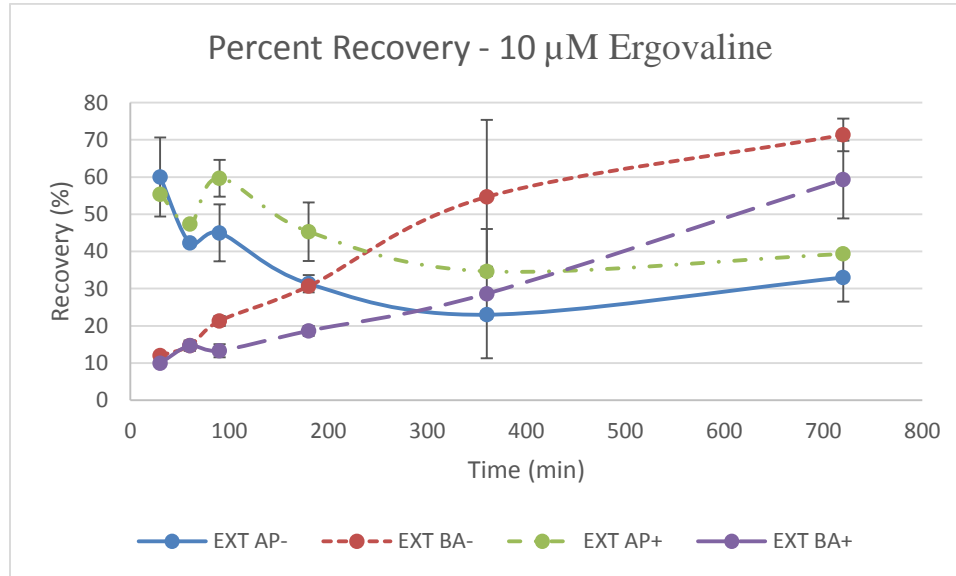


Figure 4.16 Transport of 100 μM of cephalixin (CEPH) in wells with and without 0.5 mM nateglinide (NATE). AP- (Apical chamber without NATE), BA- (Basolateral chamber without NATE), AP+ (apical chamber treated with NATE), and BA+ (Basolateral chamber treated with NATE). Values are expressed as the mean \pm SEM.

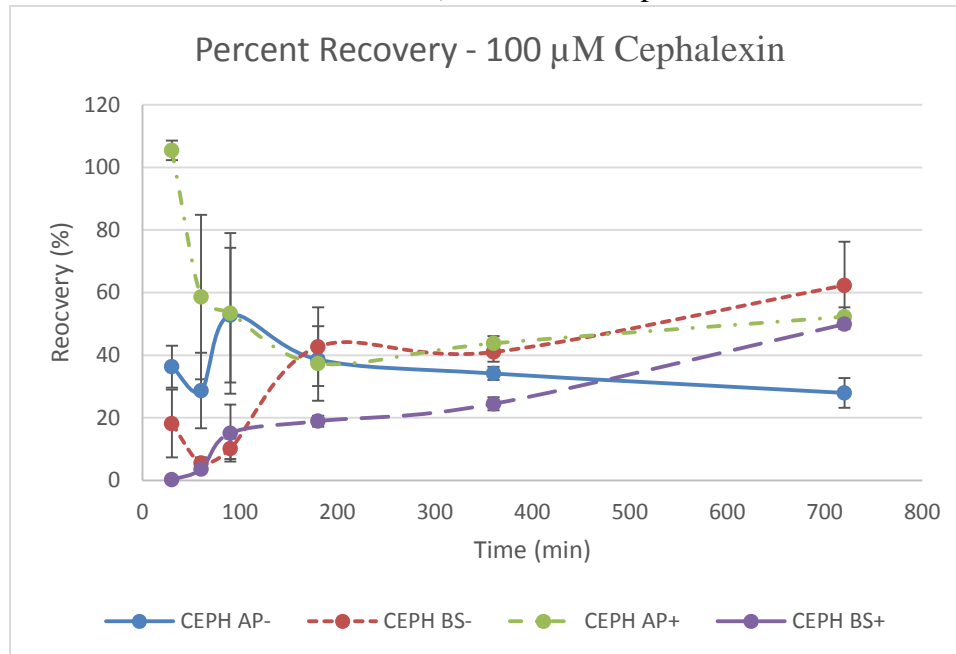


Figure 4.17 Transport of 100 μM of lysergic acid (LSA) in wells with and without 0.5 mM nateglinide (NATE). AP- (Apical chamber without NATE), BA- (Basolateral chamber without NATE), AP+ (apical chamber treated with NATE), and BA+ (Basolateral chamber treated with NATE). Values are expressed as the mean \pm SEM.

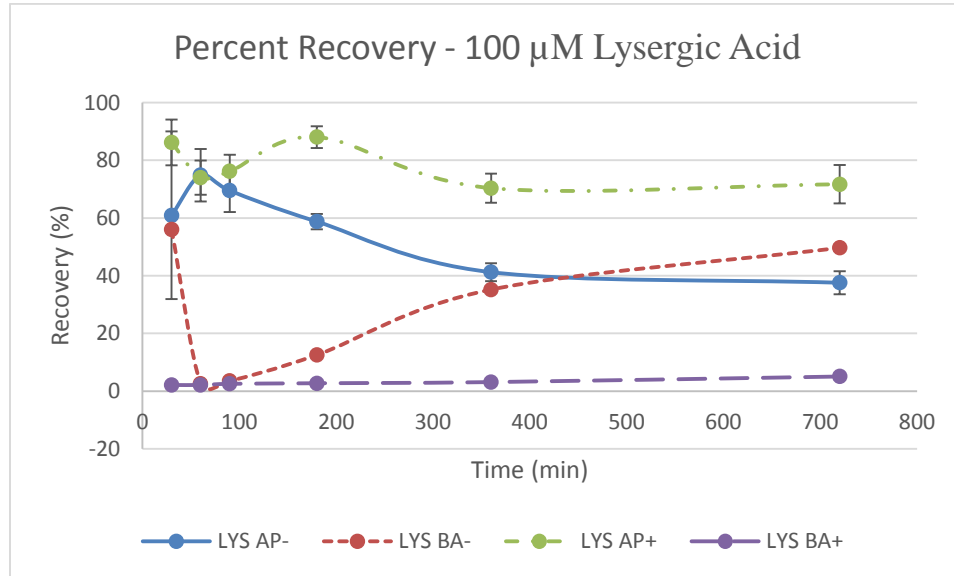


Figure 4.18 Transport of 100 μM of ergotamine (ERT) in wells with and without 0.5 mM nateglinide (NATE). AP- (Apical chamber without NATE), BA- (Basolateral chamber without NATE), AP+ (apical chamber treated with NATE), and BA+ (Basolateral chamber treated with NATE). Values are expressed as the mean \pm SEM.

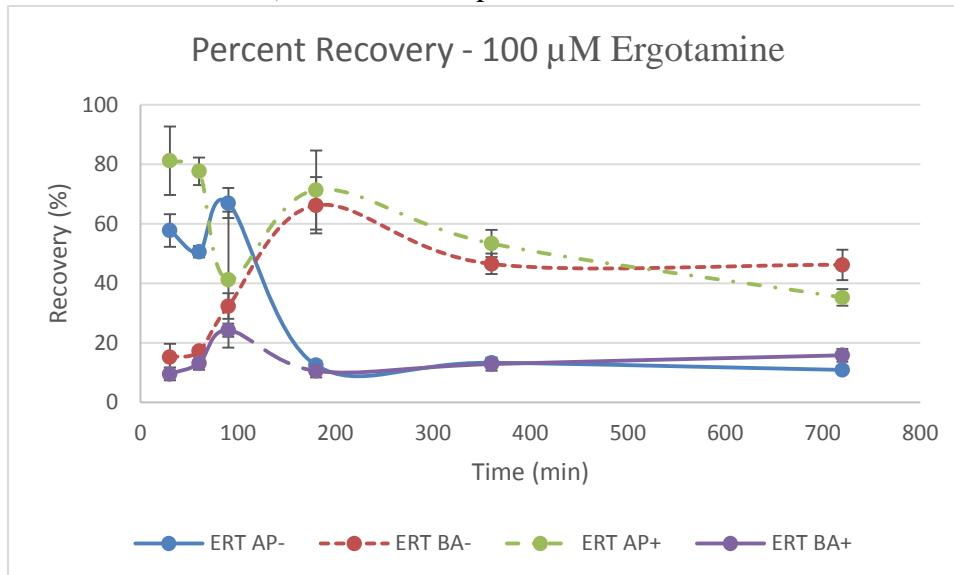
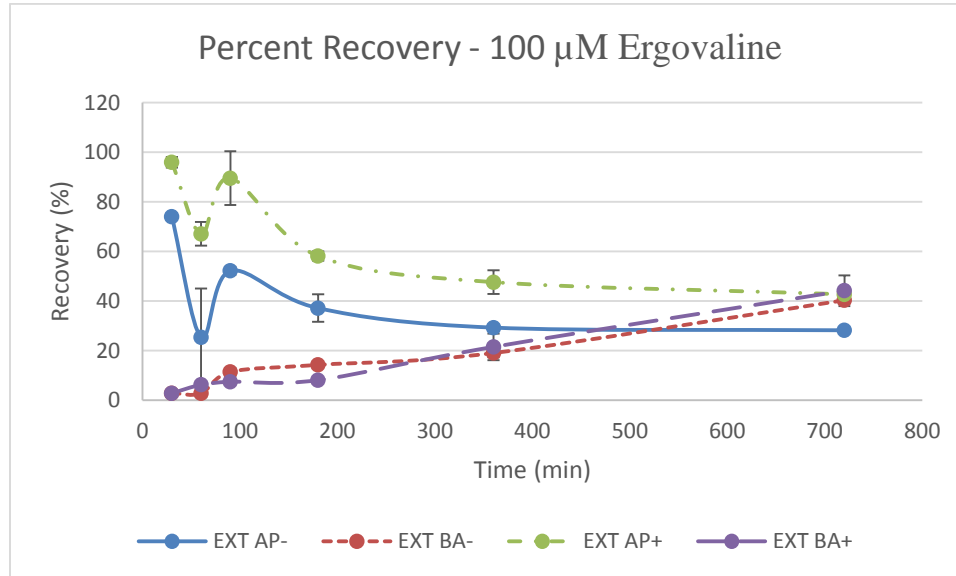


Figure 4.19 Transport of 100 μM ergovaline (EXT) in wells with and without 0.5 mM nateglinide (NATE). AP- (Apical chamber without NATE), BA- (Basolateral chamber without NATE), AP+ (apical chamber treated with NATE), and BA+ (Basolateral chamber treated with NATE). Values are expressed as the mean \pm SEM.



CHAPTER FIVE: SUMMARY AND CONCLUSIONS

Animals grazing on endophyte-positive tall fescue consume ergot alkaloids, leading to the condition known as fescue toxicosis. Symptoms include failure to thrive, reproductive failure, lack of appetite, reduced ability to regulate body temperature, and necrosis of appendages. It is thought that source of these symptoms may be due to vasoconstriction of peripheral tissues. However, vasoconstriction of core body vessels could also explain the appearance of symptoms. The mechanism or mechanisms of toxicity are still vague and have not completely identified. Nor has the causative agent been conclusively determined. It is generally thought that ergovaline is the causative agent of fescue toxicosis but there is also evidence that lysergic acid may also play a role in the disease mechanism.

There is not much available information about the metabolism of ergot alkaloids in ruminants, but that the ergopeptines are excreted through the bile and ergolines are excreted through the urine in nonruminant animals. In ruminant animals, it has been demonstrated that ergot alkaloids have the ability to cross ruminal and omasal tissues, although ergolines pass at a greater concentration. It has also been demonstrated that ergovaline is able to readily pass across Caco-2 cell monolayers used as model of the small intestine. From this evidence, it could be suggested that ergot alkaloids may pass across the small intestine and affect tissues and organs located distally to the small intestine.

The first experiment in this study was designed to identify the serotonin populations in mesenteric and ruminal vessels as ergot alkaloids are similar in structure to serotonin and may bind to the same receptors used by serotonin. Results from this

experiment indicated that the receptor 5-HT_{2A} is present in ruminal and mesenteric vasculature as a contractile response was observed as concentrations of the agonist increased. Previous work has indicated that 5-HT_{2A} is influenced by ergot alkaloid exposure. Further, 5-HT_{1D} and 5-HT_{2B} may play a role in vasorelaxation, as a relaxation response was observed in response to their respective agonists.

The second experiment focused on PepT1 transport of the ergot alkaloids ergotamine, ergovaline, and lysergic acid in the small intestine using a Caco-2 cell monolayer as a model. Because the chosen alkaloids have peptide-like structures, PepT1 was specifically the transporter of interest as its role in the small intestine is to transport dietary dipeptides and tripeptides. Cephalixin was used as the model compound because it is a known substrate of PepT1. Compounds were inhibited by the nonsulfonylurea secretagogue, nateglinide. As intestinal transport is dependent on the integrity of cell monolayers and tight junctions, it was necessary to assess compound toxicity and monitor monolayer integrity through the experiment. This was done using an alamar blue cytotoxicity assay, measuring transepithelial electrical resistance values, conducting a phenol red diffusion assay, and monitoring sucrose levels in the apical and basolateral chambers. All data indicated that ergovaline, lysergic acid, and ergotamine were non-toxic once cells were differentiated and that the cell monolayers were intact up to the time that transport studies were conducted. The results indicated that all compounds were transported at all timepoints across all concentrations. However, 10 and 100 μ M cephalixin, ergovaline, and lysergic acid transport were inhibited by nateglinide, indicating that PepT1 is involved in the transport of these compounds. Ergotamine

transport was also inhibited at the highest concentration level but inhibition was incomplete at the lower concentrations evaluated.

Previous research has indicated that ergovaline is the causative agent of fescue toxicosis. Other research has determined that lysergic acid may play as much a role as ergovaline in the disease state. In this study, we examined the role of PepT1 in ergot alkaloid transport across the small intestine. PepT1 may play a role in the transport of peptide-like alkaloids and the loline-types, but it cannot be readily confirmed as the only form of ergot alkaloid transport in the small intestine until further research is conducted. Although ergotamine was transported by PepT1 through the cell monolayer at 100 μM , based on these transport data it may not play as significant a role as the more readily transported lysergic acid and ergovaline in manifestation of symptoms of fescue toxicosis. This means that lysergic acid and ergovaline could reach the blood sooner than ergotamine and could have far-reaching effects in tissues and organs distal to their original absorption site. Furthermore, because lysergic acid and ergovaline are absorbed more readily, it is possible that these two alkaloids could contribute to vasoconstriction in the peripheral, ruminal, and mesenteric vasculature by binding to 5-HT receptors.

APPENDIX I

Table 4A Interaction and main effects of apical chambers + nateglinide v apical chambers without nateglinide exposed to cephalixin and basolateral chambers + nateglinide v basolateral chambers without nateglinide exposed to cephalixin. *P*-values of less than 0.05 are considered significant.

| Compound | Concentration (μM) | Chamber | Time effect | Nateglinide effect | Time x nateglinide interaction |
|------------|--------------------|-----------------|-------------|--------------------|--------------------------------|
| Cephalexin | 1 | AP ¹ | < 0.0001 | 0.6 | 0.9 |
| | 1 | BA ² | < 0.0001 | 0.04 | 0.6 |
| | 10 | AP | 0.002 | 0.02 | 0.9 |
| | 10 | BA | 0.001 | 0.1 | 0.4 |
| | 100 | AP | 0.1 | 0.009 | 0.1 |
| | 100 | BA | 0.0001 | 0.010 | 0.3 |

¹AP – apical chamber

²BA- basolateral chamber

Table 4B Interaction and main effects of apical chambers + nateglinide v apical chambers without nateglinide exposed to ERT and basolateral chambers + nateglinide v basolateral chambers without NATE exposed to ERT. *P*-values of less than 0.05 are considered significant

| Compound | Concentration (μM) | Chamber | Time effect | Nateglinide effect | Time x nateglinide interaction |
|------------|--------------------|-----------------|-------------|--------------------|--------------------------------|
| Ergotamine | 1 | AP ¹ | 0.0001 | < 0.0001 | 0.02 |
| | 1 | BA ² | < 0.0001 | 0.0001 | 0.2 |
| | 10 | AP | 0.0002 | 0.1 | 0.09 |
| | 10 | BA | 0.07 | 0.03 | 0.004 |
| | 100 | AP | 0.0001 | < 0.0001 | 0.002 |
| | 100 | BA | < 0.0001 | < 0.0001 | < 0.0001 |

¹AP – apical chamber

²BA- basolateral chamber

Table 4C Interaction and main effects of apical chambers + nateglinide v apical chambers without nateglinide exposed to ergovaline and basolateral chambers + nateglinide v basolateral chambers without nateglinide exposed to ergovaline. *P*-values of less than 0.05 are considered significant

| Compound | Concentration (µM) | Chamber | Time effect | Nateglinide effect | Time x nateglinide interaction |
|------------|--------------------|-----------------|-------------|--------------------|--------------------------------|
| Ergovaline | 1 | AP ¹ | 0.053 | 0.01 | 0.04 |
| | 1 | BA ² | 0.3 | 0.3 | 0.5 |
| | 10 | AP | < 0.0001 | 0.01 | 0.4 |
| | 10 | BA | < 0.0001 | -0.06 | 0.7 |
| | 100 | AP | <0.0001 | <0.0001 | 0.3 |
| | 100 | BA | < 0.0001 | 0.9 | 0.2 |

¹AP – apical chamber

²BA- basolateral chamber

Table 4D Interaction and main effects of apical chambers + nateglinide v apical chambers without nateglinide exposed to lysergic acid and basolateral chambers + nateglinide v basolateral chambers without nateglinide exposed to lysergic acid. *P*-values of less than 0.05 are considered significant.

| Compound | Concentration (µM) | Chamber | Time effect | Nateglinide effect | Time x nateglinide interaction |
|---------------|--------------------|-----------------|-------------|--------------------|--------------------------------|
| Lysergic acid | 1 | AP ¹ | < 0.0001 | 0.2 | 0.09 |
| | 1 | BA ² | 0.5 | 1.0 | 0.3 |
| | 10 | AP | < 0.0001 | 0.0001 | 0.01 |
| | 10 | BA | < 0.0001 | < 0.0001 | < 0.0001 |
| | 100 | AP | 0.1 | 0.001 | 0.4 |
| | 100 | BA | 0.3 | 0.01 | 0.3 |

¹AP – apical chamber

²BA- basolateral chamber

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- 2013 Southeast Missouri State University – Delta Tau Alpha Agriculture Honor Society
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Abstracts

M.A. Snider, D.L. Harmon, and J.L. Klotz. 2017. Assessment of bovine ruminal and mesenteric vascular serotonin receptor populations. American Society of Animal Science Conference, Baltimore, MD.

J. L. Klotz, M. F. Miller Jr., J. L. Britt, **M. A. Snider**, G. E. Aiken, N. M. Long, S. L. Pratt, A. John, and S. K. Duckett. 2017. Effects of ergot alkaloid exposure during gestation on maternal and fetal vasoactivity in sheep. American Society of Animal Science Conference, Baltimore, MD.

C. Hendrix, **M. Snider**, A. Hanson, D. Beck, and R. Kurzhals. 2017. Evaluation of genes required for telomere maintenance on HipHop dependent suppression of cell lethality after telomere loss. The Allied Genetics Conference, Orlando, FL.

M.A. Snider and R.L. Kurzhals. 2015. Survey of genes at the telomere and their function in the DNA damage repair pathway. Southeast Missouri State University Student Research Conference. Cape Girardeau, MO.

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