Effect of Stress on Mucin Expression in the Gastrointestinal Tract of Mice.

Bupe Martha Habiyambere
East Tennessee State University

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Effect of Stress on Mucin Expression in the Gastrointestinal Tract of Mice

A thesis
presented to
the faculty of the Department of Biological Sciences
East Tennessee State University

In partial fulfillment
of the requirements for the degree
Master of Science in Biology

by
Bupe Martha Habiyambere

December 2010

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Keywords: Stress, Mucin Expression, Gastrointestinal Tract
ABSTRACT

Effect of Stress on Mucin Expression in the Gastrointestinal Tract of Mice

by

Bupe Martha Habiyambere

This study investigated the effects of chronic stress on mucin expression in the GIT of mice. Sixteen ICR male mice were randomly divided into treated and control animals. Restraint stress was applied to the treated group for 21 days. Body weight (BW) changes and feed consumed (FC) were regularly recorded. After 21 days mice were euthanized and blood, GIT mucosa, and GIT sections were collected. Serum corticosterone levels were determined. The GIT sections were fixed, paraffin embedded, sectioned, and stained with AB-PAS to visualize goblet cells. Mucin expression was determined using qPCR. As expected stressed mice showed higher corticosterone than the control animals. BW decreased in the stressed group. Histology showed a decrease in goblet cells in stressed mice. Muc1 and Muc4 in the colon and Muc3 in the duodenum were upregulated in stressed animals. These findings suggest that chronic stress may affect mucin expression in the GIT.
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Last but not least, I thank my family. My “Sunshine”, you have been the best cheerleader, your trust and confidence motivated me to excel. This is my way of reminding you to “Think Big”!
DEDICATION

To Tabita and Kemo
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CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Background

The gut protective barrier is the result of interactions among the mucosal barrier, the immune system, and the gut microbiota as illustrated in Figure 1.

![Figure 1: Interactions among the mucosal barrier, the immune system, and the gut microbiota to support the gut protective barrier in the gastrointestinal tract.](image)

The mucosal barrier forms the first line of defense of the internal milieu against noxious agents. It has 3 components: the epithelial cells joined together by tight junctions to form a physical barrier between the internal and external environments of the gut with is a constant sloughing of these cells; the bicarbonate ions that neutralize the acids from the stomach; and the mucus layer. The immune system plays a critical role in the maintaining the gut protective barrier. The intestine contains more than 70% of all immune cells found in the body (Kagnoff 1993). These immune cells secrete
mainly IgA and to a lesser extent IgM and IgG antibodies. The gut microbiota comprising more than 500 bacterial species plays an important role in fermenting nondigestible foods and synthesis of biotin and vitamin K among other vitamins. The gut microbiota affects immune responses and works toward the maintenance of homeostasis (Camilleri 2008). The 3 components, the mucosal barrier, the immune system, and the gut microbiota, interact and promote the gut protective barrier. The apical side of the epithelial cells is covered by the mucus. The mucus covering is mainly composed by mucins.

Mucins are large heavily glycosylated proteins that form the major component of mucus. The protein backbones of mucins may vary in size: some mucins are small, containing only a few hundred amino acid residues, while others are quite large and contain several thousand amino acid residues (Perez-Vilar and Hill 1999). About 80% of the mass are carbohydrates to ensure high density and viscosity (Toribara et al. 1991; Moniaux et al., 2001). Mucin molecules form intertwined networks that form the major component of the mucus that protects epithelial surfaces of lumens and ducts in the body (Garrett and Grisham 1999).

Mucins have many functions including lubrication, modulation of water and electrolyte absorption, protection of the epithelial layer against mechanical and chemical stress, provide attachment sites for commensal and pathogenic microbes, and serve as ligand for the targeting of leucocytes to endothelial cells (Chang et al. 2000; Robbe et al. 2004). Therefore interference with the integrity of the mucus layer may expose epithelial cells to various insults. The mucus barrier particularly in the stomach is composed of 2 layers: one loose adherent layer and a firmly adherent layer attached to
the gastric mucosa. These 2 layers have different protective functions (Atuma et al. 2001; Phillipson et al. 2008). The firmly adherent layer counteracts the action of corrosive acids while the loosely adherent layer interacts with the swallowed toxic substances and releases nitric oxide that stimulates the accumulation of mucus by the firmly adherent layer (Atuma et al. 2001; Phillipson et al. 2008). The mucus layer does not have the same thickness throughout the digestive tract but varies along the different portions as demonstrated by Atuma et al. (2001).

There is growing evidence suggesting that mucins play a role in various other epithelial cell processes such as adhesion, renewal, differentiation, and signaling (Andrianifahanana et al. 2006). Moniaux and collaborators (2001) pointed out that mucins take part in the growth process, fetal development, epithelial integrity, carcinogenesis, and metastasis. Because mucins seem to be involved in different complex processes in the body, it is important to understand the factors that regulate the various aspects of their biology such as synthesis or secretion (Andrianifahanana et al. 2006). It has also been established that many types of cancers and inflammatory diseases cause deregulation of mucin expression (Devine and McKenzie 1992; Gendler and Spicer 1995; Hollingsworth and Swanson 2004; Moniaux et al. 2004; An et al. 2007). Based on these studies, mucins are considered potential markers in early diagnosis or detection of most tumors and inflammatory conditions in the gastrointestinal tract and may even be used in the design of mucin-based vaccines (Mall 2008) as well as in the development of appropriate therapies to correct abnormalities in their expression (Andrianifahanana et al. 2006).
Stress is the body’s nonspecific response to threats to the internal milieu (Selye 1976; Bhatia and Tandon 2005). In humans nausea and diarrhea are instinctive ways to respond to psychological difficulties and fear respectively. After severe trauma stress ulcers are a common complication, these are characterized by a disrupted gastric mucosa and bleeding (Habib et al. 2001).

This study investigated the effect of stress on the production and expression of mucins in the gastrointestinal tract. Five mucins were examined: 3 membrane-bound mucins (Muc1, Muc3, and Muc4) and 2 secreted gel-forming mucins (Muc2 and Muc5ac).

Histology of the Gastrointestinal Tract (GIT)

The stomach is the largest portion of the gastrointestinal tract. It starts at the cardiac sphincter which is the portion that joins the esophagus to the stomach and ends at the pyloric sphincter which is its junction to the duodenum. The stomach has high distention capabilities because of the presence of the rugae or folds of the mucosa and submucosa. The stomach can be divided into 5 segments: the cardia, the fundus, the corpus, the antrum, and the pylorus (Yamada et al. 2003) but Martini and Nath (2009) combine the antrum and pylorus resulting in 4 segments.

The fragmentation or breakdown of ingested food particles that starts in the mouth continues in the stomach in the presence of digestive juices. The structure of this organ is adapted to its primary function and has many tubular glands whose secretions protect the gastric mucosa and facilitate food digestion. Microscopically the stomach is
composed of 4 layers: the serosa, muscularis propria (longitudinal, circular, and oblique muscle layers), the submucosa, and the mucosa (Yamada et al. 2003). The mucosa in the stomach consists of the 3 layers: the epithelium is a simple columnar secretory layer; the lamina propria contains nerves, blood, and lymphatic vessels; and the muscularis mucosae. The gastric wall has openings of mucus-producing, parietal and chief cells (Yamada et al. 2003; Martini and Nath 2009).

The final digestion, absorption of nutrients and endocrine secretion takes place in the small intestine. The intestinal tract starts at the pyloric sphincter and ends at the ileoceleval valve for the small intestine, then the large intestine extends to the rectum. The small intestine is composed of 3 segments: the duodenum, jejunum, and ileum while the large intestine comprises the cecum, colon, and rectum. In order to increase the absorption surface, the intestinal tract has the plicae circulares, villi, and microvilli. The plicae circulares made of mucosal and submucosal invaginations are found mostly in the duodenum and jejunum (Yamada et al. 2003; Juqueira and Carneiro 2005). The villi are small fingerlike protrusions of the mucosa with variable appearance; they are leaf-shaped and large in the duodenum, thin and tall in the jejunum, while they are short and wide in the ileum (Yamada et al. 2003). The crypts of Lieberkühn found at the base of the villi are made by the epithelium that extends into the lamina propria. The microvilli are tiny protrusions that form the brush border on the apical cell membrane (Yamada et al. 2003).

The small intestine like the stomach presents 4 layers: the mucosa, submucosa, muscularis propria, and serosa. The mucosa is composed of the epithelium lubricated by the mucus, lamina propria, and muscularis mucosae; the submucosa consists of a
dense connective tissue with blood and lymphatic vessels and nerves; the muscularis propria is made of a longitudinal and circular muscular layers that control and synchronize the peristaltic movements, while the serosa is composed of connective tissue with mesothelial cells (Yamada et al. 2003; Martini and Nath 2009). The epithelium in the intestine is a simple columnar type and contains the goblet cells that are responsible for mucus production. The secretions from glands are transported to the epithelial surface by ducts (Martini and Nath 2009). The lamina propria of the mucosa harbors blood and lymphatic vessels, smooth muscle cells, nerve endings, and mucus glands. Peyer's patches are thickenings of the epithelium specifically the mucosa and submucosa of the distal portion of the ileum (Yamada et al. 2003); they play an important role in the gut immune system. The apical surface of the epithelium is covered by mucus and mucins are the major component of this covering that protects epithelial surfaces of lumens and ducts in the body (Garrett and Grisham 1999).

The gastric and intestinal epithelial cells are constantly sloughing and being replaced from the epithelial stem cells (Martini and Nath 2009).

**Classification of Mucins**

Mucins have a variable number of tandem repeats (TR) rich in proline, threonine, and serine also called PTS domain that are highly glycosylated (Gendler and Spicer 1995; Lang et al. 2007). Because of their size and complex structure, the classification of mucins has raised a number of controversies. Classification of mucins poses a number of questions such as the need to standardize the criteria considered for
classification that need to be addressed. For example, what qualifies a given gene to belong to the mucin family? Is the presence of the TR or the amount of O-glycosides good enough to determine that a gene belongs to the mucin family? It has been suggested that the mucin family of genes be renamed in accordance to their sequence homologies instead of just taking into consideration their extensive O-linked glycosylation. This is based on the fact that the members of this family are so different from one another that grouping them under the same entity poses some difficulty studying them, additionally, their size and structure are very complex (Dekker et al. 2002; Lang et al. 2007). Rose and Voynow (2006) suggested that there should be consistency in the criteria used to classify mucins. There are more than 20 human (MUC) and murine (Muc) mucins deposited in GenBank (Rose and Voynow, 2006).

Although the question of how to classify mucins remains controversial, mucins have nonetheless been classified into 2 main groups in relation to their location: the membrane-bound or transmembrane and secreted mucins. The membrane-bound mucins include MUC1, MUC3, MUC4, MUC12, MUC13, MUC16, MUC17, and MUC20 and the secreted mucins include MUC2, MUC5B, MUC5AC, MUC6, MUC7, MUC8, MUC19, and MUC21 (Perez-Vilar and Hill 1999; Escande et al. 2002; Hollingsworth and Swanson 2004; Andrianifahanana et al. 2006; Moal and Servin 2006; Lang et al. 2007; Itoh et al. 2008). The secreted mucins have been further distinguished into gel-forming such as MUC2, MUC5AC, MUC5B, MUC6, and MUC19, and non-gel forming mucins include MUC7, MUC8, and MUC9 (Andrianifahanana et al. 2006). This study focused
on the expression of 5 murine mucins (Muc): Muc1, Muc2, Muc3, Muc4, and Muc5ac in the gastrointestinal tract.

Functions and Expression Sites of Mucins

Mucins play a very important role in protection, repair, and lubrication of the epithelia of ducts and lumens particularly in the respiratory, gastrointestinal, and genital tracts and accessory organs such as pancreas and gallbladder (Reid and Harris 1998; Homsi et al. 2007). They protect the underlying tissue from different insults (Robbe et al. 2004; Andrianifahanana et al. 2006). As mentioned earlier, mucins also play a role in various other epithelial cell processes such as adhesion, renewal, differentiation, and signaling (Andrianifahanana et al. 2006) as well as in growth, fetal development, epithelial integrity, carcinogenesis, and metastasis (Moniaux et al. 2001). Mucins stimulate the presence of high concentration of IgA secretion and lysozyme on the epithelium and serve as free radical scavenger (Cross et al. 1984; Forstner and Forstner 1994). Mucins in the gastrointestinal tract play an important role in protecting the mucosa.

MUC1’s critical role in maintaining the integrity of the mucosal barrier during infection has already been established. McAuley et al. (2007) suggested that surface mucins play the role of target to the invading pathogens by limiting the interaction of pathogens with the epithelium. They demonstrated that following an infection MUC1 was upregulated and that it was highly expressed in the stomach but was low in the intestine. MUC1 has also a nonclassical expression site in many activated or nonactivated immune cells (Andrianifahanana et al. 2006). It has been also suggested
that MUC1 has the ability to inhibit T cell activation (Chang et al. 2000) as well as play a role in signaling (Songyang et al. 1994). MUC2 has a major function in maintaining the integrity of the colon (Homsi et al. 2007) and it has the ability to suppress the development of gastrointestinal tumors (Velcich et al. 2002). MUC3 (MUC3A and MUC3B) in the secreted form can inhibit the attachment of enteric pathogens (E. coli) to the epithelium of the gastrointestinal tract (Mack et al. 2003; Moal and Servin 2006). Shekels and Ho postulated in 2003 that Muc3 plays an important role in the protection of the mucosal layer. Homsi and others (2007) suggested that MUC4 together with MUC1 play a role in the mucus external static barrier such that they are able to limit pathogen access to the gut. MUC5AC acts as receptor for Helicobacter pylori in the gastric epithelial tissue (Van de Bovenkamp et al. 2003; Moal and Servin 2006). MUC6 main function is to protect the susceptible epithelia against many harmful agents (Toribara et al. 1997). The function of MUC11 and MUC12 is not well understood but evidence seems to suggest their role in mediation of epithelial cell growth and differentiation (Songyang et al. 1994). MUC12 may play a role in signaling (Songyang et al. 1994). MUC13 may be a mediator of responses of the epithelia to damage and infection (Williams et al. 1999). Its function is not clear but it is upregulated in pancreatic cancer (Moniaux et al., 2004). MUC20 mediates the mesenchymal-epithelial transition factor (Met) signaling cascade that participates in the inhibition of the Growth factor receptor-bound protein 2-Ras (Grb2-Ras) pathway (Higuchi et al. 2004). MUC21 regulates immune responses (Itoh et al. 2008).
Stress Response

The body responds nonspecifically to a demand placed on it (Selye 1936). Two axes control sequential and fast events: the hypothalamic-pituitary-adrenal axis and the sympatho-adrenal axis. Many hormones play a role in this generalized stress response including corticotrophin-releasing hormone (CRH), arginine vasopressin (AVP), corticotrophin (ACTH), glucocorticoids (cortisol or corticosterone), catecholamines (epinephrine, norepinephrine), and endorphins. The hypothalamic-pituitary-adrenal axis is critical in the initiation of the stress response (Herd 1991; Habib et al. 2001; Caso et al. 2008).

Cardiovascular Response to Stress

The aim of the cardiac response during stress is to increase the survival of the organism when threatened in preparation for a “fight or flight” reaction; it is also called “defense reaction” (Hjemdahl 2000). When exposed to stressful events, the body’s cardiovascular response is characterized by tachycardia, increase of the blood pressure and cardiac output due to the action or stimulation of glucocorticoids, and catecholamines (Hjemdahl 2000; Habib et al. 2001). Catabolism is enhanced to produce the energy that is channeled to where it is needed to ensure survival. The brain, heart, and muscles are the recipients of the much needed energy, while other nonessential functions such as reproduction and growth may be inhibited until homeostasis is re-established.

Stress triggers in the hypothalamus the release of CRH that stimulates the cells of the anterior pituitary to produce ACTH. AVP secreted by the posterior pituitary will
stimulate the anterior pituitary together with CRH to secrete ACTH as well. AVP alone does not induce the secretion of ACTH (Habib et al. 2001). Many peptides are produced in the anterior pituitary by the cleavage of proopiomelanocortin, a large precursor protein. Among peptides produced are ACTH and β-endorphin. ACTH targets the adrenal cortex, and the release of β-endorphin is directed toward the adrenal medulla. The adrenal cortex subsequently stimulates the release of cortisol in humans or corticosterone in mice, while the adrenal medulla stimulates the release of epinephrine. The presence of high corticosterone levels in the blood is a clear indication that the animal is stressed. The CRH acts on the sympathetic nerves to release norepinephrine.

The sympathetic nerves synchronizes with the adrenal medulla to release catecholamines that in the end result in shunting the blood flow toward the brain, heart, and muscles in readiness for the "fight or flight" response (Habib et al. 2001). The β-endorphin released from the anterior pituitary and the brain may have analgesic effect on the stressed body, regulate hormones, and inhibit reproduction (Lee and Wardlaw 2007).

**Stress Response in the Gastrointestinal Tract (GIT)**

When exposed to stress the body tries through nonspecific responses to maintain homeostasis and ensure its survival (Caso et al. 2008). The hypothalamus stimulates the pituitary gland and the sympathetic nervous system in response to a stressor’s action (Herd 1991). The bidirectional interactions between the brain and the gut through the parasympathetic and sympathetic pathways form the brain-gut axis, a very important regulator of many processes such as gastrointestinal motility, absorption
of nutrients, ions transport, and blood flow (Bhatia and Tandon 2005). A stressful event will trigger a cascade of signals along the brain-gut axis. The brain-gut direct connections are maintained through the vagal and the splanchnic pathways (Yamada et al. 2003). Caso and his team (2008) have emphasized the crucial role of the brain-gut axis in regard to the effects of stress on the gastrointestinal tract. The susceptibility of the gastrointestinal tract to stress has been studied extensively (Habib et al. 2001; Gabry et al. 2002; Tsukada et al. 2002; Gareau et al. 2008). If stress is prolonged, the gastric function among others will be compromised (Söderholm and Perdue 2001).

Bhatia and Tandon (2005) investigated the effect of stress on the gastrointestinal tract. They used the intestinal tissue as study sample and noted that on one hand, in the large intestine stress had stimulated intestinal permeability to large antigenic molecules thus inducing among other things mast cell activation and decrease of mucin production in the colon. On the other hand, in the small intestine stress had induced water and electrolytes absorption (Bhatia and Tandon 2005). Reber et al. (2007) studied the implications and mechanisms of psycho-social stress in mice and noted that there was a localized loss of goblet cells and crypts and antigen infiltration up to the lamina muscularis mucosae.

Because of the importance of the brain-gut axis during stress, gastrointestinal samples were chosen in this study to investigate the effects of chronic stress on mucin expression in mice to mimic the chronic exposure to stress in humans as suggested by Gareau et al. (2008). After studying stress for 40 years, Selye suggested 3 conditions that had characterized a stress response: the enlargement of the adrenal glands, abnormal development of the thymolymphatic system, and gastric ulceration (Selye
Numerous studies have pointed out that stress has adverse effects on the normal function of the body. It has been associated with a host of disease conditions such as hypertension, ulcers, congestive heart failure, and hair loss (Henry et al. 1993; Ruwof and Van der Laarse 2000; Botchkarev 2003; Zou et al. 2004, Hayoz and Mazzolai 2007; NIH September 2008; Kim et al. 2008). CRH has an effect on every cell in the body, thus its upregulation may underlie many disorders such as depression, anxiety, diabetes mellitus, and gastrointestinal dysfunctions (Habib et al. 2001).

There are controversies in regard to the etiology of ulcers in the gastrointestinal tract (Werther 2000). Stress in itself does not cause ulcers; it acts as cofactor in the breaking down of the gastric mucosa (Levenstein 1998). It has been postulated that in most cases ulcers are a consequence of defects in the mucosal barrier due to *Helicobacter pylori*, a spiral-shaped gram negative bacillus, or a prolonged use of aspirin or nonsteroidal anti-inflammatory drugs (Werther 2000). In the upper gastrointestinal tract, hydrochloric acid hypersecretion seems to be the cause instead of the breaking down of the gastric barrier (Werther 2000). Some researchers suggest that *Helicobacter pylori* bacterium causes ulcers (Marshall and Warren 1984), while others point out that *Helicobacter pylori* may not be as important in the pathogenesis of gastric ulcers. The vast majority of people infected with *Helicobacter pylori* are asymptomatic (Sidebotham et al. 1991; Markesich et al. 1995); for the people who show symptoms of gastric ulceration when treated, the cure of the *Helicobacter pylori* infection clears the ulcer disease (Sidebotham et al. 1991; Markesich et al. 1995). *Helicobacter pylori*’s involvement in the development of the disease is not clear. It is possible that *Helicobacter pylori* weaken the host’s defense machinery (Sidebotham et al. 1991;
Markesich et al. 1995). Studies have shown that stress causes dysfunction of the gastrointestinal tract system (Gareau et al. 2008), it could be inferred that there may be a change in the gene expression profile. In case of an infection with *H. pylori* an upregulation of MUC1 was noted in the stomach, yet there was very low expression in the intestine (Andrianifahanana et al. 2006). Tytgat et al. (1993) suggested that when mucin expression or composition is altered, it could lead to a change in the protective function of the mucus layer.

*Mucins in Disease*

It has been established that many types of cancers and inflammatory diseases cause deregulation of mucin expression (Devine and McKenzie 1992; Gendler and Spicer 1995; Hollingsworth and Swanson 2004; Moniaux et al. 2004; An et al. 2007). Buisine et al. (2001) investigated the mucin profile in Crohn’s disease (CD), which is a chronic relapsing inflammatory bowel disease. They analyzed the mucin expression in the ileal mucosa of 11 patients suffering from CD and 8 biopsy samples were added, this raised the total to 19 positive samples and 14 controls. They conducted a histological study, in situ hybridization, and immunohistochemistry to analyze the specimens collected. Their findings indicated that in the intact ileal mucosa the expression of MUC2 and MUC3 was similar to the control profile and MUC1 and MUC4 had a lower expression while MUC5AC, MUC5B, MUC6, and MUC7 were not expressed. The affected ileal mucosa showed an expression of MUC5AC and MUC6, but MUC2 was no longer expressed in those affected portions of the ileum. Normally, MUC5AC and MUC6 are found in the stomach and MUC6 in the duodenum. This team
of researchers suggested that MUC5AC and MUC6 might play a role in both the healing process and protection of the mucosa in bowel inflammatory diseases.

Henke et al. (2004) monitored MU5AC and MUC5B in Cystic Fibrosis (CF) in which a progressive airway obstruction is the major sign. Their study had 12 patients with CF and 11 controls. They found a decrease in MUC5B by 70% and MUC5AC by 93% in CF sputum. They suggested that this apparent decrease could be due to the presence of other components of the CF sputum such as DNA, inflammatory mediators, bacteria, and cell debris to name a few and also an altered secretion of mucins in CF.

In a review by Rose and Voynow (2006) it is suggested that on one hand in acute inflammation some mediators are able to initiate mucin hypersecretion as part of the innate immune defense to protect the airway. The mechanism by which this takes place is not yet well understood. Mucin overproduction, goblet cells, and glandular hyperplasia were initially observed and then reverted within days as homeostasis was restored to the respiratory tract as reported by Folkerts et al. (1998). On the other hand, in chronic cases mucin overproduction, goblet cells, and glandular hyperplasia that evolved to cause obstruction of the airways progressing to Asthma, CF, or Bronchitis. Rose and Voynow also pointed out that there is a susceptibility to allergens and viruses that was observed in patients with chronic airway conditions that was probably due to proliferation of goblet cells and secretory glands. These produced more mucins that resulted in mucus plug formation and airway obstruction thus increasing the morbidity rate. In the same review it was noted that MUC5AC and MUC5B were highly expressed in secretions from asthmatic airways than in normal individuals, while MUC2 was found in traces in asthmatic secretions (Kirkham et al. 2002). Watson et al. (2003) reported
the presence of MUC7 in asthmatic children patients but not in the control group. MUC6, MUC8, and MUC19 might be expressed in asthmatic secretions but no data have been published yet to validate their presence (Rose and Voynow 2006).

Voynow and Rubin (2009) noted that the expression of mucins is upregulated by pathogens, inflammatory mediators, and toxins that aggravate chronic inflammatory conditions: CF, COPD. Chu et al. (2004) used biopsy tissues from asthmatic patients to investigate the expression of mucins that was induced by Transforming Growth Factor β1 (TGF-β1) and TGF β2. They reported in their findings that Transforming Growth Factor β2 (TGF-β2) was able to upregulate mucin expression in cultured cells in control as well as in asthmatic patients and that Interleukin 13 (IL-13) could induce the release of TGF-β2 that will in turn stimulate mucin expression in asthmatic patients.

MUC5AC was investigated in gallstone disease by Finzi et al. (2006). They used specimens obtained from subjects who underwent cholecystectomy. The control group consisted of subjects without gallstone and primary cultures of gallbladder epithelial cells. They noted that the mechanisms that set off mucin overproduction are not known. However, inflammatory processes might be involved based on their findings that indicated that mucus overproduction was linked to neutrophil infiltration and upregulation of tumor necrosis factor-α (TNF-α) and epidermal growth factor-receptor (EGF-R). TNF-α and/ or EGF-R induce an upregulation of MUC5AC, while the expression of MUC1, MUC3, and MUC5B remains the same in the gallbladder.

Swartz et al. (2002) investigated the expression of MUC4 in pancreatic carcinoma. They collected specimens from 40 patients and conducted immunohistochemical analysis. Their findings established that MUC4 was
overexpressed in invasive pancreatic adenocarcinomas. They suggested that MUC4 could be used as a screening tool for invasive pancreatic carcinomas. Along the same line of thought, Moniaux et al. (2004) emphasized the role of MUC1 and MUC4 in pancreatic cancer. They indicated that these 2 mucins are upregulated and that they play a role in tumor progression. Thus these mucins could be used in the diagnosis and prognosis of the disease. Mall (2008) also indicated that mucins could be used in diagnosis of cancers and other inflammatory diseases.

Babu et al. (2006) analyzed the expression of MUC2, MUC5AC, and MUC6 in gastric infected epithelia by *H. pylori* using biopsy specimens. They used antibodies for immunohistochemical analysis after confirming *H pylori* infection. They observed that MUC5AC is present in foveolar cells of the antrum and MUC6 in the gastric glands, while MUC2 is absent in the stomach. This team of researchers suggested that *H pylori* infection may trigger the carcinogenesis cascade and that MUC2 could be used as a marker in intestinal metaplasia and early diagnosis because of its stable presence.

Velcich et al. (2002) investigated the importance of Muc2 in colorectal cancer using Muc2 knockout mice (Muc2<sup>-/-</sup>) and observed the absence of goblet cells in these mice. The mice were sacrificed at 6 months and 1 year. They observed that knockout mice had tumors in the gastrointestinal tract. In the 6-month-olds, tumors were only in the small intestine while in the 1-year-old mice, tumors were present also in the large intestine but none were found in the stomach because Muc2 is not detected in the gastric epithelium. Velcich et al. (2002) demonstrated that goblet cells were important in mucin secretion; their decrease implied a reduction of the product and also noted that Muc2 might play a role in inhibiting colorectal cancer.
Carraway et al. (1999) studied the implication of 2 membrane-bound mucins (MUC1 and MUC4) in breast cancer. They pointed out that MUC1 is upregulated in most breast cancers. Because previous studies had suggested that MUC1 might play a role in cell signaling and adhesion it has the potential to participate in tumor progression. MUC4 is upregulated in the majority of aggressive breast cancers. MUC4 is also a ligand for receptor tyrosine kinase (ErbB2) that is actively involved in breast cancer. MUC4 has a role in ErbB2 regulation (Ramsauer et al. 2006) thus its involvement in breast tumor progression or metastasis (Carraway et al. 1999; Ramsauer et al. 2006; Workman et al. 2009). Chaturvedi et al. (2008) proposed that MUC4 could be used as diagnostic tool as well as in the design of novel cancer therapies.

In view of the different roles, functions, and regulatory pathways of mucins, it has been suggested that they could be used in early detection of cancers and inflammatory diseases.

_Importance of Stress-Related Diseases_

Stress-related diseases have a worldwide distribution. According to NIH statistics 60 to 70 million people in 1996 suffered from different digestive conditions, 14.5 million people suffered from peptic ulcers in 2003; 328,000 people were disabled by the condition (NIH Sept. 2008). Patmore (2006) noted that in the US stress-management companies report revenue of approximately $18 billion a year. It is estimated that approximately 25 million Americans will contract peptic ulcers during their lifetime while NIH suggests that 1 in 10 Americans will suffer from an ulcer during their lifetimes (NIH Sept. 2008). The understanding of the changes of gastrointestinal mucins under
stressful conditions may be helpful in prevention and possibly treatment of gastrointestinal conditions associated with stress.

In view of the importance of mucins in protecting the mucosa and the potential negative effects of stress on the gastrointestinal tract, the study focus is on the following 2 questions:

1. Does chronic stress affect the quality of gastrointestinal mucins?
2. Does chronic stress affect the quantity of gastrointestinal mucin?

_Hypothesis_

Because ulceration affects the integrity of the mucosal barrier of which the mucus layer is important, it is hypothesized that stress commonly associated with gastrointestinal ulceration will alter the overall quantity and quality of mucin production from the gastrointestinal tract.

_Objectives_

The objective of the study is 2-fold: first to determine the expression of membrane-bound mucins (Muc1, Muc3, and Muc4) and secreted mucins (Muc2 and Muc5AC) in stress and nonstress conditions in mice; second to determine histological changes in the intestinal mucosa of mice exposed to stress and nonstress conditions.
CHAPTER 2

MATERIALS AND METHODS

This study focused on delineating the changes in quantity and quality of gastrointestinal mucins under stressful and nonstressful conditions in the stomach and the intestine. A portion of the tissue of interest, approximately 2 cm, was removed and immersed in Methacarn for fixation then the mucosa was immediately separated from the remaining tissue and homogenized in Trizol®.

Experimental Materials

Experimental Animals

Sixteen 4-week-old male weanling ICR Swiss strain mice were used. The mice were housed individually in cages and had a 12-hour light-dark cycle. Water and feed were available ad libitum except when the mice were being tested. The protocol was approved by the University Committee on Animal Care (UCAC) at East Tennessee State University, Johnson City, TN.

Weighing of Animals and Feed

Animal feed given was weighed at the beginning of the experiment. During the 3 weeks that followed, the mice and feed were weighed twice each week. At the end of the 21-day period, data were available to determine the body weight (BW) change during the study period and feed consumed (FC). A total of 7 repeated measures were recorded for body weight and 6 for the feed consumed.
Blood Collection

At the end of the 21-day period the mice were euthanized using carbon monoxide, and blood for the corticosterone assay was collected by retro-orbital puncture. The abdomen was opened and the stomach and the intestine were harvested, then the mucosa was collected from each segment of the GIT.

Induction of Stress

Stress was induced using a standardized stressor (restraint) as described by Bonneau et al. (1993). Stress induction was accomplished by restraining the mice for 6 hours daily for 21 days. This was done to simulate chronic stress that humans go through (Bonneau et al. 1993).

Primers Used in the Study

Specific primers that had been designed by Dr Onyango’s lab for the five mucin genes of interest and one housekeeping gene, Gapdh, were used to amplify the cDNA in the Polymerase Chain Reaction (PCR) endpoint and Real-Time Polymerase Chain Reaction (qPCR).

Experimental Methods

Stress Hormone Assay or Corticosterone Assay

Blood from the mice at the end of the study was collected by retro-orbital puncture and assayed for the stress hormone corticosterone using the Corticosterone
Enzyme Immunoassay kit (Assay design Inc, Ann Arbor, MI, Cat. No 900-097) following the manufacturer’s protocol. The blood was collected in 2 ml eppendorf tubes, left at room temperature for 40 minutes to allow coagulation to take place, then the tube was centrifuged at 1600 x g. The serum was transferred into a new clean tube and put immediately on ice then stored at -80°C until needed for the assay.

The kit which had been previously stored at 4°C was removed from storage and left at room temperature for 30 minutes before use. The tubes for the serial dilution of the 5 standards and the tubes which were going to contain the samples were labeled. The assay buffer 15, which is a Tris buffered saline with proteins and sodium azide as preservative, and the wash buffer were prepared by diluting the concentrates, the assay buffer 1:10, and the wash buffer 1:20 using double distilled water.

The standards were serial diluted by combining for standard 1, 900 µl assay buffer 15 and 100 µl stock solution to get 20 000 pg/ml corticosterone; for standard 2, 800 µl assay buffer 15 and 200 µl standard 1 to get 4 000 pg/ml corticosterone; standard 3, 800 µl assay buffer 15 and 200 µl standard 2 to get 800 pg/ml corticosterone; standard 4, 800 µl assay buffer 15 and 200 µl standard 3 to get 160 pg/ml corticosterone and for standard 5, 800 µl assay buffer 15 and 200 µl standard 4 to get 32 pg/ml corticosterone. Using the Assay layout sheet showing how the 96 well-plate could be setup, the number of wells to be used on the 96 well-plate coated with donkey anti-sheep IgG was determined and the plate was set up.

Nonspecific Binding (NSB) wells received 100 µl of the Assay buffer 15 and Maximum Binding, (B₀) wells then 50 µl in the NSB wells. The NSB wells received a total of 150 µl of the Assay buffer 15. The standard wells received 100 µl of the
standards in duplicate, while the samples wells received samples in quadruplicate. The conjugate (alkaline phosphatase conjugated with Corticosterone) 50 µl was added to all the wells except blank and total activity wells. The substrate (antibody) 50 µl, was added to all the wells except the blank, total activity, and nonspecific binding wells.

The plate was sealed and incubated at room temperature on a shaker at 300 rpm for 2 hours. After the incubation the content of all the wells was dumped, and the plate was washed thrice using the wash buffer (Tris buffered saline with detergents), the remaining buffer was carefully blotted out by tapping on a lint-free absorbent paper towel, (Kimwipes, Kimberly-Clark, Roswell, GA, Cat. No. 34256). The conjugate 5 µl, was added to the TA wells, then 200 µl of the p-nitrophenyl phosphate (pNpp) substrate was added to all the wells. The plate was incubated at room temperature for 1 hour without shaking. At the end of the incubation period 50 µl of the stop solution trisodium phosphate was added to every well. The absorbance was read immediately using the Benchmark Microplate Reader (Bio-Rad, Hercules, CA) at a wavelength of 405 nm with a correction between 570 and 590 nm.
Determination of Mucin Expression Using Real-Time Polymerase Chain Reaction qPCR

The different steps leading to the quantification of gene expression are presented in Figure 2.

Collection GIT mucosa
↓
Isolation total RNA
↓
Check RNA Concentration and Integrity
↓
Reverse transcription RNA into cDNA (Reverse Transcriptase)
↓
Generation of Positive and Negative Controls
↓
Optimization of Standards
↓
Quantitative PCR
↓
Calculation of Relative Expression

Figure 2: Overview of the steps followed to determine the expression of a gene

Collection of the Mucosa

All the mice were euthanized using carbon monoxide, and samples were collected, the mucosa among them. The abdomen was opened and the GIT was removed divided into different portions. The mucosa was separated from the rest of the underlying tissues put in a 2 ml eppendorf tube containing 1ml Trizol (Invitrogen Corp., Carlsbad, CA, Cat. No 15596-026) and homogenized immediately using an electric homogenizer (Omni TIP Homogenizing, Omni International, Marietta, GA). The homogenate was kept on ice then later stored at -80°C until the RNA isolation.
**RNA Isolation**

All the mucosal samples had already been homogenized in Trizol and kept at -80°C. Total RNA was extracted from intestinal and stomach mucosae from mice using the Trizol® kit following the manufacturer’s protocol. The mucosa was allowed to thaw on ice. The samples were incubated at room temperature for 5 minutes to permit a thorough dissociation of nucleoprotein, then 200 µl of chloroform per 1 ml of Trizol were added, capped the tube and mixed by vigorous shaking by hand of the tube for 15 seconds.

The mixture was incubated at room temperature for 3 minutes then centrifuged the tube at 12,000 x g for 15 minutes at 4°C. The mixture separated into 3 phases, a lower red, phenol-chloroform, a white interphase, and a colorless upper aqueous phase containing the RNA which represented approximately 60% of Trizol used to homogenize the tissue. The aqueous phase was carefully transferred to a fresh tube and the rest was discarded.

RNA was precipitated from the aqueous phase using 500 µl of isopropyl alcohol then incubated for 10 minutes at room temperature, followed by centrifugation at 12,000 x g for 10 minutes at 4°C and a gel-like pellet formed on the side and bottom of the tube. The supernatant was decanted into a beaker and the tube was blotted on lint-free absorbent paper towels (Kimwipes, Kimberly-Clark, Roswell, GA, Cat. No 34256).

The RNA was washed using 1000 µl of 75 % ethanol and mixed using a pipette because vortexing did not break the pellet to resuspend it then centrifuged the tube at 7,500 x g for 5 minutes at 4°C. The supernatant was quickly dumped into a beaker and the inverted tube was blotted on lint-free absorbent paper towels. The tube was air-dried
by keeping it upside down for 10 minutes. The RNA isolated was resuspended in RNase-free water then the RNA was incubated at 55°C for 1 minute. The amount of nuclease-free water used to resuspend the RNA was proportional to the pellet size.

**DNase Treatment.** The RNA that had been isolated was subjected to a DNase treatment using the Turbo DNA-free kit (Ambion Inc, Austin, TX, Cat. No 1907) to ensure that no genomic DNA was carried over to subsequent manipulations according to the manufacturer’s protocol. To the duodenal and jejunal RNA samples 5 µl of 10X Turbo DNase Buffer was added while the rest of the sections received 3 µl, then 1 µl Turbo DNase was added to all RNA samples and mixed well. The mixture was incubated at 37°C for 30 minutes in a water bath then either 5 or 3 µl of the resuspended DNase Inactivation Reagent was added depending on the amount of water used to resuspend the RNA and mixed thoroughly. The tube was flicked twice during the 5-minute incubation period at room temperature to redisperse the DNase inactivation reagent. The mixture was centrifuged at 10,000 x g (AccuSpin Micro 17R, Fisher Scientific, Germany, Cat. No 75002463) for 1.5 minutes, then the cleaned RNA was transferred to a fresh tube.

**RNA Concentration and Integrity Check**

The Agilent® 2100 Bioanalyzer (Agilent Technology Inc, Santa Clara, CA) was used to determine the RNA concentration and RNA integrity number (RIN) value for the RNA isolated. The total RNA isolated was resuspended in nuclease-free water, and an aliquot of 2 µl was sent to the Molecular Biology Core Facility (East Tennessee State
University, College of Medicine, Johnson City, TN) for analysis to determine the concentration and the RIN value of the isolated total RNA. The results were sent back in form of a gel image of the chip, an electropherogram for each sample analyzed, and chip summary. The chip could hold a maximum of 12 samples. All the RNA samples analyzed were diluted in nuclease-free water to contain 0.25 µg/µl based on their determined specific concentration.

**Reverse Transcription of RNA**

The isolated total RNA was reverse transcribed using qScript into complementary DNA using the 2-step protocol. The qScript cDNA Synthesis kit (Quanta Biosciences Inc, Gaitherburg, MD, Cat. No 95047-100) was used to reverse transcribe the RNA into cDNA. We mixed 12 µl of total RNA, 33 µl of water, 12 µl of 5X reaction mix, and 3 µl of the Reverse Transcriptase for a total volume of 60 µl per reaction tube. The reaction tube was placed in the thermal cycler (Master cycler gradient, Eppendorf, Hamburg, Germany) programmed to run one cycle at 22°C for 5 minutes, one cycle at 42°C for 30 minutes, and one cycle at 85°C for 5 minutes. Then the tube was held at 10°C, until removed for storage at -20°C.

**Negative Control or Reverse Transcriptase Minus**

Two samples from each section of the GIT representing the 2 treatments (stressed and control) were reverse transcribed with no added enzyme, the reverse transcriptase. A combination of 24 µl nuclease free water, 8 µl 5x reaction mix, and 8 µl RNA in a eppendorf tube was placed in the thermal cycler programmed to run one cycle
at 22°C for five minutes, one cycle at 42°C for 30 minutes, and one cycle at 85°C for 5 minutes just like in the regular reverse transcription described earlier. The cDNA generated without the enzyme was stored at -20°C. This step was taken to rule out any genomic DNA contamination during the actual qPCR run.

*Generation of Positive Control*

*Ligation and Transformation.* A fresh 50 µl PCR product was prepared by combining 21 µl of water, 25 µl of AccuStart PCR Supermix (Quanta Biosciences Inc, Gaitherburg, MD, Cat. No 95060-50), 2 µl of cDNA, 1 µl of forward primer, and 1 µl of reverse primer. The eppendorf tube containing the 50 µl reaction mix was placed in the thermal cycler (Master cycler gradient, Eppendorf, Hamburg, Germany), then the amplified for 40 cycles following a specific program created for each particular gene of interest.

The PCR product was run on agarose gel to check whether only a single discrete band characteristic of the amplicon in question would be present.

Luria-Bertani (LB) plates medium with ampicillin at a concentration of 50 µg/ml of were prepared in advance. The medium for LB plates was prepared by dissolving 5 g of Tryptone, 2.5 g of Yeast Extract, 2.5 g of Sodium Chloride, and 7.5 g of Agar in 500 ml of distilled water in a flask. The flask was autoclaved on liquid cycle for 40 minutes then allowed to cool in a 50°C water bath. When the content of the flask had reached 50°C, an antibiotic, ampicillin, was added just before pouring the plates. After solidification the LB plates were stacked and stored in the refrigerator at 4°C until needed. Four LB plates were placed in the incubator to warm them until needed.
LB broth was prepared using the same recipe as the LB plate without the addition of the Agar, then 5 ml aliquot in 15 ml tubes were made and autoclaved on liquid cycle as the medium for LB plates. For the LB broth ampicillin was added just before use to each tube, taking necessary precautions to avoid its contamination using aseptic techniques.

The cloning reaction was prepared using 2 dilutions 1:1 and 3:1 by mixing 1 µl of PCR product, 1 µl of vector, 1 µl of salt, and 3 µl of nuclease-free water for the 1:1 dilution while the 3:1 had 3 µl of PCR product and 1 µl of nuclease free water, salt, and vector were the same as in previous dilution. The vector used was the pCR®2.1-TOPO® (Invitrogen, Carlsbad, CA, Cat. No 4500-01). After mixing the tubes containing the cloning reaction were incubated at room temperature for 10 minutes.

During this incubation period 2 tubes of competent cells were thawed on ice while 4 LB plates were placed in the incubator at 37°C to warm them. Of the 6 µl of cloning reaction only 2 µl were added to the tube with competent *E. coli* cells to induce their transformation. The tubes with cells were mixed carefully by gently flicking them. The tubes were incubated on ice for 30 minutes.

The cells were subjected to a heat shock for 30 seconds in a water bath set at 42°C then the tubes were placed quickly on ice. To each tube 250 µl of the S.O.C. medium was added. The tubes were placed horizontally in a small open box and were incubated at 37°C for one hour on a C25 incubator shaker (New Brunswick Scientific, Edison, New Jersey) set at 200 rpm.

During this incubation period 30 µl of X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) at a concentration of 50 mg/ml (Promega, Madison, WI, Cat. No
V394A) was added on each warm LB plate. The LB plates were labeled to identify the dilution (1:1 or 3:1) and dosage (10 or 50 µl). For the 10 µl, 30 µl of S.O.C. medium was added to facilitate the spreading of 10 µl of cells, the 50 µl were spread directly without addition of S.O.C. medium. The 4 LB plates were incubated overnight for 16 hours at 37°C. The X-Gal was added to the LB plates to allow a quick screening of white and blue colonies.

After the incubation period the plates were kept at 4°C for 4 to 5 hours to reinforce the distinction between white and blue colonies. Seven LB broth tubes and one LB plate were labeled and the LB plate was placed in the incubator to warm it before adding X-Gal. To each tube containing 5 ml of LB broth, 2.5 µl of ampicillin at a concentration of 100 mg/ml was added. Six white colonies were picked to inoculate each one of the first 6 tubes and did a plate streak to keep track of the colonies used on the designated plot, the last tube was inoculated with a blue colony to serve as control to check whether the X-Gal worked well. The inoculated tubes were incubated at 37°C for 16 hours with shaking at 200 rpm using the C25 Incubator shaker (New Brunswick Scientific, Edison, New Jersey). The overnight culture was then purified using the PureLink Quick Plasmid Miniprep kit (Invitrogen, Löhne, Germany, Cat. No K 2100-10).

**Purification of Plasmids.** After a 16-hour incubation period, 2 aliquots of 2 ml of the overnight culture were made from each colony; the cells were then centrifuged at 4000 x g for 8 minutes. From the overnight culture, 400 µl of the fresh culture were mixed to an equal amount of 30% glycerol and stored at -80°C for future propagation of the plasmid.
Purification of the plasmid was accomplished using the PureLink Quick Plasmid Miniprep kit (Invitrogen, Löhne, Germany, Cat. No K 2100-10) by following the manufacturer’s procedure as outlined here. The supernatant was carefully and completely removed by pipetting. To the pellet 250 µl of RNase A buffer (R3) were added and mixed well, then 250 µl of Lysis buffer (L7) were added to the tube with cells, the tube was capped and mixed by inverting it 5 times then incubated the mixture at room temperature for 5 minutes. To the tube 350 µl of Precipitation buffer (N4) were added and homogenized quickly by inverting the tube then centrifuged at 12,000 x g for 10 minutes at room temperature to pellet the debris.

The supernatant was transferred to a labeled spin column that was placed on a 2 ml wash tube and centrifuged at 12,000 x g for 1 minute. The flow through was discarded in a beaker and placed the column back onto the wash tube. To the wash tube with the spin column 500 µl of Wash buffer (W10) were added and incubated for 1 minute at room temperature followed by a centrifugation at 12,000 x g for 1 minute. Once again the flow was discarded and 700 µl Wash buffer (W9) were added to the column and centrifuged at 12,000 x g for 1 minute and discarded the flow through. The wash tube was centrifuged again for 1 minute at 12,000 x g to remove any leftover Wash buffer (W9) then discarded the wash tube and the flow through.

The plasmid DNA was eluted using 10 mM Tris-HCl buffer to avoid any enzymatic interference in downstream manipulations of the isolated plasmid DNA (pDNA). The 10mM Tris buffer was prepared by dissolving 3.02 g of Tris in 400 ml of distilled water. The buffer was brought to pH 8 using hydrochloric acid (HCl).
Seventy-five µl of Tri-HCl buffer were directly deposited at the center of the column that was placed on a collection tube clearly labeled with the clone (colony) number and gene identification. The collection tube with column was incubated for 1 minute at room temperature then centrifuged at 12,000 x g for 2 minutes. After the incubation, the column was discarded and the isolated plasmid DNA (pDNA) was stored at -20°C.

The concentration of the pDNA was determined using a spectrophotometer, the Eppendorf BioPhotometer (Eppendorf AG, Hamburg, Germany). An aliquot of the pDNA, at a concentration of 100 µg/µl was sent to the Molecular Biology core facility (East Tennessee State University, College of Medicine, Johnson City, TN) for sequencing, to check whether the plasmid had taken in the intended insert. For sequencing vector specific primers were used, specifically M13 primers. The vector used in this study was the pCR® 2.1-TOPO® 3.9 kb.

*Optimization of Standards.* Labeled RNase-free tubes were placed in the clone zone box (USA Scientific Inc., Ocala, FL) under Ultra Violet light for 20 minutes. The 12.5 ml vial of 2x qPCR Supermix (Invitrogen, Carlsbad, CA, Cat No11730-025) thawed on ice then 2.5 µl of 20 nM Fluoroscein, a calibration dye used to spike the qPCR Supermix was added and aliquots were made to avoid constant freezing and thawing. In a 1.5 ml eppendorf tube, 100 µl 10x Sybr Green was prepared by diluting the stock solution that was 100x in nuclease-free water. In another 1.5 ml tube the set of primers to be used in the amplification process were diluted in nuclease-free water to get the mixed primer set at a concentration of 3 µM and both tubes (Sybr Green and Primer mix) were kept on ice.
Using the Eppendorf BioPhotometer (Eppendorf AG, Hamburg, Germany), the plasmid DNA (pDNA) concentration of the specific clone was determined. The pDNA concentration and the plasmid size with insert size were used to calculate the copy number, one on the parameters used to set a gradient qPCR. Different parameters, the pDNA stock copy number, and temperature range (4 different temperatures) to be used in the gradient amplification were programmed on the computer in order to have the protocol and plate setup.

In the cloning zone 10-fold serial dilutions of the pDNA were made by mixing 3 µl stock and 27 µl water for the pDNA of $10^{10}$ or $10^9$ depending on the copy number obtained then $10^8$, $10^7$, down to $10^2$. Enough mixtures for 54 wells was made; therefore, in a 1.5 ml tube the reaction mixture was made by combining 118.8 µl of water, 81 µl of Primer mix, 64.8 µl of 10x of Sybr Green, 32.4 µl of Magnesium chloride, and 405 µl of 2x qPCR Supermix with Fluoroscein. In 6 tubes that had the standards 18 µl of water was placed in the blank tube also called no template control (NTC), and 18 µl of $10^2$ in the second tube, $10^3$ in the third tube, $10^4$ in the fourth, $10^5$ in the fifth, and $10^6$ in the last tube of the pDNA dilutions then 117 µl of reaction mixture was added to each of the 6 tubes.

The wells of the 96 well-plate were loaded in duplicate with 15 µl of final reaction mix. Each one of the 6 reaction mixtures (dilutions) had 8 wells corresponding to the 4 different temperatures chosen for the gradient run. The plate was sealed and centrifuged at 1600 x g (Eppendorf 5840, Eppendorf, Hamburg, Germany) for 1 minute as many times as needed to get rid of air bubbles. The sealed plate was then placed in the thermal cycler (IQ5 Multicolor Real-Time PCR Detection System, Bio-Rad,
Hercules, CA) that was linked to a computer and the parameters were once again checked on the computer and the qPCR was set to run for approximately 2 hours and 15 minutes. This gradient run was executed for all the 6 genes used in the study using 4 different temperatures.

The purpose of running a gradient amplification was to determine the optimum temperature at which the gene in question was best expressed and construction of a standard curve by paying attention to the efficiency level, the slope of the curve generated, and presence of a single peak.

**Quantitative PCR Setup and Run**

Fragments corresponding to the mucins of interest (Muc1, Muc2, Muc3, Muc4, and Muc5AC) were amplified and quantified by Real-Time PCR to determine the expression of the 5 mucins. Gapdh, a reference gene, was also amplified and served as the internal control. After determining the melting temperature for each gene in this study, each portion of the gastrointestinal tract was run on a separate 96 well-plate to facilitate a comparison between the treated and the control groups.

Labeled eppendorf tubes were placed under UV light for 20 minutes. Serial dilutions of the pDNA were made after checking the concentration to determine the copy number. In a 1.5 ml eppendorf tube 2.2 µl of nuclease-free water, 1.5 µl primer mix, 1.2 µl of Sybr Green, 0.6 µl of MgCl\(_2\), 7.5 µl of 2x qPCR Supermix for each well to be used plus 12 extra wells were mixed when using the robot epMotion 5070 (Eppendorf, Hamburg, Germany) to dispense the mixtures. The qPCR reaction mix made was aliquoted in 8 tubes, the standards and the RT minus were placed in front rack and the
actual samples in the rear rack in the robot chamber. The robot had already been programmed to effectively dispense the reaction mixes into the 96-well plate the only variation would be the column 10 to 12 on the plate that are adapted to the number of samples placed beyond the 9th column. The samples were loaded in triplicate on the 96-well plate.

On the computer that is linked to the thermal cycler, the protocol and plate setup were set then the wells were labeled. After the robot had finished the task, the plate was taken out, sealed, and mixed by centrifugation at 1600 x g for 1 minute in the 5804 Eppendorf centrifuge (Eppendorf, Hamburg, Germany) to bring everything to the bottom of the wells and remove any air bubbles. The plate was finally placed in the thermal cycler after checking once again that the protocol and the plate setup were correct. The machine was set to run for approximately 2 hours and 15 minutes.

Histology

Collection of Tissue Samples

The harvested samples of gastrointestinal tract (2 cm per section) were fixed in Methacarn (60% methanol, 30% chloroform, 10% acetic acid) as described by Putchtler et al. (1970) for 24 hours. The fixed tissues were subsequently transferred to 70% Ethanol for holding until further processing.
**Preparation of Histological Sections**

The embedding process was done manually. A small section of the tissue, approximately 3 mm, was put into a metallic cassette with a label for identification. The tissue in the cassette was progressively dehydrated, it was immersed in 70% Ethanol for 30 minutes, then the tissue was transferred to 80% Ethanol for 30 minutes, then to 90% for 30 minutes, then to 95% for 30 minutes, then transferred to 100% for 30 minutes twice.

At this point the clearing agent (Hemo-De, Fisher Scientific, Fair Lawn, NJ, Cat No 22-143975) was progressively added to the tissue. The cassette containing the tissue was transferred to 50:50 100% Ethanol:Hemo-De for 30 minutes, then 100% Hemo-De for 30 minutes. Finally liquid Paraffin wax (TissuePrep, Fisher Scientific, Fair Lawn, NJ, Cat NoT565) was progressively added to coat the tissue in the oven at 50°C. The cassette was immersed in 50:50 Hemo-De:liquid Paraffin for 30 minutes, then transferred into pure liquid paraffin for 60 minutes, and lastly transferred into another liquid paraffin for 60 minutes. The tissue was then ready to be embedded in fresh Paraffin.

A metallic mold that had been coated with mold release solution (1:80 glycerin:ethanol 80%) was placed on a warmer at 55°C. A little bit of liquid paraffin was poured to cover the base of the metallic mold then was quickly placed on ice to start solidification of the liquid paraffin that served as glue to hold the tissue then the mold was filled with paraffin after the plastic mold had been put on top of the metallic mold. The block was allowed to solidify completely and the metallic mold was finally removed from the embedded sample.
Alcian Blue-Periodic Acid Schiff (AB-PAS) Staining

The embedded samples were sectioned, 5 µm slices mounted on slides that were AB-PAS stained to study the morphological changes of the tissues collected by quantifying intestinal mucus-secreting goblet cells and measuring the villi length.

Slides were placed on a metallic holder that was immersed into the staining container with Hemo-De for 5 minutes, then the slides were transferred into a second container of Hemo-De for 3 minutes. The slides were submerged in 100% ethanol for 2 minutes twice successively, then in 95, 80, and 70% for 2 minutes in each solution before being rinsed 3 times in double distilled water for 1 minute each time. This process was progressively rehydrating the tissue on the slide in preparation for the staining process.

The slides were then transferred into the Alcian blue solution for 30 minutes followed by 3 rinses in tap water for 2 minutes each. The slides were placed in Periodic acid solution for 10 minutes followed by 3 rinses in tap water for 1 minute each. The slides were transferred into a staining container of Schiff reagent for 10 minutes followed by 3 rinses in lukewarm water; the first 2 rinses were for 3 minutes and the last one was for 4 minutes.

The slides were progressively dehydrated for 2 minutes in 70, 80, and 95% then twice in 100% before going into Hemo-De for 3 minutes and finally in the last staining container of Hemo-De for 5 minutes. A drop of the Permunt® (Fisher Scientific, Fair Lawn, NJ. Cat. No SP15-100) was added and a cover slip was placed on each slide immediately. The mounted slides were allowed to air dry before being stored in a slide box.
The slides were examined, goblet cells as shown in Figure 3 were counted and villus length measured as shown in Figure 4. Using a micrometer ocular the slide was scanned at low magnification to look at the entire structure. Then the villi were measured at 10x while the goblet cells were counted at 40x. The measurement obtained was divided by 10 to convert it into millimeters.

Figure 3: Intestinal epithelium stained with AB-PAS to visualize Goblet cells
Figure 4: Intestinal epithelium stained with AB-PAS, arrow shows a villus length

Statistical Analysis

Corticosterone Levels

The data from the corticosterone assay were analyzed by running a one way ANOVA using PASW (Predictive Analytics SoftWare 17, SPSS Inc., Chicago, IL) to assess any differences between the treated and control mice at $P<0.05$. 
Body Weight Gain and Feed Consumed

The body weight change and feed consumed data were analyzed as repeated measures using PASW (Predictive Analytics SoftWare 17, SPSS Inc., Chicago, IL). This analysis was done to investigate whether there were significant differences within groups at the different times when the data were collected and also between treated and control groups at a P-value smaller than 0.05 (P<0.05). The individual mouse was the experimental unit.

Histological Data

Quantitative histological data of the goblet cell count and villus length were analyzed by one way ANOVA using PASW (Predictive Analytics SoftWare 17, SPSS Inc., Chicago, IL) to determine whether there was a significant difference between the treated and control mice.

qPCR Data

The iQ5 software (Bio-Rad) was used to calculate the expression and relative quantification of each gene in the present study. Data were subsequently analyzed using SAS (SAS Institute Inc. 2002, Cary, NC).
CHAPTER 3

RESULTS

Corticosterone Levels in the Sera of Stressed and Control Mice

Blood collected by retro-orbital puncture from 16 ICR mice that had been subjected to restraint-stress for 3 weeks was assayed for corticosterone in the serum. The results presented in Table 1 and Figure 3 indicated high levels of the stress hormone in the treated group. Two mice from the treated group had extremely high levels of corticosterone, the rest ranged from 3274 to 7293 pg/ml of serum for the treated mice, while in the control mice ranged from 44 to 2971 pg/ml. Without the outliers, the means ± SD were 5082 ± 1975 pg/ml for the stressed and 1565 ± 1236 pg/ml for the control mice (Table 1; Figure 5). There were highly significant differences between the stressed and control mice at a P-value of 0.001 (Table 1).

Table 1: Corticosterone levels in stressed and control mice after a 6-hour restraint period daily for 21 days

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment</th>
<th>Mean</th>
<th>SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticosterone&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Stress</td>
<td>5282</td>
<td>1975</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>1565</td>
<td>1236</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> significant difference
Figure 5: Corticosterone levels in the sera of stressed and control mice at the end of the study period

*Body Weight (BW) and Feed consumed (FC) of Stressed and Control mice*

The weight of the stressed mice at the beginning of the experiment ranged from 31.2 to 34.2 g for the stressed group with an average of 31.8 g, while the control mice weighed from 28.9 to 36.8 g with an average of 33.7 g (Table 2). After the initial exposure to stress, the stressed mice weighed on average 30.9 g. After the study period of 3 weeks, the final average weight for the stressed mice was 32.8 g and 35.7 g for the control mice. As expected the control mice increased their weight consistently throughout the 3 weeks. There was an increase in weight in both groups even though the control group weighed more than the stressed mice (Table 2; Figure 6).
The total feed consumed by the stressed mice during 21-day period ranged from 105.1 to 125.9 g with a final average of 115.2 g, while the control mice consumed feed ranging from 93.1 to 121.2 g showing a final average of 110.2 g (Table 3). It was noted that the stressed mice ate slightly more than the control mice even though the latter gained more weight that the former group. The increase in feed consumption was not linear. The statistical analysis of the feed consumed indicated no significant difference was noted between the stressed and control mice at P-value smaller than 0.05, but there were differences between measurements within treatment (Table 3; Figure 7).

Table 2: Body Weight (g) of Stressed and Control Mice taken twice weekly for 21 days

<table>
<thead>
<tr>
<th>Day of Study Period</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stressed</td>
</tr>
<tr>
<td>0</td>
<td>32.6</td>
</tr>
<tr>
<td>4</td>
<td>30.9</td>
</tr>
<tr>
<td>7</td>
<td>31.5</td>
</tr>
<tr>
<td>11</td>
<td>31.5</td>
</tr>
<tr>
<td>14</td>
<td>30.6</td>
</tr>
<tr>
<td>17</td>
<td>32.8</td>
</tr>
<tr>
<td>21</td>
<td>32.8</td>
</tr>
</tbody>
</table>
Figure 6: Body Weight (g) change of stressed and control mice during the 21-day study period. $P$-value: within treatment: 0.000 between treatments: 0.053

Table 3: Cumulative Feed Consumed by stressed and control mice for the 3-week study period

<table>
<thead>
<tr>
<th>Day of Study Period</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stressed</td>
</tr>
<tr>
<td>D4</td>
<td>20.1</td>
</tr>
<tr>
<td>D7</td>
<td>38.2</td>
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<tr>
<td>D11</td>
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<td>D14</td>
<td>78.3</td>
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<tr>
<td>D17</td>
<td>100.0</td>
</tr>
<tr>
<td>D21</td>
<td>115.2</td>
</tr>
</tbody>
</table>
Figure 7: Cumulative Feed Consumption by stressed and control mice observed for the study period of 3 weeks

**Goblet Cells Count in the Duodenum, Jejunum, and Ileum of Stressed and Control Mice**

The Goblet cells count in the control group was higher than in the stressed group. They ranged from 5 to 16 cells in the duodenum with an average of 8 for the stressed and 14 for the control mice and a standard deviation of 2. In the jejunum the mean was 7 for the stressed and 14 for the control with a standard deviation of 1 in both groups, while in the ileum they ranged from 7 to 14 goblet cells with an average of 9 for the stressed mice and 11 for the control (Table 4; Figure 8). The goblet cells counts were highly significant different between the stressed and control mice for all the 3 sections that were examined at $P < 0.001$ (Table 4).
Table 4: Goblet Cells count (GC) in the duodenum, jejunum, and ileum from stressed and control mice

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment</th>
<th>Mean</th>
<th>SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC duodenum</td>
<td>Stress</td>
<td>8</td>
<td>2</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>14</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>GC jejunum</td>
<td>Stress</td>
<td>7</td>
<td>1</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>14</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>GC ileum</td>
<td>Stress</td>
<td>9</td>
<td>1</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>11</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) significant difference between treatments (stress and nostress)
Figure 8: Goblet cells average count in the duodenum, jejunum, and ileum of stressed and control mice

*Villus Length in the Duodenum, Jejunum, and Ileum of Stressed and Control Mice*

The villus length average measurements ranged from 0.320 mm for the stressed mice to 0.430 mm for the control group with a standard deviation of 0.082 and 0.073 respectively in the duodenum; in the jejunum, they ranged from 0.260 mm for the stressed mice to 0.360 mm for the control group with a standard deviation of 0.073 and 0.035 respectively, while in the ileum they ranged from 230 mm for the stressed group to 250 mm for the control mice with a standard deviation of 0.047 and 0.035 (Table 5; Figure 9). Statistically, there were significant differences in villus length between the 2
groups only in the duodenum and jejunum. The ileum did not show significant differences between the stressed and control mice (Table 5).

Table 5: Villus Length (VL) in the duodenum, jejunum, and ileum from stressed and control mice

<table>
<thead>
<tr>
<th>Variables</th>
<th>Treatment</th>
<th>Mean</th>
<th>SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VL duodenum</td>
<td>Stress</td>
<td>0.32</td>
<td>0.08</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.43</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>VL jejenum</td>
<td>Stress</td>
<td>0.26</td>
<td>0.07</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.58</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>VL ileum</td>
<td>Stress</td>
<td>0.23</td>
<td>0.02</td>
<td>0.446</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.25</td>
<td>0.01</td>
<td></td>
</tr>
</tbody>
</table>

\(\text{a}\) significant difference between treatments (stress and nostress)
Figure 9: Villus length (mm) measured in the duodenum, jejunum, and ileum of stressed and control mice

**RNA Concentration and Integrity Check using the Agilent**

The RNA isolated had good yield and was of good quality. The concentration that ranged from 58.7 to 1119.2 ng/µl for the stressed mice while the control mice had 19.8 to 2874 ng/µl as their RNA concentration. From a total of 80 samples, 10 showed some degree of degradation. The RIN values ranged from 9.6 to 3 for the stressed mice while the control mice ranged from 3.2 to 9.8. Three samples had no RIN value determined by the software used (Table 6).

The gel image showed some degradation of the duodenum treated 7 (DT7) RNA with a RIN value of 5.1 while the rest of the sample had higher RIN value.
The 28S/18S ratio was more or less 2:1 in most RNA samples loaded on this chip except Duodenum Treated 7 (7 DT) sample (Figure 10).

Table 6: Determination of RNA Integrity Number (RIN) using the Agilent

<table>
<thead>
<tr>
<th>Section</th>
<th>Treatment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>treated</td>
<td>8.4</td>
<td>8.6</td>
<td>8.2</td>
<td>9</td>
<td>8.4</td>
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<td>8.9</td>
<td>9.6</td>
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<tr>
<td></td>
<td>control</td>
<td>8.4</td>
<td>8.2</td>
<td>8.3</td>
<td>8.4</td>
<td>8.5</td>
<td>8.9</td>
<td>8.1</td>
<td>7.5</td>
</tr>
<tr>
<td>Duodenum</td>
<td>Treated</td>
<td>8.0</td>
<td>6.7</td>
<td>7.3</td>
<td>-</td>
<td>5.7</td>
<td>4.9</td>
<td>5.1</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>7.1</td>
<td>5.6</td>
<td>6.1</td>
<td>-</td>
<td>5.6</td>
<td>9.4</td>
<td>7.0</td>
<td>8.5</td>
</tr>
<tr>
<td>Jejunum</td>
<td>treated</td>
<td>8.6</td>
<td>9.3</td>
<td>9.2</td>
<td>7.6</td>
<td>9.6</td>
<td>9.4</td>
<td>7.4</td>
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<tr>
<td></td>
<td>control</td>
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<td>7.2</td>
<td>5.4</td>
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<td>8.2</td>
<td>7.5</td>
<td>8.4</td>
<td>8.7</td>
</tr>
<tr>
<td>Ileum</td>
<td>treated</td>
<td>9.6</td>
<td>-</td>
<td>9.5</td>
<td>8.5</td>
<td>9.1</td>
<td>9.3</td>
<td>9.7</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>9.8</td>
<td>9.1</td>
<td>8.9</td>
<td>9.3</td>
<td>9.2</td>
<td>9.5</td>
<td>8.7</td>
<td>8.7</td>
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<tr>
<td>Colon</td>
<td>treated</td>
<td>9.5</td>
<td>3.0</td>
<td>7.4</td>
<td>9.3</td>
<td>9.4</td>
<td>9.3</td>
<td>9.0</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>9.6</td>
<td>3.2</td>
<td>8.7</td>
<td>9.2</td>
<td>8.3</td>
<td>8.9</td>
<td>7.7</td>
<td>3.7</td>
</tr>
</tbody>
</table>
Figure 10: Gel showing the RNA integrity captured using the Agilent. The 28S and 18S bands are within the ratio of 2:1

Expression Sites of Muc1, Muc2, Muc3, Muc4, and Muc5ac in the GIT

Before qPCR the primers that had been designed were tested to verify whether a single product would be amplified. The results of the endpoint PCR showed a single amplicon specific to each one of the target genes. Next, a mucin distribution in the GIT was assessed using endpoint PCR in order to have a general idea of the expression sites of the different mucins used in the present study as presented in the following gel images. Because the target mucins have small amplicons (below 100 bp) except Muc3
that has 125 bp, 2 different ladders were used. The first ladder on the left of the gel image started at 25 bp, while the second ladder on the right of the gel started at 100 bp.

Muc1 was expressed predominantly in the stomach of both stressed and control mice and in the colon of stressed and a little less in the control mice. Muc2 and Muc3 were found in the intestinal tract, but Muc2 was not expressed in the stomach of both groups of animals. Muc4 was predominantly expressed in the colon but found in all the intestinal tract. Muc5ac was expressed only in the stomach of stressed and control mice.

Figure 1: Expression sites of Muc1 in the GIT of mice. The arrow shows the Muc1 amplicon of 93 bp.

Figure 12: Expression sites of Muc2 in the GIT of mice. The arrow shows the Muc2 amplicon of 62 bp.
Figure 13: Expression sites of Muc3 in the GIT of mice. The arrow shows the Muc3 amplicon of 125 bp.

Figure 14: Expression sites of Muc4 in the GIT of mice. The arrow shows the Muc4 amplicon of 71 bp.

Figure 15: Expression sites of Muc5ac in the gastrointestinal tract of mice. The arrow shows the Muc5ac amplicon of 68 bp.
**Assay Optimization and Validation**

The annealing temperatures were optimized for every target gene and reference gene in the study. The temperatures ranged from 58.5 to 61.5°C. A serial dilution of the specific plasmid for each gene was run to determine the optimal temperature that gave the highest efficiency and the best slope as summarized in Table 7.

Table 7: Optimization of annealing temperatures of all the genes investigated in the study

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Annealing Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muc1</td>
<td>60.5</td>
</tr>
<tr>
<td>Muc2</td>
<td>60.5</td>
</tr>
<tr>
<td>Muc3</td>
<td>61.2</td>
</tr>
<tr>
<td>Muc4</td>
<td>59.0</td>
</tr>
<tr>
<td>Muc5ac</td>
<td>59.2</td>
</tr>
<tr>
<td>Gapdh</td>
<td>61.5</td>
</tr>
</tbody>
</table>
**Mucin Expression in the GIT of Stressed and Control Mice**

*Muc1 Expression*

Muc1 in the stomach had an expression (mean ± SD) of 0.3949 ± 0.2323 for the stressed mice and 0.2517 ± 0.2323 for the control mice (Table 8). In the duodenum Muc1 had an expression of 0.0018 ± 0.0049 for the stressed mice and 0.0026 ± 0.0049 for the control mice. In the jejunum Muc1 had an expression of almost 0.0010 ± 0.0001 for both groups of mice. In the ileum Muc1 had an expression of 0.1370 ± 0.2641 for the stressed mice and 0.1453 ± 0.2641 for the control mice. In the colon Muc1 had an expression of 0.0465 ± 0.0151 for the stressed mice and 0.0302 ± 0.0151 for the control mice. Muc1 expression was the lowest in the jejunum while it was the highest in the stomach as expected. The expression of Muc1 was significantly different between the 2 groups in the colon at P<0.05 (Table 8). The other intestinal sections did not exhibit any significant differences between the treatments (Figure 16). There was an increase in expression of Muc1 in the colon of stressed than in control animals.
Table 8: Muc1 expression in the GIT of stressed and control mice

<table>
<thead>
<tr>
<th>GIT Section</th>
<th>Variable</th>
<th>Nostress</th>
<th>Stress</th>
<th>Pooled SD</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Stomach</td>
<td>Expression</td>
<td>0.2517</td>
<td>0.3949</td>
<td>0.2323</td>
<td>0.2379</td>
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<td>Duodenum</td>
<td>Expression</td>
<td>0.0026</td>
<td>0.0018</td>
<td>0.0049</td>
<td>0.7393</td>
</tr>
<tr>
<td>Jejunum</td>
<td>Expression</td>
<td>0.0009</td>
<td>0.0010</td>
<td>0.0001</td>
<td>0.6667</td>
</tr>
<tr>
<td>Ileum</td>
<td>Expression</td>
<td>0.1453</td>
<td>0.1370</td>
<td>0.2641</td>
<td>0.9506</td>
</tr>
<tr>
<td>Colon</td>
<td>Expression(^a)</td>
<td>0.0302</td>
<td>0.0465</td>
<td>0.0151</td>
<td>0.0484</td>
</tr>
</tbody>
</table>

\(^a\) significant difference

Figure 16: Overexpression of Muc1 in the colon of stressed mice
**Muc2 Expression**

Muc2 in the stomach had an expression (mean ± SD) of 0.0005 ± 0.0022 for the stressed mice and 0.0017 ± 0.0022 for the control mice (Table 9). In the duodenum Muc2 had an expression of 0.0989 ± 0.0875 for the stressed mice and 0.0875 ± 0.0875 for the control mice. In the jejunum Muc2 had an expression of 0.5892 ± 0.4501 for the stressed mice while the control mice had 0.6959 ± 0.4501. In the ileum Muc2 had an expression of 0.5228 ± 0.3053 for the stressed mice and 0.6002 ± 0.3053. In the colon Muc2 had an expression of 1.9396 ± 1.0929 for the stressed mice and 1.4347 ± 1.0929. Muc2 was expressed consistently in the intestinal tract with the highest expression found in the colon followed by the jejunum, ileum, and duodenum. As expected, the stomach had the lowest expression of Muc2. There were no significant differences between the stressed and the control mice in all the gastrointestinal sections assessed. The expression in the colon had the largest pooled standard deviation (Table 9, Figure 17).

**Table 9: Muc2 expression in the GIT of stressed and control mice**

<table>
<thead>
<tr>
<th>GIT Section</th>
<th>Variable</th>
<th>Nostress</th>
<th>Stress</th>
<th>Pooled SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>Expression</td>
<td>0.0017</td>
<td>0.0005</td>
<td>0.00218</td>
<td>0.327</td>
</tr>
<tr>
<td>Duodenum</td>
<td>Expression</td>
<td>0.0875</td>
<td>0.0989</td>
<td>0.08750</td>
<td>0.786</td>
</tr>
<tr>
<td>Jejunum</td>
<td>Expression</td>
<td>0.6959</td>
<td>0.5892</td>
<td>0.45014</td>
<td>0.643</td>
</tr>
<tr>
<td>Ileum</td>
<td>Expression</td>
<td>0.6002</td>
<td>0.5228</td>
<td>0.30534</td>
<td>0.620</td>
</tr>
<tr>
<td>Colon</td>
<td>Expression</td>
<td>1.4337</td>
<td>1.9396</td>
<td>1.09285</td>
<td>0.370</td>
</tr>
</tbody>
</table>
Muc2 expression showing no difference between the stressed and control mice

**Muc3 Expression**

Muc3 in the stomach had an expression (mean ± SD) of 1.2239 ± 0.6488 for the stressed mice and 1.5649 ± 0.6488 for the control mice (Table 10). In the duodenum Muc3 had an expression of 0.5469 ± 0.2491 for the stressed mice and 0.2198 ± 0.2491 for the control mice. In the jejunum Muc3 had an expression of 3.5001 ± 2.2744 for the stressed mice while the control mice had 3.0356 ± 2.2744. In the ileum Muc3 had an expression of 1.1081 ± 1.0781 for the stressed mice and 1.0769 ± 1.1081 for the control mice. In the colon Muc3 had an expression of 1.2239 ± 0.6488 for the stressed mice and 1.5649 ± 0.6488 for the control mice. Muc3 was expressed in the whole gastrointestinal tract. The jejunum had the highest expression with a quite elevated
pooled standard deviation. The duodenum exhibited significant differences in Muc3 expression between the 2 treatments at $P=0.03$. The stressed mice showed a 2-fold expression increase in comparison to the control mice (Table 10, Figure 18).

Table 10: Muc3 expression in the GIT of stressed and control mice

<table>
<thead>
<tr>
<th>GIT Section</th>
<th>Variable</th>
<th>Nostress</th>
<th>Stress</th>
<th>Pooled SD</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>Expression</td>
<td>1.5649</td>
<td>1.2239</td>
<td>0.6488</td>
<td>0.328</td>
</tr>
<tr>
<td>Duodenum</td>
<td>Expression$^a$</td>
<td>0.2198</td>
<td>0.5469</td>
<td>0.2491</td>
<td>0.032</td>
</tr>
<tr>
<td>Jejunum</td>
<td>Expression</td>
<td>3.0356</td>
<td>3.5001</td>
<td>2.2744</td>
<td>0.700</td>
</tr>
<tr>
<td>Ileum</td>
<td>Expression</td>
<td>1.0767</td>
<td>1.1081</td>
<td>1.0781</td>
<td>0.954</td>
</tr>
<tr>
<td>Colon</td>
<td>Expression</td>
<td>1.5649</td>
<td>1.2239</td>
<td>0.6488</td>
<td>0.328</td>
</tr>
</tbody>
</table>

$^a$ significant difference

Figure 18: Overexpression of Muc3 in the duodenum of stressed mice
**Muc4 Expression**

Muc4 in the stomach had an expression (mean ± SD) of 0.0245 ± 0.0132 for the stressed mice and 0.0325 ± 0.0132 for the control mice. In the duodenum Muc4 had an expression of 0.0072 ± 0.0079 for the stressed mice and 0.0090 ± 0.0079 for the control mice. In the jejunum Muc4 had an expression of 0.1460 ± 0.1478 for the stressed mice while the control mice had 0.1854 ± 0.1478. In the ileum Muc4 had an expression of 0.0544 ± 0.0316 for the stressed mice and 0.0421 ± 0.0316 for the control mice. In the colon Muc4 had an expression of 1.0570 ± 0.4020 for the stressed mice and 0.4837 ± 0.4020 for the control mice. Muc4 expression in the colon showed highly significant differences between the 2 groups at $P=0.013$ (Table 1). Muc4 showed a more than 2-fold expression increase in the stressed mice than the control group (Figure 19).

Table 1: Muc4 expression in the GIT of stressed and control mice

<table>
<thead>
<tr>
<th>GIT Section</th>
<th>Variable</th>
<th>Nostress</th>
<th>Stress</th>
<th>Pooled SD</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>Expression</td>
<td>0.0325</td>
<td>0.0245</td>
<td>0.0132</td>
<td>0.245</td>
</tr>
<tr>
<td>Duodenum</td>
<td>Expression</td>
<td>0.0090</td>
<td>0.0072</td>
<td>0.0079</td>
<td>0.650</td>
</tr>
<tr>
<td>Jejunum</td>
<td>Expression</td>
<td>0.1854</td>
<td>0.1460</td>
<td>0.1478</td>
<td>0.603</td>
</tr>
<tr>
<td>Ileum</td>
<td>Expression</td>
<td>0.0421</td>
<td>0.0544</td>
<td>0.0316</td>
<td>0.450</td>
</tr>
<tr>
<td>Colon</td>
<td>Expression(^a)</td>
<td>0.4837</td>
<td>1.0570</td>
<td>0.4020</td>
<td>0.013</td>
</tr>
</tbody>
</table>

\(^a\) significant difference
Muc5ac Expression

Muc5ac in the stomach had an expression of $0.0107 \pm 0.0065$ for the stressed mice and $0.0106 \pm 0.0065$ for the control mice. In the duodenum Muc5ac had an expression of $3E^{-5} \pm 4E^{-5}$ for the stressed mice and $1E \pm 4E^{-5}$ for the control mice. In the jejunum Muc5ac had an expression of $2E^{-5}$ for the stressed mice while the control mice had $1E^{-5}$. In the ileum Muc5ac had an expression of $0.0012 \pm 0.0037$ for the stressed mice and $0.0020 \pm 0.0037$ for the control mice. In the colon Muc5ac had an expression of $0.0001 \pm 0.0001$ for the stressed mice and $3E^{-5} \pm 0.0001$ for the control mice. Muc5ac had very low expression in the colon, duodenum and jejunum. The stomach had the highest expression of Muc5ac followed by the ileum (Table 12) without showing any significant difference between the 2 treatments (Figure 20).
Table 12: Muc5ac expression in the GIT of stressed and control mice

<table>
<thead>
<tr>
<th>GIT Section</th>
<th>Variable</th>
<th>Nostress</th>
<th>Stress</th>
<th>Pooled SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>Expression</td>
<td>0.0106</td>
<td>0.0107</td>
<td>0.0065</td>
<td>0.972</td>
</tr>
<tr>
<td>Duodenum</td>
<td>Expression</td>
<td>1E-5</td>
<td>3E-5</td>
<td>4E-5</td>
<td>0.555</td>
</tr>
<tr>
<td>Jejunum</td>
<td>Expression</td>
<td>1E-5</td>
<td>2E-5</td>
<td>3E-5</td>
<td>.</td>
</tr>
<tr>
<td>Ileum</td>
<td>Expression</td>
<td>0.0020</td>
<td>0.0012</td>
<td>0.0037</td>
<td>0.687</td>
</tr>
<tr>
<td>Colon</td>
<td>Expression</td>
<td>3E-5</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.536</td>
</tr>
</tbody>
</table>

Figure 20: No significant difference in Muc5ac expression in the GIT of the mice
CHAPTER 4

DISCUSSION AND CONCLUSION

Discussion

Restraint as noted by Kvetnansky and Mikulaj (1970) is a stressful process for animals. Restraint-stress, to which the experimental mice were subjected for 6 hours daily for 3 weeks, was used to mimic chronic life stress that humans go through. To assess the effect of this stressor on the experimental mice required to determine first and foremost the corticosterone level in the serum of the mice.

The restraint-stress applied on the tested mice induced high corticosterone levels in the serum. Two mice T1 and T2 were more agitated and always resisted being restrained more than the other mice. Consistent with the observed behavior, the corticosterone assay results indicated that their stress hormone levels were very high and were considered outliers. The control mice had elevated levels of corticosterone probably due to the method used to euthanize the animals at the end of the study. A quick decapitation might have had less stressful impact than the use of carbon dioxide. Because all the mice were sacrificed the same way, the method used would not make any notable difference, it just caused a shift of the baseline.

The detection of high corticosterone levels in the serum or plasma could be an indication of stress. Findings in this study were consistent with Gu et al. (2009) when they analyzed the serum corticosterone levels of their stressed and control mice. A study that was conducted by Rich and Romero (2005) noted that in chronic stress the level of corticosterone was less than in acute stress. This could be attributed to some
degree of adaptation as suggested by Kvetnansky and Mikulaj (1970). The goal of the corticosterone assay was to validate that the protocol used was effective in inducing stress in the mice as demonstrated by the levels of corticosterone in the serum.

Mice were weighed regularly to assess whether stress would impact their body weight during the study period depending on the treatment they were subjected to. It was noted that from measurement to measurement there was a difference in body weight. The control mice as expected gained weight consistently throughout the experimental period. In the beginning the stressed mice lost weight, then they started to regain some weight and stayed somewhat on that trend. This could be due to some adaptation mechanism to cope with the stressor. There were significant differences in body weight between the 2 groups of mice. The findings in this study are consistent with Gu et al. (2009) who conducted a study in which they compared the body weight of control and stressed mice. The protocol used to induce chronic mild stress combined period of continuous illumination, mice in cage with 45° tilt, and mice in dirty cages. They noted that the body weight increased in both groups even though the stressed mice weighed less than the control mice over time. Stress had an effect on body weight.

It has been postulated that stress triggered the hunger hormone, called ghrelin. Zigman and Lutter (https://www.sciencedaily.com/release/2008/06/080615142252.htm) suggested that mice that are subjected to chronic stress have high levels of ghrelin that induces among other things an increase in feed consumed and body weight. The protocol followed in the present study mice had free access to feed except during the 6-hour restraint period and it was noted that there was no difference between the stressed
and control mice in terms of the amount of feed they ate. As expected, mice consumed more feed as they grew.

Mucin-producing cells play a very crucial role in maintaining the mucus protective layer that covers the epithelium of lumen and ducts. There was need to assess whether chronic stress had any bearing on the mucin-producing cells (goblet cells). When comparing the goblet cells in the duodenal, jejunal, and ileal sections of the gastrointestinal tract of stressed and control mice, it was noted that there were significant differences in the GC count of the 3 sections that were investigated. There was a decrease in GC counts in the stressed animals. These findings were consistent with Castagliuolo and his colleagues (1996; 1998) whose findings also support the findings of the present study. They found that rats that were subjected to immobilization-stress had an increased mucin secretion in the colon while the goblet cells were decreased in number. Pfeiffer et al. (2001) also noted that repeated exposure to restraint-stress induced reduction of mucin secretion as well as the number of goblet cells in the colon. Rubio et al. (1991) investigated the effect of stress on the mucus of the colon in rats. They observed an initial drop in goblet cells followed by a hyperproduction of mucin-producing cells.

Duodenal, jejunal, and ileal sections stained with AB-PAS were also examined to assess any difference in the morphology of the epithelium of stressed and control mice. The results revealed that there were significant differences in the heights of villi in the duodenum and jejunum of stressed and control mice. There was a decrease in height of villi of stressed mice. Nevertheless, in the ileum no significant difference was noted.
between the 2 groups. This is consistent with the findings of Burkholder et al. (2008) who noted that stress did not affect significantly the villus length of tissues from birds.

After showing that the protocol used was effective in inducing stress and assessing the effect of stress on body weight, feed consumed, and histology of intestinal tissues, the next step was to investigate whether stress had effect on gene expression of mucins in the GIT.

The quality of the RNA extracted was in general good as indicated by the ratio between the 28S and 18S bands of 2:1.

In the present study Muc1 was one of the predominant mucins expressed in the stomach and colon under normal physiological conditions. The findings in the present study were consistent with those of Audie et al. (1993), Cao et al. (1997), Corfield et al. (2000), and Wang and Fang (2003). These researchers found the same expression sites in normal conditions. Muc1 had also a moderate expression in the ileum of stressed and control mice in the present study with no significant differences between the 2 groups. Muc1 was highly expressed in the stomach with no significant differences noted between the stressed and control mice. The colon showed significant differences between the 2 groups in the present study. Corfield et al. (2000) found that mucins such as MUC1, MUC2, MUC3, and MUC4 were expressed in the colon under physiological conditions. Hoebler et al. (2006) noted an upregulation of Muc1 and Muc4 in the colon during acute colitis then diminished in chronic colitis in mice. The findings of the present study may suggest that some inflammation or pathology was going on in the colon where the 2 mucins were overexpressed in the chronically stressed mice. Wang and
Muc2 was expressed in the entire intestinal tract of stressed and control mice. No significant differences were noted between the 2 groups of mice. Ho et al. (1995) indicated in their study that MUC2 was not expressed in the normal gastric tissue, but the present study noted a low expression of this mucin in stomach of the control mice. Ho et al. (1995) noted that MUC2 was expressed in the stomach of patients with intestinal tumors. The lowest expression was in the stomach, while the highest expression site was the colon. These findings confirmed what was observed earlier when the expression sites of each mucin in the study were determined. Similar distribution was noted by Audie et al. (1993) and Corfield et al. (2000). Muc2 was mainly secreted by goblet cells and was one of the major proteins that secreted the mucus layer that protected and lubricated the epithelia of ducts and lumens (Rhaka et al. 2005). Because Muc2 was the major intestinal mucin, it was surprising to note that there were no significant differences in its expression between the control and stressed mice. Restraint-stress in the present study did not significantly alter the expression of Muc2 in the GIT probably because it was not able to trigger the specific signaling pathway through which Muc2 was controlled in this body system.

Muc3 was expressed throughout the gastrointestinal tract with the highest expression in the jejunum (Audie et al. 1993; Corfield et al. 2000). In the present study significant differences were noted in Muc3 expression in the duodenum between the 2
treatments. There was an upregulation in the stressed mice of more than 2-fold in the present study.

Leroy et al. (2003) conducted a study on MUC3 and VEGF in renal carcinoma. Their findings were comparable to Wang and Fang (2003) in regard to MUC3 implication in carcinogenesis. Shekels and Ho (2003) have pointed out that growth factors and cytokines had the ability to control the secretion of Muc3 in the GIT. Therefore an overexpression of Muc3 should be monitored closely whether in the GIT or the kidneys.

Muc4 was expressed to some extent in all the gastrointestinal tract of both groups of mice. Under physiological conditions Andrianifahanana et al. (2006) and Corfield et al. (2000) suggested similar expression sites. In the colon Muc4 expression showed highly significant differences between the stressed and control mice. Muc4 expression was upregulated in the stressed group compared to the control group. Moniaux et al. (2007) suggested that MUC4 facilitated tumor formation by allowing cancer cells to proliferate. MUC4 upregulation has been observed in many cancers (Moniaux et al. 2007; Senapati et al. 2008).

Muc5ac was expressed in the stomach of stressed and control mice with no significant differences. Song et al. (2003) suggested that MUC5AC was overexpressed in the airway epithelium under cytokines regulation but the mechanisms were not fully understood.
The data obtained in the present study suggest that the profile that emerged after chronic stress in mice was the initial stage of uncontrolled expression of mucins (Muc1, Muc3, and Muc4) that would lead to carcinogenesis in the intestinal tract. Mucins play an important role in wound healing (Buisine et al. 2001) and if no wound is present that requires repair, intact cells might proliferate uncontrolled and probably lead to carcinogenesis. The colon seems to be the most susceptible site in this study.

Conclusions

From the results of the present study, the following conclusions may be drawn:

- Chronic restraint-stress induces increased serum corticosterone level in mice.
- Stress significantly influences the body weight of mice.
- Chronic stress has an effect on the goblet cells by decreasing their count in the intestinal tract of mice.
- Chronic stress causes an increase in expression of Muc1 and Muc4 in the colon and Muc3 in the duodenum of mice.

Future Research

Determine the function of the mucins of interest by using knockout mice.

Elucidate the signaling pathways that stress triggers in the gastrointestinal tract so that mucins could be used effectively as targets in designing therapies or as screening tools.
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