Role of endothelin-1 in the gastrointestinal tract of horses in health and disease

Ramaswamy Monickarasi Chidambaram
Louisiana State University and Agricultural and Mechanical College

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ROLE OF ENDOTHELIN-1 IN THE GASTROINTESTINAL TRACT OF HORSES IN HEALTH AND DISEASE

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The Interdepartmental Program in Veterinary Medical Sciences through the Department of Comparative Biomedical Sciences

By
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May, 2003
Dedicated to my parents, Dr. S. Chidambaram Pillai and Mrs. R. Monickarasi, and my siblings for their inspiration and support toward my pursuit of higher knowledge
ACKNOWLEDGEMENTS

I express my sincere thanks and heartfelt gratitude to my mentor Dr. Rustin Moore and Dr. Changaram Venugopal, for their involvement and personal help offered toward the completion of my dissertation. I also wish to thank my other committee members, Dr. Steven Barker, Dr. Susan Eades and Dr. Leonard Kappel, for their scientific input, guidance and support throughout my studies. The assistance of Dr. Giselle Hosgood for statistical analyses and Dr. Daniel Paulsen for immunohistochemistry is much appreciated. Also, I am indebted to Dr. Gus Kousoulas for his personal help and for the use his lab for molecular studies.

This work could not have been completed without the valuable assistance of Catherine Koch, Earnestine Holmes, Frank Garza Jr., Mae Lopez, Li Huang, Dr. Lais Costa and Marian Waguespack. I am indebted to all my friends and the individuals of the School of Veterinary Medicine, Department of Comparative Biomedical Sciences, and especially members of Equine Health Studies Program and Department of CBS for their help and support. Lastly, I would like to thank my siblings, friends and family members for their support throughout my studies.

Parts of this dissertation were made possible through grants awarded by the LSU Equine Health Studies Program and LSU USDA 1433 Formula Funds.
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<td>%</td>
<td>percentile, percentage</td>
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<tr>
<td>&lt;</td>
<td>less than</td>
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<td>&gt;</td>
<td>greater than</td>
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<tr>
<td>AMP</td>
<td>adenosine mono phosphate</td>
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<td>ANF</td>
<td>atrial natriuretic factor</td>
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<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BER</td>
<td>basic electric rhythm</td>
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<td>CI</td>
<td>confidence interval</td>
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<td>centimeter</td>
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<td>carboxy terminal</td>
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<td>c-terminal fragment</td>
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<td>deoxyribonucleic acid</td>
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<tr>
<td>ECE</td>
<td>endothelin converting enzyme</td>
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<tr>
<td>EDCF</td>
<td>endothelial derived constricting factor</td>
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<td>EDTA</td>
<td>ethylene diaminitetraacyclic acid</td>
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<td>EFS</td>
<td>electrical field stimulation</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>endothelin</td>
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<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>hydrogen peroxide</td>
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<td>hypochlorous acid</td>
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<td>IFN</td>
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<td>interleukin</td>
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<td>Ile</td>
<td>isoleucine</td>
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<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>inositol-tri-phosphate</td>
</tr>
<tr>
<td>I-R</td>
<td>ischemia-reperfusion</td>
</tr>
<tr>
<td>IU</td>
<td>international units</td>
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<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>calcium ion</td>
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<td>K</td>
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<td>kg</td>
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<td>L-NAME</td>
<td>N-ω-nitro-L-arginine methyl ester</td>
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<tr>
<td>LTB</td>
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<tr>
<td>M</td>
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<td>mg</td>
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<td>Abbreviation</td>
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<tr>
<td>MLC</td>
<td>myosin light chain</td>
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<tr>
<td>MLC-P</td>
<td>myosin light chain-phosphorylated</td>
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<tr>
<td>MMC</td>
<td>myoelectric complex</td>
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<td>messenger ribonucleic acid</td>
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<td>mV</td>
<td>millivolts</td>
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<td>number</td>
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<tr>
<td>NADPH</td>
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<tr>
<td>NANC</td>
<td>non-adrenergic non-cholinergic</td>
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<td>NO</td>
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ABSTRACT

Gastrointestinal tract disease is the leading natural cause of death in horses and horses with ischemic intestinal disease have the greatest mortality. We hypothesized there is basal synthesis of endothelin-1 (ET-1) in the intestinal tract of healthy horses that is likely involved in regulating vasomotor tone, secretion and motility and that ET-1 synthesis increases with gastrointestinal tract disease, which may be involved in the pathophysiology of these disorders.

Plasma ET-like immunoreactivity was increased in horses with naturally-acquired gastrointestinal disease, compared with normal horses; values were greatest in horses with large intestinal strangulation obstruction, enterocolitis and peritonitis. There was an association between ET-1 levels and survival, PCV and duration of signs of pain.

Immunohistochemical staining for ET-1 was present in surface epithelium, villi, muscularis and serosa of numerous intestinal segments in healthy horses. Staining was also present in submucosal vessels with veins staining more intense than arteries. Staining appeared more diffuse and intense in samples from horses with intestinal strangulation obstruction. Polymerase chain reaction analysis revealed the presence of ET-1 gene expression in numerous intestinal segments of normal horses. These findings suggest ET-1 is involved in physiologic functions such as regulation of secretion, vasomotor tone and motility, and that increased ET-1 with strangulation obstruction may be involved in the pathophysiology of these disorders.

ET-1 caused sustained, concentration-dependent increases in cecal longitudinal smooth muscle tone in vitro, but the magnitude of contraction was less than that induced by carbachol. Pre-incubation of tissues with $\text{ET}_A$ (BQ-123) and $\text{ET}_B$ (IRL-1038) receptor
antagonists alone did not inhibit ET-1 induced contraction. However, contractile responses were inhibited when tissues were incubated with both antagonists (10^{-5} M) together, suggesting both ET_A and ET_B receptors mediate the contraction. Electric field stimulation did not change the contractile response.

These studies indicate a physiologic role of ET-1 in the equine gastrointestinal tract and that increased synthesis and release occurs with gastrointestinal tract disease, especially ischemic conditions, and may contribute to the pathophysiology of these disorders. Further studies involving ET-1 and ET antagonists appear warranted.
CHAPTER 1

INTRODUCTION
Endothelin (ET), a potent vasoconstrictor, was first discovered in cultured porcine aortic endothelial cells. Since its discovery, numerous studies of the role of ET in various diseases have been conducted. Endothelin is synthesized in a variety of tissues and cell types, including endothelial cells, macrophages, mesangial cells, mast cells and astrocytes, where they modulate vasomotor tone, cell proliferation and hormone synthesis. Endothelin regulates blood flow in association with endothelium-derived nitric oxide (NO). Endothelin is also proposed to be an excitatory component of the non-adrenergic, non-cholinergic innervation (NANC) of the enteric nervous system controlling gastrointestinal smooth muscle motility in conjunction with NO, an inhibitory component of the NANC system.

Endothelins are a family of vasoactive peptides that have potent vasoconstrictor actions. Of the three isoforms (ET-1, ET-2 and ET-3), ET-1 is implicated in the majority of physiological and pathological functions. Endothelin exerts its biologic activity by binding to its specific G-protein linked receptors, ET_A and ET_B. The ET_A receptors are located on the vascular smooth muscle cells and binding of ET to ET_A receptors mediates vasoconstriction. The ET_B receptors are located on endothelial cells and in non-vascular smooth muscle cells where activation by ET-1 mediates dilation and constriction, respectively. The role of ET-1 has been implicated in several pathologic states including endotoxemia, sepsis, myocardial infarction, strangulation obstruction, ulcerative disease, ischemia-reperfusion and asthma. However, there have been no reports to date demonstrating a role of ET-1 in gastrointestinal tract disease of horses.

Intestinal strangulation is a common cause of colic that accounts for approximately 10% of all the cases in horses with colic, and is universally fatal without surgical
intervention and intense medical therapy. Following surgical correction of experimental large colon volvulus in horses, the intestinal blood flow remains significantly decreased below baseline values for at least 4 hrs. This sustained reduction in blood flow is likely associated with disruption in the balance between endothelium-derived vasorelaxants (NO, PGI₂) and vasoconstrictors (ET, PGF₂α), resulting in ischemia, progressive mucosal damage and alterations in gastrointestinal motility. Although there are a plethora of mediators involved in the pathogenesis of ischemia-reperfusion injury, we propose that ET-1 is involved in the vasomotor abnormalities and subsequent ischemia. Increased plasma ET-1 concentrations have been found in various pathological conditions in other species such as low-flow states, sepsis and shock. We hypothesize that ET-1 is directly or indirectly involved in the cascade of events involving ischemia, ischemia-reperfusion injury (I-R) and motility disturbances in the gastrointestinal tract of horses.

The aim of the first study was to validate a commercially available radioimmunoassay (RIA) for measuring plasma ET-1 like immunoreactivity in horses, and to quantify and compare the circulating plasma ET-1 concentrations between healthy horses and in horses with naturally acquired gastrointestinal tract disorders. Additionally, we investigated the relationship between circulating plasma ET-1 concentrations and clinical, and clinicopathologic variables and the survival of the affected horses. We hypothesized that concentrations of plasma ET-like immunoreactivity would be greater in horses with naturally acquired gastrointestinal disorders, compared with healthy horses, and the survival of the horses would be inversely correlated with the increased plasma ET-like immunoreactivity.
The aim of the second study was to validate ET-1 immunohistochemical staining in the gastrointestinal tract of horses, and to evaluate the presence and regional distribution of ET-1 like immunoreactivity in various segments of the gastrointestinal tract of healthy horses. Further, we evaluated alterations in the intensity of ET-1 staining in intestinal segments collected from horses with naturally acquired intestinal strangulation. We hypothesized that there would be an increase in intensity of ET-1 like immunoreactivity in the affected intestinal segment of horses with naturally acquired intestinal strangulation obstruction and this would correlate with the degree of intestinal damage.

The aim of the third experiment was to develop and validate a procedure for identifying the presence of ET-1 gene expression by polymerase chain reaction in intestinal sections collected from clinically healthy horses. We hypothesized that the ET-1 would be found in variable quantities from various segments of the intestinal tract extending from the duodenum to the right dorsal colon of healthy horses. We also hypothesized that ET-1 gene expression would be greater in the mucosa of the jejunum and pelvic flexure of horses with naturally acquired small intestinal and large colon strangulation obstruction, respectively.

The aim of the fourth study was to identify and characterize the ET-1 induced in vitro responses of cecal longitudinal smooth muscle from clinically healthy horses devoid of any apparent gastrointestinal tract disorders. The roles of ET receptors, ET\textsubscript{A} and ET\textsubscript{B} in ET-1 induced changes in the gastrointestinal tract smooth muscle contractility were evaluated using specific ET\textsubscript{A} and ET\textsubscript{B} receptor antagonists, BQ-123 and IRL-1038, respectively. Additionally, we clarified the role of ET-1 in nerve-mediated contractions induced by electric field stimulation. We hypothesized that ET-1 would be a potent
contractile agent in gastrointestinal tract smooth muscle. Additionally, we hypothesized that spontaneously contracting and EFS-stimulated ET-1 induced contractile and relaxing responses would be mediated by ET\textsubscript{A} and ET\textsubscript{B} receptors, respectively.
CHAPTER 2

REVIEW OF LITERATURE
2.1 Anatomy of Equine Cecum and Colonic Viscera

Knowledge of the anatomy of equine large intestine, including its gross anatomy, physiological function and basis of intestinal motility are necessary to understand the pathophysiologic mechanisms involved in various disease processes.

2.1.1 Cecum

The cecum is a large, comma shaped structure lying along the right dorsal to right ventral abdomen with its apex near the xyphoid process of the sternum. It is divided into three regions, namely the apex, body and base with blind ends at both extremities. The cecum has four teniae that form four rows of sacculations. The cecocolic orifice is located at the ventral part of the caudal cecal base. The vascular supply to the cecum is provided by the cecal arteries (lateral and medial), which arise from the ileocolic artery (Pfeiffer and MacPherson, 1990).

The cecum is an important site of water and electrolyte absorption as well as microbial digestion of soluble and insoluble carbohydrates. The function of the cecum is to mix the ingesta constantly and retain it for a sufficient time to complete microbial digestion. There are four motility patterns recognized in the cecum (Ross et al., 1986). The first three are the haustral contraction patterns occurring every 20-30 sec that mix the contents of the cecum from the cranial base to the cranial apex. The fourth contraction is a progressive contraction pattern, which occurs once at approximately 4-minute intervals and originates every 4 minutes and originating from the pacemaker site in the cecal body or apex (Ross et al., 1986). Functional studies of the cecum have shown selective emptying of gas versus ingesta following contractions, and the prevention of reflux into the cecum from the colon due to an anatomic barrier, the cecocolic junction (Argenzio et
al., 1974). Histologically, the cecum consists of a thin layer of muscularis externa, with flattened bands of smooth muscle and a high percentage of elastic fibers. The mucosa contains short, straight cecal glands with many goblet cells and cuboidal epithelial cells. The surface epithelium consists of tall columnar cells.

2.1.2 Large Colon

The large colon or ascending colon is shaped as a double horseshoe looping from the right to left ventrally and then to the left and back to the right dorsally (Pfeiffer and MacPherson, 1990). It holds an average of 80 L and is approximately 4 m long. The diameter of the large colon varies considerably depending on the region, with the right dorsal colon being the largest. Branches of the ileocolic artery, colic branch artery and right colic arteries supply right and left ventral colon and right and left dorsal colon, respectively (Pfeiffer and McPherson, 1990). The pelvic flexure is a horse-shoe shaped segment of the large colon between the left ventral colon orally and to the left dorsal colon aborally. The pelvic flexure is situated in the left one-half of the abdomen and is in contact with the abdominal wall close to the pelvic inlet (Pfeiffer and MacPherson, 1990). The pelvic flexure cannot be unfolded as its mesentery is attached to the mesocolon, which holds the ventral and dorsal colon parallel. The pelvic flexure is devoid of sacculations and is smaller in diameter (5.25 cm) compared with the left dorsal and ventral colons (9.25 cm) (Burns and Cummings, 1991). The abrupt narrowing of the pelvic flexure predisposes to impaction. The right colic artery and colic branch artery anastomose at the pelvic flexure. Blood vessels enter the submucosa through the tunical muscularis and form the submucosal arteriolar plexus. Arterioles ascend from the
submucosal plexus to mucosa and form an extensive capillary network around the
colic glands.

The colon has a thin mucosal layer, with colonic glands separated by extensive
lamina propria. The cells lining the colonic glands are goblets cells and granular cells
(Pfeiffer and McPherson, 1990). The outer muscularis externa is thick and has bands of
smooth muscle and elastic fibers (Pfeiffer and McPherson, 1990). A thin-layered
muscularis mucosa is present, and villi are absent. Few lymphoid follicles are also seen
in the colon.

The main function of large intestine or ascending large colon is microbial
fermentation of carbohydrates. Up to 75% of the energy requirement is met by the
production of volatile fatty acids through the microbial digestion process. Further, a large
quantity of fluid is secreted and resorbed (upto 95%) in the large colon (Argenzio et al.,
1974).

2.2 Physiology of Gut Motility

The control of gut motility is complex but well coordinated. Gastrointestinal
motility is controlled and regulated by the enteric nervous system and modulated by a
delicate interaction between myogenic, neural and humoral mediators. Gastrointestinal
motility is affected by the ability of gastrointestinal smooth muscle to generate cyclic
changes in the resting membrane potential. A basic pattern of electromyographic activity
in the intestine characterized by a low frequency, low amplitude wave or basic electric
rhythm (BER), with superimposing action potentials (spikes of high frequency and
amplitude) has been established (Davies and Gerring, 1985).
The membrane potential of smooth muscle depends on the selective ion permeability of ions and NaK-ATPases within the membrane. The resting membrane potential (-60mV) triggers smooth muscle contraction through the influx of calcium ions (Casteels, Droogmans, Reaymaekers, 1989). Calcium ion channels are sensitive to change in the membrane potential. If the membrane potential exceeds threshold, these channels are active and result in a rapid increase in calcium concentration in the sarcolemma and initiate smooth muscle contraction. In the gastrointestinal tract, the resting membrane potential varies between –35 and –75mV, causing the BER. The ion movements across the smooth muscle membranes generate the BER. Although the BER originates in the smooth muscle, it can be mediated by neural and humoral factors (Davies, 1989).

Two recognized patterns of contractility are segmentation and peristalsis. Segmentation creates the slow aboral transit of ingesta and mixing ingesta with gastrointestinal secretions, whereas peristalsis promotes transit in an aboral direction (Davies, 1989). Peristalsis is accomplished by a combination of circular and longitudinal muscle contractions. During peristaltic movements, the longitudinal muscle acts first for long stretches and subsequently circular smooth muscle contract/relax initiating propulsive movements of the intestine. Other factors such as digesta viscosity, pressure gradients and gravitational forces are important in influencing gastrointestinal motility in horses (Weems, 1982). During the preparatory phase of peristaltic movement, circular smooth muscle relaxes and longitudinal muscle contracts and when filling pressure increases both circular and longitudinal smooth muscle contracts. Thus, shortening and constricting movements of the equine cecum occur by the synchronized contractions of
circular and longitudinal smooth muscle, occurring approximately every 4 minutes (Sellers and Lowe, 1986).

The small intestinal contractions have an organized cyclical pattern, termed the migrating myoelectric complex (MMC). The motility pattern of the large intestine is more varied because the BER is present intermittently and at a variable frequency. Based on the duration of spike activity, two major patterns of electrical activity have been observed in the equine large colon. Short spike bursts (lasting < 5 sec) and long spike bursts (10-20 sec) are involved in mixing contractions, and in cecocolic retropulsion and colic propulsion, respectively (Ross, Rutkowski, Cullen, 1989). Extrinsic innervations of the large colon include parasympathetic fibers from vagal nerves and sympathetic fibers from the cranial mesenteric ganglia. Generally, parasympathetic stimulation tends to increase contractile activity of the gastrointestinal tract and sympathetic stimulation causes the opposite effect. In addition to its extrinsic innervation, the enteric nervous system plays an important role in the maintenance of gut motility. The enteric nervous system has two main plexi, namely the myenteric (Auerbach’s) plexus and the submucosal (Meissner’s plexus). The myenteric plexus is located between the circular and longitudinal smooth muscle layers and the submucosal plexus is positioned within the submucosa. The myenteric and the submucosal plexuses contain excitatory cholinergic neurons, excitatory and inhibitory adrenergic neurons, and excitatory and inhibitory non-adrenergic, and non-cholinergic (NANC) neurons (Boeckxstaens et al., 1993).

Gastrointestinal hormones or gut peptides such as gastrin, cholesytokinin, secretin, glucagon, motilin and pancreatic polypeptide tend to influence intestinal
motility, secretion, and blood supply. Prostaglandins (E and F) alter smooth muscle tone, and are found to be associated with visceral pain during mild colic (Roger and Ruckebusch, 1987). Compared with other intestinal segments, a greater number of ganglia and neurons per cm were found in the wall of the pelvic flexure suggesting the presence of a ‘pacemaker’ in the region (Schusser and White, 1997). Neurotransmitters such as substance-P, methionine-enkephalin, calcitonin gene-related peptide and vasoactive intestinal peptide (VIP) were found in the wall of the pelvic flexure (Burns and Cummings, 1993). Studies have shown that injury to the myenteric plexus during strangulation obstruction may permanently reduce the neuronal density, leading to compromise of function and possibly development of an impaction (Schusser, Scheidemann, Huskamp, 2000). This finding is in agreement with a higher incidence of colic in horses with history of large colon impaction (Dabareiner and White, 1995). Sellers & Lowe (1986) proposed that malfunction of the pelvic flexure pacemaker is one of the reasons for large colon displacement and torsion.

2.2.1 Motility Disturbances

In horses, various gastrointestinal tract disorders such as ischemia, ischemia-reperfusion (I-R) injury, impaction, endotoxemia, and parasitism result in disturbances of gastrointestinal motility. Various endogenous mediators, electrolyte imbalances and neural factors are involved in the pathogenesis of intestinal dysmotility associated with abdominal disorders. Postoperative ileus results from disruption in the balance between the excitatory and inhibitory neurotransmitters secondary to inflammation, endotoxemia and intestinal distention (Bilkslager et al., 1994). Disruption in cecal motility and emptying may be a key factor in the etiopathogenesis of cecal impaction (Collatos and
Romano, 1993). However, the mechanism has not been fully elucidated mainly due to an insufficient understanding of neurohumoral control of gastrointestinal motility.

Experimentally-induced pelvic flexure impactions in ponies caused mild persistent colic characterized by increased large colon contractile activity (Lowe, Sellers, Brondum, 1980). In experimentally-induced extraluminal obstruction in ponies, the motility of intestine proximal to the obstruction was increased. In horses, endotoxemia has been shown to cause cecal and proximal colonic ileus and cecal hypoperfusion through a mechanism involving alpha-2 adrenergic receptors (Eades and Moore, 1993).

Ischemia-reperfusion injury leads to profound functional and structural alterations in the activity of the gastrointestinal tract. In rats, I-R injury is characterized by functional dysmotility of the duodenum and jejunum with prolonged duration of contraction and a decreased number of contractions, respectively (Takahashi et al., 2001). In addition, the interdigestive MMC cycling time was found to increase during the ischemic period in experimentally induced I-R model in piglets, with no change during reperfusion (Hebra et al., 1993). The impaired motility during I-R is attributed to the release of superoxide and other vasoactive mediators such as ET and NO.

2.3 Regulation of Intestinal Blood flow

2.3.1 Role of Endothelium

Endothelium is a metabolically active layer, consisting of a single layer of flattened and polygonal cells lining the circulatory system (Bennet, Luft, Hampton, 1959). These cells are coated with a carbohydrate-rich cell coat, glycocalyx, consisting of glycosaminoglycans and polysaccharide chains of the plasma membrane glycoproteins and glycolipids (Borsum, 1991). Vascular endothelium is increasingly recognized as the
central mediator of a number of important processes in vascular biology and disease. Until recent years, it was considered simply as a barrier between blood and extravascular tissue. However, endothelial cells play a pivotal role in the balance between coagulation and fibrinolysis, immune responses, regulation of blood pressure and perfusion, and mediate local inflammatory responses (Pearson, 1991).

Recent studies have demonstrated that endothelium plays a critical role in regulation of vascular tone by releasing various vasoactive mediators such as endothelial-derived vasorelaxant (NO, prostacyclin) and vasoconstrictor (ET, prostaglandins) agents, which are major determinants in maintaining blood flow. Endothelial damage can disrupt the balance between vasorelaxant and vasoconstrictor substances, which lead to increased vasosmotor tone, resulting in increased intestinal vascular resistance and subsequent reduction of blood flow. The most likely endothelium-derived vasoactive mediators involved are NO and PGI2 (vasorelaxants) and ET-1 (vasoconstrictor).

2.3.2 Endothelin and Nitric Oxide

Various endothelial-derived contracting and relaxing factors are released following humoral, mechanical and neural stimulation of the endothelium, and the balance between these factors is responsible for the control of vascular tone and blood flow (Brenner, Troy, Ballerman, 1989). Endothelial cells synthesize NO (vasodilator, inhibits platelet aggregation and neutrophil adhesion) and ET-1 (vasoconstrictor and platelet aggregator). The opposing effects of NO and ET on vascular smooth muscle suggests a regulatory role of endothelium regarding blood flow and vasomotor tone. Inactivation of NO synthase and prostaglandin synthesis by N-ω-nitro-L-arginine methyl ester (L-NAME) and indomethacin, respectively, augmented ET-1 induced contractility in rabbit coronary
smooth muscle, which impaired coronary blood flow (Berti et al., 1993). The impaired blood flow during intestinal obstructive disorders in various species may be due to an imbalance of these mediators (increased ET-1 and decreased NO).

Because of its high metabolic activity and subsequent requirement for blood flow, the gastrointestinal tract mucosa is a primary target tissue of low-flow conditions such as hemorrhagic or endotoxin shock (Patel, Kaleya, Sammartano, 1992). One of the principal homeostatic mechanisms for the host’s response to reduced circulating blood volume (hypovolemic or endotoxin shock) is to shunt blood away from the gastrointestinal tract in an attempt to increase the effective blood volume and maintain perfusion of vital organs (Patel, Kaleya, Sammartano, 1992). The maintenance of blood flow and cardiac output is achieved mainly through intense sympathetic stimulation, and by other humoral and vasoactive mediators. The release of sympathetic mediators (epinephrine, norepinephrine) during these conditions causes strong vasoconstriction of arterioles, and large capacity intestinal and mesenteric veins, thereby displacing the blood volume from other parts of the circulation (splanchnic) and increasing total peripheral resistance and venous return. The gastrointestinal tract tissues have high resting O₂ uptake (20-25 mL/kg/min) that is normally met by a high blood flow rate (300-500 mL/kg/min) (Guyton, 2000). If blood flow falls below critical values and the O₂ uptake is compromised, intestinal damage ensues. Thus, gut circulation appears to be more susceptible to the vasoconstrictor properties of ET-1 than many other peripheral vascular beds.

The role of splanchnic ischemia or hypoxia in the pathogenesis of shock has stimulated much interest in the mechanism of intestinal ischemia. Haglund & Lundgren
(1987) suggested the presence and induction of unknown vasoactive substances that reduced the intestinal perfusion or caused systemic shock during low-flow conditions in the intestine and even after surgical correction of intestinal strangulation obstruction. This reduction in blood flow is probably due in part to splanchnic vasoconstriction, where humoral mediation is likely critical and is achieved through the release of endothelial-derived mediators. The sensitivity of the intestinal vasculature to ET-1 may be of considerable importance considering the fact that ET-1 levels are increased in a variety of instances such as chronic hypoxia, ischemia and sepsis (Cernacek and Stewart, 1989; Weitzberg et al., 1991; Murch et al., 1992; Fevang et al., 1998).

Non-selective agents should be avoided during pharmacological intervention of splanchnic circulation. Various experimental data suggest that therapeutic approaches using selective ET blockers and/or NO donors are beneficial in the treatment of I-R injury (Oktar et al., 2002; Kalia et al., 2001). The prevention of splanchnic vasoconstriction using vasodilators has been used in human gastrointestinal surgery (Gentilini et al., 1999). Selective agents, such as prostaglandin E and glucagons are used to increase mucosal blood flow. Studies have shown that captopril, an angiotensin antagonist, can also be used to disrupt angiotensin (vasoconstrictor) secretion during I-R (Guo et al., 1998). Anticoagulants have been advocated for use in gastrointestinal surgery to prevent intravascular thrombosis and or/emboli. Heparin administration was found to improve colonic blood flow, vascular resistance and systemic arterial blood pressures in experimentally-induced colonic transmural and vascular obstruction for 1 hour in ponies (Provost et al., 1991). The mechanism of action of heparin lies in its ability to act as a co-factor together with antithrombin III (AT-III), causing increased AT-III as an inhibitor of
activated clotting factors. However, few studies have shown the beneficial effects of using these agents to improve equine intestinal blood flow after ischemia.

2.4 Endothelin

The role of endothelium in the regulation of vasomotor tone was elucidated by the discovery of vasorelaxants synthesized from endothelial cells (prostacyclin, NO) (Moncada et al., 1976; Furchgott and Zawadzki, 1980). Studies have shown that endothelium also releases a vasoconstricting peptide, termed endothelial-derived constricting factor (EDCF) (Hickey et al., 1985). This peptidergic substance was later purified from supernatant of porcine aortic endothelial cell culture medium, and was termed endothelin (Yanagisawa et al., 1988). Cloning and sequence analysis has since revealed three distinct endothelin-related genomic loci in human, pig and rat that encodes for three similar but distinct peptides, ET-1 (formerly called porcine or human endothelin), ET-2 and ET-3 (formerly called rat-ET) (Yanagisawa et al., 1988; Itoh et al., 1988; Inoue et al., 1989). In addition, sarafotoxin (STX) has also been included in the ET family due to its remarkable similarities in homology and bioactive properties. The presence of ET in invertebrates and fish indicates its long evolutionary history (Kasuya et al., 1989).

Endothelins are a family of 21-amino acid peptides (MW 2492) and are the most potent vasoconstrictors known to date. Endothelins are multifunctional peptides that have vasoactive, ionotropic, and mitogenic properties (Rubanyi and Polokoff, 1994), and have the ability to modulate other hormone systems as well as intestinal, renal and pulmonary function. Expression of ET in non-vascular cells/tissues suggests its diverse role in physiological and pathological processes (Levin, 1995; Rubanyi and Polokoff, 1994).
These diverse and complex functions include neurotransmission, stimulation of intestinal contraction (motility), synthesis of atrial natriuretic factor (ANF), counter-balancing NO release, inhibition of renin release and control of sympathetic tone (Levin, 1995).

2.4.1 Biology of ET

The human ET-1 gene sequence found in chromosome 6 is 6.8kb in size and encompasses 6 exons and 5 introns. Each portion of the exon encodes a portion of ET-1, and exon 2 encodes the majority of prepro-ET-1 (Lee et al., 1990). The promotor region has the typical CAAT and TATA sequences regulating transcription and has several responsive elements, which provide regulatory sites for various stimuli (Lee et al., 1990). The genes for ET-2 and ET-3 are located on chromosome 1 and 20, respectively. The equine ET-1 has not been completely sequenced, however, a group of scientists have cloned and published the partial sequence of chromosomal ET-1 gene (Gene Accession # AF130760).

2.4.2 Endothelin Isoforms

Each of the isoforms of ET is a product of separate genes that encode the large precursor, prepro-ET. Endothelin-1, is the predominant type of ET, but ET-2 and ET-3 also exist. Vasointestinal constrictor (VIC), also known as β-endothelin, has been demonstrated in murine intestine (Inoue et al., 1989) and differs from ET-1 by three amino acids (in positions 4, 6 and 7).

Endothelin-1 is the most abundant circulating ET isoform, and has been best characterized (Rubanyi and Polokoff, 1994). Of all the endothelin types, the principal ET of importance in vascular diseases is ET-1, and is the only isoform synthesized by the endothelium. Endothelin-1 is also found in smooth muscle, airway epithelium and other
cell types. The ET-1 and ET-3 isoforms have been found in nearly every organ examined including different segments of the gastrointestinal tract of rats (Matsumoto et al., 1989; Firth and Ratcliffe, 1992). The ET-3 isoform also circulates in the plasma and is found in the central nervous system, kidney and lung, however, the cellular source is not clear (Hemsen and Lundberg, 1991). The presence of ET-3 in the brain suggests its role in important functions including astrocyte proliferation and development (Shinmi et al., 1989). However, the expression of ET-2 is much more limited and found predominantly in the kidney and intestine, where its function remains unclear (Levin, 1995).

2.4.3 Biosynthesis of ET

Endothelin levels are controlled at the level of transcription of its precursor, preproendothelin (preproET). Endothelin isoforms are processed from prepro-ET to form pro-ET, which is then post-translationally converted into the active peptide, ET. Endothelins are derived from unusual proteolytic cleavage of large pre-proET (approximately 203 amino acid) by specific dibasic endopeptidases. Each pre-proET has a distinct gene and amino acid sequence processed to the polypeptide prohormone, big ET, of various lengths (37-41 amino acids). The polypeptides (big ETs) are cleaved in the cytoplasm by the proteolytic action of membrane-bound metalloproteinases, endothelin-converting enzymes (ECE), into ET and a C-terminal fragment-1 (CTF-1) (Figure 2.1). The big ET-1 has very low biologic activity. The enzymatic process of the synthesis of mature ET-1 occurs by unusual proteolytic cleavage of big ET-1 between Trp21 and Val22 by ECE (in case of big ET-3 it is between Trp21 and Ile22) (Opgenorth, Wu-Wong, Shiosaki, 1992). The conversion of big ET-1 to ET-1 by ECE is essential for the expression of full vascular activity. A group of five distinct ECE isoforms, ECE-1a, ECE-
**Figure 2.1** Biosynthesis of endothelin-1. Abbreviations: S-S – disulfide bond; CR- conserved region; VR- variable region.
1b, ECE-1c, ECE-2 and ECE-3 have been cloned and classified (Turner and Murphy, 1996; Schweizer et al., 1997), each with a different tissue distribution and pH requirement for activity. Each bioactive form of the ET isoforms is synthesized by specific conversion between Trp\textsuperscript{21}-Val\textsuperscript{22} and Trp\textsuperscript{21}-Ile\textsuperscript{22}. The ECE isoforms are found in many cell types including endothelium, epithelium and alveolar macrophages (Takahashi et al., 1993; Ohnaka et al., 1990). However, ECEs have not yet been identified that are selective for ET-2 and ET-3. Greater than 80% of the ET-1 synthesized by the endothelium is secreted abluminally toward the tunica media away from the luminal surface of the airway or vessel (Wagner et al., 1992). Directional secretion allows ET-1 to act in a paracrine or autocrine manner whereas secretion into the circulation allows ET-1 to act as a hormone.

The half-life of ET mRNA is 20-30 minutes, whereas the ET peptide has an extremely short half-life of 4-7 minutes. Endothelins are stable in plasma, predominantly cleared or metabolized in the lung during their first passage (90%), and excreted in small amounts via the kidney and liver (de Nucci et al., 1988). In rats and humans, the lung is responsible for ET-1 elimination; however, in pigs the renal and splanchnic circulation are involved in ET-1 clearance. Some studies suggest that ET is eliminated from blood quickly and is likely followed by the internalization of the peptide into the tissue parenchyma and/or vasculature of various tissues (Anggard et al., 1989; Shiba et al., 1989). The pathway of ET synthesis is well defined, but its complex process of degradation, which also determines its biologic activity, is not completely understood.

2.4.4 Endothelin Structure

Endothelin is a family of 21 amino acid peptides with free amino and carboxy-
terminal, which include four cysteine residues at positions 1, 3, 11 and 15, forming two intra-molecular disulfide linkages between Cys\(^1\) and Cys\(^{15}\), and Cys\(^3\) and Cys\(^{11}\) (Yanagisawa et al., 1988). The presence of the disulfide bonds provides a compact NH\(_2\) terminal core region consisting of outer and inner loops. The disulfide bonds present in ET are vital to its high affinity binding to one class of ET receptors, but are of less importance in recognition by the other class of ET receptors. Amino acid residues 4-7 comprise the variable region, residues 12-14 are highly unpolar regions, and residues 16-21 are hydrophobic. The hydrophobic C terminus in all three ET isoforms is strictly conserved. The terminal tryptophan region of ET has been shown to be essential for its full biologic activity. The ET-2 isoform exhibits the closest structural similarity to ET-1, differing by only two amino acid residues at positions 6 and 7, whereas ET-3 differs from ET-1 by six amino acids at positions 2, 4, 5, 6, 7 and 14 (Figure 2.2). These peptides exhibit different degrees of vasoactivity and it has been suggested that the overall charge of the inner Cys-Cys loop may be an important determinant of this property (Kloog and Sokolovsky, 1989).

### 2.4.5 Endothelin Receptors

The use of radiolabelled ET in autoradiographic and standard ligand binding studies has demonstrated that the ET isopeptide binding sites are distributed in a wide variety of both adult and fetal organs and tissues in different species (Davenport et al., 1989; Takayanagi et al., 1991). The binding sites have been shown in locations such as the central nervous system, lung, gastrointestinal tract (stomach, small intestine and colon) and within the cardiovascular system (Power et al., 1989; MacCumber, Ross, Snyder, 1990). Two independent groups have reported the cloning, sequencing and functional
expression of endothelin receptors (Arai et al., 1990; Lin et al., 1991). Two types of ET receptors were cloned, termed endothelin A ($\text{ET}_A$) and endothelin B ($\text{ET}_B$) with size ranging between 45,000-50,000 daltons. The $\text{ET}_A$ and $\text{ET}_B$ receptor genes are located on human chromosomes 4 and 13, respectively (Arai et al., 1990; Sakurai et al., 1990). There is a high degree of sequence identity at the amino acid level (90%) between the receptor subtypes, however, little homology is found between the two receptor types, $\text{ET}_A$ and $\text{ET}_B$. The greatest diversity occurs in the extra-cellular domain and in the cytoplasmic COOH region. Both ET receptors ($\text{ET}_A$ and $\text{ET}_B$) belong to a family of heptahelical G-protein-coupled receptors. The ET receptors have an extracellular NH$_2$ terminal region, transmembrane helices separated by 3 extracellular and 3 cytoplasmic loops with a terminal cytoplasmic COOH region. Endothelin is hydrophilic and thus unable to cross plasma membranes. The saturable binding sites are present in the cell wall, and it is presumed that ET acts by binding to these receptors.

All three ET isoforms bind to their distinct receptors in mammalian cells that have different affinities to endogenous ligands. The two main types of ET receptors found in smooth muscle and endothelium are $\text{ET}_A$ and $\text{ET}_B$, respectively. These receptor types originally identified in the vasculature have also been found in many other organs such as the liver, brain, kidney and gastrointestinal tract, with the greatest concentrations in heart and lung; this suggests ET’s pleiotropic function. The $\text{ET}_A$ receptor is principally found in vascular and airway smooth muscle cells and has 10 times more binding affinity for ET-1 than for ET-3 (Rubanyi and Polokoff, 1994). The affinities for binding of the ET isoforms to $\text{ET}_A$ receptors are in the order: ET-1 > ET-2 > ET-3 (Rubanyi and Polokoff, 1994). Currently two subtypes of $\text{ET}_B$ have been cloned and characterized, namely $\text{ET}_{B1}$
Figure 2.2 Endothelin isoforms. The dark colored circle represents variable amino acid region compared to ET-1.
and ET<sub>B2</sub>. The ET<sub>B1</sub> receptors are found in the endothelium and mediate the release of NO and prostacyclin causing vasodilatation (de Nucci et al., 1988; Sakurai et al., 1990), whereas ET<sub>B2</sub> receptors on the smooth muscle mediate vasoconstriction. Thus, binding of ET-1 with ET<sub>A</sub> receptors leads to constriction and with ET<sub>B1</sub> causes dilatation. The difference in tissue-specific expression between ET receptor types and subtypes contributes to the different actions of endothelin. Within a particular tissue, the distribution of ET<sub>A</sub> and ET<sub>B</sub> receptors varies. Consistent with functional activity of ET-1 in intestinal smooth muscle, ET-1 binding sites have been documented in the stomach, ileum, jejunum, and colon of rats (Koseki et al., 1989; Takahashi et al., 1990) and guinea pig (Bolger et al., 1992). In the kidney, the ET<sub>A</sub> receptors are found predominantly in the vasa recta whereas ET<sub>B</sub> is found mostly in the collecting ducts (Simonson, 1993). In horses, a genetic polymorphism of the ET<sub>B</sub> receptor is linked to the Overo Lethal White Foal Syndrome in paint foals, characterized by spastic colon and intestinal aganglioneurosis (Yang et al., 1998).

**2.4.6 Endothelin Signal Transduction Mechanism**

Endothelins bind to receptors and elicit their effects, such as vasoconstriction and vasodilatation, via signal transduction pathways through G-protein activation (Figure 2.3). The G-proteins have various subunits such as G<sub>s</sub>, G<sub>i</sub> and G<sub>q</sub>. The G<sub>s</sub> alpha subunit primarily activates adenylyl cyclase that catalyzes the formation of cAMP from ATP. The G<sub>i</sub> alpha subunit inhibits adenylylate cyclase. The G<sub>q</sub> alpha subunit activates phospholipase C (PLC) that cleaves phosphotidylinositol-4, 5-bisphosphate (PIP<sub>2</sub>) in the cell membrane to release diacylglycerol (DAG) and inositol-(1, 4, 5)-triphosphate (IP<sub>3</sub>). The principal protein affected by the activation of phospholipases is protein kinase-C
(PKC), which is maximally active in the presence of calcium ion and DAG. The PLC contains SH2 domains that allow it to interact with tyrosine phosphorylated receptor kinases. This allows PLC to be intimately associated with the signal transduction complexes of the membrane as well as membrane phospholipids that are its substrates.

The phosphoinositide cascade is mediated by membrane bound enzyme PLC, which is activated by ET binding to its receptors (Simonson et al., 1989). The IP3 causes the release of Ca\textsuperscript{2+} stores in the endoplasmic reticulum and sarcoplasmic reticulum, increasing the levels of Ca\textsuperscript{2+} in the cytosol. The increased level of cytosolic Ca\textsuperscript{2+} then causes smooth muscle or vascular contraction. The DAG and Ca\textsuperscript{2+} activate PKC, catalyzing phosphorylation of serine-threonine residues of various target proteins mediating mitogenic action.

Endothelin-induced contraction is mediated by the activation of nonselective cation channels permeable to calcium (Zhang et al., 2000). The binding of ET to its receptor leads to the activation of Gs\textsubscript{α} subunit of adenylyl cyclase, forming cAMP from ATP. The cAMP acts as an intracellular messenger and catalyzes various other cellular functions. The ET-induced vasoconstriction is a biphasic response, whereby a period of transient vasoconstriction is followed by a distinct and sustained vasoconstriction. The transient vasoconstriction observed with ET release is attributed to the release of Ca\textsuperscript{2+} from the intracellular calcium stores and the characteristic sustained vasoconstriction is mediated mainly through the persistent entry of extracellular calcium through calcium channels of the plasma membrane (Masaki, 2000). The vasodilator action of the ET-activated receptor, ET\textsubscript{B1}, is attributed to the release of NO and prostacyclin or increased levels of cGMP through activation of G\textsubscript{i}. The G\textsubscript{i} activation has been shown to cause
inhibition of adenylate cyclase and activation of the Na\(^+\)-H\(^+\) antiporter (Aramori and Nakanishi, 1992; Koh et al., 1990). Another function of the ET receptor is to mediate the growth activity of cells such as smooth muscle or endothelial cells (mitogenic). This activity is mediated through various mechanisms, i.e., classical PKC-dependent and -independent pathways.

2.4.7 Endothelin Receptor Antagonists

A number of ET receptor antagonists have been developed and tested in animal models. The first ET antagonist developed for human testing was a natural by-product of *Streptomyces misakiensis*. However, this ET antagonist has low binding and functional properties. A series of peptide antagonists for endothelins selective toward ETA (BQ 123), ET\(_B\) (BQ 788, IRL-1038) and both ET-A/ET-B receptors (PD 142893, TAK-044, RO 46-2005) have been designed. A competitive ET antagonist, BE-18257, a cyclic pentapeptide was shown to have very low potency in binding and functional assays (Benigni and Remuzzi, 1999). These compounds are hydrolyzed by circulating peptidases, and do not penetrate the blood brain barrier when given orally (Benigni and Remuzzi, 1999). Recently, synthetic non-peptide antagonists have been developed (PD 155080-Bosentan, BMS 182874, SB 209676) and they produced mixed results (Benigni and Remuzzi, 1999).

2.4.8 Factors Involved in ET-1 Expression and Secretion

Endothelin-1 levels are controlled at the level of transcription of its precursor, preproendothelin-1. The upstream regulatory element of the ET gene contains an acute phase response element, which is activated by various important stimuli (Inoue et al.,
Figure 2.3 Mode of action involved in ET-1 induced constriction, relaxation and mitogenesis. VOC-voltage operated channel, PKC-protein kinase C; DAG-diacylglycerol; IP3- inositol triphosphate; NO- nitric oxide; PLC- phospholipase C; PIP2- phosphatidylinositol biphosphate; MLC-myosin light chain; MLC-P- phosphorylated myosin light chain; PGE2-prostaglandin E2; PGI2-prostoglandin I2 and RER-rough endoplasmic reticulum.
The ET-1 synthesis is controlled by a number of physical and humoral factors (Kurihara et al., 1989; Emori et al., 1989; Kourembanas et al., 1991; Kanse et al., 1991; Malek and Izumo, 1992). The physical factors include shear stress and hypoxia. The humoral factors involved in ET-1 synthesis are hormones (vasopressin, angiotensin, epinephrine, insulin), bradykinin, cytokines (tumor necrosis factor-(TNF)-α, interleukin-(IL)-1), growth factors such as transforming growth factor (TGF)-β, platelet activating factor (PAF) and endotoxin. The suppressors include NO, atrial natriuretic factor (ANF) and cGMP.

2.4.8.1 Physical Factors

Low shear stress causes increased ET-1 mRNA expression and ET release in cultured aortic endothelial cells (Yoshizumi et al., 1990). However, bovine aortic endothelial cells exhibited a dose-dependent decrease in ET-1 mRNA expression with increased shear stress (Malek and Izumo, 1992). Also, Kourembanas et al. (1991) demonstrated increased ET-1 mRNA expression in cultured human umbilical vein endothelial cells to low oxygen tension.

2.4.8.2 Humoral Factors

Cytokines such as TNF and IL-1 are identified as the primary mediators in the pathogenesis of sepsis and are important modulators of ET-1 secretion (Kahaleh and Fan, 1997, Kruse-Elliot, Whorton and Olson, 1998). In vitro ET synthesis increases in cultured porcine aortic endothelial cells stimulated with TNF and IL-1 (Kanse et al., 1991; Yoshizumi et al., 1990). Also, IL-1 and IL-1β increase ET-1 mRNA levels and ET synthesis in a dose-dependent manner (Yoshizumi et al., 1990). It is speculated that an IL-1-endothelin axis exists between macrophages and smooth muscle cells, which
implicates its role in vasospasm. But additional studies have shown that IL-1β, IL-6 and IL-8 have no effect on ET-1 synthesis and release from cultured endothelial cell, whereas interferon-γ and TNF have only a small effect (Kanse et al., 1991).

Cytokines released by activated platelets such as TGF-β induces ET-1 gene expression by binding to nuclear factor-1 (NF-1) (Kanse et al., 1991), but the role of TGF in modulation of ET-1 secretion during I-R is unknown. Various hormones like vasopressin and angiotensin-II induce an increase in endothelial ET-1 mRNA levels (Emori et al., 1989). The induction and secretion of ET-1 by TGF-β in endothelial cell cultures is in a manner similar to thrombin (Kurihara et al., 1989). Thus, during vascular injury and thrombin formation, ET-1 may play a role in local constriction via the release of TGF-β from activated platelets. Taken cumulatively, these results suggest that the production of ET-1 in endothelial cells is regulated by factors associated with platelet aggregation and macrophage infiltration.

Tumor necrosis factor, an important primary mediator of endotoxemia, increases the transcriptional rate of the ET-1 gene (Marsden and Brenner, 2000). Endotoxin stimulates monocytes/ macrophages to release TGF, IL-1 and IL-6 (Morris, 1991, Morris et al., 1992). These mediators initiate and perpetuate a cascade of inflammatory events that can lead to endotoxic shock and multiple organ failure. Stimulation of cultured vascular smooth muscle cells with TNF-α (10 ng/mL), IFN-γ (1,000 U/mL), IL-1β (500 U/mL) and lipopolysaccharide (10 micrograms/mL) for 48 hrs markedly increase the ET-1 mRNA expression and ET-1 secretion (Woods et al., 1998). This suggests that these mediators, TNF and IL-1, released during endotoxemia may stimulate and augment synthesis and release of ET from the vascular endothelium.
2.5 Colic

Gastrointestinal tract disease is the leading natural cause of death in adult horses, (Baker and Ellis, 1981) and most horses are afflicted by colic sometime during their lifetime. Acute gastrointestinal tract diseases of the equine abdomen associated with pain are termed ‘colic’. The mortality rate for colic was shown to be the highest of any cause of death in horses, including old age and injury (Tinker et al., 1997). The etiology and pathophysiology of colic is complex and varied.

Small intestinal strangulating obstruction secondary to twisting around its mesentery or incarceration and strangulating large colon volvulus are common causes of colic in horses, and are associated with high mortality (Barclay, Foerner, Phillips, 1980; Harrison, 1988; MacDonald et al., 1989). The poor prognosis for survival following surgical correction of intestinal strangulation obstruction in horses despite appreciable advances in medical care is associated with a sustained reduction in intestinal blood flow and mucosal perfusion with changes in microvascular permeability leading to edema, congestion, hemorrhage, and ultimately, necrosis of the mucosal layer (Snyder et al., 1990; Wilson et al., 1994; Darien et al., 1995).

Most cases of naturally acquired intestinal strangulation obstruction in horses involve simultaneous arterial and venous occlusion, with venous occlusion being more predominant. Strangulating obstruction of the large colon and small intestine are the most common causes of vascular occlusion in horses with colic (Barclay, Foerner, Phillips, 1980; Huskamp, 1982). Vascular occlusion of the intestine may be induced by parasitism, with subsequent thromboembolism, and or by strangulation obstruction. Intestinal ischemia (strangulating large colon volvulus and small intestinal strangulation
obstruction) is universally fatal without surgical intervention. Up to one-half of the deaths attributed to colic are associated with lesions in the large intestine (Pascoe et al., 1983).

Ischemic injury to the mucosal epithelium occurs predominantly during strangulation obstruction; however, intestinal injury can progress after surgical correction owing to either continued ischemia associated with microvascular thrombosis or splanchnic vasoconstriction during the postoperative period. Intestinal blood flow has been shown to remain significantly depressed below the baseline values for at least four hours after correction of experimentally induced complete arteriovenous occlusion of the large colon in ponies (Wilkins et al., 1994). Thus, even after surgical correction and/or resection of affected intestine the remaining intestine is predisposed to continued ischemic damage resulting in increased mortality (Fischer and Meagher, 1986). Alternatively, damage induced by ischemia likely continues after blood flow is restored, which is initiated by cytotoxic reactive oxygen metabolites and perpetuated by neutrophils and other mediators (Moore, Muir, Granger, 1995). This process is known as ischemia-reperfusion injury. Endotoxemia is estimated to occur in nearly 25% of horses with colic; most of these cases involve intestinal ischemia or inflammation (King and Gerring, 1988). The high mortality subsequent to intestinal ischemia is associated with disruption of the intestinal mucosal barrier, which allows translocation of luminal endotoxin and bacteria into the systemic circulation, resulting in endotoxemia and circulatory shock. Thus, eventually horses succumb to the effects of endotoxic and hypovolemic shock.
2.5.1 Classification of Colic

Colic can be classified into various types based on the etiology and the intestinal segment involved. Colic has been anatomically and functionally classified as simple obstruction, strangulating obstruction, non-strangulating infarction, enteritis, peritonitis, ulceration and ileus (Tinker et al., 1997). The non-specific colic cases without definitive diagnosis are referred commonly as medical, spasmodic, flatulent, or mild colic; and may be attributed to numerous factors including diet or parasites (Parry, 1983).

2.5.1.1 Simple Obstruction - Impaction

Simple obstruction of the intestine involves luminal obstruction without vascular compromise, the most common of which is the impaction of the intestinal tract. This type of simple obstruction is more common in horses, and accounts for nearly 30% of all colic cases admitted to referral centers (Dabareiner and White, 1995). Impaction results from accumulation of ingesta in the intestinal tract, which may be due to abnormalities in myogenic, humoral and neurogenic control of gut motility (Stanghellini, 1988), thereby disrupting aboral propulsion of ingesta. Obstruction and dehydration of ingesta in the gastrointestinal tract also lead to impaction.

Impaction often develops at sites of narrowed luminal diameter such as the pelvic flexure or just proximal to the transverse colon in the right dorsal colon. The common sites for impaction in the horse include the ileum, cecum, pelvic flexure, right dorsal colon and small colon (White and Dabareiner, 1997). Partial obstruction is more common in the cecum, large colon and small colon, where gas and fluid often pass around the ingesta. Cecal impaction is the most common cause of cecal disease and obstruction, accounting for up to 5% of colic cases admitted to US veterinary teaching hospitals.
Cecal impaction is more common in the cranioventral part of the cecal base (White, 1980). Local distention due to the impacted mass can cause alterations in circulation to the bowel wall, thereby increasing capillary permeability and loss of fluid and protein into the peritoneal cavity and bowel. Likewise, focal areas of mural ischemia can occur due to distention and or pressure from the ingesta mass.

2.5.1.2 Strangulation Obstruction

Occlusion of the intestinal blood flow with simultaneous blockade of the intestinal lumen is defined as intestinal strangulation obstruction (Schwartz and Stores, 1969). Strangulation obstruction can be further divided into hemorrhagic and ischemic strangulation obstruction. Hemorrhagic strangulation obstruction is characterized by arteriovenous occlusion and luminal obstruction; however, venous occlusion predominates and with some patent arterial blood flow (White, 1990; Snyder JR, 1989). This often results in severe mural hemorrhage, edema and eventually necrosis. Clinically, this is the most common type of strangulation obstruction in the horse. Ischemic strangulation obstruction is characterized by simultaneous venous and arterial occlusion together with luminal obstruction. Despite the type, strangulation obstruction leads to rapid injury to the intestinal mucosa, a highly metabolically active layer. Depending on the extent of ischemia, strangulation obstruction can also be further classified into warm and cold type. In the ‘cold’ type there is a complete blood loss (no flow, as in organ transplantation), whereas in the ‘warm’ type there is reduced flow (Southard, 1990).

Strangulating large colon volvulus is a common and frequent cause of fatal colic requiring surgical intervention (Barclay Foerner, Phillips, 1980; Harrison, 1988; Snyder et al., 1989). Approximately 80% of the colon is unanchored and able to move freely in
the abdomen; because of this anatomical arrangement, it is prone to volvulus, especially at the base of the colon. Unfortunately, the major blood vessels of the colon, the dorsal and ventral colonic arteries, enter the mesentery at this location. With the onset of colonic volvulus, the twisting and stretching of the vessels in the mesentery compresses the vasculature (Fischer and Meagher, 1986), resulting in colonic mucosal ischemic injury and epithelial barrier disruption owing to reduced or absent blood flow.

Vascular occlusion is commonly associated with large colon volvulus, which accounts for 7-40% of horses requiring colic surgery (Barclay, Foerner, Phillips, 1980; Huskamp, 1982). If the mucosa is not irreversibly damaged during the volvulus, the epithelium may re-establish an intact mucosal barrier through various mechanisms (Blikslager et al., 1997). Intestinal strangulation obstruction leads to characteristic lesions such as gas and fluid distention, edema, congestion, hemorrhage, necrosis and eventually gangrene or rupture (or both) (Van Hoogmoed et al., 2000). The extent of damage varies from mild mucosal injury to complete loss of mural wall integrity and subsequent endotoxemia, hypovolemia and shock. The magnitude of injury depends on the degree and duration of lack of blood supply and the metabolic status of the animal. Despite improvement in surgical techniques and post-operative medical care, the survival rate after correction of large colon volvulus is low (21-42%) (Fisher and Meagher, 1986).

Small intestinal strangulation causes acute, severe colic pain due to intestinal ischemia. Small intestinal strangulation may occur from small intestinal volvulus, incarceration through a mesenteric rent or a natural opening such as epiploic foramen or inguinal ring. The incidence and frequency of small intestinal strangulation obstruction
varies depending on age and other risk factors (Morris, Moore, Ward, 1989).

2.6 Ischemia-Reperfusion Injury

2.6.1 Ischemia

Ischemia is defined as a critical reduction of blood flow that occurs either due to functional constriction or mechanical obstruction of blood vessels (thrombus), leading to inadequate tissue perfusion and oxygenation (Fantone, 1990; Grum, 1990). Reduction in blood flow causes a reduced oxygen supply to the tissues, resulting in disruption of normal cellular and bioenergetic pathways (Fantone, 1990; Grum, 1990). Oxygen is essential for normal cellular function and survival. Oxygen deprivation leads to failure of oxidative phosphorylation and a decrease in ATP production, which is detrimental to the metabolic needs of the tissues.

As the oxygen concentration decreases, anaerobic glycolysis occurs in an attempt to maintain cell function (Grum, 1990). These changes cause a decreased intracellular pH due to the accumulation of lactic acid and hydrogen ions from ATP hydrolysis. The accumulated lactic acid further inhibits ATP production and results in increased intracellular acidosis. Due to the lack of ATP, the Na\(^+\)-K\(^+\)-ATPase pump function is altered resulting in increased intracellular calcium concentration. Thus, increased intracellular pH, and accumulation of Ca\(^{2+}\) together with the release of proteases, lipases and other degrading enzymes results in autolytic destruction of cellular organelles and eventually results in cell death (Moore, Muir, Granger, 1995). Thus, a reduction in blood supply to the intestinal tract results in rapid injury and death of the highly energy-dependent mucosal epithelial cells.
2.6.2 Reperfusion Injury

Reperfusion injury is defined as tissue damage sustained on restoration of blood flow after a period of ischemia (Moore, Muir, Granger, 1995). Reperfusion injury occurs as a result of a complex series of cellular metabolic events that are initiated during the ischemic period and are aggravated during restoration of blood flow. Predominant lesions or alterations associated with intestinal I-R injuries include alterations in cell membrane permeability, tissue edema, inflammatory cell influx, hemorrhage and necrosis of the intestinal wall (Moore et al., 1994). Horses with intestinal compromise from prolonged or severe ischemia secondary to strangulation obstruction probably do not undergo a relevant reperfusion injury. However, horses with ischemia of less severity or shorter duration may be susceptible to reperfusion injury. There is experimental evidence to show that the small intestine and large colon of horses are capable of undergoing I-R injury (Horne et al., 1994; Moore et al., 1994;). Moore et al. (1994) showed histopathologic evidence of reperfusion injury in the large colon of adult horses after reestablishment of colonic arterial blood flow after 3 hrs of low-flow ischemia. In horses, even after surgical correction of volvulus or strangulation obstruction, horses may recover well for a few hours and then begin to deteriorate due to continued ischemia or reperfusion injury (Moore et al., 1994).

Provost et al. (1991) showed that after correction of a 1-hr 720º experimental large colon torsion in ponies, colonic blood flow increased above ischemic flow, but remained well below the preocclusion flow. Similar observations of reduced blood flow below pre-ischemic values after the release of arteriovenous occlusion was shown in the large colon of horses (Wilkins et al., 1994). This observed reduction in blood flow after
correction of experimentally-induced large colon volvulus (Provost et al., 1991) or combined arteriovenous occlusion (Wilkins et al., 1994) in ponies was attributed to vasoactive mediators released during obstructive disorders.

2.6.3 Mechanism of I-R injury

Several mechanisms seem to be essential for the development of post-ischemic lesions of the gut. The injury is triggered by both molecular and enzyme-mediated mechanisms (Figure 2.4). Initially, oxygen radicals initiated by the hypoxanthine-xanthine oxidase system are the molecular triggers. Second, activation of phospholipase A$_2$, possibly due to influx of calcium, constitutes an enzymatic trigger. This deleterious cascade is perpetuated by phospholipids-derived mediators and neutrophils.

2.6.3.1 Production of Oxygen Free Radicals

Under normal conditions, cellular oxidative phosphorylation involves a 4-electron reduction of oxygen to water (Flaherty and Weisfeldt, 1988). This occurs as a series of reactions resulting in the formation of superoxide anion and hydroxyl radical (OH\(^{-}\)). The superoxide anion and OH\(^{-}\) are highly reactive free radicals that can react with cell membranes and cause loss of cellular integrity and tissue damage (Flaherty and Weisfeldt, 1988). Further, hydrogen peroxide (H$_2$O$_2$), which is not highly reactive, can be converted into OH\(^{-}\) by ferrous iron, which is made available from the superoxide-mediated conversion of ferric to ferrous iron (Flaherty and Weisfeldt, 1988). Naturally occurring antioxidants protect the tissues from the oxygen free radicals (OFRs) formed during normal cellular metabolism. Endogenous antioxidants include enzymatic antioxidants (superoxide dismutase, catalase and glutathione peroxidase) and non-enzymatic antioxidants (alpha-tocopherol, ascorbate and beta-carotene).
During reoxygenation of ischemic tissues, rapid formation of these radicals can overwhelm the protective effects of endogenous antioxidants, resulting in further injury. During ischemia, several events occur that set the stage for OFR production upon reperfusion. Hypoxanthine accumulates in endothelial cells and in intestinal mucosal cells and is catalyzed by xanthine oxidase (XO), an enzyme present in the cytosol, to form xanthine and uric acid, with the generation of a superoxide radical. Increased intracellular Ca^{2+} during ischemia, in conjunction with calpain, converts xanthine dehydrogenase (XDH) to XO (Cohen, 1989). The hypoxanthine that accumulates during ischemia provides large amounts of substrate for superoxide synthesis during reoxygenation. Superoxide radicals are converted to H_2O_2 by endogenous superoxide dismutase.

The synthesis of OH radicals through iron-dependent mechanisms is called the Haber-Weiss reaction (O_2^- + H_2O_2^- → OH^-). During reoxygenation, the production of free radicals such as OH overwhelms the endogenous antioxidant systems. These hydroxyl radicals initiate cellular damage via lipoperoxidation and release of phospholipids-derived mediators (leukotriene-B_4 and platelet activating factor), which are chemoattractants of neutrophils. Thus the overabundance of OFRs produced during reperfusion leads to oxidant-induced tissue injury (Flaherty and Weisfeldt, 1988).

2.6.4 Mucosal Injury

Studies have shown strangulation obstruction in adult horses can induce gross changes in the intestinal wall, necrosis of villus tips in strangulated small intestinal segment, and to a lesser degree, in the non-strangulated jejunum orad to the strangulation (Dabareiner et al., 1993). Experimentally-induced large colon ischemia causes
Figure 2.4 Proposed etiopathogenesis of ischemia-reperfusion injury. During ischemia, hypoxanthine accumulates in epithelial cells and intestinal mucosal cells simultaneous with the conversion of xanthine dehydrogenase (XDH) to xanthine oxidase (XO). On reperfusion, hypoxanthine is metabolized by XO. The superoxide and hydrogen peroxide (H$_2$O$_2$) react in the presence of an Fe$^{3+}$ catalyst to form the hydroxyl ion (OH$^-$), which initiates lipoperoxidation, and causes release of phospholipid-derived mediators (LTB$_4$, PAF), which are chemoattractants of neutrophils. On adhesion to the endothelium, neutrophils release elastase and lactoferrin, causing extravasation. O$_2$ is converted to O$_2^-$, which is further metabolized to H$_2$O$_2$ by nicotinamide adenine nucleotide phosphate (NADPH) oxidase. Myeloperoxidase then catalyzes the reaction of H$_2$O$_2$ and a chloride ion resulting in toxic hypochlorous acid (HOCl). [Redrawn with the permission of Moore et al., 1995]
accumulation of fluid in the intestinal wall, lumen and mesentery and the serosa becomes dark purple (Snyder et al., 1988). The thickening of the intestinal wall is due to hemorrhage, edema and congestion (Snyder et al., 1989). The observed changes are attributed to increased capillary hydrostatic pressure associated with venous obstruction and increased microvascular permeability due to inflammatory cell infiltration and mediator release during ischemia.

During small intestinal hemorrhagic strangulation obstruction, fluid sequesters in the subepithelial space, and the epithelium separates from the underlying attachment to the basement membrane at the villus tip (White, Moore, Trim, 1980). Sloughing of epithelial cells develops due to severe intracellular alterations. The villi are completely denuded in 3 hrs, and the villi contract to the levels of the crypts (Freeman et al., 1988). During various periods of ischemia, complete necrosis of the mucosal epithelium extends to the base of the crypt, which occurs by 4 to 5 hrs, and by 6 to 7 hrs degeneration extends beyond the muscular layer (White, Moore, Trim, 1980). Combined ischemia and reperfusion of 2-hrs duration induces moderately severe mucosal injury in the equine jejunum, characterized principally by disruption of enterocyte attachment from the basement membrane to the lamina propria (Arden et al., 1990). Eventually, the progression of mucosal epithelial injury leads to mucosal necrosis, with disruption of the mucosal barrier, allowing migration of luminal bacteria and endotoxin into the systemic circulation.

2.7 Role of ET-1 in the Pathophysiology of The Gastrointestinal Tract Disorders

The role of ET-1 in etiopathogenesis of various disorders such as ischemia, I-R injury, vasospasm, motility disorders and endotoxic shock is well documented in several
species (Murch et al., 1992; Chou et al., 1995; Wanecek et al., 1997; Soda et al., 1999; Tekin et al., 1999; Ozel et al., 2001). High concentrations of circulating plasma ET-1 concentrations and increased tissue ET-1 expression have been shown in these pathological conditions, suggesting a direct or indirect role of ET in the pathogenesis of these disorders. The observed increase in plasma ET-1 is believed to be exacerbated by the reduced intestinal blood flow caused by increased vascular resistance, resulting in ischemia and I-R injury.

Endothelial damage occurs in the colonic vasculature of the large colon and small intestine of horses subsequent to I-R and is further injured by endotoxemia (Henninger et al., 1992; Dabareiner et al., 1993). The sustained reduction in blood flow and/or reperfusion injury may be attributable to the release of endothelial-derived contractile factors (EDCF), such as ET-1, from the damaged vasculature or due to imbalance between ET-1 and NO release.

Horses with strangulation obstruction (low-flow states) and endotoxemia have increased levels of circulating IL-1, catecholamines, TNF-α and thrombin, which are known to cause increased ET-1 synthesis and secretion (Barton and Callatos, 1999; Morris, 1991). The potent contractile effects of exogenous ET-1 were characterized in isolated, endothelium-intact and-denuded colonic blood vessels of horses (Venugopal et al., 2001). This dose-dependent contraction of colonic vasculature was abolished by prior administration of ET receptor antagonists. Thus, the observed reduction in blood flow and I-R injury associated with surgical correction in intestinal strangulation obstruction in horses may be due to increased circulating levels of ET-1, altering vasomotor tone and reducing blood flow.
Endothelin-1 promotes leukocyte rolling, expression of adhesion molecules, platelet aggregation, thromboxane formation, superoxide generation and arachidonic acid release (Boros et al., 1998). In rats, ET-1 induces a dose-dependent increase in vascular permeability through the activation of $\text{ET}_\text{A}$ receptors due to disruption of the endothelial barrier (Filep et al., 1991). Use of ET receptor antagonists decreased ET-induced endothelial-leukocyte interactions and other pro-inflammatory responses (Boros et al., 1998). Wolfard et al. (1999) found that $\text{ET}_\text{A}$ receptor antagonism attenuated small bowel injury due to I-R in dogs. This clearly suggests that activation of ET receptors is a major factor in the mediation of ischemia and I-R injury. Another possibility is that ET-1 induced vasoconstriction leads to the no-reflow phenomenon or post-ischemic hypoperfusion.

Endothelin immunoreactivity is over-expressed or up-regulated with various inflammatory conditions, low-oxygen tension, hypoxia, and peritonitis (Murch et al., 1992; Dikranian et al., 1994; Chou et al., 1995). Hypoxia induces an increase in ET-1 immunoreactivity in large intestinal endothelial and epithelial cells of rats (Dikranian et al., 1994; Chou et al., 1995). The ET-1 immunolabelling was identified as single particles in normoxic rat large intestine, but only in low levels; this labelling was predominantly confined to the cytoplasm of endothelial cells of mucosal arterioles and post-capillary venules. Hypoxia was associated with increased ET-1 immunolabelling in the mucosal vasculature and epithelium of the rat large intestine.

Endothelin also has potent contractile activity in non-vascular smooth muscle such as vas deferens, uterus, trachea, stomach and ileum of various species (Takahashi et al., 1990; Rae, Calixto, D’Orleans-Juste, 1995). Abundant ET-1 binding sites have been
found in the stomach, intestine and colon of rats, and other species (Takahashi et al., 1990; Koseki et al., 1989). All three endothelin isoforms (ET-1, -2 and -3) and both receptor types (ET\textsubscript{A} and ET\textsubscript{B}) are expressed throughout the gastrointestinal tract, where they likely have some physiological role. The ET-1 action mediated by the ET\textsubscript{A} receptor un masks the inhibitory ET-1 action mediated through the ET\textsubscript{B} receptor. Repetitive peristalsis in fluid-perfused isolated segments of guinea pig small intestine showed that propulsive motility is mediated by ET\textsubscript{A} receptors on enteric neurons, whereas its action is masked by activation of ET\textsubscript{B} receptors (Shahbazian and Holzer, 2000).

Several studies suggest ET-1 as a potential candidate in the pathophysiology of strangulation obstruction and other disorders. Endothelin-1 is synthesized in the vascular endothelium (Yanagisawa et al., 1988) and causes contraction of intestinal vasculature in horses (Venugopal et al., 2001). Furthermore, ET receptors are present in both vascular and non-vascular smooth muscle of the gastrointestinal tract in horses and other species. Although it is highly unlikely that ET-1 alone can induce intestinal damage, it may act synergistically with other endogenous mediators in the pathogenesis of gastrointestinal tract disorders. Overall, its potent vasoconstrictor nature, widespread distribution and synthesis in various organs, its regulatory role with NO in regulating blood flow and vasomotor tone, and interrelationships with inflammatory mediators suggest its potential involvement in these disorders.

**2.8 Methods of Quantification of Equine Endothelin-1**

Measurement of plasma or tissue expression of ET-1 has been achieved by various techniques such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), competitive polymerase chain reaction and by real time polymerase chain
reaction (TaqMan assay) (Benamou et al., 1998; Uchide et al., 2000; Seligman et al., 2000). Even though most techniques have advantages and disadvantages, the quantification of ET-1 poses a unique problem, due to its inherent nature and its mode of secretion into the circulation. Further, numerous problems are associated with measurement and interpretation of the measured ET-1, which can be due to either ET-1 itself and/or the techniques used.

Circulating concentrations of ET-1 are only a few pg/mL and any assay designed to measure the ET-1 must be quiet sensitive. The circulating half-life of ET-1 is 5-7 min and it is rapidly cleared mainly through the lungs and kidneys. This depends on a variety of factors such as renal or lung function, enzymatic degradation and the binding properties of the circulating ET. Further, secretion of ET-1 is mostly abluminal (>80%) so the measured plasma ET-1 simply reflects its synthesis and overspill. Commercially, there is no anti-equine ET antibody or sera available for the measurement of ET-1. Endothelin is highly conserved among mammalian species and a high (>80%) degree of homology at the nucleotide level among different species, including bovine, rat, human and pig. The ET-1 is the most important and active isoform in blood and is usually the one measured. Cross-reactivity of ET-1 to ET-2 and ET-3 should be taken into consideration while quantifying the plasma ET-1. Thus, there are a myriad of factors involved in the measurement and interpretation of circulating ET levels.

2.8.1 Immunoassays

Measurement techniques such as RIA and ELISA are widely employed. Because the circulating levels of ET are quite low, the plasma needs to be concentrated or extracted before these assays. The extracting process might degrade the ET-1 and also the
variation between and within assay may vary depending upon the technique and the personnel involved (Morganti et al., 2000). The sensitivity of these assays should also be considered. Even though most of the good immunoassays are sufficient to detect levels of 0.5 pg/mL, the sensitivity of the methods varies and usually falls in the range of 2-8 pg/mL (Goddard and Webb, 2000). The inter- and intra-assay variation are high, with >10% having been reported, reducing the accuracy of the measurement (Morganti et al., 2000). In addition, sampling and type of sample (icteric, lipemic or hemolyzed) have also been found to affect the measurement of the ET-1. Regardless of these problems, the RIA is widely employed with careful interpretation and using more appropriate terms like ‘ET-like immunoreactivity’.

2.8.2 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a powerful tool for amplifying specific DNA or RNA targets. Apart from cloning and sequencing DNA, PCR is used in a variety of scientific applications. The exquisite sensitivity and specificity of the PCR reaction (it can detect and amplify even one DNA molecule) renders it a unique and valuable diagnostic tool in medicine. Various procedures such as quantitative PCR and southern blots are used to quantify the PCR products. However, these methods are cumbersome and labor intensive. Recently, techniques such as life cycler and TaqMan assays have been used.

2.9 Statement of Hypotheses and Specific Objectives

2.9.1 Global Hypothesis

The major physiologic functions of the intestine are motility, secretion and absorption, all of which are directly or indirectly controlled by blood flow. So any
impairment in blood flow has a deleterious effect on gastrointestinal tract function. Reduction in blood flow due to vasoconstrictor agents seems to be of primary importance during various occlusive/non-occlusive disorders in horses. Studies have shown that the progressive intestinal damage observed in colic during both pre- and post-operative conditions could be due to increased levels of vasoconstrictor mediators, resulting in a sustained reduction of blood flow. I hypothesize that this sustained reduction in intestinal blood flow contributes to intestinal tract injury and is due to increased circulating levels of ET-1 and that there will be increased ET-1 immunostaining in horses with intestinal strangulation obstruction, compared with gastrointestinal segments from normal horses. We also hypothesize that ET-1 will cause a concentration-dependent contraction of the cecal smooth muscle of healthy horses. Our studies also included the identification of ET-1 gene expression and immunohistochemical localization and distribution of ET-1 in the gastrointestinal tract of clinically healthy horse and those with naturally acquired intestinal strangulation obstruction. Additionally we characterized ET-1 response in the equine gastrointestinal smooth muscle mediated by ET\textsubscript{A} and ET\textsubscript{B} receptors.

2.9.2 Specific Objectives

1. To determine basal circulating plasma ET-like immunoreactivity in healthy horses.

2. To determine circulating plasma ET-like immunoreactivity in horses with naturally acquired gastrointestinal tract disorders.

3. To investigate any relationship between circulating concentrations of plasma ET-like immunoreactivity and clinical and clinicopathological variables and survival of horses affected with naturally acquired gastrointestinal tract disorders.
4. To evaluate the presence and regional distribution of ET-1 like immunoreactivity in various intestinal segments of healthy horses.

5. To determine and compare the alterations in intensity and distribution of ET-like immunoreactivity in the intestinal segments collected from horses with naturally acquired small intestinal and large colon strangulating obstruction with those from healthy horses.

6. To identify the presence of the ET-1 gene expression in various segments of gastrointestinal tract of healthy horses and those with naturally acquired intestinal strangulation obstruction.

7. To determine the concentration-response relationship of ET-1 on cecal smooth muscle and compare the responses with carbachol, a muscarinic agonist.

8. To determine ET receptor types involved in the ET-1 mediated smooth muscle response in cecal smooth muscle by using specific ET receptor antagonists.

2.10 References


CHAPTER 3

PLASMA CONCENTRATIONS OF ENDOTHELIN-LIKE IMMUNOREACTIVITY IN HEALTHY HORSES AND HORSES WITH NATURALLY ACQUIRED GASTROINTESTINAL TRACT DISORDERS*

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3.1 Introduction

Gastrointestinal tract disease (colic) is the leading cause of death in adult horses (Baker and Ellis, 1981). Although mortality rates vary among studies, a substantial number of horses with large-colon strangulating obstruction failed to survive despite advances in equine medicine and surgery (Phillips and Walmsley, 1993). Colonic blood flow remains significantly less than baseline values for at least 4 hours after correction of experimentally-induced complete arteriovenous occlusion of the large colon in ponies (Wilkins et al., 1994). Sustained reduction of intestinal blood flow after surgical correction is postulated as a cause for the progression of mucosal injury resulting from a continuation of ischemic injury associated with microvascular thromboses, splanchnic vasoconstriction or the resulting cascade of events related to restoration of blood flow (Moore, 1997).

Endothelium synthesizes and releases vasorelaxing (eg, nitric oxide [NO], prostacyclin) and vasoconstricting (eg, endothelin [ET], prostaglandins) substances that regulate vasomotor tone (Katusic and Shepherd, 1991; Shepherd and Katusic, 1991). Several intrinsic vasoregulatory mechanisms control tissue blood flow. Reduced blood flow after the correction of colonic volvulus could occur secondary to the generation of endogenous vasoconstrictor substances that induce contraction of the colonic vascular smooth muscle. Thus, sustained hypoperfusion may be mediated by a disruption in the balance between endothelium-derived vasoactive substances. For example, a decrease in nitric oxide (NO) and/or and an increase in ET-1 could lead to increased intestinal vascular resistance (Moore et al., 1995; Turk, 1998).

Endothelins are a family of peptides (ET-1, ET-2, ET-3 and vasoactive intestinal contractor peptide) that exert numerous biological and pathophysiologic effects by binding
Endothelins, the most potent vasoconstrictors known, are synthesized in several tissues where they act to mediate or modulate vasomotor tone, cell proliferation and hormone synthesis (Levin, 1995). Production of ET-1 by endothelial cells increases during periods of reduced blood flow and hypoxia in canine vascular endothelium (Rubanyi and Polokoff, 1994). In addition, circulating concentrations of ET increase in several species during certain pathological conditions such as endotoxemia, cardiogenic shock, and asthma (Myhre et al., 1993; Takao et al., 1994; Cernacek and Stewart, 1989). The sustained hypoperfusion reported after arteriovenous occlusion of the large colon in horses (Moore, 1997) could thus be mediated by increased synthesis of ET-1 in the intestinal vasculature.

We hypothesized that ET, and specifically ET-1, may play a central role in regulating regional blood flow in the gastrointestinal tract of horses, particularly during pathological conditions such as intestinal strangulation obstruction and low-flow states. The purposes of the study reported here were to measure and compare plasma concentrations of ET-like immunoreactivity between healthy horses and those with naturally acquired gastrointestinal tract disease and to investigate the relationship between plasma ET-1 like immunoreactivity concentrations and clinical and clinico-pathologic variables and survival in affected horses.

3.2 Materials and Methods

3.2.1 Animals

This study was approved by the Clinical Animal Care and Use Committee of Louisiana State University. Horses of various light breeds ranging in age from 1 to 24 years (median, 7.5 yrs) were used. Control horses (n = 29) were determined to be healthy on the
basis of medical history and results of a thorough physical examination and clinical evaluation. Affected horses (n=142) were those admitted to the Veterinary Teaching Hospital at Louisiana State University because of gastrointestinal tract disorders. The magnitude and cause of abdominal pain in affected horses was assessed by a thorough physical examination and clinical evaluation. Temperature, respiratory rate, heart rate, capillary refill time, mucous membrane color, and degree and duration of signs of abdominal pain were recorded. For the purposes of this study, we categorized all horses into 1 of 10 groups. Group 1 (n = 29) comprised the healthy control horses. Group 2 (n = 6) consisted of horses with a large intestinal strangulation obstruction (ie, large-colon volvulus and cecocolic intussusceptions). Group 3 (n = 26) comprised affected horses with simple obstruction of the large intestine (ie, impactions, cecal and colon displacements, enterolithiasis). Group 4 (n = 12) comprised horses with a small-intestinal strangulating obstruction. Group 5 (n = 9) comprised horses with a simple obstruction of the small intestine (ie, ileal impaction). Group 6 (n = 12) and 7 (n = 8) consisted of horses with large and small intestinal inflammatory bowel disease, respectively (ie, enterocolitis and anterior enteritis, respectively). Group 8 (n = 13) consisted of horses with peritonitis, gastric rupture or cecal perforation), Group 9 (n= 11) consisted of horses with gastric disorders (ie, ulcers, impactions), and Group 10 ( n =45) consisted of horses with idiopathic gastrointestinal tract disorders (ie, tympany or undiagnosed medical colic). Affected horses were assigned to a group on the basis of the clinical diagnosis made from the results of clinical, clinicopathologic, and where applicable, surgical and postmortem examinations.

3.2.2 Sample Collection

Blood samples were obtained from control horses and affected horses immediately of
admission to the Veterinary Teaching Hospital and prior to the initiation of treatment. Blood (10 ml) was collected via jugular venipuncture into prechilled collection tubes containing EDTA\(^a\) (500 kU/ml) and aprotinin\(^b\) (1 mg/ml). Samples were kept cold and centrifuged within a few minutes of collection at 1,500 X g for 10 in a refrigerated (4˚C) centrifuge. Plasma was then transferred into plastic tubes and stored at –70˚C until assayed. Additional blood samples (3 ml) were collected into tubes containing EDTA for determination of CBC and plasma fibrinogen determination and into tubes containing heparin for plasma biochemistry analyses, including determination of creatinine concentration.

### 3.2.3 Measurement of Plasma ET-Like Immunoreactivity

Plasma ET-like immunoreactivity was quantified by use of a radioimmunoassay\(^c\) designed to measure ET-1 concentration in human plasma. The antibody used in this assay principally recognizes human ET-1. However, the manufacturer reports that the antibody has 67 and 84 % cross-reactivity with human ET-2 and ET-3, respectively. Before assaying plasma samples collected from control and affected horses, the assay was validated for use with equine plasma. Recovery of ET-like immunoreactivity was determined by adding known amounts of synthetic human ET-1 to pooled plasma from clinically normal horses. Concentrations of ET in multiple replicates of spiked samples were measured within and between assays to determine intra-and interassay variations, respectively.

To prepare plasma samples for the assay, they were thawed and extracted according to the manufacturer’s recommended protocol by use of reverse-phase columns\(^d\). Prior to extraction, columns were activated with 5 ml of 100% methanol, washed with 5 ml of deionized water, and pre-treated with 5 ml of 4% acetic acid in distilled water; plasma samples were acidified by mixing with an equal volume of 4% acetic acid in distilled water.
After the addition of acidified plasma samples (1.5 ml) to pretreated columns, columns were washed with 25% ethanol in distilled water, and ET was eluted with 4% acetic acid in a solution of 86% ethanol in distilled water. The eluant was evaporated to complete dryness in a 37°C water bath under a stream of clean, dry nitrogen gas. Dried extracts were reconstituted in 0.5 ml of assay buffer (borate buffer, pH 8.4).

Standard curves for the radioimmunoassay were prepared by the use of synthetic human ET-1 according to the manufacturer’s guidelines. Each standard and extracted sample was assayed in duplicate. Rabbit 125I–labeled ET-1 tracer and rabbit anti-endothelin antiserum were added to each extracted sample and incubated for 18 hrs at 4°C. A donkey anti-rabbit cellulose precipitant was added to each tube and was incubated at room temperature (20 to 22°C) for 30 minutes. Tubes were then centrifuged at 2,000 X g for 15 minutes. The supernatant containing unbound 125I-labeled ET-1 was separated from the pellet (containing antibody-bound 125I-labeled ET-1) by vacuum aspiration. Radioactivity in the pellet was counted in a gamma counter for 2 minutes. Concentrations of ET-like immunoreactivity in extracted plasma samples were determined by extrapolation from a cubic spline standard curve.

3.2.4 Statistical Analyses

Continuous variables considered in the statistical analyses included plasma concentrations of ET-like immunoreactivity, heart rate, total plasma protein and creatinine concentration, PCV, platelet and neutrophil counts, WBC, durations of signs of pain, and outcome (ie, survival vs nonsurvival). Continuous data were summarized by the use of median, mean (± SD), and range.

Data was tested for normality by use of the Shapiro-Wilk statistic, with normality
determined by failure to reject the null hypothesis at $P < 0.05$. Many variables did not follow a normal distribution. Thus, continuous data were compared between control and affected horses by use of the Kruskall-Wallis test for comparison of multiple groups of ranked data. When a significant difference ($P < 0.05$) was detected, selected ad hoc comparisons were made by the use of the Kruskall-Wallis procedure and by correcting the significance level for the number of comparisons to maintain a type I error at 0.05.

For affected horses, an association between ET-like immunoreactivity and other continuous variables was explored by use of stepwise linear regression. Variables were maintained in the regression equation at $P < 0.05$. Pearson’s correlation coefficients were calculated for the variables in the model. In addition, associations between survival and both the continuous variables and the categorical groupings were explored by use of logistic regression. Any horse that died or was euthanatized after a diagnosis was made was assigned to the nonsurvival group. Stepwise selection of variables was performed, with variables maintained in the model if the Wald statistic was significant at $P < 0.05$. The 95% confidence interval (CI) of the conditional odds ratio for the variables included in the model was calculated, and variables whose CI excluded 1 were considered significant. All analyses were performed with the use of computer software programs.

3.3 Results

3.3.1 Validation Assay

Mean percentage recovery of ET-1 recovered was 80% (range, 64 to 102%). Inter-assay and intra-assay variation were < 5% and 10%, respectively. The lowest detectable limit was 0.1 pg/ml of ET-like immunoreactivity/ml of plasma.
3.3.2 Plasma Concentrations of ET-Like Immunoreactivity

Median plasma concentrations of ET-like immunoreactivity were significantly greater in horses with various gastrointestinal tract disorders, compared with healthy horses (Table 3.1). In affected horses, the greatest median values for the ET-like immunoreactivity were observed for horses with large intestinal strangulating obstruction (median, 10.02 pg/ml; range, 3.8 to 22.62 pg/ml), peritonitis (9.19 pg/ml, 7.9 to 25.83 pg/ml), and enterocolitis (8.89 pg/ml, 6.30 to 18.36 pg/ml).

3.3.3 Results of Clinical, Hematologic, and Biochemical Analyses

Packed cell volume (PCV), platelet count, heart rate, and plasma creatinine concentration were slightly greater, and WBC and neutrophil count were slightly lower in affected horses, compared with healthy horses ($P>0.05$). However, none of these differences were statistically significant. In addition, none of the other clinical, hematologic, or biochemical variables differed significantly between healthy and affected horses.

3.3.4 Association Between Plasma ET-Like Immunoreactivity and PCV and Duration of Signs of Pain

Considering results from all affected horses, a significant association was found between the plasma concentration of ET-like immunoreactivity and PCV and duration of signs of pain (Figures 3.1 and 3.2). However, when these variables were considered separately, the positive correlation was only modest (PCV, $r = 0.344$; duration of signs of pain, $r = 0.216$). Correlation increased when both PCV and duration of pain were added to the regression model ($r = 0.407$). No other variables were significantly associated with plasma ET-like immunoreactivity concentration.
Table 3.1 Plasma concentrations of endothelin-(ET)-like immunoreactivity in healthy horses and horses with naturally acquired gastrointestinal tract disorders.

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>ET-like immunoreactivity (pg/ml)</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy (29)</td>
<td></td>
<td>1.8\textsuperscript{a}</td>
<td>1.09-3.12</td>
</tr>
<tr>
<td>LI strangulating obstruction (6)</td>
<td></td>
<td>10.02\textsuperscript{c}</td>
<td>3.8-22.62</td>
</tr>
<tr>
<td>LI simple obstruction (26)</td>
<td></td>
<td>3.97\textsuperscript{bc}</td>
<td>3.27-8.01</td>
</tr>
<tr>
<td>SI strangulating obstruction (12)</td>
<td></td>
<td>5.53\textsuperscript{bcd}</td>
<td>1.72-9.48</td>
</tr>
<tr>
<td>SI simple obstruction (9)</td>
<td></td>
<td>6.14\textsuperscript{bcd}</td>
<td>4.97-9.70</td>
</tr>
<tr>
<td>Enterocolitis (12)</td>
<td></td>
<td>8.89\textsuperscript{bcd}</td>
<td>6.30-18.36</td>
</tr>
<tr>
<td>Anterior enteritis (8)</td>
<td></td>
<td>3.29\textsuperscript{bc}</td>
<td>1.06-7.23</td>
</tr>
<tr>
<td>Peritonitis (13)</td>
<td></td>
<td>9.19\textsuperscript{ed}</td>
<td>7.89-25.83</td>
</tr>
<tr>
<td>Gastric disorders (11)</td>
<td></td>
<td>4.72\textsuperscript{bd}</td>
<td>3.51-7.20</td>
</tr>
<tr>
<td>Idiopathic colic (45)</td>
<td></td>
<td>6.24\textsuperscript{bc}</td>
<td>3.62-9.67</td>
</tr>
</tbody>
</table>

LI = Large intestinal. SI = Small intestinal
\textsuperscript{a-d} Median values with different superscript letters are significantly \((P < 0.05)\) different.
Figure 3.1 Packed cell volume versus plasma concentrations of endothelin- (ET)-like immunoreactivity in 142 horses with naturally acquired gastrointestinal tract disorders. The association between the 2 variables was significant \((P < 0.01)\), but the variables were only modestly positively correlated \((r = 0.344)\).
Figure 3.2 Duration of signs of abdominal pain versus plasma concentrations of endothelin-(ET)-like immunoreactivity in 142 horses with naturally acquired gastrointestinal tract disorders. The association between the 2 variables was significant ($P < 0.01$), but the variables were only modestly positively correlated ($r = 0.216$).
3.3.5 Association Between Outcome, ET-Like Immunoreactivity and PCV

Logistic regression analysis revealed a significant association between outcome (survival vs non-survival) and plasma concentration of ET-like immunoreactivity (odds ratio, 0.942; 95% CI, 0.890 to 0.998) and PCV (0.945, 0.896 to 0.996) (Figure 3.3). Survival was best explained by addition of both variables (ET-like immunoreactivity and PCV) to the logistic model. No other variables were significantly associated with outcome.

3.4 Discussion

Results of our study indicate that the circulating concentration of ET-like immunoreactivity is increased in horses with gastrointestinal tract disorders, compared with healthy horses. Plasma ET-like immunoreactivity in 29 healthy horses ranged from 1.09 to 3.2 pg/ml, which is in agreement with values reported previously for horses (Benamou, et al., 1998). Within the affected group, median plasma concentration of ET-like immunoreactivity was greatest in horses with large intestinal strangulating obstruction, enterocolitis, and peritonitis. Logistic regression analysis revealed an association between the ET-like immunoreactivity and outcome, whereas regression analysis revealed an association between plasma ET-like immunoreactivity, PCV, and duration of signs of pain.

Two ET receptors have been identified and cloned: ET$_A$ receptors on the vascular smooth muscle mediate vasoconstriction, and a subtype of the ET$_B$ (ET$_{B2}$) receptors located on the endothelium mediate vasodilatation via release of prostacyclin or NO (Clozel et al., 1992). The expression of the ET family of vasoactive peptides increases in different tissues such as intestine and heart of rats, humans, and dogs during low-flow ischemia, increased shear stress, hypoxia, and inflammatory disorders (Rubanyi and Polokoff, 1994; Rubanyi
Figure 3.3 - Logistic regression analysis showed a significant association between outcome (survival vs non-survival) and plasma concentration of ET-like immunoreactivity (odds ratio, 0.942; 95% CI, 0.890 to 0.998) and PCV (0.945, 0.896 to 0.996). Survival was best explained by addition of both variables (ET-like immunoreactivity and PCV) to the logistic model.
and Vanhoutte, 1986). Endothelin-1 is believed to be the most important isoform, although all isoforms have been identified in the intestine of people and mice (Inoue et al., 1989; Saida, Mitsui, Ishida, 1989). Moreover, there is a growing body of evidence that ET-1 serves as a principal mediator in pathophysiological events involving ischemia-reperfusion (I-R) injury associated with intestinal strangulation obstruction and myocardial infarction in pigs and human patients (Battistini and Dussault, 1998; Fevang et al., 1998). Because of the reported cross-reactivity of the anti-ET-1 antibody used in the radioimmunoassay with ET-2 and ET-3, we could not specifically determine which ET isoform was present in the plasma samples collected from affected and control horses. However, we presumed that the immunoreactivity detected was primarily attributable to ET-1 because this is not only the predominant isoform in the intestine of many animal species but it is also the circulating isoform found during various pathologic conditions in several species (Fevang et al, 1998; Weitzberg et al., 1991).

Because of its high metabolic activity and substantial blood flow requirements, the gastrointestinal mucosa is the primary target tissue of many types of low-flow induced circulatory shock associated with intestinal obstruction, hemorrhage and sepsis (Fevang et al, 1998; Weitzberg et al., 1991; Massberg et al., 1998) Occlusive vascular diseases and non-occlusive states associated with I-R lead to endothelial synthesis of ET (Rubanyi and Polokoff, 1994). Increased intestinal vascular resistance during circulatory shock shunts blood away from gastrointestinal tissues to more vital organs (Patel, Kaleya, Sammartaon, 1992), resulting in intestinal hypoperfusion. Increased plasma concentrations of ET-1 in pigs with an intestinal strangulation obstruction may be associated with reduced intestinal blood flow, intestinal hypoperfusion, and shock (Fevang et al., 1998). Nitric oxide, an endothelium-
derived vasorelaxant, inhibits the reduction ET synthesis (Boulanger and Luscher, 1990). Further, during I-R injury and other low-flow states, endothelial production of NO is compromised and responsiveness to ET-1 is enhanced (Thompson, Valeri, Lieberthal, 1995; Wood et al., 1995). Thus, disruption of the balance between ET-1 and NO may lead to prolonged vasoconstriction, resulting in impaired intestinal blood flow, ischemic injury and ultimately mucosal barrier disruption.

Greater than 80% of the ET-1 synthesized by the endothelium is secreted abluminally toward the tunica media where it functions in a paracrine fashion (Wagner et al., 1992). Therefore, measurement of plasma ET-1 concentrations may not truly reflect the overall synthesis and release of ET-1 during pathophysiologic events. Despite these limitations, measurement of plasma ET-1 concentrations reportedly is a useful indicator of congestive heart failure, pulmonary hypertension and endotoxemia in humans and other animals (Nakamura et al., 1991; Lerman et al., 1991; Cody et al., 1992). The increase in plasma concentrations of ET-like immunoreactivity that we detected in horses with gastrointestinal tract disorders supports similar findings in pigs with strangulation obstruction (Fevang et al., 1998). In addition, ET-1 mRNA expression was shown to be increased in the small intestine of rats with peritonitis (Chou et al., 1995). Although we detected significantly more plasma ET-like immunoreactivity in horses with gastrointestinal tract disorders, compared with healthy horses, we could not determine whether this increase was reflected solely by an increase in ET-1 or whether this increase was specific to horses with colic. The increase in circulating ET-like immunoreactivity could have been attributable to other factors related or unrelated to the primary disease.

The increase in plasma concentration of ET-like immunoreactivity that we detected
in affected horses may have resulted from specific stimuli leading to increased synthesis and release of ET-1 from the vasculature of the affected intestinal segment or from the affected gastrointestinal tract tissues. Shock-related stimuli may have also induced an increase in ET-1 synthesis from tissues distant to the gastrointestinal tract. Alternatively, an increase in concentration of circulating ET-1 may have simply resulted from hemoconcentration. Lipopolysaccharides, tumor necrosis factor (TNF), and interleukin-1 (IL-1) are principal mediators of sepsis and are also important modulators of ET-1 secretion (Battistini, Forget, Laight, 1996; Dinarello, 1991). These and other regulatory peptides may activate the ET-1 gene through an acute phase response element of an upstream regulatory element (Marsden and Brenner, 1992). In vitro ET-1 synthesis increases in cultured porcine aortic endothelial cells stimulated with TNF and IL-1 (Kanse et al., 1991; Yoshizumi et al., 1990). Although speculative, increased plasma concentrations of ET-like immunoreactivity that we detected in affected horses may have resulted from endothelial injury or colic-associated stimuli (interleukins or TNF) that lead to an increased release of ET-1 from the intestinal vascular endothelium and subsequent spillover into the systemic circulation. However, the exact mechanism for the increase in plasma ET-like immunoreactivity in horses with gastrointestinal tract disorders could not be determined.

Median plasma concentration of ET-like immunoreactivity in horses was greatest in horses with large intestinal strangulation obstructions, enterocolitis, and peritonitis. Clinically, the magnitude of tissue damage and severity of shock are typically more severe in horses with these conditions, compared with other causes of colic. In horses with obstructive pulmonary disease, bronchoalveolar lavage fluid ET-1 concentrations were strongly correlated with the severity of disease (Benamou et al., 1998). Thus, the high plasma
concentrations of ET-like immunoreactivity in horses with the large-intestinal strangulating obstruction, enterocolitis, and peritonitis, may also correlate with the severity of endothelial damage and tissue injury, and may indicate involvement of ET-1 in the etiopathogenesis of these disorders. Alternatively, high ET-like immunoreactivity concentrations may have simply reflect a more severe systemic illness in these horses. In rats, administration of ET-1 leads to hemoconcentration, edema, and enhanced protein extravasations in various organs including the duodenum (Valentin et al., 1991). In our study, the significant association between plasma ET-like immunoreactivity concentration and PCV and the duration of signs of pain may have been attributable to severe and long-standing disease that resulted in a greater synthesis and release of ET-1. However, the increase in plasma ET-like immunoreactivity concentration may also have been associated with the degree of dehydration and altered cardiovascular status of the horses with severe gastrointestinal tract disease.

Circulating concentrations of ET-1 associated with pathologic conditions can affect systemic hemodynamics, resulting in increased systemic vascular resistance and decreased cardiac output (Lerman et al., 1991). Logistic regression analyses revealed a strong positive correlation between the plasma ET-like immunoreactivity concentration and outcome (survival vs nonsurvival) in the horses of the present study. Similarly, in humans with congestive heart failure, plasma ET concentrations are positively correlated with the extent of pulmonary hypertension (Cody et al., 1992). However, from the present study, we cannot determine whether the increase in plasma concentration of ET-like immunoreactivity simply represents a biological marker for the disease severity, and potentially, prognosis for horses with gastrointestinal tract disorders, or whether it
contributes to the etiopathogenesis of these disorders. The use of ET-1 receptor antagonists may help clarify the role of ET-1 as a biological marker or pathologic mediator of colic in horses.

3.5 Product Information

\[\text{Sigma Chemical Co, St Louis, Mo.}\]

\[\text{Endothelin, Nichols Institute Diagnostics, San Juan Capistrano, Calif.}\]

\[\text{Sep-Pak C18 columns, Waters Associates, Milford, Mass.}\]

\[\text{SAS for windows 95, version 7.0, SAS Systems Inc, Cary, NC.}\]

3.6 References


CHAPTER 4

DISTRIBUTION OF ENDOTHELIN-1 IMMUNOHISTOCHEMICAL STAINING IN THE INTESTINAL TRACT OF CLINICALLY HEALTHY HORSES AND IN THOSE WITH SMALL INTESTINAL AND LARGE COLON STRANGULATION OBSTRUCTION
4.1 Introduction

The gastrointestinal tract is the major target tissue of low-flow induced circulatory shock such as occlusion, volvulus, hemorrhage and or sepsis (Alexander et al., 1990; Massberg et al., 1995). Small intestinal and large colon strangulating obstruction account for approximately 10% off all horses with colic, and these conditions are universally fatal without surgical correction (Phillips and Walmsley, 1993). Despite surgical correction and intensive medical care, most horses die due to the rapidity with which the intestinal mucosa undergoes irreversible damage during ischemia, and subsequent endotoxemia and hypovolemic shock. Increased plasma levels of endothelin-1 (ET-1) have been shown during low-flow ischemia of the small intestine in various species like humans, pigs and horses (Sharefkin et al., 1991; Fevang et al., 1998; Ramaswamy et al., 2002), suggesting involvement of ET-1 in regulation of mucosal hypoperfusion and in the pathogenesis of intestinal ischemia.

Endothelins are a family of vasoconstrictor peptides synthesized by various cells that exert numerous biological and pathophysiologic actions via binding to G-linked protein receptor subtypes, $\text{ET}_A$ and $\text{ET}_B$ (Yanagisawa et al., 1998; Rubanyi and Polokoff, 1994). All three ET isoforms (ET-1, ET-2 and ET-3) have 21 amino acids and 2 disulfide bridges, each with different affinities for the ET receptors (Inoue et al., 1989). Each ET isoform is a product of separate genes that encode the large precursor prepro-ET. Of all ET isoforms present in the intestine (Matsumoto et al., 1989; Chou et al., 1995; de la Monte et al., 1995), ET-1 is suggested as the most important isoform in the regulation of intestinal functions.
Endothelin-like immunoreactivity has been observed in endothelial cells, vascular smooth muscle cells, mast cells, epithelial cells and various other cell types in the intestinal tract of different species (Eaker et al., 1995; Liu, Yamada, Ochi, 1998; Minchenko et al., 1999). In the human colon, ET and its binding sites were observed in the enteric neurons, submucosal plexus, mucosal layer and in blood vessel walls (Inagaki et al., 1991; Egidy et al., 2000). Expression of ET in vascular and non-vascular cells and tissues suggests its diverse role in various physiological functions such as vasomotor tone, ion secretion and closure of the ductus arteriosus (Coceani and Kelsey, 2000; Levin, 1995; Rubanyi and Polokoff, 1994). However, to our knowledge there are no studies reported regarding the distribution of ET-1 in the gastrointestinal tract of horses.

Increased expression of ET-1 and intensity of staining in epithelial and endothelial cells of the rat colon were correlated with the degree of mucosal injury (Sugimachi et al., 2000). Hypoxia has been shown to induce an increase in ET-1 like immunoreactivity in large intestinal endothelial and epithelial cells of rats (Dikranian et al., 1994). It has been hypothesized that a sustained reduction in intestinal blood flow and progressive mucosal injury after intestinal arteriovenous occlusion in horses could be mediated principally by increased local expression and synthesis of ET-1 in the intestinal microvasculature and tissues.

We hypothesized that there would be ET-1 staining in the surface epithelium and vasculature of the gastrointestinal tract of normal horses, and this immunohistochemical staining would increase in horses with naturally acquired intestinal strangulation obstruction. The presence of ET-1 like immunoreactivity in the normal horse will suggest its role in normal physiological functions, whereas increased expression of ET-1 in blood
vessels and intestinal tissues during strangulation obstruction may indicate its involvement in the pathogenesis of these disorders. The objectives of the study were to evaluate the regional distribution of ET-1 in different segments of the gastrointestinal tract of clinically healthy horses, and to determine and compare the expression of ET-1 like immunoreactivity in the intestinal segments of horses with naturally acquired small intestinal and large colon strangulating obstruction.

4.2 Materials and Methods

4.2.1 Tissue Collection

This study was approved by the Clinical Animal Care and Use Committee of Louisiana State University. Gastrointestinal tract biopsy specimens were collected from 6 clinically healthy adult horses with no history or clinical or laboratory evidence of disease involving the gastrointestinal tract or cardiovascular system (control, n =6) destined for euthanasia for other reasons. Samples were collected from the control horses immediately after euthanasia with an overdose of pentobarbitala (100 mg/kg).

Sections were collected from the following sites from the gastrointestinal tract of control horses: greater curvature of stomach, duodenum (1 foot from pylorus), ileum (1 foot from ileocecal junction), cecal body (half way between apex and base), right ventral colon (1 foot from the cecocolic junction), left ventral colon (1 foot from pelvic flexure), left dorsal colon (1 foot from pelvic flexure), right dorsal colon (1 foot from diaphragmatic flexure), transverse colon (6 inches from duodenocolic ligament), and the small colon (4 feet from duodenocolic ligament). All biopsy specimens were collected from the anti-mesenteric side of the intestinal segments from control and affected horses. Biopsy specimens were also collected from horses admitted to the LSU Veterinary
Teaching Hospital with naturally acquired small intestinal (n=25) and large colon (n=13) strangulation obstruction at the time of exploratory surgery or after euthanasia. Tissues were fixed in 4% aqueous zinc buffered formalin for approximately 24 hours and embedded in paraffin for immunohistochemistry.

4.2.2 ET-1 Immunohistochemistry

Paraffin-embedded tissues were serially sectioned at 4µm and attached to silanized slides and dried overnight (37°C). Tissues sections were deparaffinized, hydrated through graded alcohol solutions, and equilibrated in phosphate buffered saline (PBS, pH 7.6). Immunostaining was performed, using a modified three-step Avidin-Biotin complex (ABC) method with a Vector Elite ABC Rabbit IgG kit (Hsu, Raine Fanger, 1981). The entire staining procedure was performed at room temperature (20-22°C).

After rehydration, slides were immersed in 0.3% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase activity. Slides were rinsed and pre-incubated with normal goat serum\(^b\) (1:50) for 45 minutes to block non-specific binding. After incubation, the excess serum was removed and the primary antibody (Ab), rabbit-anti-ET-1 antisera\(^c\) at a dilution of 1:700, was added and incubated at room temperature for 60 minutes. After addition of primary Ab, the slides were washed and incubated with secondary biotinylated goat anti-rabbit-IgG antisera (1:200) for 30 minutes. The ABC mixture\(^d\) was applied for 30 minutes. The horse-radish-peroxidase substrates were then developed by staining with freshly prepared chromogen, diaminobenzidine (DAB), for 5 minutes. The slides were counterstained with Mayer’s heamatoxylin for 30 sec,
dehydrated with ethanol and xylene, cleared and mounted in a resinous media and evaluated by light microscopy. Brown staining was considered positive.

The specificity for ET-1 like immunoreactivity was evaluated: 1) by the omission of primary antibody; 2) by the substitution of normal rabbit IgG for primary antibody; and 3) by pre-incubation of the primary antibody overnight at room temperature with excess ET-1° peptide. The blocked antibodies were then used on tissue sections and run parallel with immunostaining.

Three sections from each slide were evaluated twice to minimize variation. A numerical value ranging from 0 to 3 was assigned to each slide based on the relative amount of staining, with control slides (blocked control). The intensity and distribution of the ET-1 like immunoreactivity for the following variables was evaluated: surface epithelium, crypt epithelium, mucosal vasculature, submucosal arterioles and venules (smooth muscle, endothelium), muscularis and serosa. A zero score was assigned if there was no staining present. Grade 1 - indicated a mild increase brown staining compared with blocked antibody; Grade 2 - indicated a moderate increase brown staining compared with blocked antibody; and Grade 3 - indicated an intense increase in brown staining over that with blocked antibody.

The affected segments (duodenum, jejunum, ileum, pelvic flexure) of horses with small intestinal and large colon strangulating obstruction were compared with the duodenum, jejunum, ileum, and pelvic flexure scores from control horses. The modal (most frequent) scores were reported as a summary measurement for each tissue, stain, and variable.
4.3 Results

In healthy horses, the intestinal surface epithelium and large vessels, mainly the veins and the muscular layer, were positive for ET-like immunoreactivity (Figures 4.1, 4.3 and 4.4). No staining was observed in mucosal mucus glands, and Brunner’s glands in duodenum and jejunum, respectively (Figure 4.2). The observed ET-like immunoreactivity was completely abolished by pre-absorption of antiserum with an excess amount of ET-1 (Figure 4.1B and 4.3B). Under high magnification, ET-like immunoreactivity was observed in the cytoplasm of the surface epithelial cells and endothelial cells with moderate staining intensity. The staining was more intense in the apical region of the surface epithelium. The surface epithelial cells also showed a mosaic distribution pattern of ET-like immunoreactivity (Figure 4.4). Staining of crypt epithelium and endothelial cells were variable and inconsistent. Veins stained more intensely than arteries (Fig 4.5). ET-like immunoreactivity was diffuse and variable in the endothelium of the blood vessels. The ET-like immunoreactivity staining in muscularis was diffuse and variable, with greatest intensity of staining observed in the outer muscular and serosal layers.

Heavy hemorrhage, congestion and lack of structural details in affected tissue segments from horses with naturally acquired intestinal strangulation obstruction made it difficult to subjectively evaluate ET-like immunoreactivity. Overall, staining intensity was greater in the serosal and outer muscular layers of the affected intestinal segments (Figure 4.6). Diffuse staining was also noted in the mucosa and in blood vessels of affected intestinal segments. The staining was intense and diffusely distributed in affected
Figure 4.1 Photomicrograph showing ET-like immunoreactivity staining in the surface epithelium of the duodenum of a clinically healthy normal horse (A). The ET-1 immunoreactivity was abolished by pre-absorption of anti ET-1 with excess amount of ET-1 (B).
Figure 4.2 Photomicrograph illustrating the lack of ET-like immunoreactivity in the mucus of the duodenal glands (↑).
Figure 4.3 Photomicrograph illustrating intense ET-like immunoreactivity in the surface epithelium and lamina propria of the ileum of a clinically healthy horse (A). Pre-absorption of anti-ET-1 antibody with ET-1 abolished the ET-like immunoreactivity (B)
Figure 4.4 Photomicrograph illustrating patchy distribution of ET-like immunoreactivity (arrow heads) with anti-ET-1 Ab staining in the pelvic flexure of clinically healthy horses (A). Note the intense staining in the blood vessels (↑) in the lamina propria.
Figure 4.5 Photomicrograph illustrating intense ET-like immunoreactivity in the tunica media and endothelial lining of the blood vessels in a healthy horse (A) and in horse with large colon strangulation obstruction. B) ↑- indicate the endothelial lining of blood vessels.
Figure 4.6 Photomicrograph illustrating diffuse and intense ET-like immunoreactivity in the muscular and serosal layer of pelvic flexure of horse with colon torsion. Note the intense staining in the endothelial lining of the blood vessels (↑).
Figure 4.7 Photomicrograph illustrating diffuse ET-like immunoreactivity in the mucosal layer, due to extensive mucosal damage in the pelvic flexure of horse with colon torsion.
intestinal segments from horses with large colon volvulus, compared with control tissues (Figure 4.7). The modal scores for clinically healthy horses varied from 0 and 1 depending on tissue type and variable (Table 4.1). Overall, the modal (most frequent) scores of ET-1 staining intensities were higher (2) compared with the ET-1 immunoreactivity staining in clinically healthy horses (Table 4.2)

**4.4 Discussion**

The distribution of ET-like immunoreactivity was examined in the gastrointestinal tract of healthy horses and in intestinal segments from horses with naturally acquired intestinal strangulation obstruction. The most important findings of this study were the presence of ET-1 like immunoreactivity in the villi and surface epithelium of the small intestine and large intestine, respectively, in healthy horses. This suggests a role for ET-1 on normal gastrointestinal tract function. The ET-like immunoreactivity was predominantly confined to the cytoplasm of the surface epithelium with less staining in the crypts, and variable, diffuse ET-like staining in the muscularis layer. A similar pattern of ET-immunoreactivity has been reported in rat colon (Sugimachi et al., 2000). The endothelial lining of blood vessels in different segments of the gastrointestinal tract stained positive for ET-1, suggesting its involvement in the regulation of regional intestinal blood flow.

Localization of ET-1 was investigated using apolyclonal antibody against ET-1 (rabbit). Due to the cross reactivity of anti-ET-1 antibody used in the study (ET-1-100%; ET-2 - 91%; ET-3-0.05%), we could not specifically determine the ET isoforms present in the gastrointestinal tract. However, it is mostly ET-1 because of its predominant
Table 4.1 Modal values for ET-1 immunoreactivity staining scores in different sections and tissue types of the gastrointestinal tract of clinically healthy horses

<table>
<thead>
<tr>
<th>Tissue Sites</th>
<th>Epithelium</th>
<th>B.V</th>
<th>T.media</th>
<th>Endothelium</th>
<th>SM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Surface</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Crypt</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duodenum (n =5)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Jejunum (n =5)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Ileum(n =6)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Cecum (n =6)</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Pelvic flexure(n =4)</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Right ventral colon(n =6)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Right dorsal colon (n =5)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Left ventral colon (n =6)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Left dorsal colon (n =6)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Small colon (n =6)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Transverse colon (n=6)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

B.V – blood vessels, T. Media – tunica media, SM – smooth muscle

Table 4.2 Modal values of ET-1 immunoreactivity staining scores in affected intestinal segments from horses with naturally acquired small intestinal and large colon strangulation obstruction.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Epithelium</th>
<th>Blood Vessels</th>
<th>SM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Artery (M)</td>
<td>Vein (M)</td>
</tr>
<tr>
<td>SI strangulation (n =25)</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>LI strangulation (n =13)</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

SI- small intestinal, LI- large intestinal, M- muscle, SM-smooth muscle
distribution in the intestinal tract and the fact that ET-like immunoreactivity was abolished by pre-absorption of anti-ET-1 with excess amount of ET-1. Endothelins, 21 amino acid peptides, are present in nearly every organ examined. Of the three isoforms, ET-1 and ET-2 were expressed in the gastrointestinal tract (de La Monte et al., 1995); and are potent constrictors of intestinal smooth muscle. The ET receptor types (ET\textsubscript{A} and ET\textsubscript{B}) have been identified in the intestine of pigs, humans and other species and have been found to have increased expression in young animals (Nankervis, Dunaway, Miller, 2001; Egidy et al., 2000). The roles of ET-1 in the regulation of intestinal hemodynamics and oxygenation of tissues have been studied (Nankervis, Dunaway, Miller, 2001). The presence and identification of ET-1, and its receptor types in the intestinal tissues suggest a physiological role of ET-1 in intestinal function.

The release of ET-1 occurs in discontinuous pulses from the blood vessels and helps in maintenance of vasomotor tone. The inconsistent ET-1 staining of endothelial cells within different sections of the intestine may thus reflect the state/change in ET-1 production by the blood vessels. Similar variable staining intensity and the diffuse nature of ET-1 staining was observed in previous studies in rat colon (Sugimachi et al., 2000) and human placenta (Barros et al., 2001).

The ET-1 immunolabelling was identified as single particles in normoxic rat large intestine, but only in low levels with labeling predominantly confined to the cytoplasm of endothelial cells of mucosal arterioles and post-capillary venules (Dikranian et al., 1994). The expression of ET-1 like immunoreactivity in the intestinal surface epithelium of the horses in this study suggests that ET-1 might play a role in epithelial cell differentiation or secretory activity as suggested by others in different species (Takahashi et al., 1990; de
la Monte et al., 1995; Shichiri, Marumo, Yukio, 1998). The gastrointestinal tract epithelial cells and intestinal vasculature possess ETA and ETB receptors (Inagaki et al., 1991; Nankervis, Dunaway, Miller, 2001). Therefore, the observed ET-1 like immunoreactivity in the villi and surface epithelial cells may reflect a paracrine type of uptake of ET secreted by endothelial or lamina proprial cells.

During shock or low-flow states, ET-1 release likely plays a role in helping to shunt blood away from the splanchnic circulation to more vital organs (Fevang et al., 1998). Although this is a needed defense mechanism, it likely contributes to the continued ischemia in the post-operative period, or subsequent to shock resuscitation, and could result in mucosal barrier disruption. Further, the functional role of ET-1 in gastrointestinal functions like intestinal motility and ion secretion has been elucidated (Moummi et al., 1992; Hosokawa et al., 1995). Recently, we reported an increase in plasma ET-like immunoreactivity in horses with acquired gastrointestinal tract disorders such as large intestinal strangulation obstruction, peritonitis and enterocolitis (Ramaswamy et al., 2002). The mechanism of increased plasma ET-1 concentrations in various gastrointestinal tract disorders (Murch et al., 1992; Ramaswamy et al., 2002) though unclear, is speculated to be an increased synthesis or release of ET-1 by injured or stimulated endothelial cells from affected intestinal tissues and subsequent absorption into the circulation, rather than the systemic release of ET-1 from other tissues. Submucosal injection of ET-1 induces gastric ulceration secondary to a decrease in gastric mucosal blood flow, ischemia and increased acid secretion (Watanabe et al., 2000).
In our study, increased intensity of ET-like immunoreactivity in the outer muscular layer and blood vessels of the intestinal segments collected from horses with acquired intestinal strangulating obstruction disorders was observed. The increased expression of ET-1 may enhance epithelial damage because of its ability to cause vascular contraction and ischemia. However, the lack of structural details due to advanced disease conditions and variable staining intensity between viable and non-viable sections of affected tissues makes it difficult to confirm this assumption. Involvement of ET-1 in the pathogenesis of various intestinal disorders has been previously indicated by immunohistochemistry (Chou et al., 2001; Sugimachi et al., 2000). Similarly, increased ET-1 staining in various cancer cell lines, in human patients with prostate cancer and pituitary adenoma, and in ischemic rat colon tissues, compared with control tissues, has been reported (Sugimachi et al., 2000; Asham et al., 2001; de Matteis et al., 2001). Investigating the role of ET-1 in intestinal disorders using ET antagonists could further extend the clinical implications of this study.

In conclusion, this study demonstrated the presence and distribution of ET-1 in the intestinal tract of normal horses suggesting its role in physiologic functions. The increased expression of ET-1 observed in affected intestinal segments supports the hypothesis that ET-1 is involved in the pathogenesis of ischemic injury in horses.

4.5 Product Information

aBeuthanasia-D®, Schering-Plough Animal Health, New Jersey.
bNormal Goat serum, Vector Laboratories, Burlingame, Calif.
cRabbit-anti endothelin, Peninsula Laboratories, Belmont, Calif.
dVectastain, Elite ABC Kit, Vector Laboratories, Burlingame, Calif.
4.6 References


Nankervis CA, Dunaway DJ, Miller CE. Endothelin ET\textsubscript{A} and ET\textsubscript{B} receptors in postnatal intestine. *Am J Physiol Gastrointest Liver Physiol* 2001;280:G555-G562.


CHAPTER 5
DETECTION OF ENDOTHELIN-1 GENE EXPRESSION IN THE GASTROINTESTINAL TRACT OF CLINICALLY HEALTHY HORSES USING POLYMERASE CHAIN REACTION
5.1 Introduction

Endothelins (ET), a family of highly conserved 21 amino acid peptides, possess potent vasoconstrictor activity and numerous other functions in mammals (Rubanyi and Polokoff, 1994). They were originally isolated from cultured porcine endothelial cells (Yanagisawa et al., 1988) and later expressed in other cell types in the nervous, urinary, respiratory and gastrointestinal systems (Rubanyi and Polokoff, 1994; Inagaki et al., 1991). Three different isoforms of ET, including ET-1, ET-2 and ET-3, each encoded by a separate gene, have been identified in humans and other species (Inagaki et al., 1991; Inoue et al., 1989; Shiba et al., 1992). Endothelin isoforms are processed as large pre-proforms and post-translationally processed to active peptides. Endothelin synthesis is regulated by various stimuli (hypoxia, hormones, cytokines) at the transcriptional level (Shiba et al., 1992; Levin, 1995; Elton et al., 1992; Yoshizumi et al., 1990). Endothelins exert their pharmacological action by binding to two distinct high affinity receptors subtypes, ET$_A$ and ET$_B$, which are seven-transmembrane G-protein-coupled receptors (Inagaki et al., 1991) distributed widely in a variety of cell types including vascular smooth muscle, endothelial cells and macrophages.

Endothelins are widely expressed in various mammalian tissues and organs including the gastrointestinal tract, and localization of ET receptors in the intestinal tract of bovine, rats, and other laboratory animal species has been documented (Inagaki et al., 1991). These isopeptides have a variety of biological roles, including involvement in vasoconstriction, cell growth, and intestinal and bronchial smooth muscle contraction (Levin, 1995; Elton et al., 1992; Yoshizumi et al., 1990). Several inflammatory mediators such as vasopressin, angiotensin, lipopolysaccharide, tumor necrosis factor-$\alpha$ and
interleukins stimulate the synthesis of ET. Endothelin synthesis is also enhanced by a variety of other stimuli such as low shear stress and hypoxia (Levin, 1995; Elton et al., 1992). Several studies have emphasized the gastrointestinal tract as a major target of the endothelins (Chou et al., 1995). High concentrations of plasma ET-1 have been observed in various pathological conditions such as sepsis, ulcerative colitis, Crohn’s disease and endotoxemia (Murch et al., 1992; Myhre et al., 1993). Recently, we observed increased circulating plasma ET-like immunoreactivity in horses with naturally acquired gastrointestinal tract disorders such as strangulation obstruction, enterocolitis and peritonitis (Ramaswamy et al., 2002). To our knowledge, no studies have reported ET-1 gene expression in the gastrointestinal tract of horses. The expression and relative amounts of ET-1 gene expression in gastrointestinal segments should enable us to further determine the physiological and pathological role of ET-1 in the gastrointestinal tract in horses.

We hypothesized that the ET-1 would be found in variable quantities from various segments of the intestinal tract extending from the duodenum to the right dorsal colon of healthy horses. The primary objective of the study reported here was to determine the presence of the equine ET-1 gene expression in different sections of the gastrointestinal tract of clinically healthy horses, using reverse-transcriptase polymerase chain reaction.

5.2 Materials and Methods

This study was approved by the Institutional Animal Care and Use Committee of Louisiana State University. Tissue samples from 6 clinically healthy horses donated for reasons unrelated to gastrointestinal tract disorders were obtained immediately after euthanasia with intravenous sodium pentobarbital\(^a\) (100 mg/kg, IV). Samples were
collected from various sections of the gastrointestinal tract extending from the stomach to the large colon. For uniformity in tissue collection, the following landmarks were used: stomach (halfway around the greater curvature), duodenum (1 foot from pylorus), jejunum (25 foot from ileocecal junction), ileum (1 foot from the ileocecal junction), right dorsal colon (1 foot orad to diaphragmatic flexure), pelvic flexure and right ventral colon (1 foot from cecocolic orifice). A section of heart (left ventricular wall) tissues were also collected. Full-thickness tissue samples were collected, immediately snap frozen in liquid nitrogen and stored at –80°C until analyzed.

Total RNA was extracted using TRI REAGENT LS according to the manufacturer’s instructions. The RNA samples were heat inactivated at 65°C for 10 min and chilled on ice 2 min. The RNA samples were reverse-transcribed by adding 25 µL of DEPC treated water, 1 µL of oligo(dT) primers and Ready-To-GOTM You-Prime First-Strand Beads.

Oligonucleotide primer pairs specific for the equine ET-1 gene were designed and synthesized in our laboratory. The ET-1 gene was then amplified from the cDNA by use of primers specific for equine ET-1, 5’-AAGCAGGAAAAAGAAGTCAGGGTGGAG-3’ (FP) and 5’-CAGCCTTTCTCCATAGTGTCTTGG-3’ (RP). The ET-1 amplification product was predicted to be 202 bp in length. The PCR reaction mixture consisted of 3 µL of cDNA, 1 µL of forward and reverse primers (20 µM), 10 µL of PCR buffer, 10 µL of dNTP, 0.5 µL of Taq polymerase, and a sufficient amount of distilled water to have a final volume of 100 µL. The PCR reactions were performed in a thermal cycler programmed as follows: initial denaturation at 96°C for 3 min; cDNA was amplified in 35 cycles (94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec). Subsequently, final
extension was performed at 72°C for 10 min. A negative control (no template) was run to avoid any false positive reactions from exogenous contaminating DNA. The gels were visualized with an ultraviolet transilluminator and the gels were photographed with a Fluor-S™ Multi-imager.

5.3 Results

We confirmed the identity of the PCR products by sequencing after extensive purification, using an automated DNA sequencer. The resultant sequences were confirmed by comparison to the known published partial sequence of equine ET-1. Agarose gel electrophoresis of PCR products from the various sections of the gastrointestinal tract tissues, including the stomach, duodenum, jejunum, ileum, right ventral colon, pelvic flexure and right dorsal colon, demonstrated the expected product sizes, (Figure 5.1) and are identical to the published nucleotide partial sequence. No amplification signal was noticed in the negative control samples.

5.4 Discussion

To our knowledge, this is the first study to demonstrate the presence of constitutively expressed of equine ET-1 gene in various sections of the gastrointestinal tract of horses. Although this study showed the presence of the ET-1 gene expression in the gastrointestinal tract of clinically healthy horses, it does not reveal any information regarding the cellular localization or differential expression of ET-1 within the gastrointestinal tract tissues or alterations with disease conditions. Endothelins are multifunctional, vasoconstricting peptides exhibiting vasoactive, inotropic, and mitogenic properties, and have the ability to modulate other hormone systems as well as intestinal, renal and pulmonary function (Rubanyi and Polokoff, 1994; Inagaki et al., 1991; Inoue et
Figure 5.1 - Agarose gel (1.5 %) electrophoresis pattern of RT-PCR amplification products of equine ET-1 in tissues collected from different sections of gastrointestinal tract tissues of clinically healthy horses. Lane 1- stomach, Lane 2- duodenum, Lane 3-jejunum, Lane 4- ileum, Lane 5- right ventral colon, Lane 6- Marker (100bp), Lane 7-pelvic flexure, Lane 8- right dorsal colon, Lane 9-Lung, and Lane 10-heart.
al., 1989; Shiba et al., 1992). Induction of ET-1 synthesis is controlled at the level of transcription of its precursor, preproendothelin-1. Therefore, the presence of ET-1 gene should directly reflect the relative amount of ET-1 peptide based on the assumption that there is no posttranscriptional regulation of ET-1. Endothelin isoforms are initially synthesized as large precursor polypeptides, pre-proET, that are cleaved at two pairs of basic amino acids to generate intermediate peptides, big ET-1. The big ET-1 is post-translationally processed to active peptide, ET-1, by endothelin converting enzyme (ECE) present in the cell surface (Rubanyi and Polokoff, 1994). A number of studies on the distribution of the ET-1 mRNA in a variety of tissues such as stomach have been reported (Egidy et al., 2000; Guan, Chen and Qian, 1998). Expression of ET-1 mRNA has been reported in wide variety of cell types such as macrophages, vascular smooth muscle cells, myocytes, astrocytes and mast cells (Liu, Yamada, Ochi, 1998; Dikranian et al., 1994). The ET-1 and ET-3 peptides were shown to be present in the gastrointestinal tract of other species; they have been identified in rat gut mucosa by radioimmunoassay and by in situ hybridization (Takahashi et al., 1990, MacCumber et al., 1989).

Studies have shown the presence of ET-1 mRNA expression in a variety of tissues such as lung, uterus, ovary, stomach and intestinal tissues of rats (Takahashi et al., 1990). In rat large intestine, ET-1 has been identified in the lamina propria as single particles suggesting that this peptide has a role in villus motility (Dikranian et al., 1994). Studies have found ET-like immunoreactivity, binding sites for ET-1 and the presence of ECE mRNA in the human colon, and ET-1 seems to be a potent intestinal secretagogue that increases colon contraction by direct stimulation of smooth muscle (Mac Cumber et al., 1989). However, sites of ET-1 synthesis in the equine intestinal tract and its relation to
physiological function (motility, secretion) and in pathological conditions such as ischemia-reperfusion injury, inflammatory bowel disease or post-operative ileus have yet to be determined.

The finding of ET-1 gene expression in different sections of the equine gastrointestinal tract is of particular importance owing to the multifunctional role of ET-1 in the gastrointestinal tract of other species (Chou et al., 1995; Murch et al., 1992). Additionally, involvement of ET-1 in a variety of gastrointestinal tract disorders such as ischemia-reperfusion injury, inflammatory bowel disease, peritonitis, and paralytic ileus has been suggested in various species (Egidy et al., 2000). In horses, studies have implicated the role of ET-1 in the pathophysiology of chronic obstructive airway diseases and laminitis (Benamou et al., 1998; Katwa et al., 1999). In our recent study, we found increased plasma ET-like immunoreactivity in naturally acquired gastrointestinal disorders, compared with clinically healthy horses; specifically horses with strangulation obstruction, enterocolitis and peritonitis had the greatest concentrations, suggesting its potential role in these disorders (Ramaswamy et al., 2002).

Identification of ET-mRNA in the gastrointestinal tract of healthy horses suggests that intestinal segments may be a source of ET-1 production during gastrointestinal tract disorders as observed in our previous study (Ramaswamy et al., 2002). The RT-PCR conditions, including the optimization of primers used in this study, may be useful in analyzing the relative levels of ET-1 gene expression in pathophysiological conditions of horses using further quantitative or semi-quantitative PCR methods such as the ribonuclease protection and TaqMan assays. Taken together with the fact that ET-1 has specific receptors and pharmacological activities in these ET-1 producing tissues, our
study suggests that ET-1 may play an important role as a locally (and perhaps systemically) acting regulating/modifying physiological modulator and might be an important pathological mediator.

In conclusion, the present study identified the presence of the ET-1 gene expression in various sections of the gastrointestinal tract in healthy horses, suggesting a role of ET-1 as a regulator of intestinal function. Studies showing the presence of ET-1 and the correlation with other findings with various disease conditions make it important to perform further studies such as in situ hybridization and quantitative studies (TaqMan assay) to determine the distribution and differential expression of ET-1 throughout the different segments of the gastrointestinal tract and within different components of the intestinal wall of clinically healthy horses.

5.5 Product Information

a Beuthanasia-D®, Schering-Plough Animal Health, NJ.
b TRI REAGENT-LS®, Molecular Research Center Inc, Cincinnati, OH.
c Ready-To-GO™ You-Prime First-Strand Beads, Amersham Pharmacia Biotech Inc, Piscataway NJ.
d GenBank Accession AF130760, National Center for Biotechnology Information (NCBI) National Library of Medicine (NLM) at National Institute of Health (NIH), Bethesda, Md.
e Gene Lab, College of Veterinary Medicine, Louisiana State University, Baton Rouge
f ABI PRISM 377 DNA sequencer, Perkin Elmer, Foster City, Calif.
5.6 References


CHAPTER 6

CHARACTERIZATION OF ENDOTHELIN-1 MEDIATED IN VITRO RESPONSES OF CECAL LONGITUDINAL SMOOTH MUSCLE IN HORSES
6.1 Introduction

Endothelin-1 (ET-1), a 21 amino acid vasoconstrictor peptide, is expressed in a variety of cell types including endothelial cells, mast cells, macrophages, mesangial cells, and central and peripheral neurons (Takahashi et al., 1990; Rubanyi and Polokoff, 1994). Endothelins have been suggested to play a broader role in diverse physiological functions such as intestinal secretions, motility, maintenance of vasomotor tone, and closure of the patent ductus arteriosus (Shahbazian and Holzer, 2000; Rubanyi and Polokoff, 1994; Coceani, Kelsey, Seidlitz, 1992). Endothelin-1 exerts its biologic activity by binding to its receptors, ET\textsubscript{A} and ET\textsubscript{B}. The ET\textsubscript{A} receptors are located on smooth muscle and mediate contraction. On the other hand, the vascular ET\textsubscript{B} receptors, ET\textsubscript{B2} are located on endothelial cells and mediate relaxation by the release of nitric oxide (NO). The ET\textsubscript{B} receptors, ET\textsubscript{B1} on non-vascular smooth muscle mediate constriction (Clozel et al., 1992). Actions of ET-1 in intestinal smooth muscle are complex, and involve both excitatory and inhibitory effects depending on the type of receptor, section of tissue and species involved (Takahashi et al., 1990; Bolger et al., 1990; Eglen et al., 1989). Most of the actions of endothelins in the gastrointestinal tract are contractile and occur via their direct actions on the smooth muscle (Okabe et al., 1995).

Gastrointestinal motility is regulated by the enteric nervous system together with excitatory and inhibitory neurons of the nonadrenergic- noncholinergic (NANC) system (Van Hoogmoed et al., 2000; Malone et al., 1999). Endothelins are shown to be localized in the enteric nervous system (Takahashi et al., 1990; Inagaki et al., 1991) and have been proposed to exert important modulatory actions on gastrointestinal motility (Takahashi et al, 1990; Allcock et al., 1995; Miasiro et al., 1999). The involvement of ET-1 in intestinal
motor regulation is due to the presence of both types of ET receptors, ET<sub>A</sub> and ET<sub>B</sub>, in the gastrointestinal tract (Inagaki et al., 1991; Yoshinaga et al., 1992). Endothelin-1 caused concentration-dependent contraction in isolated longitudinal smooth muscle of rat cecum and in opossum internal anal sphincter smooth muscle (Okabe et al., 1995 Chakdar and Rattan, 2001).

The role of NANC innervation in the gastrointestinal tract has been studied in a variety of species, including rats, sheep, cats and horses (Venkova and Krier, 1994; Reid and Titchen, 1988; Van Hoogmoed et al., 2000). Functions of the NANC inhibitory components have been detected in jejunum and ventral colon of horses (Van Hoogmoed et al., 2000; Malone et al., 1999). The role of nitric oxide (NO) and vasoactive intestinal peptide (VIP) in inhibitory neurotransmission in the equine jejunum and ventral colon has been reported using pharmacological agents (Van Hoogmoed et al., 2000; Rakestraw et al., 1996). The NO released from NANC allows receptive relaxation aboral to bolus during peristalsis. In horses, motility disorders are associated with a variety of pathologic conditions, including impaction, postoperative ileus, endotoxemia, and peritonitis (Blikslager et al., 1994; Alican et al., 1998). Luminal distention associated with impaction, strangulation obstruction, and ischemic injury results in gastrointestinal motility disturbances by interfering with the functions of various neurotransmitters such as NO, acetylcholine (ACh), norepinephrine (NE) and VIP. No studies have been reported regarding the role of ET-1 in the gastrointestinal tract of healthy horses. A study on isolated cecal tissue will allow a more definitive evaluation of the presence of ET receptors on cecal smooth muscle and its potential role in cecal motility.
We hypothesized that ET-1 would cause concentration dependent contraction of cecal longitudinal smooth muscle, mediated by ET\textsubscript{A} and ET\textsubscript{B} receptors. Also, we hypothesized that ET-1 will cause contractions similar to those produced by carbachol in equine longitudinal smooth muscle. The purposes of the study reported here was to characterize the ET-1 mediated responses in isolated equine cecal longitudinal smooth muscle and compare them with carbachol, a well-known intestinal smooth muscle contractile agent. Additionally, the role of ET\textsubscript{A} and ET\textsubscript{B} receptors in the mediation of ET-1 induced responses in equine cecal longitudinal smooth muscle was studied using specific ET receptor antagonists.

6.2 Materials and Methods

6.2.1 Animals and Tissue Collection

The study was approved by the Louisiana State University Institutional Animal Care and Use Committee. Tissues collected from adult healthy horses (n=36) that were destined for euthanasia for reasons unrelated to any apparent gastrointestinal tract or systemic diseases were used. Tissues were collected immediately after euthanasia with an overdosage of pentobarbital sodium (100 mg/kg, IV). A 15-cm long segment from the cecal base along the ventral taenia was collected, ingesta were gently removed by lavage with 0.9% NaCl and the tissue was placed in a beaker containing oxygenated, modified Kreb’s solution. The tissue segments were pinned flat in a dissecting bath containing sufficient warm (37°C) oxygenated Kreb’s buffer solution to ensure that tissues were completely immersed, and bubbled with a mixture of 95% O\textsubscript{2} and 5% CO\textsubscript{2}. The intestinal mucosa and submucosa were removed gently with sharp dissecting scissors and strips measuring 2-mm wide and 10-mm long were cut parallel to the taenia to obtain
longitudinal muscle sections and perpendicular to the taenia to obtain circular smooth muscle strips. All samples were tested in duplicate.

6.2.2 Drugs and Reagents

All solutions were prepared on the day of the experiment. Endothelin-1, ETA receptor antagonist (BQ-123) and ETB receptor antagonist (IRL-1038), carbachole, atropine, and guanethidine were used. The drugs were dissolved according to manufacturer’s instructions with distilled water. The ET-1 was reconstituted in distilled water and stored in aliquots of $10^{-4}$M at –80°C. Aliquots were thawed immediately prior to use and further diluted to the desired concentration. Carbachol was dissolved in distilled water immediately before use. The composition of modified Kreb’s solution was as follows [mM]: NaCl, 118; MgSO$_4$, 1.2; CaCl$_2$, 2.5; KCl, 4.7; NaHCO$_3$, 24.9; and dextrose [pH 7.3 to 7.4].

Preliminary studies (n=6) were conducted to determine the appropriate resting tension and equilibration period for tissues to determine the effect of ET-1. Results from our study showed that a resting tension of 2 g was required to elicit a consistent contractile pattern, which is similar to previously reported studies (Van Hoogmoed et al., 2000; Rakestraw et al., 1996; Malone et al., 1999). Both longitudinal and circular muscle strips exhibited spontaneous contractile activity. The spontaneous activity of longitudinally-oriented smooth muscle was cyclic and occurred at a steady rate of approximately of 2-3 contractions per minute (25mm/min). The circular smooth muscle occurred in a pattern typical to that of colonic tissue, however, the pattern of contractions during the 3-minute period didn’t sufficient enough to evaluate the response of
exogenous agents added. So further experiments were conducted on longitudinal smooth muscle.

6.2.3 Experimental Protocol

One end of each strip was mounted to the floor of the organ bath (10 ml) and the other end was attached to a force-displacement transducer, interfaced with a polygraph, to record the change in tension. For evaluation of tissue responses, intestinal smooth muscle tissues were equilibrated for 45-minutes in the organ baths containing Kreb’s solution maintained at 37°C by a circulating water and oxygenated with a gas mixture of 95% O₂ and 5% CO₂. Resting tension was set at 2 grams and tissues were allowed to equilibrate for an additional 60 minutes. Throughout the experiment, Kreb’s solution was changed at 15-minute intervals.

Based on preliminary studies, further experiments were performed to characterize the role of ET-1 in the longitudinal smooth muscle. Tissues from 6 horses (48 strips) were used for determining the concentration-response (C-R) relationships of both ET-1 and carbachol and 18 horses (104 strips) were used for C-R relationships of ET-1 in the presence of ETA and ETB receptor antagonists, separately and together.

6.2.3.1 Concentration-Response Relationships of Carbachol and ET-1 on Cecal Longitudinal Smooth Muscle

After a 95-minute equilibration period, cumulative C-R relationships for ET-1 and carbachol at graded concentrations of 10⁻⁹, 10⁻⁸, 10⁻⁷ and 10⁻⁶ M were performed in separate baths. Each dose was added at 3-minute intervals and changes in tension were recorded.
6.2.3.2 Effect of ET Antagonists, BQ-123, IRL-1038, Separately and Together on ET-1 Induced Response

After the application of 2 g tension, the tissues were incubated separately with ET$_A$ (BQ-123) and ET$_B$ (IRL-1038) antagonists, at 3 concentrations of 10$^{-9}$, 10$^7$ and 10$^{-5}$ M, for a period of 45 minutes, and a C-R relationship of ET-1 (10$^{-9}$ to 10$^{-6}$M) was then determined. Similarly, cecal muscle strips were incubated with 3 individual concentrations of 10$^{-9}$, 10$^7$ and 10$^{-5}$M of the combined antagonists for a period of 45 minutes, and a C-R relationship of ET-1 (10$^{-9}$ to 10$^{-6}$M) was determined as before.

6.2.3.3 Electrical Field Stimulation (EFS)

The electrodes used for the EFS consisted of a pair of platinum wires fixed at both sides of the smooth muscle strip. Electric field stimulation was generated by a stimulator and applied to all tissues simultaneously. Repetitive stimuli (trains) at 3-minute intervals of millisecond duration pulses were tested at intensities of 40V and 60V, with frequency of 5Hz, 10 Hz and 20Hz to the cecal longitudinal smooth muscle preparations. These stimulation parameters have been shown to cause facilitate intestinal smooth muscle responses in horses (Malone et al., 1999). In our study, tissues were given repetitive stimuli at 3-minute trains of 10 millisecond duration pulses at intensities of 60V and 20Hz to the longitudinal smooth muscle preparation. Tissues from 5 horses were prepared as before and allowed to equilibrate for 1 hr, after which a resting tension of 2 g was set and EFS applied. In a separate set of experiments, after equilibration, tissues were incubated with atropine (10$^{-5}$M) and guanethidine (10$^{-5}$M) for 45 minutes and EFS was applied. Tetrodotoxin (10$^{-3}$M), a sodium channel blocker, was added to test the role of nerve conduction in the EFS induced contractions.
6.3 Data analysis

The percentage changes in baseline tension were quantified by measuring percentage change in the area under the curve (AUC) using Image Analysis Software. The active change in tissue responses was calculated as the percentage change from baseline tension. The percentage change in baseline tension for ET-1 responses was calculated by measuring the AUC 3-minutes prior to the addition of the drug compared with the AUC during the 3-minute period after each dose was added (Rakestraw et al., 1996). The changes in tension due to ET-1 were compared with carbachol and with and without the ET receptor antagonists.

All data were considered continuous and followed a normal distribution as noted by failure to reject the null hypothesis of normality at $P < 0.05$, using the Shapiro-Wilk test. The data were summarized as mean ± SEM and was evaluated for an effect of antagonist and concentration, using a mixed linear model accounting for the random variance of horse. Where there were significant interaction effects at $P < 0.05$, predetermined multiple comparisons were made across concentrations within each antagonist group, and between groups at specified concentrations, using least squares means maintaining type I error at 0.05. PROC UNIVARIATE and PROC MIXED were used for the analysis using software.

6.4 Results

Both longitudinal and circular smooth muscle showed spontaneous contractile activity (Figure 6.1). The addition of ET-1 produced a slow sustained, concentration-dependent contractile response in equine cecal longitudinal muscle (Figure 6.2). The carbachol-induced contractile effects were significantly greater than that induced by ET-1
Figure 6.1 Physiograph recording (25 mm/sec) of cecal circular (A) and longitudinal (B) smooth muscle spontaneous contractile responses, - 2g tension.
Figure 6.2 - Physiograph recording (25 mm/sec) of cecal longitudinal muscle contractile response to increasing log molar concentrations of ET-1.
Pre-incubation of cecal longitudinal muscle strips with the ETA receptor antagonist, BQ-123, did not inhibit the contractile response of ET-1 (Figure 6.4). A slight non-significant increase in contractile activity was observed in tissues pre-incubated with BQ-123 (10^{-9}, 10^{-7} M), compared with that of control tissues ($P>0.05$). Similarly, pre-incubation of tissues with the ETB receptor antagonist, IRL-1038, did not abolish ET-1 induced response (Figures 6.4 & 6.5). The contractile responses of longitudinal tissue to ET-1 were significantly decreased when tissues were pre-incubated with BQ-123 and IRL-1038 together at 10^{-5}M concentrations, compared with tissues incubated with lower dose of the both receptor antagonists (10^{-7}, 10^{-9} M) and compared with control tissues receiving no antagonists (Figure 6.6). Contractile activity of the longitudinal muscle strips was not affected by EFS in both control tissues and those incubated with atropine and guanethidine. Also, the addition of tetrodotoxin did not alter the contractile activity of cecal longitudinal smooth muscle.

6.5 Discussion

This study demonstrated that ET-1 causes a concentration-dependent contraction of longitudinal smooth muscle of the equine cecum. The response of longitudinal smooth muscle to ET-1 was significantly less than with the muscarinic agonist, carbachol, on an equimolar concentration basis. It appears that the magnitude of ET-1 induced contractile response is of lesser magnitude in intestinal smooth muscle compared to that of ET-1 effect in the large intestinal vasculature of horses (Venugopal et al., 2001). Pre-incubation of longitudinal muscle strips with the ET antagonists, BQ-123 and IRL-1038, at any concentration did not cause a significant reduction in the response induced by ET-1. However, when tissues were pre-incubated with both antagonists together, the
Figure 6.3- Mean ± SEM % change in area under the curve (AUC) in equine longitudinal smooth muscle to log molar concentrations of endothelin-1 and carbachol. *Significant differences ($P<0.0001$) in response for ET-1 and carbachol were observed at log molar concentrations of $10^{-7}$ M and $10^{-6}$ M.
Figure 6.4 - Mean ± SEM % change in area under the curve (AUC) in equine longitudinal smooth muscle to log molar concentrations of endothelin-1 before (control) and after incubation of tissues with different log molar concentrations (10^{-9} M, 10^{-7} M and 10^{-5} M,) of an endothelin receptor-A antagonist (BQ-123).
Figure 6.5 - Mean ± SEM % change in area under the curve (AUC) in equine longitudinal smooth muscle to log molar concentrations of endothelin-1 before (control) and after incubation with different log molar concentrations ($10^{-9}$ M, $10^{-7}$ M and $10^{-5}$ M) of an endothelin receptor-B antagonist (IRL-1038).
Figure 6.6 - Mean ± SEM percentage change AUC in equine longitudinal smooth muscle to log molar concentrations of endothelin-1 before (control) and after incubation with different log molar concentrations ($10^{-9}$ M, $10^{-7}$ M and $10^{-5}$ M) of an endothelin receptor-A and B antagonist (BQ-123 and IRL-1038) together. Contractile responses to ET-1 were *significantly decreased when tissues were incubated with both antagonists at $10^{-5}$ M log molar concentration ($P <0.001$).
contractile effects of ET-1 were significantly inhibited. These findings suggest that both endothelin receptors, ET\(_A\) and ET\(_B\), mediate contraction in response to ET-1 in the cecal longitudinal smooth muscle.

Along with branches of both autonomic nervous systems, intestinal peristalsis is coordinated and controlled by the enteric nervous system (Van Hoogmoed et al., 2000). The shortening and constricting movements of the equine cecum occur by the synchronized contractions of circular and longitudinal smooth muscle, occurring approximately every 4 minutes (Sellers and Lowe, 1986). Any lack of coordination between activities of the smooth muscle layers can result in motility dysfunction. Endothelin-1 is the most potent vasoconstrictor known to date, and its effect on intestinal muscle varies depending on the type of tissue, section of the gastrointestinal tract and species tested (Borges et al., 1989; Bolger et al., 1992; Takahashi et al., 1990; Eglen et al., 1989). The ET-1 mediated contraction depends on the release of both intracellular calcium and the entry of extracellular calcium (Yoshinaga et al., 1992). Both ET\(_A\) and ET\(_B\) receptors are involved in the activation of gastrointestinal motility in guinea pig ileum (Miasiro et al., 1995). Elevated plasma endothelin-1 like immunoreactivity has been implicated with intestinal dysmotility in human patients with acute pancreatitis (Chen et al., 1999). However, no studies have reported the role of ET-1 in intestinal motility or dysmotility of horses.

Endothelins are found in enteric neurons with binding sites in the myenteric and submucosal plexus, mucosa, muscular layers and in the vasculature of rat colon (Takahashi et al., 1990). Endothelin receptors have the greatest distribution in cecum and stomach, compared with other sections of the guinea pig intestine (Kuwahara et al.,
In the gastrointestinal tract, the contractile effect of ET-1 has been reported in the guinea pig ileum (Bolger et al., 1992; Ishida et al., 1989) and the stomach and colon of rats (Takahashi et al., 1990). Endothelin-1 causes a marked biphasic response in guinea pig ileal longitudinal smooth muscle with a similar magnitude to that observed in vascular smooth muscle (Lin and Lee, 1990; Miasiro et al., 1999). Okabe et al. (1995) reported direct contractile effect of ET-1 in both longitudinal and circular smooth muscle cells. In our study, ET-1 caused a slow, sustained concentration-dependent contractile response (10^{-9} \text{ M} \text{ to } 10^{-6} \text{ M}). The concentrations tested for ET-1, may or may not of physiological concentration range, however, various neurotransmitters are found in macromolar concentration. Also the degree of contraction of longitudinal smooth muscle to ET-1 was significantly lower compared with that of muscarinic agonist, carbachol. The contractile response of ET-1 in longitudinal smooth muscle was lower than the reported effect of ET-1 in the colonic vasculature of horses (Venugopal et al., 2001). This difference in the magnitude of the contractile response of ET-1 between vascular and non-vascular smooth muscle has been documented in other species (Fulginiti et al., 1993), suggesting that ET-1 has more potent contractile effects in vascular smooth muscle than in intestinal smooth muscle.

Endothelin-1 has been implicated in regulation of gastrointestinal motility and in the pathogenesis of ischemia and ischemia-reperfusion (I-R) injury (Tekin et al., 1999; Anadol et al., 1998). Treatment with bosentan (ET_{B} receptor antagonist) abolished I-R injury induced intestinal transit delay suggesting that I-R associated delays in intestinal transit involve an endothelin-dependent mechanism (Alican et al., 1998). Also, a beneficial effect of ET_{A} antagonists in reducing ET-1 induced gastric and intestinal
mucosal damage in rats has been reported (Massberg et al., 1998). The ET antagonists BQ-123 and IRL-1038 used in our study are specific competitive endothelin receptor antagonists that block $\text{ET}_A$ and $\text{ET}_B$ receptors, respectively. In our study, the epithelial mucosal and submucosal layer of the cecal tissues was removed for evaluating ET-1 response in the longitudinal smooth muscle. The ET receptors present in the muscular layer of gastrointestinal smooth muscle are $\text{ET}_A$ and $\text{ET}_{B2}$ receptor types, and both mediate contractile response on binding to ET-1.

Pre-incubation of tissues with $\text{ET}_A$ receptor antagonist, BQ-123 ($10^{-9}$ M, $10^{-7}$ M, $10^{-5}$ M) did not significantly alter the ET-1 induced contractile response. However, the contractile responses were slightly reduced on tissues pre-incubated with $\text{ET}_A$ receptor antagonist at $10^{-5}$ M though it was not significantly different from other treatments (Figure 6.4). Also the contractile response of ET-1 was slightly greater in tissues treated with $10^{-7}$ and $10^{-9}$ M compared to control. No significant difference in contractile response to ET-1 was seen in tissues pre-incubated with $\text{ET}_B$ receptor antagonist, IRL-1038 at any concentration (Figure 6.5). Thus, pre-incubation of tissues separately with BQ-123 and IRL-1038 did not significantly alter the ET-1 induced changes in cecal longitudinal muscle tension. The contractile response to ET-1 was completely abolished when tissues were incubated with both antagonists combined at $10^{-5}$ M concentration. This type of response may be explained by the fact that when one receptor type is blocked ($\text{ET}_A$) the other receptor ($\text{ET}_{B2}$) effects are unmasked to induce a contractile effect, and vice versa. This could also suggest that both $\text{ET}_A$ and $\text{ET}_B$ receptors mediate contraction of cecal longitudinal smooth muscle when ET-1 binds. Similarly, studies using an $\text{ET}_A$ receptor antagonist (BQ 123) and a combined $\text{ET}_A / \text{ET}_B$ receptor
antagonist (PD145065) showed that both endothelin $\text{ET}_A$ and $\text{ET}_B$ receptor types mediate contraction in the guinea pig ileum (Miasiro et al., 1995).

Under our experimental conditions, the contractile activity of the cecal longitudinal smooth muscle layer was unaffected with EFS. Similarly, unlike in circular smooth muscle, the contractile activity of the ventral colon longitudinal muscle strips of horses was unaffected by EFS (Van Hoogmoed et al., 2000). The lack of EFS response in our study can be speculated to be due either an insufficient release of neurotransmitter to elicit a response or the effects of neurotransmitters were not sufficient to override the intrinsic contractile (spontaneous contraction) responses.

In summary, ET-1 caused a concentration-dependent contractile effect on cecal longitudinal smooth muscle of clinically healthy horses, which appears to be mediated by both $\text{ET}_A$ and $\text{ET}_B$ receptors. However, the ET-1 induced contraction in cecal longitudinal smooth muscle was not as pronounced as observed in equine vascular smooth muscle and was less than carbachol-treated cecal smooth muscle. The observed effects of ET-1 on cecal longitudinal smooth muscle contraction may suggest that alterations in cecal motility might be associated with changes in ET-1 synthesis and release during naturally acquired gastrointestinal tract disorders. Further studies are needed to determine the role of ET-1 in the smooth muscle responses of diseased or affected tissues.

6.6 Product Information

\(^a\)Beuthanasia-D, Schering-Plough Animal Health Corp, Union, NJ.

\(^b\)Endothelin-1, American Peptide Company, Sunnyvale, Calif.
BQ-123, American Peptide Company, Sunnyvale, Calif.

IRL-1038, American Peptide Company, Sunnyvale, Calif.

Carbachol, Sigma-Aldrich Inc, St Louis, Mo.

Atropine, Sigma-Aldrich Inc, St Louis, Mo.

Guanethidine, Sigma-Aldrich Inc, St Louis, Mo.

Tetrodotoxin, Sigma-Aldrich Inc, St Louis, Mo.

Model 7D polygraph, Grass instruments, Quincy, Mass.

Chart recorder model 25-60, Grass instruments, Quincy, Mass.

Grass S48 stimulator, Astro-Med Inc, Grass Instruments Division, West Warwick, RI.

Sigma Scan Pro 4.0, Jandel Scientific Software, San Rafael, Calif.

SAS for windows 95, version 7.0, SAS Systems Inc, Cary, NC.

6.7 References


CHAPTER 7
DISSERTATION SUMMARY
Endothelin-1, the most potent vasoconstrictor known to date, has been implicated in a variety of physiological processes ranging from maintenance of vasomotor tone, ion secretion, closure of the patent ductus arteriosus and control of gastrointestinal tract motility. Also, ET-1 has been found to play a major role in the pathophysiology of intestinal ischemia, cerebral vasospasm, peritonitis, megacolon and cardiogenic shock. The maintenance of vascular tone and smooth muscle homeostasis is achieved mainly by ET-1 in conjunction with NO. An imbalance in the release of ET-1 and NO are believed to cause alterations in vasomotor tone and gastrointestinal motility. We investigated the role of ET-1 in the gastrointestinal tract of horses by measuring circulating concentrations of plasma ET-like immunoreactivity and characterized the role of ET-1 mediated responses in equine cecal longitudinal smooth muscle. Endothelin-1 gene expression was determined in different sections of the gastrointestinal tract of clinically healthy horses. We examined the regional distribution of ET-1 like immunoreactivity using immunohistochemical staining in the gastrointestinal tract of clinically healthy horses and compared this to intestinal segments collected from horses with acquired strangulation obstruction.

In the first study, we found increased concentrations of plasma ET-like immunoreactivity in horses with acquired gastrointestinal disorders, compared with clinically healthy horses. Median plasma concentrations of ET-like immunoreactivity were 1.80 pg/ml (range, 1.09 to 3.2 pg/ml) in healthy horses. Plasma ET-like immunoreactivity was greatest in horses with strangulating large intestinal obstruction (median, 10.02pg/ml; range, 3.8 to 22.62 pg/ml), peritonitis (9.19pg/ml; 7.89 to 25.83 pg/ml), and enterocolitis (8.89 pg/ml, 6.30 to 18.36 pg/ml). Also, the concentration of
ET-like immunoreactivity was significantly associated with survival, PCV, and duration of signs of pain. The source of measured ET-like immunoreactivity in plasma remains unclear. The increased circulating concentrations levels of ET-1 may be from the damaged endothelium, from affected tissues or may be a result of hemoconcentration in affected horses. Greater than 80% of the ET-1 synthesized by the endothelium is secreted abluminally toward the tunica media where it functions in a paracrine fashion. Therefore, measurement of plasma ET-1 concentrations may not truly reflect the overall synthesis and release of ET-1 during pathophysiologic events. However, the significant increase in ET-1 like immunoreactivity in severe intestinal disorders such as strangulating obstruction and peritonitis, compared with other less severe disorders, indicates a potential involvement of ET-1 in these disorders.

Immunohistochemical studies revealed regional distribution of ET-1 like immunoreactivity in different segments of the equine gastrointestinal tract extending from the duodenum to the right dorsal colon. The ET-1 like staining is confined to the cytoplasm of the surface epithelium, villi, the muscular layer, and less intense staining in the epithelial crypts. The endothelial lining of the blood vessels stained with veins stained more intensely than arteries. The presence of ET-1 in the surface epithelial cells suggests the potential physiological role of ET-1 in ion secretion and intestinal motility. This function of ET-1 has previously been shown in rat and human colon. This is the first study to report the presence of ET-1 in the gastrointestinal tract of horses. An increase in the intensity of ET-1 staining from intestinal segments from horses with acquired small intestinal and large colon strangulation obstruction, compared with intestinal segments from healthy horses, was observed.
We identified ET-1 gene expression in various sections of the gastrointestinal tract of the clinically healthy horse, suggesting a role of ET-1 in the regulation of intestinal function. Therefore, the intestinal segments may be a source of ET-1 synthesis and release during gastrointestinal tract disorders as observed in our previous study. Studies showing the presence of ET-1 and the correlation with other findings in various disease conditions justifies the need to perform further studies such as in situ hybridization and quantitative studies to determine the differential expression of ET-1 in acquired gastrointestinal tract disorders of horses.

We characterized the role ET-1 mediated responses in equine cecal longitudinal smooth muscle in vitro. Despite the dose-dependent increase in contractile activity of ET-1 in spontaneously contracting equine longitudinal smooth muscle, the magnitude was much less compared with the muscarinic agonist, carbachol. The effects of ET-1 on intestinal smooth muscle occur by binding of ET-1 to its specific ET receptors types, ET_A and ET_B. Binding of ET-1 to ET_A in smooth muscle, and ET_B in vascular endothelium mediate contraction and dilatation, respectively. However, these responses vary depending upon the tissues and species tested. Incubation of cecal longitudinal muscle separately with BQ-123 (ET_A blocker) or IRL-1038 (ET_B blocker) did not significantly inhibit the contractile activity to ET-1 at any concentration (10^-9M, 10^-7M, 10^-5M). However, contractile responses of cecal longitudinal smooth muscle to ET-1 were significantly inhibited when tissues were incubated simultaneously with both antagonists at a concentration of 10^-5M (P<0.001). This suggests that both receptors ET_A and ET_B receptors are likely involved in the contractile effect. The results from this study suggest the presence of ET_A and ET_B receptors in the gastrointestinal tract and that they likely
mediate the contractile effect of ET-1 in cecal smooth muscle of horses. However, findings from these studies do not explain the importance of ET-1 in intestinal motility disorders, which will require further evaluation in affected intestinal segments.

In summary, the circulating concentrations of plasma ET-1 may be a potential indicator of severity of the gastrointestinal tract disorders in horses. Endothelin-1 gene expression was detected in different sections of intestine including the duodenum, jejunum, ileum, pelvic flexure and colon; as well as in the heart and lung. Immunohistochemical localization of ET-1 like immunoreactivity in the surface epithelium, villi and blood vessels in various segments of intestinal tract of healthy horses and those with intestinal strangulation obstruction indicates its potential role in intestinal secretions and motility. In horses, both $\text{ET}_A$ and $\text{ET}_B$ receptors mediate the ET-1 induced contractile response in cecal longitudinal smooth muscle. Further investigation is warranted to further characterize the role of ET-1 in the gastrointestinal tract of horses during health and disease. Additionally, the benefit of ET antagonists in attenuating motility disturbances and other disorders involving the intestinal vascular and nonvascular smooth muscle of horses to be evaluated.
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September 5, 2002

American Journal Of Veterinary Research
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