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The Recovery of Gut Barrier Function With Selenium Rich Diet in Acute DSS-Induced Colitis

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THE RECOVERY OF GUT BARRIER FUNCTION WITH SELENIUM RICH DIET IN ACUTE DSS-INDUCED COLITIS

by

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DEDICATION

This thesis is dedicated to my sister. For without her I would not have had the courage to keep going. She is a strong and highly motivated individual that encourages all to pursue their dreams no matter what problems lie in their wake. She gives me a sense of passion for the work I do in the lab and elsewhere in my life. She is the example person I wish to become in this world. For she is going for her dreams, conquering the obstacles, and achieving what was thought to be impossible. She has gone above and beyond the call of sisterhood to help me prepare my thesis. She has taught me to incorporate a higher level of thinking and given me the ability to do what I thought I couldn't.

I would also like to dedicate this thesis to Dr. Raja Fayad and my lab mates, Dr. Arpit Saxena, Kamaljeet Kaur, and Alex Sougiannis. Dr. Fayad was a great teacher and mentor. I will never forget his kind words and helpful guidance in my undergraduate and masters degrees. He taught me to keep going, to never give up, and to push forward even when times are rough. He will always be remembered. To my lab mates: I will never forget all of your guidance. You have helped me in so many ways that I cannot express my thanks enough. You have listened to all my questions and answered all my phone calls when experiments go wrong. You have been there for me every step of the way. I hope to make you all proud with this thesis.

ABSTRACT

Background: Acute Dextran Sodium Sulfate (DSS)-induced colitis is an inflammatory ailment limited to the colon. It works to destroy the morphology and gut barrier goblet and epithelial cells that aid in providing homeostasis. Selenium (Se) is an essential micronutrient that has anti-inflammatory and antioxidant properties and is known to play a role in reducing inflammation in areas elsewhere in the body. The current study is focused on how Se alters gut barrier permeability and functionality related to the recovery of tight junction regulation and mucin secretion. *Methods:* C57BL/6 mice were randomly placed into control (normal water) and 2% DSS water receiving groups and within these groups they were randomly given either a Se rich diet or a control diet *ad libidum*. Hemotoxylin-Eosin and Alcian Blue staining was used to study the colon morphology and to quantify the goblet to epithelial cell ratio. Western Blot was used to analyze protein expression levels for MUC-2 and ZO-1. Gut barrier permeability was assessed by administering FD4 and determining its plasma concentration by spectrofluorescence. ELISA was used to study the colon-secreted cytokine levels of TNF-α and IL-1β. *Results:* DSS + Se mice showed significantly lower clinical scores, histopathology, and higher goblet to epithelial cell ratios compared to DSS mice given a control diet. It is interesting to note that there was a main effect of diet and DSS treatment with ZO-1 expression. We found no significant difference between the groups for gut permeability as well as for MUC-2 expression, and IL-1β or TNF-α

secretion. *Conclusion:* The data suggests that Se works to reduce the severity of colitis by increasing ZO-1 expression and goblet cell content.

Keywords: Selenium; Acute Colitis; Inflammation; Gut Barrier; Mucin; Tight Junction

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CHAPTER 1: PROPOSAL

INTRODUCTION

Inflammatory bowel disease (IBD) has been a growing concern in the United States and all over the world. IBD can be classified as either Ulcerative Colitis (UC) or Crohn's Disease (CD) and has been characterized by chronic uncontrolled inflammation that results in damage to the lining of the gastrointestinal tract, blood in stools, diarrhea, and weight loss (Abraham, 2009; Matter, 2011). It is estimated that as many as 1.4 million American's, or 1 in every 200 people, suffer from IBD and as many as 70,000 new cases appear each year (Hanauer, 2006). The peak onset of the disease has been shown to be from 15 to 30 years of age with most recent data suggesting a higher incidence before the age of 20 (Abraham, 2009; M'Koma 2013). Studies have shown that individuals suffering from IBD for at least 6-8 years are six times more likely to develop colorectal cancer (M'Koma 2013; Mattar, 2011). Acute colitis is a single inflammatory flare-up that presents the same symptoms of IBD except on a much lower scale (Keshavarzian, 2003). These acute flare-ups have been attributed to genetic and non-genetic factors. However, genetics have only been able to account for 20-25% of susceptibility, while environmental (diet, exercise, drugs, smoking, and social stress), immunological, and microbial factors play a larger role (Keshavarzian, 2003). Acute colonic flare-ups that are frequent and reoccurring are more indicative of chronic inflammation, which may lead to the development of IBD.

The GI tract must prevent leakage of gut bacteria into the abdominal cavity for if it does not, acute inflammatory immune responses may occur (Matricon, 2008; McGuckin, 2009). The intestines are home to trillions of commensal bacteria that make up a microbiome. This population of bacteria is tightly regulated and the immune system is highly responsive in distinguishing harmful bacteria from commensal (Johannson, 2013). Studies examining IBD development have found concomitant intestinal barrier dysfunction and increased intestinal permeability allowing bacteria to leak outside of the intestines (Matricon, 2010). The gut barrier is made of an outer mucus layer and an inner single layer of epithelial cells that are held together by tight junctions (TJ) (Antoni, 2014). TJ are composed of zona occludens (ZO) that are located at the apical surface of the epithelial cells. These cell junctions are the rate-limiting step in paracellular permeability (Clayburgh, 2004). Inflammation can downregulate their overall expression and translocate them to the inside of the cell away from the surface causing increased paracellular leakage of noxious bacteria (Ma, 2004). Inflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin factor 1 beta (IL-1β have been shown to be able to alter intestinal TJ permeability (Ma, 2004; Wang, 2005). Furthermore, reactive oxygen species (ROS) may also cause oxidative stress-induced inflammation and lead to decreased intestinal barrier function by downregulation of TJ proteins (Keshavarzian, 2003). TJs are the main regulatory site for paracellular permeability and are highly investigated in inflammatory diseases (Clayburgh, 2004). However, further research is needed in examining nutritional effects on TJ expression as well as how these effects may alter other portions of the gut barrier in acute colitis.

In addition to tight junction regulation, the secretive functions of gut epithelial cells can have a role in intestinal permeability. The mucus layer is composed of a two-layered system that regulates the luminal bacterial environment and is important for the protection of the barrier epithelial cells (Johansson, 2013). The inner layer is formed by mucins (Muc 2, 3, and 4) that are secreted by goblet cells. In this layer there is relatively no bacteria, which provides the protective function to the epithelial cells. On the other hand, the outer layer has the same mucins as the inner layer, but here is where the bacteria in the gut thrive (Johansson, 2013). UC cases show diminished outer layer of mucus causing the bacteria and other noxious agents to move to the inside layer putting them in direct contact with apical epithelial surface. The diminished outer layer has been correlated with a loss of goblet cells and their secreted mucins (Dorofeyev, 2013). While mucous production is important for gut protection, further work is needed to determine how nutrition impacts the secretory function of gut epithelial cells.

Selenium (Se) is an essential micronutrient that exerts its anti-inflammatory and antioxidant effects through many families of selenoproteins. Dietary supplementation of Se has been shown to play a role in thyroid hormone metabolism, cardiovascular health, prevention and reduction of cancer, and immune function (Huang, 2012). A deficiency in Se has been negatively correlated with IBD, which suggests a crucial role of Se in inflammatory pathology (Barrett, 2013). Research has shown that Se can shift macrophage polarization from an M1 pro-inflammatory state to an M2 anti-inflammatory state after an insult of injury (Nelson, 2011). Additionally, glutathione peroxidases (Gpx), a major family of selenoproteins, have been found to decrease inflammation, reduce ROS, and decrease cancer incidence in mouse models of inflammation-associated

carcinogenesis (Krehl, 2012). Deficiency in subtypes of Gpx, Gpx-1, Gpx-2, or both, have shown development of spontaneous intestinal inflammation and increased apoptosis of intestinal epithelial cells (Edelblum, 2006; Krehl, 2012). While Se has shown beneficial effects in various disease states the effects on gut barrier dysfunction, especially tight junction regulation and mucus production, is not very well understood.

Se has demonstrated positive effects in reducing inflammation and preserving epithelial cells. In cell models of human breast cancer, Se has enhanced the function of TJs by relocation of ZO-1 proteins to the apical surface, thus decreasing permeability (Martin, 2007). Additionally, rat models examining stress and chemically induced gastric ulcers have found Se to prevent gastric wall mucus depletion. (al-Moutairy, 1996). The current study is focused on how Se alters gut barrier functionality related to the recovery of tight junction regulation and mucin secretion. However, there are currently gaps in our understanding or how Se can impact intestinal barrier function in a mouse model of acute colitis. Thus, we examined Se function with a widely used mouse model of intestinal inflammation, dextran sodium sulfate (DSS) - induced colitis (Perse, 2012). This model has demonstrated acute, chronic, and relapsing experimental inflammation and has been shown to closely resemble human IBD (Okayasu, 1990; Perse, 2012).

Purpose and Aims

The purpose of this study is to examine the preventive and restorative effect of Se on acute DSS-induced colitis in C57BL/6 mice. The severity of colitis in the mice will be observed through assessment of clinical score and histopathology. Inflammatory mediators that will be studied include expression of inflammatory cytokines TNF-α and IL-1β and localization of pro-inflammatory transcription factor Nf-κβ. Variables of gut

barrier function that will be studied include ZO-1 expression and localization, gut permeability, mucus protein content, and goblet cell content. The overall hypothesis is that a Se rich diet will decrease the severity of colitis by decreasing inflammation, decreasing gut permeability, and increasing mucus protein content.

Specific Aim #1 will determine the effect of Se rich diet on the severity of colitis in mice with DSS-induced colitis. The primary outcomes measured will be clinical score and histopathology to indicate severity of colitis. The score consists of weight loss, diarrhea, and hemoccult. We hypothesize that a Se rich diet will decrease the severity of colitis by decreasing clinical score and histopathology as compared to control diet.

Specific Aim #2 will determine the effect of Se rich diet on gut barrier function in mice with DSS-induced colitis. Primary outcomes measured will be gut barrier permeability. Secondary outcomes include mucus protein expression, goblet cell content, tight junction expression, and secreted tissue expression of inflammatory cytokines. We hypothesize that a Se rich diet will improve gut barrier function by decreasing gut permeability as compared to control diet. We also hypothesize that these positive changes will be associated with increased mucus protein expression, goblet cell content, and tight junction protein expression and decreased inflammatory cytokine secretion in the colon.

WORKING MODEL

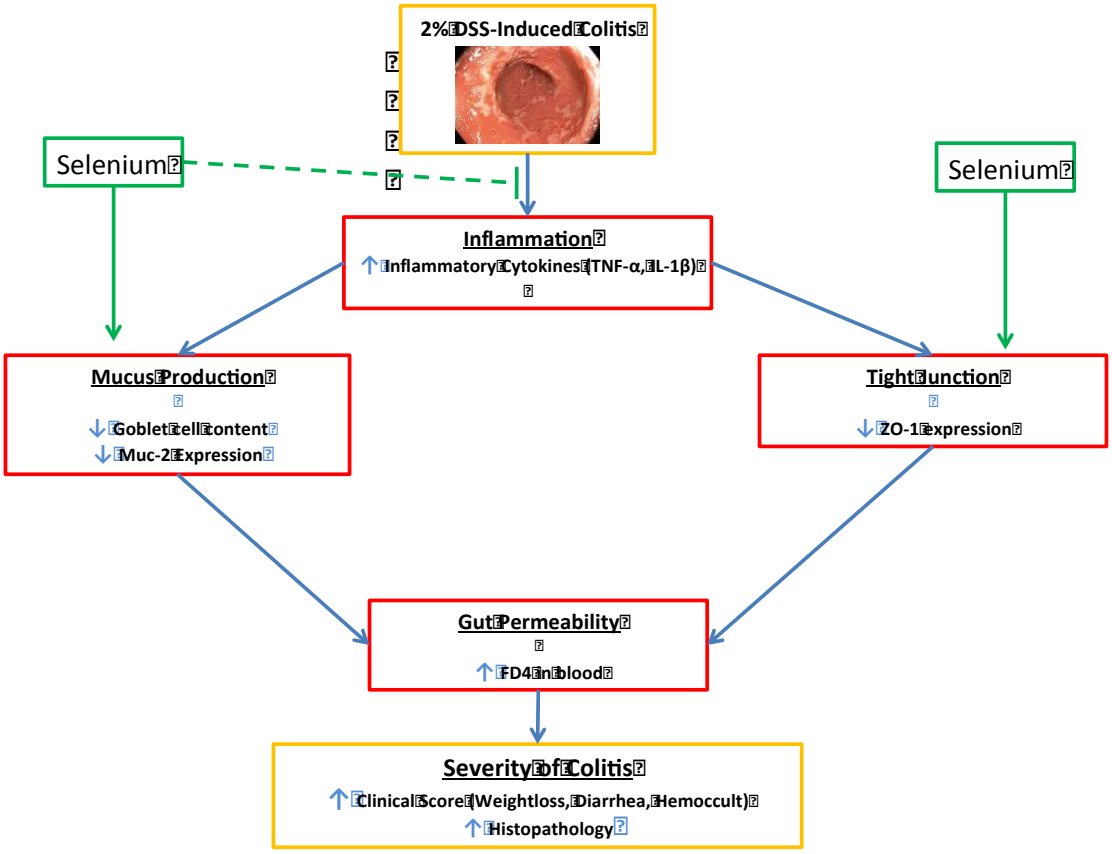

Figure 1.1 Working Model: This study is aimed to uncover the proposed model of the protective effect of Se on gut barrier function in C57BL/6 mice with acute DSS-induced colitis. The overall hypothesis is that a Se rich diet will decrease the severity of colitis by decreasing inflammation, decreasing gut permeability, and increasing mucus protein content.

METHODS

Animals and Housing

Four to five week old male and female C57BL/6 mice (n=33) were bred and maintained in the animal resource facility at the University of South Carolina. They were housed three-five per cage and maintained on a 12:12 light-dark cycle in a low stress environment (22°, 50% humidity, low noise). Mice were split into two main groups: control and experimental. The control group consisted of a mixed population, while only males were placed in the experimental groups. Each main group was subdivided into mice receiving either a Control diet (0.02ppm Se) or a Se rich diet (0.75ppm Se). After 1 week of either diet, experimental mice were given 2% Dextran Sodium Sulfate (DSS) (MP Biochemicals, MW 36,000 – 50,000) dissolved in their drinking water for 5 days followed by 5 days of normal drinking water to induce acute colitis. Control mice were given normal drinking water ad libitum throughout the duration of the study. All animal experiments were approved by the University of South Carolina's Institutional Animal Care and Use Committee.

Monitoring Animal Health

Food and water intake, as well as body weight, were measured every alternate day for all mice throughout the length of the study. During and following DSS treatment in experimental groups, mice were observed every alternate day for clinical signs of disease, which included weight loss, diarrhea, and positive fecal hemoccult. Weight loss was

ranked by a point system as follows: 0= 0-5% weight loss; 1=6-10% weight loss; 2=11-15% weight loss; 3=16-20% weight loss; and 4=>20% weight loss. The appearance of diarrhea was ranked as 0= well-formed pellets, 2= pasty and semi-formed stools that do not adhere to the anus, 4= liquid stools with no form that do not adhere to the anus. Positive hemoccult was scored as follows: 0= no blood or negative hemoccult, 2= some blood (<50%) or positive hemoccult, and 4= gross bleeding (>50%) using hemoccult kit (Beckman Coulter). The clinical score was then determined by adding and totaling the scores of weight loss, diarrhea, and hemoccult.

Tissue Collection

All mice were sacrificed 17 days after initial induction of Se rich or Control diet. The mice were sacrificed within 2 hours by cervical dislocation and tissue collection was performed as a non-survival surgery. The mouse colon was excised and flushed with PBS (EMD Chemicals) and three 1cm sections of each were cut. The first section was stored at -80°C for protein expression studies. The second section was fixed in formalin (Fisher Scientific) and stored in 70% ethanol, which was later cut into 5-6μm thin sections for use in Hemotoxylin and Eosin (H&E) and Alcian Blue and Nuclear Fast Red staining. The last section was placed in 12-well plates containing 1ml of RPMI 1640 media that included 1% Penicillin-streptomycin (Mediatech, Inc) per well and was incubated for 24 hours at 37°C and 5% CO2. The RPMI media containing tissue cytokines was centrifuged at 10,000g for 10 minutes at 15°C and the supernatant was collected and stored at -20°C until further analysis.

Histology

The histopathology of the colon was observed through H&E staining. A standard protocol for H&E staining was used. The severity of colitis was quantified by a scale of 0 to 4, where 0= no infiltration and inflammation; 2= moderate infiltration and inflammation; and 4= severe infiltration and inflammation with distorted crypts. Alcian Blue and Neutral Fast Red staining was used to detect goblet and epithelial cells in the colon. Goblet cells were stained blue with Alcian Blue and epithelial cells were stained pink with Neutral Fast Red. They were quantified by goblet to epithelial ratio using 6 crypts per colon section from each mouse for all treatment groups.

Gut Permeability

Gut barrier integrity was assessed in all mice by permeability to FITC-dextran (MWav= 4000; FD4) (Sigma Aldrich). The FD4 was administered by gavage based on the animal's body weight in grams and diluted with 125mg/ml of PBS to five hour fasted mice. Plasma was sampled before and 1 h after FD4 administration and measured for florescence as previously described by Yang et al., 2003.

Enzyme-linked Immunosorbant Assay

The RPMI medium supplemented with secreted colon cytokines was used to quantify the local concentrations of TNFα and IL-1β (BD biosciences) using commercially available BD OptEIA enzyme-linked immunoabsorbant assay (ELISA) kits, according to the manufacturer's instructions. The local cytokine concentrations were normalized by the estimated protein content in the colon supernatant by a Bradford protein assay.

Western Blotting

Colon tissue samples frozen at -80°C were homogenized in RIPA buffer that was supplemented with protease and phosphatase inhibitors (SIGMA). The samples were then centrifuged at 10,000 rpm for 15 minutes and the supernatant was collected for protein analysis by a standard Bradford assay. Protein homogenate from all groups was electrophoresed on 7% SDS-PAGE gels and transferred to a nitrocellulose membrane for 3 hours at 4°C. The membrane was blocked by 1X PBS and 0.1% Tween 20 for 1 hour and subsequently probed for ZO-1 (Abcam) and MUC-2 (Abcam) overnight. The bands were detected using chemiluminescence and normalized relative to GAPDH expression (Genetex). The bands were quantified by densitometry and expressed as mean area density by using Image J software (Image J).

Statistical Analysis

XLStat for Windows (verson 2009.4.07) statistical software was used to perform all statistical analysis. Independent two-tailed t-tests were used to determine significance for all single variables. Statistical significance was considered at $P < 0.05$ level of confidence

REVIEW OF LITERATURE

The literature review for this study is divided into three main sections with further subcategories to explore knowledge more in depth on specific topics. The first section divulges inflammation and its role within injury and the immune response. The second section draws attention to the gut barrier, its components, and how it relates to inflammatory events. The final section sheds knowledge on the dietary nutrient called selenium. This section will explore in detail the functions and roles of selenium in the body and how it may be used as a therapeutic treatment. Furthermore, the current

knowledge of links between gut barrier dysfunction, inflammation, and selenium will be reviewed in this section.

I. Inflammation

Injury or infection of tissues sets off chemical and physical signals that allow infiltration of blood cells and fluid in order to promote healing. These events are called an inflammatory response, which is a type of defense mechanism housed within the immune system of the body. Its primary purpose is to contain, neutralize, dilute, or wall off deleterious agents and has been characterized by distinct cardinal signs, such as heat, swelling, redness, pain, and sometimes loss of function (Anderson, 2001; Medzhitov, 2008). Inflammation may seem to be more degenerative than reparative, but if closely regulated it plays a pivotal role in the wound healing process. An acute response has been characterized by immediate and nonspecific events. The immediate inflammatory action calls for the activation of local macrophages and mast cells that release inflammatory mediators, such as chemokines and cytokines, in order to attract white blood cells and clotting agents to divest infectious agents for wound repair (Medzhitov, 2008). The white blood cells, mainly neutrophils, and plasma proteins are normally housed within the cardiovascular system and may venture into local tissue if the endothelial membrane becomes leaky or slightly more permeable than normal. This infiltration of inflammatory agents is necessary for repairing tissue damage, but it must be tightly regulated and resolved quickly in order to decrease the risk for increasing mortality and morbidity for diseases such as rheumatoid arthritis, diabetes, Crohn's disease, and atherosclerosis (Tracey, 2002). A chronic response has been characterized by

delayed and highly specific events, which is more of an unregulated inflammatory response with an abundance of inflammatory mediators and white blood cells as compared to acute inflammation (Eming, 2007; Ryan, 1977). Moreover, chronic inflammation has been widely studied as a mechanism of the development of modern human diseases, such as cancer (Shacter, 2002), Inflammatory Bowel Disease (Zhang, 2012), Alzheimers (Bibi, 2014), and Chronic Obstructive Pulmonary Disease (Maclay, 2013).

A. The Pathway of Inflammation

Injury promotes the immediate activation of an immune mediated response that allows infiltration of polymorphonuclear (PMN) leukocytes, mainly neutrophils, through the endothelial membrane. This process occurs via the activation of the membrane by local proinflammatory cytokines: Tumor Necrosis Factor-α (TNF-α), Interleukin -1β (IL-1β), and Interferon-γ (IFN-γ) (Eming, 2007). After several days, the neutrophils are joined by larger populations of activated macrophages via attraction by monocyte chemoattractant protein -1 (MCP-1). Macrophages are widely studied phagocytes that are thought to have primarily deleterious effects on tissues. However, not only do they encompass a proinflammatory phenotype (M1), but they also present an antagonist side, the anti-inflammatory phenotype (M2). Studies have shown that M1 is the predominant phenotype during the initial phase of acute inflammation due to its high T helper cell-1 (Th-1) cytokine response (Mantovani, 2013; Romagnani, 2000). The Th-1 response is activated by TNF-α, which binds to toll-like receptors and TNF-α receptors

located on the plasma membrane of macrophages. The binding stimulates a cascade affect that activates protein kinase C (a major regulatory Phosphorylation protein). PKC may phosphorylate Iκβ, which is bound to nuclear factor kappa beta (Nfκβ) (Aveleira, 2010). Phosphorylation of this complex allows Nf-κβ to dislocate into the nucleus, which promotes the transcription of proinflammatory mediators, such as TNF-α, IL-1β, IL-6, reactive oxygen species (ROS), inducible Nitric Oxide Synthase (iNOS) CXCL9 and CXCL10 (Chazaud, 2014; Dohi, 2014; Lawrence, 2009; Mantovani, 2013). After the wound has been cleansed of debris, further leakage of cell contents has ceased, and neutrophils are phagocytized, the macrophage will undergo polarization to an M2 phenotype to initiate the second phase – healing (Ramaiah, 2007). The M2 response is of Th-2 type and is activated by transforming growth factor-β (TGF-β), IL-1, IL-4, and IL-10 and its primary role is to promote tissue repair and regeneration in order to recover functionality of the damaged tissue (Chazaud, 2014). These activating factors bind to the cell surface receptors on the macrophage and illicit transcription and release of anti-inflammatory mediators (IL-4, IL-13, IL-10, arginase, proline, CCL17, CCL22, and CCL24, VEGF, and MMPs), which dampen inflammation and promote tissue repair (Mantovani, 2013; Martinez, 2008).

II. Inflammation and the Gut

A. Epithelial Barrier

The gastrointestinal tract involves a large tube-like structure that runs from the mouth to the anus. Consumed nutrients travel on the luminal side and remain there unless excreted or selectively absorbed by the single layer of epithelial cells that line this tract. The intestines are the primary areas in the gastrointestinal tract that allow absorption of nutrients that are vital for homeostatic functionality. Moreover, selective permeability is one of the key protective functions of the intestinal epithelial cells. This feature allows the uptake of nutrients and minimal exposure to various toxins, antigens, and microorganisms (Lennernas, 1998). Molecules and ions may be selectively taken into the epithelial cells through two types of transport. The first is transcellular – the intake of substances through the apical membrane on the extracellular surface or the basolateral membrane on the luminal side. However, substances taking this route of transport require either a lipophilic composition or a specific mechanism of ATP-dependent transport across the membrane. Substances that do not have access to either of these requirements may take the second route of transport – paracellular. This is the route, by which the substances may travel into the intercellular space between adjacent epithelial cells. However, this pathway is tightly regulated by cell junctions (Gonzalez-Mariscal, 2007; 2008).

1. Cell Junctions

i. Tight junctions – A type of epithelial and endothelial cell junction located on the apical surface of the membrane. Tight junctions play a vital role in paracellular transport by tightly regulating permeability of essential molecules and ions and keeping out noxious agents (Gu, 2011). The degree of permeability may fluctuate due to the local mucosal or luminal environmental stimuli as well as various physiological and pathological conditions. Tight junctions are integrated via an array of proteins as well as with an association of scaffolding proteins. Occludins are transmembrane proteins that have a PDZ domain that directly regulates paracellular trafficking or permeability (Hwang, 2013). Zona Occludins (ZO) are cytoplasmic scaffolding proteins that hold occludins in proper orientation through interaction with the PDZ domain and binding to actin located in the cell cytoskeleton. These PDZ domains allow scaffolding and structural interaction of ZO and occludins by spacing these proteins in close proximity (Gonzalez-Mariscal, 2000). The final proteins of interest that play a pivotal role in permeability are the claudin family. These proteins vary in their degree of leakiness and this function allows permeability of certain types of ions (i.e claudin-8 reduces Na2+ permeability) (Gonzalez-Mariscal, 2008).

ii. Other Cell Junctions – Tight junctions may be the most apical junction and highly important in permeability; however, there are other

junctions to consider in regard to cell-cell environmental homeostasis. The adherens junctions are located directly below the tight junctions. The main structural protein components in these junctions consist of cadherins (Dejana, 1995). They come in a variety of types, the most important being E-cadherin. It comprises specific roles of cell-cell adhesion and interaction with the actin cytoskeleton components. In order for E-cadherin to exhibit these roles, it must be joined in a complex with catenins (alpha, beta, or gamma)(Dejana, 1995). Gap junctions reside below adherens junctions and are involved in cell-cell exchange of ions and low molecular weight molecules. The passage of molecules and ions is conducted through hemichannels made of proteins called connexins. In the gastrointestinal tract, they are found in abundance in the inner smooth muscle and studies have shown that they may influence the contractile activity of the gut (Nielsen, 2012).

B. Mucus Layer

The GI lining in the mouth and the esophagus have multiple layers of squamous epithelial cells. However, the stomach, small intestine, and large intestine have a single, thin layer of these cells causing increased vulnerability to damage and pathology. Aiding in protective defenses to these epithelial cells in the lower part of the GI tract is the mucus layer. Mucus is a transparent liquid layer that covers epithelial cells lining the GI tract and it is made of proteins known as mucins. There are two different types of mucins: classic gel forming (MUC2, MUC5AC, MUC5B, and MUC6) and

transmembrane (MUC1, MUC3, MUC4, MUC12, MUC13, MUC16, and MUC17) (Johansson, 2011). The small intestine has a single mucus layer, while the large intestine is composed of 2 distinct layers with separate functionality. The single layer of mucus in the small intestine is composed of the mucus protein MUC2, as well as antibacterial peptides, which allows this layer to be unattached to the epithelial cells and easily removable (Johansson, 2011). The function of the mucus in this region is to keep the surface of the epithelia as well as within the crypts where stem cells lie as sterile as possible. The colon is doubly lined with an outer mucus layer that is composed of MUC2 that resembles the single mucus layer of the small intestine, while the inner mucus layer is composed of MUC2 that is bound to the surface of the epithelia by attachment of goblet cells (Johansson, 2013). The MUC2 from the inner layer gradually becomes the outer layer in order to renew damaged mucin proteins from noxious agents. The functionality of the two layers remains separate. The inner layer protects the single layer of epithelial cells from consumed deleterious agents and is considered a sterile environment, while the outer layer houses the large populations of bacteria that make up the microbiome of the large intestine. The bacteria found here is ultimately essential for normal functionality of the colon and as long as it is regulated and within the outer layer of mucus, the colon may function without developed pathologies (Johansson, 2013). Like the epithelial cells, the mucus layer is constantly renewed in order to keep up proper functionality. The notch signaling pathway promotes differentiation of stem cells into absorptive cells

(epithelial cells) via hairy and enhancer of split-1 (HES-1) and when this pathway is inhibited the secretory linage of cells or goblet cells are differentiated by MATH-1 (Jeon, 2013).

C. Barrier Dysfunction via Inflammation

Inflammation is not only a process, by which healing occurs, but it has also been studied as a mechanism for the development of disease. Studies have shown that low grade inflammation involving infiltration of activated T cells that release TNF-α and other noxious agents may be a primary contributing factor in disease development, especially in the colon (Piche, 2014). Not only does TNF-α stimulate the activation of Nfκβ to promote further inflammation, but it also stimulates the disassembly of tight junction proteins (Aveleira, 2010). Studies have elucidated that PKC is an important regulator of tight junction permeability. Activation of PKC may target downstream proteins, such as Nfκβ or it may circle back and target the tight junctions themselves (Aveleira, 2010; Gonzalez-Mariscal, 2008). PKC is not the only protein that regulates tight junctions; myosin light chains (MLC) may alter their permeability as well. TNF-α and IL-1β stimulate the phosphorylation of MLCs by activating myosin light chain kinases (MLCK). This promotes the rearrangement of actin filaments in the cytoskeleton and loss of structural support for the tight junctions, thus allowing increased permeability (Turner, 2009; Wang, 2005). Beta-Catenin (β-Catenin) is a cytoplasmic protein that allows adherens junctions to be connected to the actin cytoskeleton for structural support, thus maintaining their ability to keep cells in close

proximity and aiding in tight regulation of permeability (Hurst, 1999). Stimulation of the MLCK causes a contraction of the actin filaments in the cytoskeleton, which results in rearrangement of not only ZO-1, but also β-catenin (Hurst, 1999). These rearrangements lead to increased permeability and allow infiltration of immune cells into the lumen of the GI tract and subsequent inflammation. The goblet cell proteins HES-1 and MATH-1 are important in UC and CD disease development (Zheng, 2011). Studies have found that the expression of the transcription factor Hath-1 is essential for goblet cell differentiation and that HES-1 suppresses Hath-1 leading to suppression of goblet cell formation, which results in decreased mucus layers. In UC there is an abnormal expression of HES-1, which promotes further suppression of goblet cells (Zheng, 2011).

III. Selenium, Inflammation, and the Gut

A. Selenium

Selenium (Se) is an essential trace element apart of the semimetals that is incorporated into proteins – selenoproteins – via co-translational modification by selenocysteine (SeCys). It is found within many food sources such as egg noodles (34.7ug), beef liver (57.0ug), brazil nuts (839.2ug), canned tuna (80.4ug), plain yogurt (8.1ug), and mushrooms (4.3ug) as well as a variety of others (Holben, 1999). Intake of Se is an average of 40ug per day in Europe and between 93ug (women) and 134ug (men) in the USA (Rayman, 2012). Selenoproteins are a growing area of research and have so far been found to reduce cancer, cancer-related mortality, and virulence associated with viral

infections, regulate thyroid hormones and function, improve mental health and reproductive performance, and act as a potent antioxidant. Recently, multiple studies have elucidated the role of Se on reducing the mortality of a variety of cancers as well as possible metastasis (Clark, 1996; Harris, 2012; Nagy, 2013; Rayman, 2012; Wrobel, 2013). The mechanism of action has been found to be through sufficient activation of c-Jun NH2-terminal kinase 1 (JNK1) leading to decreased β-catenin signaling with subsequent decreased cell proliferation (Fang, 2010).

Dietary or supplemental Se-species are absorbed in the GI tract and are routed to the liver where it is sorted into either the pathway for excretion by the kidneys or for synthesis into other Se metabolites. Regulation of these distribution pathways as been postulated as both active and passive; however, it has not been elucidated as to which has primary control. Most Se is transported out of the liver to other various locations as selenoprotein P (SeP), where it may be metabolized into other families of selenoproteins. The main family of importance is the glutathione peroxidases (GPxs) whose primary properties include antioxidant functions. There are four GPx members: Gpx1 (cytosolic) – functions to reduce retroviral virulence, GPx2 (gastrointestinal) – shows anti-apoptotic functions in colon crypts and maintains intestinal mucosal integrity, GPx3 (plasma) – antioxidant within extracellular fluids, and finally GPx4 (phospholipid) – present in high concentrations in the testis and is essential for sperm motility and viability (Rayman, 2012).

B. Selenium and inflammation

The immune system relies on many defense mechanisms, such as generation of reactive oxygen species (ROS) and inflammatory responses to protect the body from pathogens and noxious agents. During an immune reaction ROS may rise above normal concentrations, which may damage cell membranes, proteins, or DNA causing mutations and possibly dysfunction (Huang, 2012). H2O2 is a species of ROS that acts as a signaling molecule in the activation of cysteine residues. GPxs target the H2O2 species and metabolizes them into non-harmful agents. Preventative studies in humans have shown TNF-α stimulated immune cells having markedly higher expression of GPxs and are dependent on ROS concentration (Defi, 2011; Huang, 2012). Additionally, Se may downregulate cytokine and adhesion molecule expression that are released by macrophages (Roman, 2014). Studies looking at Se deficiency and/or GPx Knockout (KO) mice show higher incidence for cancer as well as spontaneous development of intestinal inflammation (Krehl, 2012; Roman, 2014). Inflammatory responses may not be a single event, but may be termed as a flare-up and occur frequently or more gradually over a period of time. During these flare-ups the actively inflamed mucosa shows reduced GPx activity and longer duration of inflammation, especially in Crohn's disease (Pinto, 2013). Se supplementation studies suggest use as a therapeutic target for these tissues to lessen the damage of colitis.

C. Selenium and Barrier Dysfunction

Cell-cell junction dysfunction and delocalization associated with inflammatory events, especially in the gut, have been widely researched (Al-Sadi, 2007; Groschwitz, 2009; Vaziri, 2012). Tight junctions are thought to be the main target for therapy, since they play a major role in sealing the membrane to prevent leakage of harmful agents into surrounding tissues. Acute inflammation creates cracks in these tight seals to allow immune defenses to clean the area of potential threats. However, if these junctions are not resealed, the inflammation may not fully resolve and may develop into chronic inflammation and/or cancer. Multiple therapies have been studied to directly and indirectly restore these junctions to proper functionality: Berberine (Gu, 2011), Moxibustion (Bao, 2011), Carbachol (Zhang, 2014), and Vasoactive intestinal peptides (Conlin, 2009). Se is a dietary nutrient that has shown plentiful benefits in healthcare and has been considered as a therapeutic strategy in a colitis model as well as in a cancer model (Martin, 2007). However, research on tight junction restoration after an inflammatory event with the use of Se is minimal. Studies suggest that Se may be used to decrease ROS, which helps to eliminate a potential threat for tight junction dysfunction (Keshavarzian, 2003). Furthermore, one study has observed the effects of a Se, gamma linolenic acid, and iodine on tight junctions in human breast cancer cells. This study revealed that supplementation with any of these alone or in combination enhanced tight junction function by relocation of ZO-1 to the apical surface with subsequent decreased paracellular permeability

(Martin, 2007). There are currently few to no studies looking at Se and mucus production. There is one study that shows Se protects the mucosal layer by an unknown mechanism in rats given diaspirin cross-linked hemoglobin (DBBF-Hb) (Baldwin, 2002). More research is needed to elucidate the role of Se in restoring barrier function and possible prevention of prolonged acute inflammation in the gut.

D. DSS-Induced Colitis Mouse Model

Dextran sodium sulfate (DSS) is a negatively charged sulfated polysaccharide that when ingested may induce damage. The DSS model of intestinal inflammation in mice was developed by Okayasyu and collegues (1990) and has been a widely used representative and reproducible model for IBD and acute colitis for many years (Okayasu, 1990). This model promotes epithelial damage by way of the toxic sulfate groups, which creates a large inflammatory response in the intestines, especially the colon, for several days after administration. The advantages to this model are that the dosages may be varied in order to bring about acute injury, chronic injury, or a specific time course of injury by dissolving DSS in drinking water. Another advantage to this model is that the development of disease is slow and steady, which is optimal for studying the different stages of disease pathogenesis. Furthermore, it is known that DSS-induced colitis brings about disruption and changes the expression of tight junction proteins as well as disrupts the mucosal layer of the intestines by depletion of goblet cells (Chassaing, 2014). The typical exposure of DSS to induce significant acute colitis is 4-7 days of low dose

DSS (2-3%), which brings about weight loss, immune cell infiltration, and barrier dysfunction. Studies looking into barrier restoration allowed mice to have 2-3 days of regular drinking water following DSS treatment (Chassaing, 2014).

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CHAPTER 2

THE RECOVERY OF GUT BARRIER FUNCTION WITH SELENIUM RICH DIET IN ACUTE DSS-INDUCED ACUTE COLITIS[1]

[1] S Depaepe, J Carson, R Thompson, and X Wang. To be submitted to *Nutrition*.

ABSTRACT

Background: Acute Dextran Sodium Sulfate (DSS)-induced colitis is an inflammatory ailment limited to the colon. It works to destroy the morphology and gut barrier goblet and epithelial cells that aid in providing homeostasis. Selenium (Se) is an essential micronutrient that has anti-inflammatory and antioxidant properties and is known to play a role in reducing inflammation in areas elsewhere in the body. The current study is focused on how Se alters gut barrier permeability and functionality related to the recovery of tight junction regulation and mucin secretion. *Methods:* C57BL/6 mice were randomly placed into control (normal water) and 2% DSS water receiving groups and within these groups they were randomly given either a Se rich diet or a control diet *ad libidum*. Hemotoxylin-Eosin and Alcian Blue staining was used to study the colon morphology and to quantify the goblet to epithelial cell ratio. Western Blot was used to analyze protein expression levels for MUC-2 and ZO-1. Gut barrier permeability was assessed by administering FD4 and determining its plasma concentration by spectrofluorescence. ELISA was used to study the colon-secreted cytokine levels of TNF-α and IL-1β. *Results:* DSS + Se mice showed significantly lower clinical scores, histopathology, higher goblet to epithelial cell ratios compared to DSS mice given a control diet. It is interesting to note that there was a main effect of diet and DSS treatment with ZO-1 expression. We found no significant difference between the groups for gut

permeability as well as for MUC-2 expression or IL-1β and TNF-α secretion.
Conclusion: The data suggests that Se works to reduce the severity of colitis by increasing ZO-1 expression and goblet cell content.

Keywords: Selenium; Acute Colitis; Inflammation; Gut Barrier; Mucin; Tight Junction

INTRODUCTION

Inflammatory bowel disease (IBD) has been a growing concern in the United States and all over the world. IBD can be classified as either Ulcerative Colitis (UC) or Crohn's Disease (CD) and has been characterized by chronic uncontrolled inflammation that results in damage to the lining of the gastrointestinal tract, blood in stools, diarrhea, and weight loss (Abraham, 2009; Matter, 2011). It is estimated that as many as 1.4 million American's, or 1 in every 200 people, suffer from IBD and as many as 70,000 new cases appear each year (Hanauer, 2006). The peak onset of the disease has been shown to be from 15 to 30 years of age with most recent data suggesting a higher incidence before the age of 20 (Abraham, 2009; M'Koma 2013). Studies have shown that individuals suffering from IBD for at least 6-8 years are six times more likely to develop colorectal cancer (M'Koma 2013; Mattar, 2011). Acute colitis is a single inflammatory flare-up that presents the same symptoms of IBD except on a much lower scale (Keshavarzian, 2003). These acute flare-ups have been attributed to genetic and non-genetic factors. However, genetics have only been able to account for 20-25% of susceptibility, while environmental (diet, exercise, drugs, smoking, and social stress), immunological, and microbial factors play a larger role (Keshavarzian, 2003). Acute colonic flare-ups that are frequent and reoccurring are more indicative of chronic inflammation, which may lead to the development of IBD.

The GI tract must prevent leakage of gut bacteria into the abdominal cavity for if it does not, acute inflammatory immune responses may occur (Matricon, 2008; McGuckin, 2009). The intestines are home to trillions of commensal bacteria that make up a microbiome. This population of bacteria is tightly regulated and the immune system is highly responsive in distinguishing harmful bacteria from commensal (Johannson, 2013). Studies examining IBD development have found concomitant intestinal barrier dysfunction and increased intestinal permeability allowing bacteria to leak outside of the intestines (Matricon, 2010). The gut barrier is made of an outer mucus layer and an inner single layer of epithelial cells that are held together by tight junctions (TJ) (Antoni, 2014). TJ are composed of zona occludens (ZO) that are located at the apical surface of the epithelial cells. These cell junctions are the rate-limiting step in paracellular permeability (Clayburgh, 2004). Inflammation can downregulate their overall expression and translocate them to the inside of the cell away from the surface causing increased paracellular leakage of noxious bacteria (Ma, 2004). Inflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin factor 1 beta (IL-1β have been shown to be able to alter intestinal TJ permeability (Ma, 2004; Wang, 2005). Furthermore, reactive oxygen species (ROS) may also cause oxidative stress-induced inflammation and lead to decreased intestinal barrier function by downregulation of TJ proteins (Keshavarzian, 2003). TJs are the main regulatory site for paracellular permeability and are highly investigated in inflammatory diseases (Clayburgh, 2004). However, further research is needed in examining nutritional effects on TJ expression as well as how these effects may alter other portions of the gut barrier in acute colitis.

In addition to tight junction regulation, the secretive functions of gut epithelial cells can have a role in intestinal permeability. The mucus layer is composed of a two-layered system that regulates the luminal bacterial environment and is important for the protection of the barrier epithelial cells (Johansson, 2013). The inner layer is formed by mucins (Muc 2, 3, and 4) that are secreted by goblet cells. In this layer there is relatively no bacteria, which provides the protective function to the epithelial cells. On the other hand, the outer layer has the same mucins as the inner layer, but here is where the bacteria in the gut thrive (Johansson, 2013). UC cases show diminished outer layer of mucus causing the bacteria and other noxious agents to move to the inside layer putting them in direct contact with apical epithelial surface. The diminished outer layer has been correlated with a loss of goblet cells and their secreted mucins (Dorofeyev, 2013). While mucous production is important for gut protection, further work is needed to determine how nutrition impacts the secretory function of gut epithelial cells.

Selenium (Se) is an essential micronutrient that exerts its anti-inflammatory and antioxidant effects through many families of selenoproteins. Dietary supplementation of Se has been shown to play a role in thyroid hormone metabolism, cardiovascular health, prevention and reduction of cancer, and immune function (Huang, 2012). A deficiency in Se has been negatively correlated with IBD, which suggests a crucial role of Se in inflammatory pathology (Barrett, 2013). Research has shown that Se can shift macrophage polarization from an M1 pro-inflammatory state to an M2 anti-inflammatory state after an insult of injury (Nelson, 2011). Additionally, glutathione peroxidases (Gpx), a major family of selenoproteins, have been found to decrease inflammation, reduce ROS, and decrease cancer incidence in mouse models of inflammation-associated

carcinogenesis (Krehl, 2012). Deficiency in subtypes of Gpx, Gpx-1, Gpx-2, or both, have shown development of spontaneous intestinal inflammation and increased apoptosis of intestinal epithelial cells (Edelblum, 2006; Krehl, 2012). While Se has shown beneficial effects in various disease states the effects on gut barrier dysfunction, especially tight junction regulation and mucus production, is not very well understood. Se has demonstrated positive effects in reducing inflammation and preserving epithelial cells. In cell models of human breast cancer, Se has enhanced the function of TJs by relocation of ZO-1 proteins to the apical surface, thus decreasing permeability (Martin, 2007). Additionally, rat models examining stress and chemically induced gastric ulcers have found Se to prevent gastric wall mucus depletion. (al-Moutairy, 1996). The current study is focused on how Se alters gut barrier functionality related to the recovery of tight junction regulation and mucin secretion. However, there are currently gaps in our understanding or how Se can impact intestinal barrier function in a mouse model of acute colitis. Thus, we examined Se function with a widely used mouse model of intestinal inflammation, dextran sodium sulfate (DSS) - induced colitis (Perse, 2012). This model has demonstrated acute, chronic, and relapsing experimental inflammation and has been shown to closely resemble human IBD (Okayasu, 1990; Perse, 2012).

METHODS

Animals and Housing

Four to five week old male and female C57BL/6 mice (n=33) were bred and maintained in the animal resource facility at the University of South Carolina. They were housed three-five per cage and maintained on a 12:12 light-dark cycle in a low stress environment (22°, 50% humidity, low noise). Mice were split into two main groups:

control (n=15) and experimental (n=18). The control group consisted of a mixed population, while only males were placed in the experimental groups. Each main group was subdivided into mice receiving either a Control diet (0.02ppm Se) (n=17) or a Se rich diet (0.75ppm Se) (n=16). After 1 week of either diet, experimental mice were given 2% Dextran Sodium Sulfate (DSS) (MP Biochemicals, MW 36,000 – 50,000) dissolved in their drinking water for 5 days followed by 5 days of normal drinking water to induce acute colitis. Control mice were given normal drinking water ad libitum throughout the duration of the study. All animal experiments were approved by the University of South Carolina's Institutional Animal Care and Use Committee.

Monitoring Animal Health

Food and water intake, as well as body weight, were measured every alternate day for all mice throughout the length of the study. During and following DSS treatment in experimental groups, mice were observed every alternate day for clinical signs of disease, which included weight loss, diarrhea, and positive fecal hemoccult. Weight loss was ranked by a point system as follows: 0= 0-5% weight loss; 1=6-10% weight loss; 2=11-15% weight loss; 3=16-20% weight loss; and 4=>20% weight loss. The appearance of diarrhea was ranked as 0= well-formed pellets, 2= pasty and semi-formed stools that do not adhere to the anus, 4= liquid stools with no form that do not adhere to the anus. Positive hemoccult was scored as follows: 0= no blood or negative hemoccult, 2= some blood (<50%) or positive hemoccult, and 4= gross bleeding (>50%) using hemoccult kit (Beckman Coulter). The clinical score was then determined by adding and totaling the scores of weight loss, diarrhea, and hemoccult.

Tissue Collection

All mice were sacrificed 17 days after initial induction of Se rich or Control diet. The mice were sacrificed within 2 hours by cervical dislocation and tissue collection was performed as a non-survival surgery. The mouse colon was excised and flushed with PBS (EMD Chemicals) and three 1cm sections of each were cut. The first section was stored at -80°C for protein expression studies. The second section was fixed in formalin (Fisher Scientific) and stored in 70% ethanol, which was later cut into 5-6μm thin sections for use in Hemotoxylin and Eosin (H&E) and Alcian Blue and Nuclear Fast Red staining. The last section was placed in 12-well plates containing 1ml of RPMI 1640 media that included 1% Penicillin-streptomycin (Mediatech, Inc) per well and was incubated for 24 hours at 37°C and 5% CO2. The RPMI media containing tissue cytokines was centrifuged at 10,000g for 10 minutes at 15°C and the supernatant was collected and stored at -20°C until further analysis.

Histology

The histopathology of the colon was observed through H&E staining. A standard protocol for H&E staining was used. The severity of colitis was quantified by a scale of 0 to 4, where 0= no infiltration and inflammation; 2= moderate infiltration and inflammation; and 4= severe infiltration and inflammation with distorted crypts. To ensure reliability, the scoring was method was repeated three times on three tissue sections. The coefficient of variation for these measures was 1.4%. Alcian Blue and Neutral Fast Red staining was used to detect goblet and epithelial cells in the colon. Goblet cells were stained blue with Alcian Blue and epithelial cells were stained pink with Neutral Fast Red. They were quantified by goblet to epithelial ratio using 6 crypts

per colon section from each mouse for all treatment groups. The optimal number of crypts was determined by assessing goblet cell content in 2, 4, 6, 8, 10, 12, and 14 crypts per tissue section. The variance decreased from 2 to 6 crypts and did not substantially differ after 6 crypts with increased sampling. This measure was repeated three times on three tissues to ensure reliability. Furthermore, several tissue sections were recounted for goblet cell content and we found the coefficient of variation to be 1.4%.

Gut Permeability

Gut barrier integrity was assessed in all mice by permeability to FITC-dextran (MWav= 4000; FD4) (Sigma Aldrich). The FD4 was administered by gavage based on the animal's body weight in grams and diluted with 125mg/ml of PBS to five hour fasted mice. Plasma was sampled before and 1 h after FD4 administration and measured for florescence as previously described by Yang et al., 2003.

Enzyme-linked Immunosorbant Assay

The RPMI medium supplemented with secreted colon cytokines was used to quantify the local concentrations of TNFα and IL-1β (BD biosciences) using commercially available BD OptEIA enzyme-linked immunoabsorbant assay (ELISA) kits, according to the manufacturer's instructions. The local cytokine concentrations were normalized by the estimated protein content in the colon supernatant by a Bradford protein assay.

Western Blotting

Colon tissue samples frozen at -80°C were homogenized in RIPA buffer that was supplemented with protease and phosphatase inhibitors (SIGMA). The samples were then centrifuged at 10,000 rpm for 15 minutes and the supernatant was collected for protein

analysis by a standard Bradford assay. Protein homogenate from all groups was electrophoresed on 7% SDS-PAGE gels and transferred to a nitrocellulose membrane for 3 hours at 4°C. The membrane was blocked by 1X PBS and 0.1% Tween 20 for 1 hour and subsequently probed for ZO-1 (Abcam) and MUC-2 (Abcam) overnight. The bands were detected using chemiluminescence and normalized relative to GAPDH expression (Genetex). The bands were quantified by densitometry and expressed as mean area density by using Image J software (Image J).

Statistical Analysis

SAS/STAT statistical software version 9.3 was used to perform all statistical analysis. Two-way repeated measures analysis of variance (ANOVA) was used to determine significance and interactions in clinical scores between experimental groups. In this analysis, the repeated measure was time. Two-way ANOVA with Tukey post hoc analysis were used to determine significance and interactions for all other variables in control and experimental groups. Pearson correlations were used to determine links between clinical score and goblet cell content. Statistical significance was considered at $p < 0.05$ level of confidence.

RESULTS

Clinical Score

Clinical score is a tool to to assess the severity of DSS-induced colitis. Experimental mice were observed and assigned a clinical score every alternate day during the study. Clinical scores increased from the start of DSS administration until day eight in mice treated with DSS alone. DSS treated mice given a high Se diet showed similar clinical scores as the control diet group during the first six days of DSS treatment.

However, we saw that Se reduced clinical score significantly in the last two days of the study ($p < 0.01$) (Figure 2.1). To elucidate the role of Se diet in reducing clinical score in the last few days of the experiment, we looked at colon tissue sections to assess the histopathology.

Colon Morphology

DSS-induced colitis is associated with increased inflammation, distorted crypts, and high immune cell infiltration. Colon tissues were sectioned for investigation of the degree of inflammation and infiltration of immune cells. Figure 2.2 shows representative images of colon morphology and analysis of histopathology. Control mice without DSS treatment showed no inflammation immune cell infiltration. Furthermore, mice administered only a high Se diet showed no change in inflammation and immune cell infiltration compared with controls. DSS treatment alone showed a significant increase in inflammation and immune cell infiltration over controls. However, mice treated with DSS and given a Se rich diet showed 60% less of a degree of inflammation and infiltration of immune cells as compared to their control diet counterparts (Figure 2.2B). In order to understand how Se attenuates colitis, we studied gut barrier integrity by looking at goblet cell content.

Goblet cell content

Mucus plays a primary role in protecting the gut barrier. It is secreted from goblet cells that lie within the colon crypts. Goblet cells were stained blue with Alcian Blue and epithelial cells were stained red with Nuclear Fast Red. The cells were quantified histologically at 40X, but are represented at 20X (Figure 2.3A). Mice treated with DSS alone showed significantly reduced goblet to epithelial cell ratios compared to controls.

Treatment with high Se diet alone showed no change over controls. However, mice given DSS and a high Se diet showed higher goblet cell content compared to controls (Figure 2.3B). Due to these positive findings, we ran a correlation between goblet cell content and clinical score. We found a strong negative trend between the two outcomes (r = -0.677) (Figure 2.4). To further elucidate Se ability to attenuate colitis we measured ZO-1, which is another protein associated with gut barrier integrity.

Expression of ZO-1

Tight junctions adhere to the apical surface of epithelial cells and prevent passage of noxious agents. ZO-1 is a scaffolding protein associated with the assembly of tight junctions and expression is reduced in DSS-induced colitis (Poritz et al., 2007). Figure 2.5 shows the expression of ZO-1 in control and experimental mice. There was a main effect of Se rich diet to increase the tight junction expression of ZO-1 regardless of DSS treatment. Furthermore, there was a main effect of DSS to increase the expression of ZO-1 irrespective of diet. These findings are interesting, but may reflect the tissue sampling and that it was taken 5 days post DSS treatment and some repair has been initiated.

Colon tissue-secreted inflammatory cytokines

Colitis is associated with increased secretion of inflammatory cytokines and immune cell infiltration. Previous research studies have shown Se to have an anti-inflammatory nature through its families of selenoproteins (Huang et al., 2012). We measured the levels of two cytokines that can be secreted during DSS-induced colitis in order to establish Se effect on these cytokines within this model (Figure 2.6A-B). We found similar levels of cytokine expression between all groups. Further analysis revealed no significant main effect of either diet or DSS treatment on the secretion of cytokines.

Gut barrier permeability

Another way to assess gut barrier integrity is to assess its permeability to fluorescent-labeled sugars. FITC (or FD4) is a sugar that is too large to pass through the tight junctions or epithelial membranes. Colitis has been associated with damage to the gut barrier and higher gut permeability. In the current study, integrity of the gut barrier was assessed by administering FD4 to mice 1 hour before sacrifice and measuring its concentration in the plasma. Gut permeability was found to be at similar concentrations between the groups. In this experiment, we found no main effect of DSS treatment or Se rich diet. Furthermore, due to high variability within each group the results could not be accurately interpreted (Figure 2.7).

Mucus protein expression

Goblet cells are known to secrete different mucins that aid in building the mucus layer of the gut barrier. MUC-2 relative expression levels were studied to see if the rise in goblet cell content relayed higher mucus secretion. MUC-2 expression levels were found to be similar between the groups. In this experiment, we found no main effect of DSS treatment or Se rich diet. Furthermore, duet to high variability between the groups these results cannot be accurately interpreted (Figure 2.8).

DISCUSSION

Inflammation is becoming a larger part of research, as it is known to be involved in the pathways leading to other diseases, such as cancer. Inflammatory research in the gastrointestinal tract has been focused on IBD and its mechanisms of development (Xavier, 2007). The appearance of IBD usually occurs after several acute, unresolved bouts of colitis. These acute instances may be brought about by genetic factors, microbial

factors, or more recently investigated environmental factors (Xavier, 2007). Studies are broadening the scope to include environmental causes, since only 20% percent of CRC cases are related to genetics (Keshavarzian, 2003; Clayburgh 2004). More specifically, they are delving into how inflammation stemming from diet or lifestyle effects gut barrier function. The gut barrier is involved in regulating the permeability of ions and solutes from the lumen to the blood stream. Higher permeability of the gut has been associated with inflammation (Clayburgh, 2004). Moreover, prolonged dysregulation of the gut barrier has been linked to IBD (McGuckin, 2009). Treatments let alone preventions for colitis-associated barrier dysfunction are minimal. Se is a well-known antioxidant that has been linked with decreased inflammation in mouse models of chronic inflammation induced colon cancer (Krehl, 2012). However, there are gaps in the literature that elucidate the role of Se in gut barrier function in models of acute inflammation. The present study aims to reveal how Se impacts the severity of DSS as well as how it may impact the gut barrier function in a mouse model of acute colitis.

The experimental model used in the current study was administration of Se rich diet (0.75 ppm) one week before DSS treatment as a preventative to acute colitis and compare findings to mice given a control diet (0.02 ppm). The first aim of the current study was to assess the severity of colitis in mice given a Se rich diet and compare to control diet. The severity of colitis was assessed by assigning clinical scores to experimental mice after the induction of DSS. Mice given a Se rich diet revealed lower clinical scores during the last few days of the experiment when compared to their control diet counterparts (Figure 2.1). These findings indicate that DSS onslaught in the colon is not eradicated by Se, but that Se supplementation may help restore homeostasis faster

than a control diet. One possible mechanism by which Se may reduce the severity of colitis is by increasing activity of GPx enzyme activity. Tham et al., 2002, saw GPx activity rise in mice after induction of DSS for 7 days even though this study did not include Se rich diets (Tham, 2002). These findings suggest that inflammation allows endogenous sources of GPx enzymes to aid in resolving damage and allow restoration to occur. The current study findings may indicate that Se rich diets are adding to the endogenous sources of GPx and thus aiding in a faster recovery from colitis. In order to see if the mice are recovering from the onslaught of DSS the morphology of the colon was studied. Figure 2.2 indicates that experimental mice receiving a Se rich diet showed less inflammation and immune cell infiltration of the colon as compared to control diet. Furthermore, the morphology of the colon in these mice showed an appearance quite similar to the control mice receiving no DSS. These findings reveal that Se may be helping to reduce the severity of colitis, indicated by clinical score, by improving the morphology of the colon to a control-like appearance. In order to fully understand the role of Se in restoring colon morphology we examined the gut barrier function and its associated structures and proteins.

The second aim of the current study was to examine Se role in gut barrier function. Goblet cells are considered a large part of the gut barrier, since they provide the secreted mucins that protect the single layer of epithelial cells at the lumen surface (Johansson, 2013). Goblet and epithelial cells were quantified under a microscope to see if Se played a role in varying goblet cell content. Figure 2.3 shows that experimental mice receiving a Se rich diet had a large effect on increasing goblet cell content. Furthermore, colitis-induced mice receiving the Se rich diet had similar goblet cell

content as the control mice. Studies suggest that less secreted mucins compromise the thin epithelial layer and may cause colon inflammation by bacterial translocation (Johansson, 2013). The current findings could indicate that Se helps increase goblet cell content in order to provide more mucus secretion for gut barrier protection. These results are similar to ones seen in mice given trinitrobenzenesulfonic acid-induced colitis with either vitamin E and/or Se administration (Ademoglu, 2004). However, since there was interaction between Se diet and DSS treatment we cannot be certain of these interpretations. To see if change in goblet cell content induced a change in secreted mucus the intestinal mucus secretion protein MUC-2 was analyzed in expression studies. Figure 2.8 shows similar MUC-2 expression levels across all groups. These data occurred high variability and thus cannot be accurately interpreted. However, in our lab we have found that Se increases goblet cell content and MUC-2 secretion in chronic inflammation-induced colon cancer. (Saxena, Unpublished Data). A Pearson correlation was ran between clinical score and goblet cells and it shows a strong negative trend (Figure 2.4). These findings suggest that Se works to reduce clinical score by increasing goblet cell content. However, this is only representative of a trend and should be further investigated.

Additionally, tight junctions are a critical part of the gut barrier function as they provide a tightly sealed barrier against lumen pathogens. ZO-1 is a major scaffolding protein of tight junctions and when expression of this protein is dysregulated permeability of the gut barrier tends to increase (Poritz, 2007). Figure 2.5 shows significantly higher expression of ZO-1 in mice given Se rich diets as compared to those on control diets. Furthermore, there was higher ZO-1 expression in experimental mice as compared to

control mice. The latter was an unexpected finding. Martin et al found that Se may increase ZO-1 expression in a model of human breast cancer (Martin, 2007). However, to date there are no studies involving Se and ZO-1 expression in the colon. The current study findings may indicate that Se may be healing the gut barrier by expressing more ZO-1, which could be linked to less inflammation and decreased clinical score seen in these mice. However, further analysis is needed in order to elucidate the findings of increased ZO-1 expression in mice with DSS and Se rich diets. Gut barrier structures and associated protein expressions are good indicators of in tact gut barrier function, but investigation in the permeability of the gut barrier will provide more insight to its integrity. Gut barrier permeability was assessed to observe the integrity of the gut and compare these findings with gut barrier associated proteins. Figure 2.7 shows no significant difference between any of the groups. These findings were very unexpected possibly due to a miscalculation resulting in ten times more FD4 administered than required. Furthermore, due to high variability between the groups these results cannot be accurately interpreted.

Inflammation is known to cause increased circulating and secreted cytokines. The current study observed specific cytokines TNF-α and IL-1β, since they have been found to disrupt tight junction proteins and allow higher gut permeability (Turner 2009 and Wang 2005). Figure 2.6 shows no significance across the groups. However, control mice given a control diet showed higher cytokine secretion as compared to those mice on a Se rich diet. Furthermore, experimental mice given Se rich diet showed higher cytokine secretion as compared to their control diet counterparts. Increased inflammation may have a dual role. Acutely, it is needed to bring about immune cells and clotting agents to

drive resolution (Medzhitov 2008). These data suggest that Se is working in favor of higher secreted cytokines to bring down clinical score and aid in the recovery of colon morphology. However, further investigation is needed to clarify these findings.

There were some limitations in this study. Some of the measurements taken were extremely variable. In order to get smaller standard errors one must input more mice to each group. The timing of this study was staggered due to limitations in mice received per week from the breeding colony. The 33 mice obtained for this study were obtained over four months. Another limitation was a miscalculation in the amount of FD4 given to the mice, which resulted in ten times the amount administered.

In conclusion, Se was shown to have beneficial effects on the morphology of the colon as well as to some extent the gut barrier proteins. These positive effects may be the reason we saw decreased severity of colitis seen in C57BL/6 mice given DSS-induced colitis. However, future experiments need to focus on gut permeability and its relation to inflammatory cytokines and tight junction expression in order to fully understand the mechanism behind Se role in gut barrier recovery in colitis.

FUTURE DIRECTIONS

Some future directions would be to study Se on gut barrier function in a chronic inflammatory setting and compare those findings to the ones presented in the current study. Additionally, it would be interesting to include multiple sacrifices at multiple time points throughout the study to see how the gut barrier is changing with Se rich diet. Another direction would be to further investigate the timing of Se administration. The present study gave Se diets as a preventative one week before DSS treatment. It would be

interesting to study the severity of colitis if Se diet was given as a preventative for three to four weeks before DSS treatment. This could possibly reveal a larger reduction in the severity of colitis.

FIGURE LEGENDS

Figure 2.1. Effect of Se rich diet on clinical score. Clinical score for DSS treated Control (Co + DSS) and Selenium (Se + DSS) WT mice during the last 10 days of the study. Weightloss, diarrhea, and fecal hemoccult were used to calculate the clinical score. * $p < 0.01$ (DSS vs DSS + Se).

Figure 2.2 A-B. Effect of Se rich diet on the morphology of the colon. (A) Representative Hematoxylin and Eosin stained images (20X) of the transverse colon section for all treatment groups. **(B)** The graph shows quantitative measure of the degree of inflammation and infiltration of immune cells in the transverse colon for all treatment groups. Data are expressed as a means ± SE. # $p < 0.05$ (Different from all other groups).

Figure 2.3 A-B. **Effect of Se rich diet on goblet cell content**. **(A)** Representative Alcian Blue and Nuclear Fast Red stained images (20X) of the transverse colon section for all treatment groups. **(B)** The graph shows quantitative measure of the ratio of goblet to epithelial cells. Quantification performed on colon images at (40X). Data expressed as a means ± SE. # $p < 0.01$ (Different from all other groups); * $p < 0.05$ (DSS + Se vs Control).

Figure 2.4. **Correlation between clinical score and goblet cell content.** Clinical Score negatively correlates with higher goblet cell content measured in experimental mice. Data are expressed as a means ± SE. $r = -0.677$

Figure 2.5 A-B. **Effect of Se rich diet on tight junction protein ZO-1. (A)** Representative western blot image of the expression levels of ZO-1 as well as a ponceau stain. **(B)** Protein expression level of ZO-1 in colon tissues from all groups of mice. Data are expressed as a means ± SE. * $p < 0.05$ (Represents a main effect of Se diet or DSS treatment)

Figure 2.6 A-B. **Effect of Se rich diet on colon tissue-secreted cytokines.** The graph above shows profiles of cytokines **(A)** TNF-α and **(B)** IL-1β that were secreted from colon tissue of mice belonging to all treatment groups. Data are expressed as a means ± SE. No significant difference was detected between groups.

Figure 2.7. Effect of Se rich diet on gut permeability. Gut permeability measured by administration of FD4 by gavage 1 hour before sacrifice in C57BL/6 mice for all treatment groups. Data are expressed as means ± SE. No significant difference was detected between groups.

Figure 2.8. **Effect of Se rich diet on MUC-2 expression. (A)** Representative western blot image of the expression levels of MUC-2 as well as a ponceau stain. **(B)** Protein expression level of MUC-2 in colon tissues from all groups of mice. Data are expressed as a means ± SE. No significant difference was detected between groups.

FIGURES

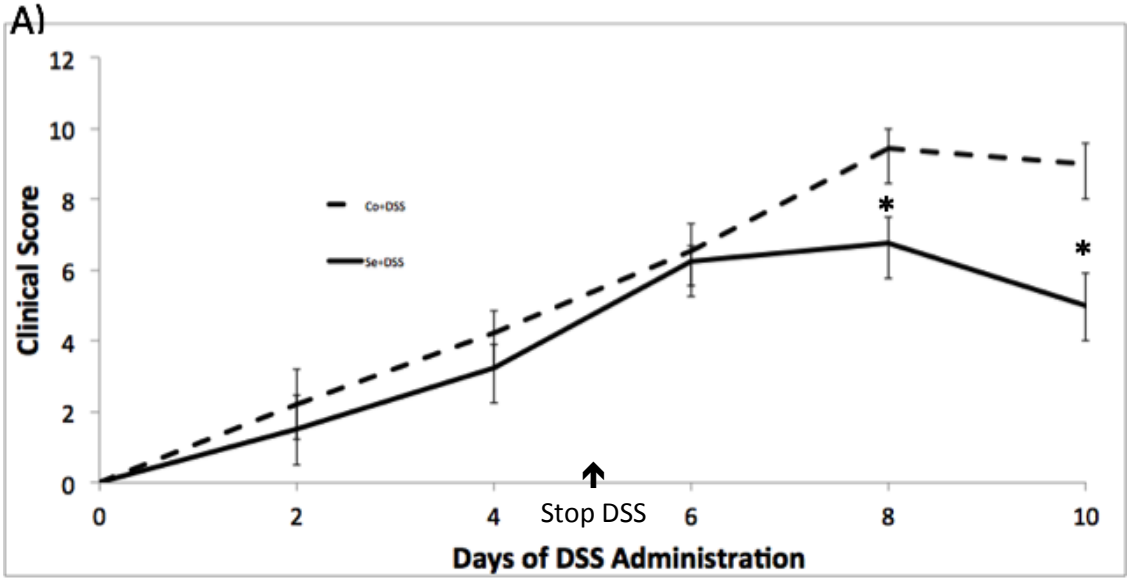

Figure 2.1. Effect of Se rich diet on clinical score.

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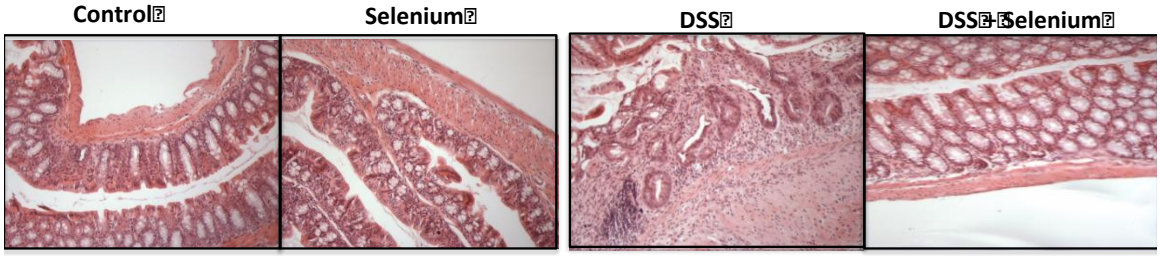

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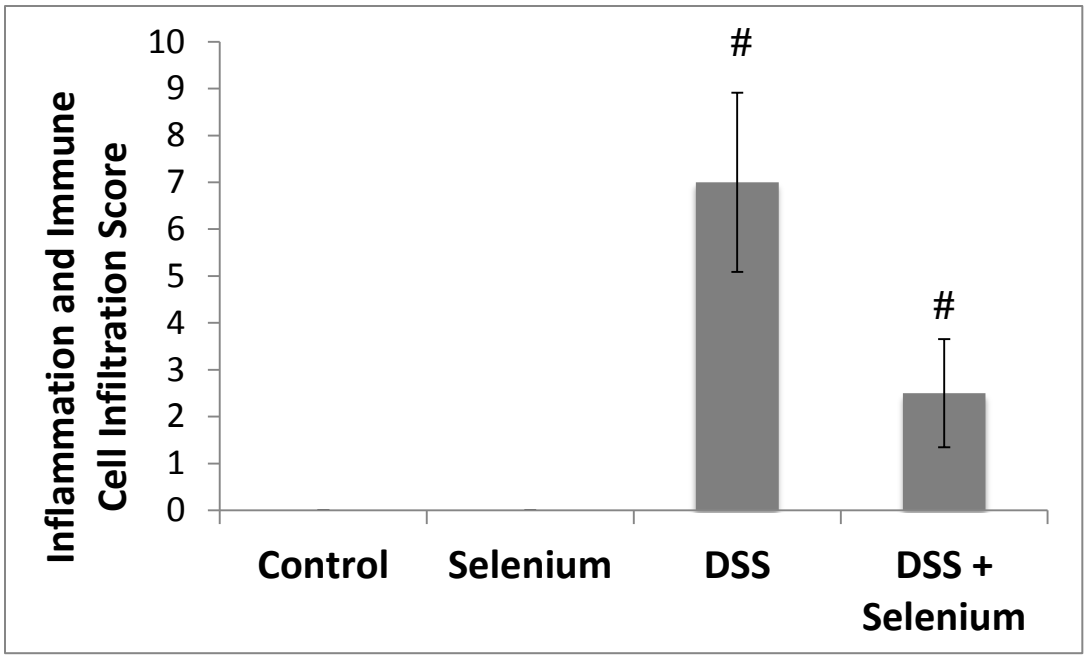
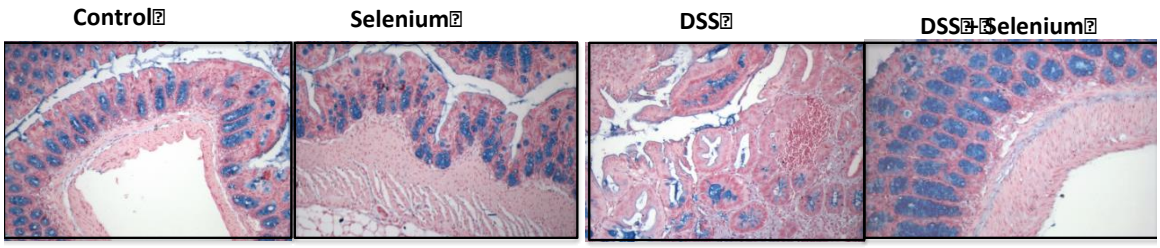


Figure 2.2 A-B. Effect of Se rich diet on the morphology of the colon.

A)



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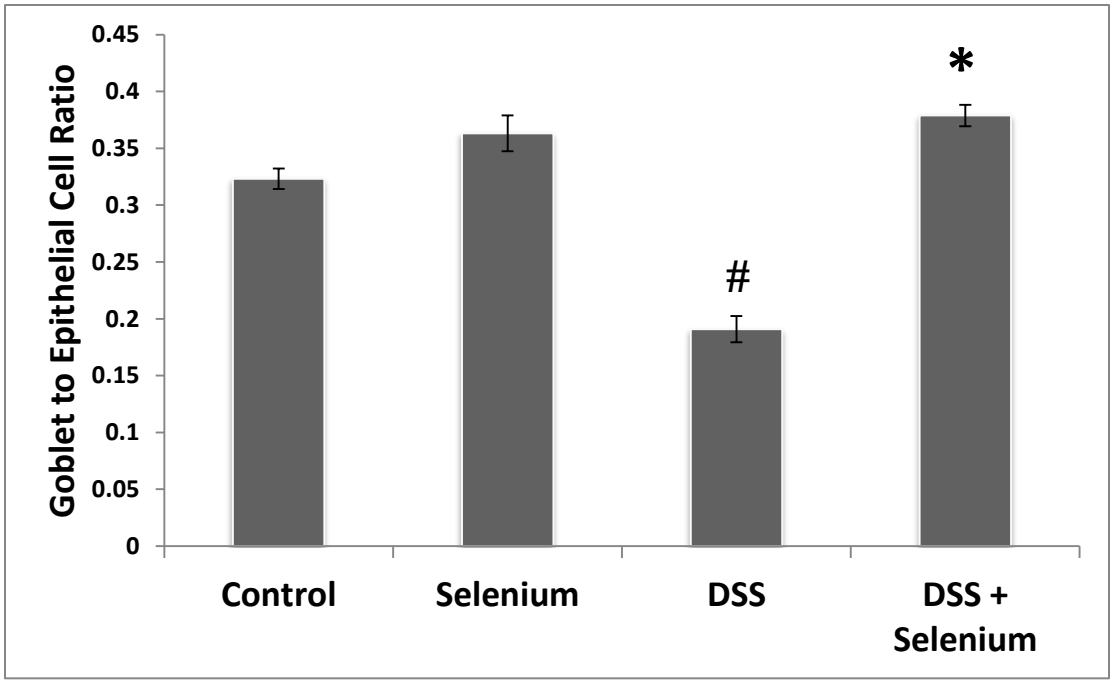


Figure 2.3 A-B. **Effect of Se rich diet on goblet cell content**.

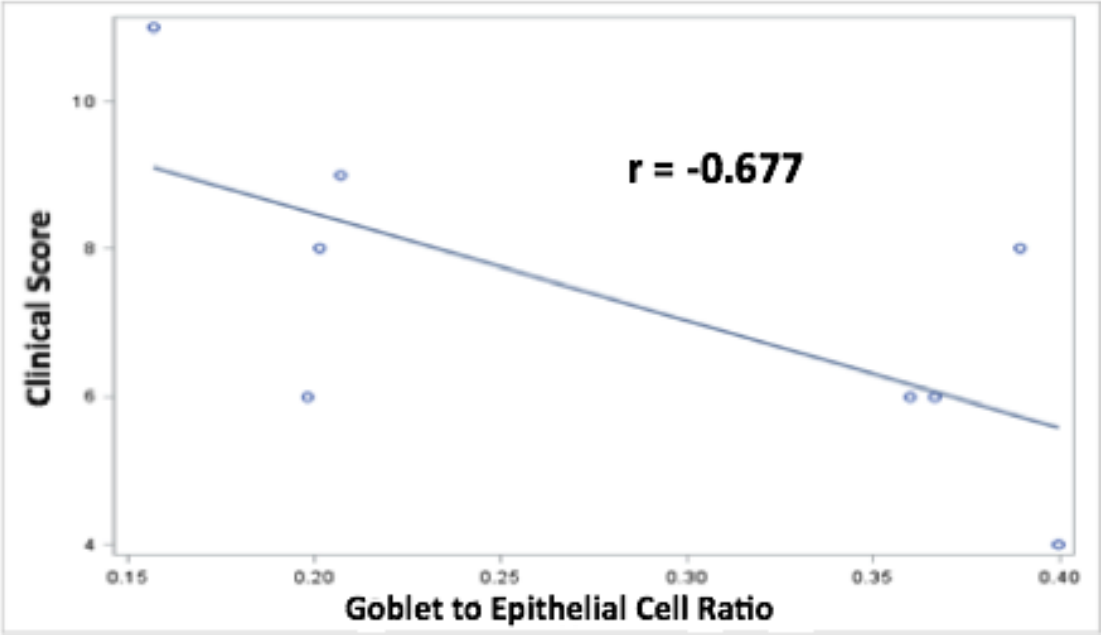
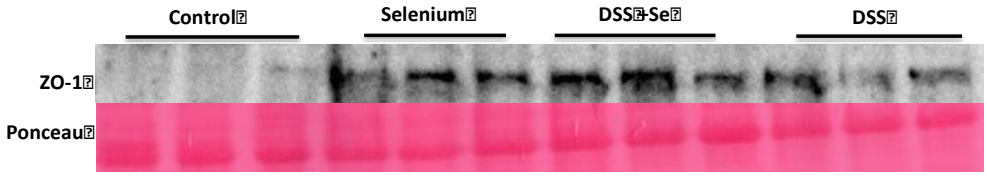


Figure 2.4. **Correlation between clinical score and goblet cell content**

A)



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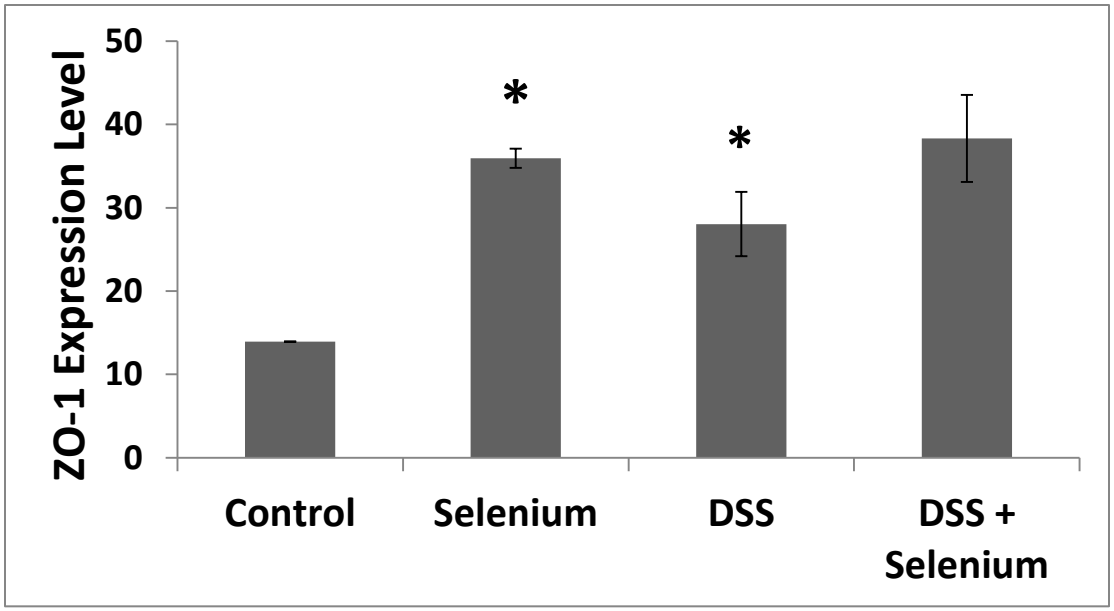
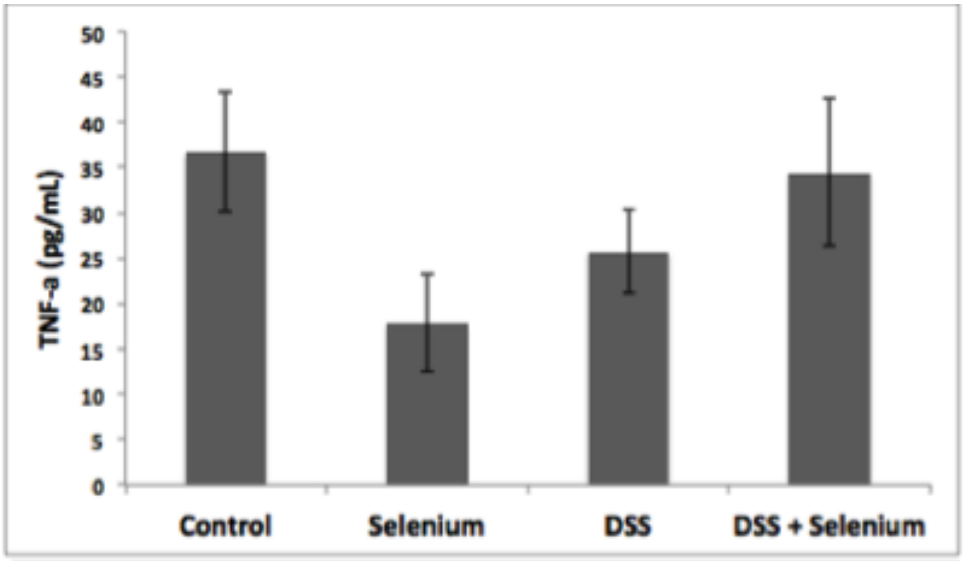


Figure 2.5 A-B. **Effect of Se rich diet on tight junction protein ZO-1**

A)



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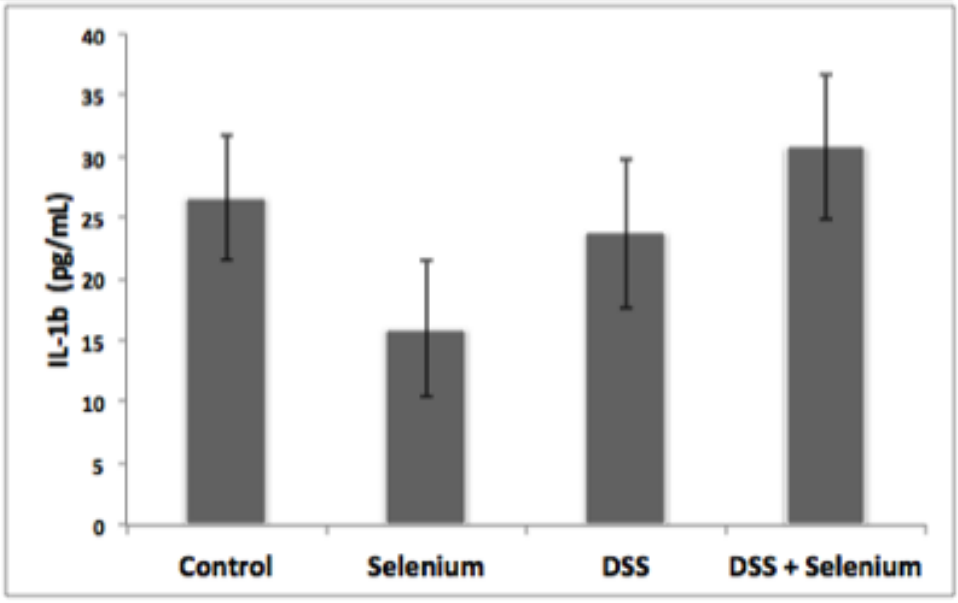


Figure 2.6 A-B. **Effect of Se rich diet on colon tissue-secreted cytokines.**

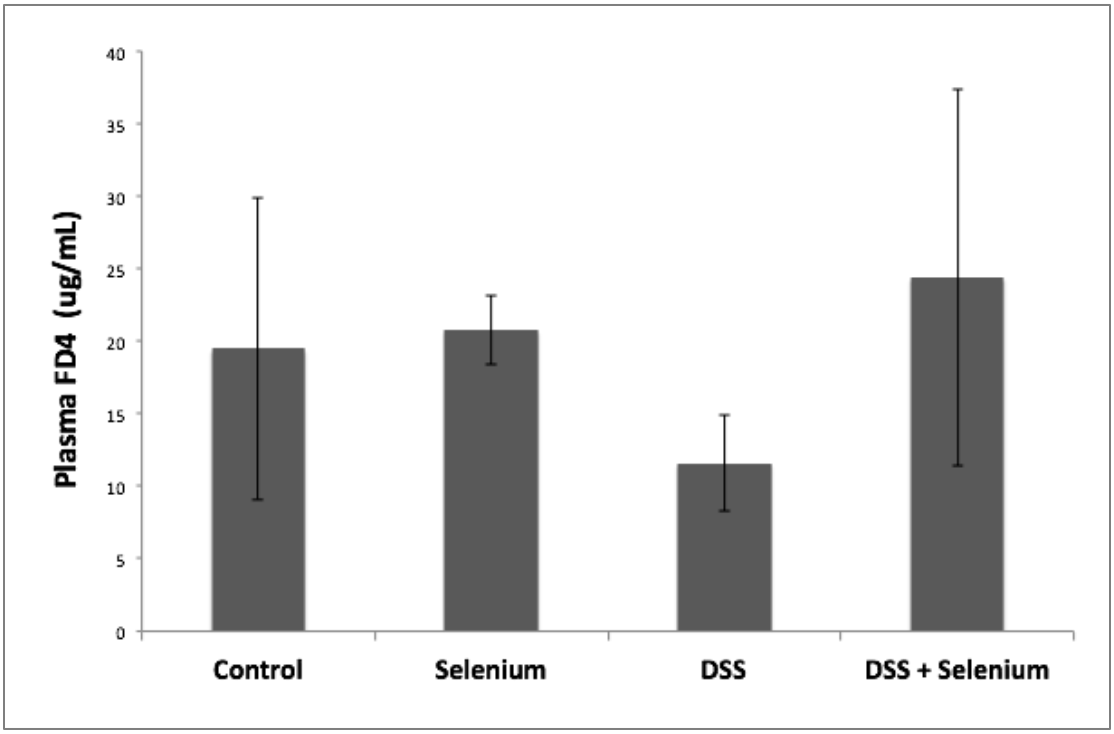
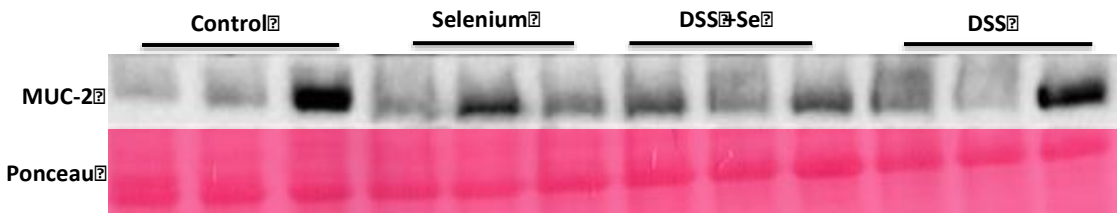


Figure 2.7. Effect of Se rich diet on gut permeability.

A)



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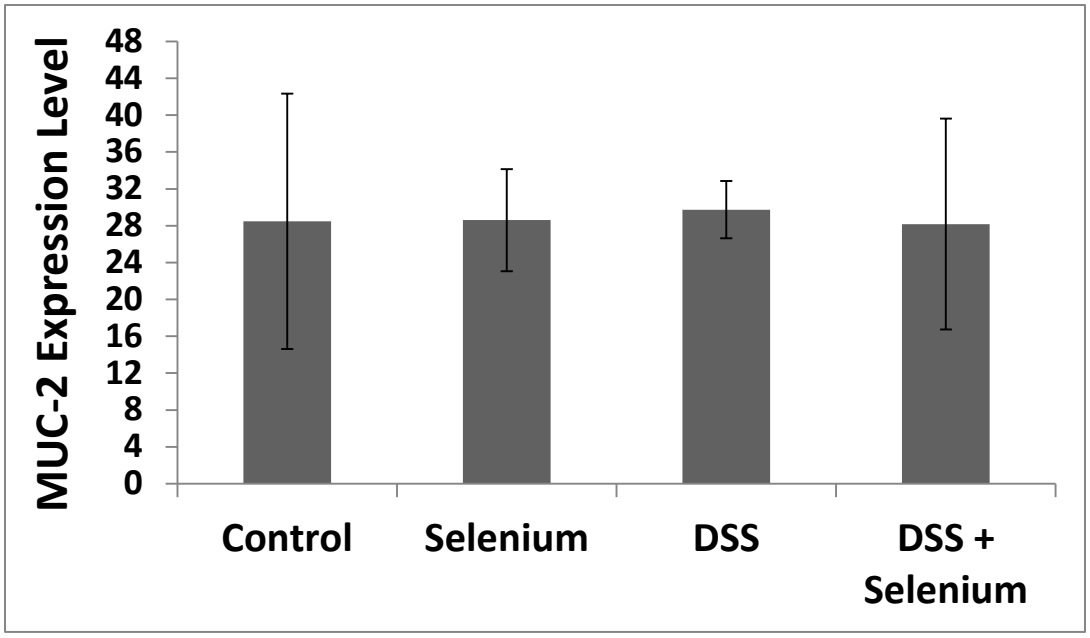

Figure 2.8 A-B. **Effect of Se rich diet on MUC-2 expression level**

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