Effects of Zinc Deficiency and Supplementation on

Dextran Sodium Sulfate (DSS)-Induced

Intestinal Inflammation in Growing Rats

By

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A thesis submitted to the Faculty of Graduate Studies
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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THE UNIVERSITY OF MANITOBA

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ABSTRACT

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Dextran sodium sulfate (DSS) treatment in rats has been found to be a human colitis-like model; it exhibits many morphological and pathophysiological features that resemble human ulcerative colitis. This study investigated the effects of Zn deficiency (ZD), energy restriction (PF), normal Zn (ZN), acute Zn treatment (ZT), and chronic Zn supplementation (ZS) on intestinal inflammation, trace mineral status, hematology, and metallothionein and caspase-3 (apoptosis marker) in small intestine and colon of DSS-challenged and unchallenged growing rats.

Providing 5% DSS in the drinking water for 4 days reduced body weight, feed intake and liver copper concentration, elevated white blood cell count, and resulted in diarrhea and bloody stool. The histology showed that DSS was specific for colon since intestinal inflammation was not present in the small intestine.

Dietary Zn did not alter the severity of ulceration in the colon. DSS-challenge attenuated the elevation of serum Zn in the ZT and ZS groups. Serum haptoglobin was elevated after DSS challenge in the ZS and ZN rats but not the other groups.

DSS-induced inflammation increased MT immunostaining in the colon submucosa, while MT immunostaining remained steady in the small intestine after challenge with DSS. MT immunostaining was almost absent in ZD while Zn supplemented rats had the strongest MT immunostaining in small intestine and colon.

In the small intestine, ZS rats had the lowest apoptosis and the ZD, ZT and PF rats had the most apoptosis after DSS challenge. In the colon, apoptosis was elevated after DSS challenge in all the groups except ZD and ZS.

The results of this study indicated that acute Zn treatment or chronic Zn supplementation did not reduce acute intestinal inflammation induced by DSS in growing rats.

ACKNOWLEDGEMENTS

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AE	Acrodermatitis enteropathica
AIN-93G	American Institute of Nutrition-93 growth diet for rodents
AIN-93M	American Institute of Nutrition-93 mature diet for rodents
ANOVA	Fisher's analysis of variance
BW	Body weight
Ca	Calcium
CAC	Colorectal carcinoma
CBC	Complete blood count
Cd	Cadmium
CD	Crohn's disease
CO_2	Carbon dioxide
CRIP	Cysteine-rich intestinal protein
CS	Corticosterone
CTL	Control group
Cu	Copper
DNA	Deoxyribonucleic acid
DNBS	Dinitrobenzene-sulphonic acid
DRI	Dietary reference intake
DSS	Dextran sulfate sodium
Fas	Fas-ligand

Fe	Iron
GC	Goblet cells
Hb	Hemoglobin
Hct	Hematocrit
H&E	Hematoxylin and eosin
Hg	Mercury
HGD	High grade dysplasia
IBD	Inflammatory bowel disease
IHC	Immunohistochemistry
IL-1	Interleukin-1
IL-18	Interleukin-18
kDa	Kilodalton
LGD	Low grade dysplasia
LP	Lamina propria
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MPO	Myeloperoxidase
MT	Metallothionein
MW	Molecular weight
PC	Paneth cells
PF	Pair-fed group
PCR	Polymerase chain reaction

RBC	Red blood cell
RDA	Recommended daily allowance
SEC	Surface epithelial cells
SEM	Standard error of the mean
SAS	Statistical analyses software
UC	Ulcerative colitis
UL	Upper limit
WBC	White blood cell
WT	Weight
ZD	Zinc-deficient group
Zn	Zinc
ZN	Zinc-normal group
ZS	Zinc-supplemented group
ZT	Zinc-treated group

I. LITERATURE REVIEW

Zinc (Zn) is important for many physiological and biochemical functions including maintenance of intestinal barrier, gut immune function, antioxidant activity, and inhibition of apoptosis (Blanchard et al, 2001). Zn deficiency impairs immune function and resistance to infection (Walker et al, 2004). Zn deficiency can be detected through a positive response to supplementation.

Zn supplementation has a therapeutic effect in patients with inflammatory bowel disease (IBD)(Luk HH et al, 2002). Although Zn absorption is decreased in patients with IBD (Banares et al, 1990), the therapeutic effect of Zn supplementation may involve other protective mechanisms.

Many studies have been done to understand the role of Zn in IBD, either by studying its role in antioxidant activity, or cell apoptosis, or inflammation. The focus of this study is to investigate effects of dietary Zn deficiency and supplementation on intestinal inflammation, metallothionein (MT) and apoptosis in a rat model of dextran sulfate sodium (DSS) induced colitis. The following sections will give a brief overview of the previously mentioned topics.

Zinc (Zn)

Zinc (Zn) was identified as a separate element in 1509 and its essentiality was demonstrated in plants and animals in 1934 (Shills, 1999). Zn has an atomic number of 30, and an atomic weight of 65.37. In pure form Zn is a bluish white metal. It occurs as five stable isotopes in nature (Shills, 1999). Zn complexes to amino acids, proteins, peptides, and nucleotides. Zn has an affinity to hydroxyl and thiol groups and for ligands

containing electron-rich nitrogen as a donor (Shills, 1999).

Because it is widespread in food, naturally occurring Zn deficiency was considered unlikely until 1955. Zn deficiency was suggested in malnourished Chinese patients during World War II. In 1956, Zn deficiency syndrome in humans was demonstrated (Shills, 1999). In rural Iran, the endemic hypogonadism and dwarfism were suggested to be derived from Zn deficiency, and this increased the appreciation of the public health significance of Zn deficiency (Shills, 1999).

Zn content in food varies widely ranging from 0.02 mg/100 g in egg whites, 1 mg/100 g in chicken to 75 mg/100 g in oysters (Shills, 1999). The total dietary Zn intake is influenced by food choices. Animal products provide almost 70% of the zinc consumed (Shills, 1999). Red meats (especially organs), eggs, and seafood (especially mollusks and oysters) are the best sources of Zn because of the absence of compounds that inhibit Zn absorption (phytate, oxalate, polyphenols, fibers, some vitamins and minerals) and the presence of compounds that maintain zinc solubility (citric acid, picolinic acid, prostaglandins, histidine, cysteine and glutathione (Groff et al, 2000; Shills, 1999). Drinking water provides only 2% of daily Zn intake (WHO, 1996).

In a typical adult Western diet, the Zn content is between 10 and 15 mg/day (Food and Nutrition Board, 1989). Some researchers suggest that the mean Zn intake for women is less than 10 mg/day and for men is less than 15 mg/day. The low intakes for women may be related to lower total energy by women rather than to the density of Zn in the diet (Groff et al, 2000).

The 2001 Dietary Reference Intake (DRI) recommends 11 mg/day for males and 8 mg/day for women because of their lower body weight. The Recommended Daily

Allowance (RDA) for male and female adolescents is 8 mg/day. The requirement for children is estimated at 3 mg/day for 1-3 year olds, and 5 mg/day for 4-8 year olds. The RDA for pregnancy is 11 mg/day due to the storage of zinc in the maternal bones which makes Zn somewhat unavailable. The Zn requirement for lactating women is 12 mg/day. The upper limit (UL) that is likely to cause no adverse effects is 7 mg/day for children 1-3 years old and 12 mg/day for children 4-8 years old and . 23 mg/day for adolescents. The UL for all other groups (male, female, pregnancy, lactation) is 40 mg/day (Anonymous (a), 2001).

Zn is present in all organs, body fluids, tissues, and secretions of the body. It is mainly an intracellular ion. More than 95% of total body Zn is found in the cell. Total body Zn content in adults is 1.5 g in women and 2.5 g in men (Shills, 1999). The majority of Zn is absorbed in the small intestine with smaller amounts absorbed in the stomach and large intestine (Zn absorption will be discussed in more detail in the next section). There are no specific Zn stores; high Zn intakes elevate bone, liver, and intestinal stores (Shills, 1999). The major route of Zn excretion is through feces with some urinary loss and skin loss (Shills, 1999). The total body Zn content is partially controlled by regulating the intestinal absorption and excretion from Zn pools (Shills, 1999). An inverse relationship exists between plasma Zn concentrations and its absorption; Zn deficiency stimulates gastrointestinal zinc absorption (Shills, 1999).

Zn is present in all body organs, mainly liver, kidney, muscle, bones and skin. Zn serves two main functions in the human metabolism: (1) as a component of metalloproteins (metallothionein, steroid receptors, thymulin and gene regulatory proteins), and (2) as a component of enzymes. There are more than 300 enzymes that

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need Zn as an indispensable structural part of the enzyme as catalytic factors or coactive associates (Jarres, 2001). When dietary Zn is inadequate to meet the body needs, zinc containing enzymes and metallothionein must supply the Zn (Groff et al, 2000).

Zn deficiency is defined as a low total body Zn mass, as calculated with Zn tracer studies. Zn deficiency is corrected by increased dietary Zn or Zn supplementation (Walker et al, 2004). Acrodermatitis enteropathica (AE), a genetic disorder affecting Zn absorption, is the most dramatic manifestation of Zn deficiency. If left untreated, AE can result in an overall mortality of 18% to 20% (Walker et al, 2004). Milder forms of Zn deficiency are more common and more difficult to recognize, but could result in impaired immune function and increased susceptibility to infection (Walker et al, 2004). Zn deficiency could occur when there is an increase in demand that is not met by dietary intake, for example, pregnancy, lactation, protein energy malnutrition and other disease states like inflammatory bowel disease (IBD) (Shills, 1999).

Zn absorption and transport

The small intestine, most likely the jejunum, is the main site for Zn absorption, however, the contribution of each segment of the intestine towards Zn absorption has not been established (Groff et al, 2000). Zn absorption varies from 12% to 59%. Recent studies suggest approximately 33% Zn absorption from non-vegetarian diets (providing approximately 11 mg of Zn), and 26% Zn absorption from lactovegetarian diet (providing almost 9 mg Zn) (Hunt et al, 1998).

Zn absorption appears to be enhanced by low Zn status (Mahon and Stump 2000). Zn absorption also depends on the presence of inhibitors or enhancers. Possible

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enhancers are citric acid, picolinic acid, prostaglandins and amino acids (e.g. lysine and glycine) which serve as ligands for Zn. Products of protein digestion could also serve as ligands. Pancreatic secretions are thought to have unidentified enhancers for Zn absorption.

Zn absorption involves a saturable carrier mechanism that works best at low Zn intake and low luminal Zn concentrations. A non-saturable passive mechanism involving paracellular movement is predominant with increased Zn intakes (Mahon and Stump 2000). Zn absorption reacts rapidly to changes in dietary Zn as it increases at low Zn intakes and it decreases with high dietary Zn intakes (Cousins et al, 1985).

In the lumen of the intestine, Zn ions are bound to amino acids or short peptides and the Zn ions are released at the brush border for absorption by a carrier mechanism (Mahon and Stump 2000). Two carriers have been identified: metallothionein (MT) and cysteine-rich intestinal protein (CRIP) which serve as intracellular binding ligands for Zn (Cousins et al ,1985). The Zn absorbed into the enterocyte has two possible fates: (1) stored or used within the enterocyte, or (2) bound to CRIP or MT and transported through the cell and basolateral membrane into plasma (Groff et al, 2000).

Zn passing from the intestinal cells to the portal blood is mainly transported bound to albumin. Transfer of Zn from the liver to blood is by albumin as well as α-2 macroglobin, transferrin, and immunoglobin IgG.. The albumin bound Zn seems to be the most readily taken up by tissues (Groff et al, 2000).

Several Zn transporters have been identified which function in the cellular uptake of Zn and intracellular movement of Zn. One family of Zn transporters, the hZIP proteins have 14 members identified in the human genome (Kambe et al, 2004), and they function

to move Zn into cells across the plasma membrane (Franklin et al, 2003). Another group of Zn transporters are the cation diffusion facilitator family (CDF, also named ZnT, and recently named SLC30A) (Seve et al, 2004). The SLC30A includes 10 members which have a distinct tissue distribution and subcellular localization and they play separate roles in intracellular movement of Zn (Zalewski et al, 2005).

Zn and other trace elements

Zn interacts with several nutrients, for example, copper (Cu), iron (Fe), and this is relevant to studies including a Zn supplementation phase.

Large quantities of Zn could interfere with **Cu** bioavailability because increased intake of Zn will induce metallothionein (Cu- binding ligand) synthesis. This protein will trap Cu and make it unavailable for absorption and Cu metalloproteins (Shills, 1999). One of the trials assessing impact of Zn supplementation (10 mg elemental Zn) on Cu levels found that six weeks of daily Zn supplementation of 83 adults in India and Pakistan resulted in decreased Cu levels from 1.01+/- 0.11 mg/L to 0.86 +/-0.04 mg/dl in the highest supplemented group. Six weeks after the end of supplementation, levels returned to normal (Abdulla et al. 1998). On the other hand, Sazawal et al. (2004) assessed a sample of 115 infants who received 10 mg Zn per day for 120 days. After the supplementation period, they found that there was no significant difference in Cu concentration among Zn supplemented or control infants (2.5 µg/dl and 5.5 µg/dl respectively) (Sazawal et al, 2004).

Fe supplementation inhibits Zn absorption. The two elements have chemically similar absorption and transport mechanisms and there is a limit for the

bioavailability of each (Walker et al, 2004). Many studies have been done to assess the interaction of Zn and Fe by looking at biochemical indicators. Zavaleta et al. (2000) studied the effect of Fe supplementation on hematologic changes during pregnancy, and the effect of adding Zn; they did not find an adverse affect of Fe and Zn supplementation. Zoltkin et al. (2003) evaluated the efficacy of Sprinkles formulated with Fe and Zn in anemic infants, compared with Fe alone. They found that in a controlled setting, home-fortification using micronutrient Sprinkles with Fe, or Fe and Zn, was very successful in the treatment of anemia; however, this intervention alone was not enough to improve Zn status or promote catch-up growth. Kaganda et al. (2003) studied the effects of Fe deficiency on changes in Fe and Zn metabolism and its possible interactions with dietary Zn. With development of severe Fe deficiency, rats fed the Zn-adequate diet had elevated levels of Zn concentration in the liver, plasma, kidney, spleen, and femur, and as a result, decreased Zn absorption due to increased levels of Zn in the tissues. The authors speculated that the decrease in Zn absorption and the increase in tissue Zn concentration of the rats might be due to a decreased Zn requirement and depressed Zn metabolism associated with Fe deficiency.

The focus of this thesis is the potential roles of Zn in preventing cellular damage using a model for intestinal inflammation. Although Zn may protect cells through several mechanisms, the next sections will focus specifically on the relationships of Zn with metallothionein and apoptosis.

Zn and metallothionein (MT)

Metallothionein (MT) is low molecular weight metal binding protein. It has 61

amino acids and 20 of them are cysteine (Bremner et al, 1990). Cadmium MT was the first MT to be isolated by Margoshes and Vallee in 1957 and it was considered to act via a detoxifying mechanism after exposure to heavy metals. The in vitro binding affinity of metals to MT in vitro is Zn< Cd< Cu< Hg (Holt et al, 1980). Zn is the primary MT inducer followed by Cu (Coyle et al, 2002).

MT plays a key role in Zn uptake, distribution, release, storage and absorption by competing with other transporter proteins or supplying them with Zn (Vasak et al, 2000). MT is present in the liver, pancreas, kidney, red blood cell and intestine. In the small intestine, MT is believed to regulate trace element absorption and excretion (Groff et al, 2000). Liver and red blood cell MT is reduced when dietary Zn decreases, hence MT has been proposed to reflect Zn status or stores.

Clarkson et al (1984) developed an immunohistochemical technique that was sufficiently sensitive to detect physiological quantities of MT in tissues from rats and humans. Their results indicated that MT is present in the hepatic blood vessels, liver, and ileum in both rats and humans, and that fasting and Zn injection affected the distribution and intensity of immunoreactivity. The staining variation could be related to fasting induced MT synthesis. Immunoreactivity was seen in portal veins in rats and humans suggesting the transport of Zn from the intestine to the liver involved MT (Mahon and Stump, 2000)

Szczurek et al. (2001) investigated the effect of dietary Zn deficiency and repletion on MT immunolocalization and MT concentration in the liver and small intestine of growing rats. They found that when the rats were fed a Zn deficient diet, they had no detectable MT immunostaining in the liver and the small intestine, and after 24-hr

Zn repletion, they found strong MT staining in the intestinal paneth cells and the surface epithelial cells in the proliferative region of villi. The pair fed control had strong MT staining in the liver localized around central veins and after 24-hr energy repletion, the hepatic MT staining faded. Szczurek et al. (2001) demonstrated that MT immunolocalization and concentration in the small intestine and liver were responsive to dietary Zn and cell-type-specific localization of MT in the small intestine. The MT localization in the small intestine suggested a role for Zn and MT in gut immunity and intestinal mucosal turnover.

A study by Paski et al (2003) assessed the relationship between histochemically reactive Zn and metallothionein bound Zn in intestine and liver under different Zn nutritional status. They used male Wister rats and fed them diets with three different Zn concentration (3 mg Zn/kg, 30 mg Zn/kg, 155 mg Zn/kg) for 2 or 6 weeks. After 6 weeks of dietary treatment, they found that dietary Zn intake had no effect on the abundance of the intestinal histochemically reactive zinc even though dietary Zn was associated with increased levels of MT (Paski et al, 2003).

Coyle et al (1999) studied the effect of MT on Zn transfer into non-gut tissues in MT null mice [(MT) +/+] and MT normal mice [(MT)-/-]. Each mouse was given an oral gavage of ^{65}ZnSO$_4$ solution containing 154, 385, 770, or 1540 nmol Zn per mouse. The mice were investigated 4 hours after giving the Zn solution. They found that the percentage of oral Zn dose retained in the body was not significantly different between MT null and MT normal mice. Blood and plasma ^{65}Zn concentrations increased progressively in the MT null mice at doses more than 154 nmol Zn whereas the corresponding values for the MT normal mice were approximately half. The MT null

mice had more ^{65}Zn in muscle, heart, brain and skin while the normal MT mice retained more ^{65}Zn in the liver. MT induction in the small intestine was maximal the 385 nmol oral dose of Zn. When ^{65}Zn (770 nmol) was provided in a solid egg white diet, MT normal and MT null mice absorbed one-fourth and one-eighth, respectively, less ^{65}Zn compared to the same amount of ^{65}Zn in the oral solution. The MT normal mice absorbed 2-fold more Zn from the egg white diet compared to the MT null mice. This study indicates the importance of MT for intestinal absorption of Zn and that gut MT presents an absorptive advantage only when Zn is incorporated into solid food (Coyle et al, 1999)

In 2000, the same group examined the effect of MT on intestinal Zn loss during acute endotoxin inflammation and 2 d food deprivation in MT normal and MT null mice (Philcox et al 2000). They used intraperitonial injections of bacterial endotoxin lipopolysaccharide (LPS) for endotoxin inflammation. Plasma Zn was reduced in the MT normal but not in the MT null mice. LPS injections produced a higher liver Zn (677 ±27 nmol) and a higher MT (106±2 nmol Cd bound/g) in MT normal mice than starvation for 2 days. LPS injections in MT null mice did not increase liver Zn or decrease plasma Zn. MT null mice fed the Zn deficient diet (0.8 mg Zn/kg) lost 10% of body weight over 25 days whereas there was no loss in weight in the MT normal mice. Philcox et al (2000) concluded that MT inhibits Zn loss from the intestine when MT is highly expressed but when uninduced (in Zn deficiency) MT appears to protect Zn and body mass decreasing only urinary and nonintestinal Zn losses.

There has been extensive investigation regarding MT and Zn, and the role of MT in Zn absorption, transport and storage. It is quite evident that MT plays a major role in

Zn maintenance, however, MT has an essential role with other minerals. These areas require more investigation.

Zn and apoptosis

Apoptosis is a major mechanism of cell death in the body and in cells. Apoptosis is a form of cell suicide characterized by a decrease in cell volume, dramatic condensation of the cell chromatin and cytoplasm, and nuclear DNA fragmentation (Calder et al, 2002). Apoptosis is a normal physiological process that enables important processes ranging from epithelial turnover to B- and T-cell development (Calder et al, 2002).

Several studies have indicated that cell apoptosis plays a role in Zn deficiency. Zn deficient animals have enhanced spontaneous and toxin-induced apoptosis in many cell types (Zalewski and Forbes, 1993; Shankar and Prasad, 1998). Thymic atrophy is a main feature of Zn deficiency and this atrophy is accompanied by apoptotic cell death of thymocytes (Calder et al, 2002).

Many studies have demonstrated that Zn is a regulator of lymphocyte apoptosis in vivo and in vitro (Zalewski and Forbes, 1993; Shankar and Prasad, 1998). In vitro, more lymphocytes and thymocytes undergo apoptosis when cultured in a Zn-free medium or with Zn chelators (Calder et al, 2002). On the other hand, apoptosis of T-lymphocytes induced by in vitro exposure to toxins and other agents is inhibited by the addition of high concentrations of zinc salts (Calder et al, 2002). It has been suggested that Zn is an important intracellular regulator of apoptosis, since lymphocytes keep intracellular Zn at levels somewhat above those needed to suppress apoptosis. In addition, a dose response

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relation exists between intracellular Zn levels and the degree of vulnerability to apoptosis (Calder et al, 2002). The mechanisms by which Zn affects apoptosis are not well understood, but it is likely that Zn acts at many levels. There is a correlation between inhibition of Ca^{2+}/Mg^{2+} DNA endonuclease activity and the inhibition of apoptotic DNA fragmentation (Shankar and Prasad, 1998).

Fraker (2001) has suggested that the primary reason for lymphopenia in Zn deficiency is decreased production of lymphocytes and increased cell death among precursor cells. King et al. (2002) studied the role of apoptosis in the loss of precursor lymphocytes in Zn deficient mice. They found that Zn deficiency increased apoptosis in the pre-T cell population. They also found that the rate of death in the pro-T-cells (CD4-, CD8-) was greater in Zn deficient mice. On the other hand, Zn deficiency did not enhance death in mature T-cells (King et al, 2002).

Another study by Fraker et al. (2000) demonstrated that Zn deficiency stimulates the hypothalamic- pituitary-adrenal cortex stress axis to produce corticosterone(CS) and that adrenalectomy provides protection from corticosterone induced apoptosis in Zn deficient mice. They demonstrated both in vivo and in vitro, that CS at concentrations seen in ZD mice would produce very similar losses of pre-B and pre-T cells. These data indicate the role of glucocorticoids in inducing apoptosis and disrupting lymphopoiesis.

The relationship between histopathological changes and apoptosis was investigated in Zn deficient rats in the thymus, testis, skin, esophagus, kidney, and liver. The seven week old male Sprague Dawley rats were given diets containing 0% Zn (50 μg Zn/100 g feed) or 0.02% Zn (20 mg Zn/100 g feed). Significant morphological changes were seen only in the 0% Zn diet group. There was atrophy of the thymus after 4 weeks,

oligospemia after 5 weeks, testicular atrophy with the loss of sperm cells and spermatocytes and thickening of the epithelium of the skin and esophagus after 10 weeks in the Zn-deficient rats. Increased apoptosis was observed in the thymus and testis at 1 and 3 weeks, respectively, and in the kidney and liver, apoptotic cells appeared after 13 and 34 weeks, respectively. It was concluded that the functional and morphological changes in the testis and the thymus of rats on the 0% Zn diet were due to elevated apoptosis and that even if the supply of Zn is stopped for only a short period of time, immunocytes and germ cells cannot survive sufficiently (Nodera et al, 2001).

The role of Zn in apoptosis could be summarized in three main areas (Chimienti et al, 2001): (1) Zn prevents DNA fragmentation by inhibiting Ca/Mg dependant endonuclease (Cohen et al 1984), (2) Zn chelation induces apoptosis in many cell lines (MacCabe et al 1993), and (3) Zn inhibits caspase-3 (Perry et al 1997). Caspases are cysteine proteases that are specifically activated in apoptosis. They are called executioners of apoptosis (Nicholson et al, 1999). Caspase-3 is an important member of the caspase family (Kumar et al, 1999). The human small intestine shows evidence of the presence of caspase-3 in the tip of the villi and the crypt base. In the colon, only the region of shedding at the luminal surface shows caspase-3 apoptosis with minimal apoptosis seen at the base of the crypt (Grossmann et al, 2002).

Inflammatory bowel disease (IBD)

Crohn's disease (CD) and Ulcerative colitis (UC) are the most common IBD disorders. They were first recognized in the late nineteenth/early twentieth century. Their etiology remains unclear; however, they are a subject of active research (Hugot, 2004).

Both are chronic, life long medical conditions. They are the most common cause of chronic gastrointestinal disease in the developed world (Longobardi et al, 2003). Although the incidence of IBD has stabilized in high incidence areas such as North America and northern Europe, IBD continues to rise in low incidence areas such as Asia, Southern Europe and some of the developing countries. As many as 2.2 million persons in Europe and 1.4 million persons in the U.S suffer from this disease (Loftus et al, 2004).

IBD is common in young people and has a major affect on quality of life (Russell et al, 2004). The causes of the disease are not well known but genetic, immune, and autoimmune phenomena are involved. Triggers of the disease are also unknown but likely involve viral or bacterial interactions with immune cells lining the mucosal wall of the intestinal tract, or environmental factors.

The episodes of the disease vary from mild to severe and unremitting. Patients suffering from IBD have a high incidence of food intolerance, and relatively low food allergy incidence (Mahon and Stump 2000).

Crohn's Disease (CD)

The pathogenesis of CD is complex, and genetic and environmental factors contribute to its etiology. CD involves an inflammatory response that begins under the mucosa and spreads outwards, penetrating all layers of the intestine, causing edema and submucosal inflammation leading to thickening of the bowel wall and scarring causing obstruction. Usually, it affects the lower part of the ileum but may occur in any part of the gastrointestinal tract (Vega et al, 2003).

The clinical course of CD is marked by periods of remission and exacerbation.

CD is characterized by abdominal pain, diarrhea, weight loss, and fever. Patients with CD frequently develop fistulas extending to the bladder and/or the surface of the skin. CD affects about 56% of patients before the age of 22 with similar incidence in both sexes (Vega et al, 2004).

Ulcerative colitis (UC)

UC is characterized by ulceration and inflammation occurring on the surface mucosa of the large intestine, which results in the passage of pus and blood in stool. The inflammation includes the rectum and extends in a continuous manner into the colon. Symptoms of UC include fever, bloody diarrhea, cramping, abdominal pain, rectal bleeding, anemia, and hemorrhage leading to toxic megacolon which could be fatal. Treatment of UC in acute stages is an elemental diet to decrease fecal volume. Drug therapy may include corticosteroids, sulfasalazine, antibiotics, antidiarrheal drugs, immunosuppressive agents and anti-inflammatory drugs. Surgical removal of the inflamed part is potentially curative (Mahon and Stump 2000).

IBD and MT

It has been shown in models of IBD that free radicals are involved in mediating intestinal damage. MT plays a protective role by attenuating free radical induced injury. In order to examine the protective role of MT against IBD, Bruwer et al. (2001) used an immunohistochemistry technique to assess the staining of MT in CD and UC patients. Their results showed increased expression of MT in the fibroblasts of all ulcerative and/or fissural lesions in UC and CD.

The over-expression of MT may lead to sequestration of Zn ions and as a result functional inactivity of the p53 tumor suppressor gene (Bruewer et al, 2002). The aim of Breuwer et al. (2002) study was to determine the potential role of MT in the carcinogenesis of ulcerative colitis (UC) as well as the possible effect on p53 function. Positive MT staining was seen in UC and in low grade dysplasia (LGD); there was less MT staining in high grade dysplasia (HGD) and colorectal carcinoma (CAC). Positive immunoreactivity of p53 was predominant in HGD and CAC but not in LGD and UC. They found that in histologically normal tissues near CAC there was significant MT staining in six of seven patients with simultaneous lack of p53 expression. Bruewer et al. (2002) suggested that MT over-expression could represent an important early step in CAC development independent of p53 expression and it should be investigated as an independent cancer risk factor in UC in the long term.

IBD and apoptosis

Tumor necrosis factor (TNF) is a common pathogenic factor in IBD (Kontoyiannis et al, 2001). It can trigger enterocyte cell death by apoptosis or necrosis depending on the activation or blockage of specific caspases (Ruemmele et al, 1999).

Studies in IBD animal models suggest that activated macrophage play an important role in mediating the disease process. One mechanism of macrophage loss from normal mucosa and of recruited macrophages from IBD mucosa is by apoptosis through the expression of the active form of IL-1 converting enzyme (McAlindon et al, 1999). This activation also leads to release of IL-1 and IL-18 which both contribute to intestinal inflammation (Siegmund et al, 2002).

IBD and Zn

Naveh et al. (1993) found that acetic acid-induced intestinal inflammation reduces absorption of zinc in the small and large intestine. They also found that a surgical procedure in the intestine reduces the absorption of Zn. Valberg et al. (1986) measured zinc absorption in 29 patients with IBD and found impairment in $^{65}ZnCl_2$ absorption in undernourished persons with severe or moderate disease activity. The IBD patients did not have biochemical evidence of zinc deficiency, or clinical signs of Zn depletion. Fernandes-Banares et al. (1990) studied serum levels of Zn, Cu, Se, and alkaline phosphatase activity in patients with IBD, and they found that more than 50% of the patients with active colonic or small bowel disease showed Zn levels less than the 15th percentile of the control group, and that the serum Zn levels remained low even after total enteral nutrition.

Many studies have investigated the effect of Zn supplementation on IBD. Luk et al. (2002) studied the protective action of Zn sulfate and the pathogenic mechanism of 2, 4-dinitrobenzene sulfonic acid-induced colitis in rats. They found that Zn given rectally has a therapeutic effect against colitis. Di Leo V et al (2001) evaluated the effect of Zn supplementation at a different dose in a model of experimental colitis in rats. Colitis was induced by intra-rectal instillation of dinitrobenzene-sulphonic acid. Rats received Zn sulphate, 2 mg/kg or 30 mg/kg, twice a day by gavage for 9 days, 3 days before the induction of colitis, or intrarectal instillation of Zn 20 mg/kg once daily 8 hours after the induction of colitis, for 6 days. They found that Zn treated rats had less diarrhea, lower colonic weight, and higher body weight than untreated rats but there were no differences

in macroscopic inflammation, adhesion, colonic distention, and neutrophil infiltration of the colonic mucosa. Zn supplementation did not affect mucosal Zn and Fe concentrations or plasma Zn levels in rats with colitis. MT synthesis in the colon was elevated in Zn supplemented control rats and to a lesser extent in Zn supplemented colitic rats. They concluded that Zn administration induces metallothionein synthesis, but it had little effect on the short term course of experimental colitis.

In an interesting study by Sturniolo et al. (2002), the effect of Zn supplementation on small and large intestine tight junctions in rats with induced colitis was evaluated. Rats were given 2 or 30 mg/kg body wt Zn or glucose by gavage starting 3 days before colitis was induced by intrarectal administration of dinitro-benzene-sulfonic acid, for 7 days. It was found that the number of perfused tight junctions complexes were significantly higher in rats with colitis than in controls, and higher in the rats with colitis given high dose Zn. They concluded that Zn could regulate tight junction permeability with possible beneficial implications for healing processes in IBD.

Experimental models of IBD

Many efforts have been made to study experimental animal models of colitis in order to improve the medical treatment of this disease. Experimental models have proven to be important for detecting therapeutic agents and investigating the mechanism of pathogenesis. Over the past two decades more than 20 experimental models with different ranges of clinical manifestation similar to human IBD have been developed (Jurjus et al. 2003). An appropriate animal model should have certain key characteristics: the gut should display morphological alterations, inflammation, signs and symptoms,

pathophysiology, and a course similar to human IBD. In addition, it is recommended that the animal of interest should have a well defined genetic background and a well characterized immune system (Jurjus et al. 2003). Rat induced colitis is one of the ideal models because of its similarity to human IBD (same causal factors, pathology, histopathology, pathophysiology, and clinical spectrum). Most commonly used models are induced by administering toxic chemicals for example, acetic acid, formalin, indomethecin, carrageenan, trinitrobenzene sulfonic acid or polysaccharides such as dextran sulfate (Vowinkel et al. 2004).

Dextran sodium sulfate (DSS) induced colitis

After reviewing all the various models, DSS was chosen as the model for this study because of the short time of the exposure to the chemical for the rapid induction of inflammation, and the safety of using DSS in comparison to other more toxic chemicals. DSS is a heparin-like polysaccharide that contains three sulfate groups per glucose molecule. It is a sulfated polymer that is thought to induce mucosal injury and inflammation initially through a direct toxic effect on epithelial cells that resembles human inflammation (Vowinkel et al. 2004). Depending on the time course of oral administration in the drinking water, DSS induces colitis. Animals show different responsiveness to DSS-induced colitis depending on DSS concentration, molecular weight, and the duration of exposure, and the age and inbred strain of the animal (Vowinkel et al. 2004). For a reliable induction of colitis in mice, the following calculation was used to measure the DSS load in mice: (drinking volume (ml) x [DSS (g)/100 ml])/body weight (g). DSS load of $\geq$ 30 mg/g body weight for 7 days resulted in

a significantly elevated colonic myeloperoxidase (MPO) activity, if compared to mice receiving less DSS and controls.

The DSS- induced colitis model exhibits many morphological and pathophysiological features that resemble human UC, including mucosal damage, superficial ulceration, production of cytokines, leukocyte infiltration, and other inflammatory mediators. The exact pathogenesis of DSS-induced colitis is not clear. Nevertheless, a direct toxic action on colonic epithelium, macrophage enhancement by DSS, and altered colonic microflora have all been shown in the pathogenesis of DSS colitis. Many studies have used the DSS model for the induction of IBD.

Korkina et al. (2003) gave 200-220 g male Wister rats 5% DSS (40 KD M.W.) in the drinking water for 3 days followed by 4 days of treatment with 1% w/v DSS solution. They found that by the second day of 5% DSS administration, the rats had loose stool, swollen and reddish anus, weakness and slow movement. By the third day, all the rats suffered severe diarrhea, bloody stool, severely inflamed anus, and extreme weakness, and one rat died on day 3 of DSS administration. After switching to 1% DSS, the clinical symptoms were moderately improved, and diarrhea and bleeding in the stool remained unchanged until cessation of the experiment. Upon exposure to DSS, the colonic tissues demonstrated many changes in the parameters of oxidant/antioxidant status. Specifically, intensity of lipid peroxidation, $O_2^{\cdot-}$ production and GSH content were significantly increased. Glutathione peroxidase activity was elevated. On the other hand, tissue superoxide dismutase and catalase were sharply suppressed. The formation of OCL^- and $OH^{\cdot}$ and $O_2^{\cdot-}$ release was significantly inhibited upon treatment with DSS.

Masubuchi et al (2004) induced colitis by feeding 2 month old male Wistar

rats 3% DSS (M.W. 5000) dissolved in drinking water for 7 days. Treatment of the rats with DSS resulted in UC-like symptoms including severe bloody diarrhea. Mucosal injury and inflammation were demonstrated by a marked increase in MPO activity. Endotoxin levels detected in the portal blood of the rats treated with DSS were higher (endotoximia) than control ones, despite no signs of liver injury judged by liver weight and serum alanine aminotransferase leakage. The body weights of DSS-treated rats were not different from controls.

Osman et al (2004) investigated the modulation of DSS-induced acute colitis using different probiotic strains of lactobacillus and bifidobacterium. Sprague Dawley rats (200 g) were given 5% w/v DSS (36,000-50,000 KD) in drinking water for 7 days. There was no mortality and the disease activity index (body weight, stool consistency, rectal bleeding) were increased in severity on a scale of (0-4), but disease activity was decreased on days 4, 5, 6 and 7 due to the lactobacillus and bifidobacterium treatment.

Shimizu et al. (2003) administered 2%, 3%, and 4% DSS to 4 week old weanling rats and compared their clinical findings with control weanling rats and 8 week old rats given 4% DSS. The clinical symptoms in weanling rats given 4% DSS were more severe than the adult rats given the same treatment: 2 of 10 rats given the 4% DSS died during the experiment and 3 of 10 rats given the 2% DSS had no bloody stool. From this experiment they concluded that disease severity can be adjusted in UC for young animals by giving different concentrations of DSS.

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II. STUDY RATIONALE

There have been some studies about the effect of Zn on inflammation but the exact role has not yet been established. In our study, we would like to investigate the effects of Zn deficiency and supplementation on intestinal inflammation and examine the potential relationships with metallothionein and apoptosis in the small intestine and colon, hematology, and liver Cu and Fe.

There have been some studies in the literature about the effect of Zn on MT. For example, Szczurek et al (2001) demonstrated that MT immunolocalization and concentration in the small intestine and liver were responsive to changes in dietary Zn in growing rats but they did not investigate what happens during inflammation.

Zn involvement in apoptosis is not very clear, and as discussed earlier, apoptosis has two pathways. In our study, we will use caspase-3 to determine apoptosis as Didier et al (2004) provided the first evidence that moderate Zn deficiency elevated caspase-3 in several tissues including small intestine and colon.

There are conflicting opinions about the influence of intestinal MT on Zn absorption. Some researchers have proposed that mucosal MT sequesters Zn in the intestinal wall thereby reducing absorption and allowing Zn transfer back into the gut lumen. It has also been proposed that MT synthesis in mucosal cells is activated by increased luminal Zn concentrations but this is not significantly induced at normal dietary Zn intakes indicating that the influence of MT on Zn absorption is minimal (Cousin, 1985; Vallee et al, 1993; Lönnerdal, 1989).

There is not much research in the literature regarding zinc and hematology or the effects of DSS challenge despite presence of bloody stool. A study done by El-Hendy et al (2001) which investigated the effect of Zn deficiency on hematological parameters found that Zn deficiency had a negative effect on hematological parameters. Another study by Donmez et al (2002) on broiler chicks found no significant differences between control and Zn supplemented groups for hematological parameters. There does not seem to be any study reporting effects of Zn supplementation and deficiency on hemotology in DSS-challenged or normal rats.

To complete the picture, we chose to study the effect of Zn on Fe and Cu liver in normal and DSS-challenged states. Zn, Fe and Cu compete for absorption and transport in the intestine and high levels of Zn could have adverse effects.

Hypothesis

Our hypothesis is that acute and chronic Zn supplementation will increase MT immunostaining, and decrease cell apoptosis in the colon and small intestine of DSS-challenged rats resulting in less intestinal inflammation and injury. Conversely, Zn deficiency will decrease MT immunostaining and increase cell apoptosis, resulting in greater intestinal inflammation.

Objectives

1. To determine the effect of dietary Zn deficiency and Zn supplementation on mucosal injury (histology), inflammation (haptoglobin), MT (immunostaining), apoptosis (caspase-3), complete blood count and trace

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mineral status during acute DSS-challenge in growing rats.

2. To determine the efficacy of Zn treatment one day after induction of acute DSS-induced inflammation in Zn-deficient rats on mucosal injury, inflammation, MT, apoptosis, complete blood count and trace mineral status.

There are many examples in the literature of weanling rat models for developing Zn deficiency and for intestinal inflammation based on DSS-challenge. In our study, we wanted to study the effect of dietary Zn on intestinal inflammation. We completed 2 pilot studies of DSS (2% and 5% in drinking water). The 2% DSS produced very mild intestinal inflammation and the stool was soft with some blood. The 5% DSS produced moderate to severe intestinal inflammation, diarrhea and bloody stool. However, we could not prolong the study for more than 4 days after the 5% DSS treatment because during the pilot study some rats had to be euthanized between the fifth and sixth day. To ensure that all rats ended the study on the same day, we chose a 4 day exposure to DSS treatment.

The Zn-deficient diet contained 3 mg Zn/kg diet to have a moderately severe Zn deficiency. Since Zn deficiency decreases appetite and thus body weight, a pair-fed (PF) group was included. The PF rats had the normal amount of Zn in the diet, however, they were given the same amount of diet that was eaten by the ZD rats on the previous day. Since the amount of diet given to PF rats was restricted, they are an energy restricted group. Using the PF group helps us to determine if differences are due to Zn or the lack of energy. The control group was given a normal amount of Zn (30 mg Zn/kg diet)

based on the amount of Zn in the AIN-93G diet. The Zn-supplemented group (300 mg Zn/kg diet) was given 10 times the normal amount of Zn, an amount which increases Zn content of tissues without major adverse effects on other trace minerals.

Water intake was measured during DSS challenge to determine whether water intake and thus DSS consumption was similar among the dietary groups and whether any of the rats would develop dehydration due to the inflammation or Zn intake.

It is known that trace elements interfere in absorption, thus, liver iron, copper and Zn were assessed to determine the effects of deficient and supplemental amounts of dietary zinc. Fe, Cu and Zn were assessed in liver as it is the main organ for storing trace minerals. Serum and femur Zn were measured to confirm Zn status.

Complete blood counts were obtained as abnormality in the white blood cell count would be an indicator of inflammation, and this is used clinically by physicians. Haptoglobin is an acute phase protein for assessing severity of inflammation. It was used to determine whether Zn affects inflammation.

Histology of the small intestine and colon using H&E stain was used to assess the effect of DSS and Zn on mucosal injury. Since we used DSS as the inflammatory challenge and DSS is reported in the literature to be specific for the intestine, the tissues that we used for histology and immunohistochemistry were the small intestine and colon.

The intestine has a fast turnover of epithelial cells (3-5 days), thus caspase-3, an early marker of apoptosis was used for the immunohistochemical assessment of apoptosis. Immunohistochemistry was used for metallothionein detection so we could localize the cells present in the tissue with positive staining.

III. RESEARCH DESIGN AND METHODOLOGY

Animals, Diet and Study Design

Sixty-four 3 week old male Sprague Dawley rats (Charles River, St Constant, PQ) were acclimatized for 5-7 days and randomly assigned to one of four groups: Zinc-deficient (ZD, n= 32, 3 mg Zn/kg diet), Zinc-normal (ZN, n= 16, 30 mg Zn/kg diet), Zinc-supplemented (ZS, n= 16, 300 mg Zn/kg diet), or pair-fed (PF, n= 16, 30 mg Zn/kg diet) for 17 days (Figure 1). The PF group was given the same amount of feed as consumed by the ZD rats daily but were provided with the zinc normal diet (30mg Zn/kg diet). On day 17, half the rats in each dietary group (n= 8) received 5% DSS (M.W. 40 KD; MP Biomedicals) in their drinking water for 4 days. On day 18, half of the rats fed the Zn-deficient diet until one day after DSS induction were given the Zn-supplemented diet for the remaining 3 days and designated the Zn-treated (ZT) group (Figure 1). Thus, there were n=8 rats/group either receiving DSS treatment (ZD+, ZN+, PF+, ZS+, ZT+) or not receiving DSS treatment (ZD-, ZN-, PF-, ZS-, ZT-).

All diets (Table 1) were based on the AIN-93G (Reeves et al, 1993) except the diet contained egg white as the protein source (to formulate zinc-deficient diets), additional biotin (due to avidin in egg white) and additional potassium phosphate (for the AIN-93M-MX Mineral Mix to be equivalent to the AIN-93G-MX Mineral Mix). Feed cups were refilled 3 times weekly. Feed consumption was measured and corrected for spillage. The pair-fed rats were fed daily.
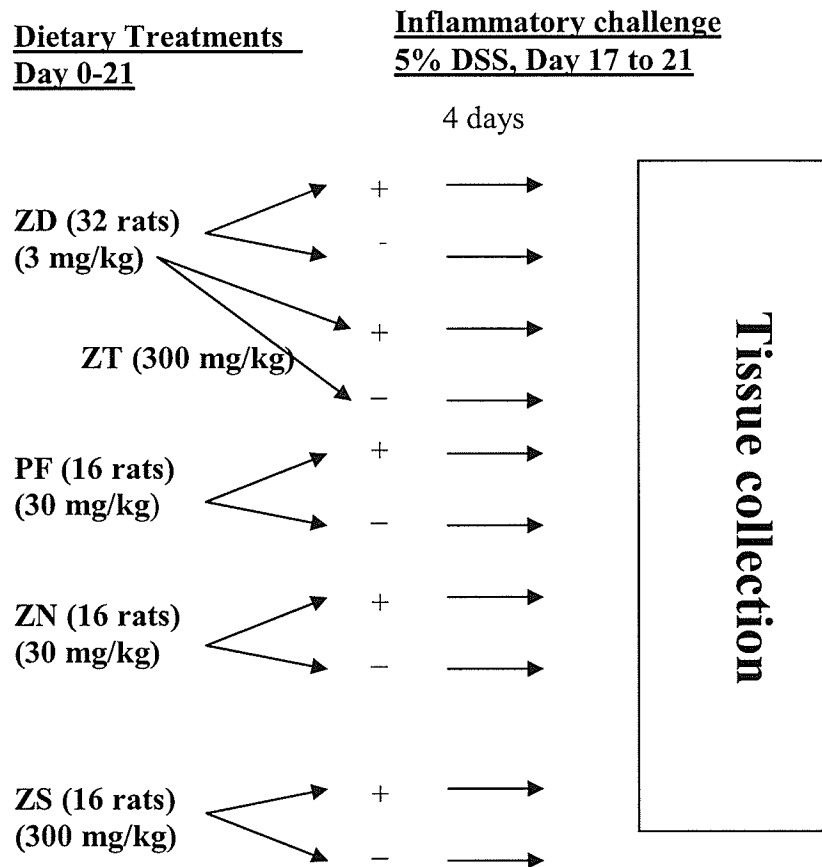
Dietary Treatments
Day 0-21

Inflammatory challenge
5% DSS, Day 17 to 21

4 days

ZD (32 rats)
(3 mg/kg)

ZT (300 mg/kg)

PF (16 rats)
(30 mg/kg)

ZN (16 rats)
(30 mg/kg)

ZS (16 rats)
(300 mg/kg)

Tissue collection

Figure 1 - Study design. ZD=zinc-deficient, ZT=zinc-treated, PF=pair-fed, ZN=zinc normal, ZS=zinc-supplemented groups, (+) = DSS challenge, (-) = without DSS challenge.

All rats were housed individually in a controlled environment (21-23°C; humidity 55%) in a 14:10-h light-dark cycle. Rats were housed in stainless steel wire-bottom cages to avoid mineral contamination and to collect food spillage. Deionized water was provided in plastic bottles with stainless steel sipper tubes to avoid mineral contamination. After DSS induction, water intake was measured daily. Body weight was

measured weekly, and after DSS induction, body weight was measured daily. Animal

care was provided in accordance with a protocol approved by the University of Manitoba

Protocol Management and Review Committee.

Table 1. Diet Formulation

Diet Ingredients[1] (g/6 kg diet)	ZD (3 mg Zn/kg diet)	ZN (30 mg Zn/kg diet)	ZS (300 mg Zn/kg diet)
Dextrose (cerelose)	3622.6	3567.6	3027.6
Egg white	1275	1275	1275
Fiber (cellulose)	300	300	300
Mineral mix (AIN-93M-MX Zn-free)	210	210	210
Potassium phosphate	32.4	32.4	32.4
Vitamin mix (AIN-93-VX)	60	60	60
Choline	15	15	15
Biotin mix[2]	60	60	60
Zn premix[3]	5	60	600
Soybean oil	420	420	420

[1]Diet ingredients were obtained from Harlan Teklad, except the dextrose (Moonshiners) and potassium phosphate (Fisher Scientific)
[2]200 mg biotin/kg dextrose
[3]5.775 g zinc carbonate/kg dextrose

Tissue collection and storage

Rats were euthanized by CO_2 asphyxiation. Body weight, body length and tail

length were recorded. Trunk blood was collected in tubes without coagulant for serum

collection, and in heparinized tubes for complete blood count (CBC) analysis, and both

tubes were immediately placed on ice. Blood in tubes without coagulant was centrifuged

for 8 min at 1400 rpm to separate serum, and the serum was stored at -80°C. The

heparinized blood was sent to the Hematology Laboratory, Health Science Centre for a

CBC which included total erythrocytes, leukocytes, lymphocytes, platelets, neutrophils, monocytes plus hemoglobin and hematocrit.

Liver, spleen, small intestine and colon were dissected and weighed, and length of the small intestine and colon was recorded. The mid-point of small intestine and first section of colon were placed in phosphate buffered formalin and sent to the Pathology Laboratory, Health Sciences Centre for preparation of paraffin-embedded blocks and tissue sections for immunohistochemistry and histology. Livers were dissected, weighed, immediately frozen in liquid nitrogen and stored at -80°C. Femurs were dissected and frozen for mineral analyses.

Mineral analyses

Zinc, copper and iron were analyzed using atomic absorption spectrometry. For this technique, tissues are accurately weighed and then dissolved using strong acids. In atomic absorption spectrometry, the resulting solution is injected into the flame of the instrument and atomized. Light of an appropriate wavelength for a particular element is shone through the flame, and some of the light is absorbed by the atoms of the sample. The quantity of light absorbed is proportional to the concentration of the element (Henry, 1996).

Serum samples were diluted with deionized water and then analyzed for Zn using atomic absorption. Femur and liver tissues were dried to obtain the dry weight, wet-ashed using trace element grade nitric acid, and then appropriately diluted for mineral analysis. Liver tissues were analyzed for zinc, copper and iron. Femurs were analyzed for zinc. Bovine liver standard reference material 1577b (US Department of Commerce,

National Institute of Standards and Technology, Gaithersburg, MD) was used as the quality control.

Haptoglobin

Haptoglobin is an acute phase protein found in the blood of humans and animals (Larsson et al, 2006) and under normal conditions, it is either absent from the blood or present at very low levels. Haptoglobin can increase significantly in response to acute infection, trauma or inflammation. The haptoglobin assay is based on the principle that haptoglobin at low pH preserves peroxidase activity of hemoglobin. Preservation of the peroxidase activity is directly proportional to the amount of haptoglobin present in the specimen (Anonymous (c), 2004).

Haptoglobin was measured using a kit (Tridelta Development Ltd.) Briefly, 7.5 µl of calibrator was pipetted in duplicate into designated wells, and 7.5 µl of serum was pipetted in duplicate into wells of a 96 well microplate. Diluted hemoglobin (100 µl) was added to all wells and the plate was mixed gently. Chromogen/Substrate (140 µl) was added to each microwell and the plate was incubated for 5 min at room temperature. The absorbance was read spectrophotometrically at 630 nm with a microplate reader (SpectraMax 340, Molecular Devices Corp.) and the SOFTmax Pro software (version 1.2.0, Molecular Devices Corp., Sunnyville, CA) was used to determine the concentrations of unknowns using the calibration curve of absorbance versus haptoglobin concentration.

Histology

The Pathology Laboratory, Health Sciences Centre, prepared hemotoxylin and eosin stained tissue sections for small intestine and colon. All slides were blinded, examined microscopically and scored for inflammation as indicated in the figure legends.

Metallothionein (Immunohistochemistry)

Immunohistochemistry (IHC) combines immunological, biochemical and anatomical techniques to identify specific tissue components by means of specific antibody/antigen reaction tagged with a visible label. IHC allows for visualization of the localization and distribution of specific cellular components (Anonymous (b), 2002).

The procedure for MT immunostaining has been previously published by Szczurek et al (2001). Briefly, slides for small intestine and colon were incubated for 1 h at room temperature with monoclonal mouse anti-MT antibody (clone E9; Dako, Carpinteria, CA; diluted 1:25 in phosphate buffered saline). Prediluted Dako Envision System goat anti-mouse/anti-rabbit peroxidase labeled polymer was added for 30 min. The reaction product was visualized with 3, 3'-diaminobenzidine tetrahydrochloride (DAB.4HCL; Polysciences, Warrington,PA). Tissues were counterstained with Harris hematoxylin and eosin. Computer images of immunostained sections are obtained using Nikon Coolscope. Slides were blinded and viewed at 10x magnification under the microscope. The intensity of MT staining was estimated using an arbitrary subjective scale of minuses and pluses according to Jasani and Elmes (1991) as described in the figure legends.

Apoptosis (Immunohistochemistry)

Caspase-3 is one of the major key executioners of apoptosis; it is partially or totally responsible for the proteolytic cleavage of many important proteins (Enari et al, 1998).

Apoptosis was assessed with the Apoptosis Marker: Signalstain Cleaved Caspase-3 (ASP 175) IHC Detection kit (Cell Signaling). This kit is designed to detect the activation of caspase-3 by immunohistochemistry. It utilizes the ABC immunoperoxidase method to detect endogenous levels of caspase-3 protein. Briefly, slides for small intestine and colon were deparaffinized, rehydrated and then boiled for 10 min for antigen unmasking. Peroxidase quench was added followed by a wash step and blocking solution was added followed by a wash step. The primary antibody and the negative control were added overnight and the slides were washed with three changes of buffer. Slides were covered with biotinylated secondary antibody for 30 min, and premixed AB reagent was added for 30 min before another wash step. Substrate chromagen was added and the slides were monitored for red brown color, and then washed with distilled water. Finally, slides were dehydrated then mounted. The slides were blinded and viewed by 10x -40x magnification under the microscope. The number of apoptotic cells was counted in the whole tissue section of small intestine and colon for each animal.

Statistical Analysis

Data were analyzed with the Statistical Analysis System (SAS; The SAS System V9.1 for Windows, SAS Institute Inc., Cary, NC) using a two-way ANOVA for the main

effects of DSS challenge and diet, and the interaction of DSS x diet. Duncan's Multiple Range test was used for means testing. The level of significant was set at $P<0.05$. All values were reported as mean±standard error of the mean (SEM).

There were only two parameters (haptoglobin and serum Zn) with a significant interaction between DSS and diet and these data are shown in figures with all the individual groups. For all the other parameters with a significant main effect of DSS challenge or a significant main effect of diet, data from individual groups was pooled as described in the figures [n=40 for DSS challenge and n=16 for each dietary treatment].

IV. RESULTS

Growth Assessment

Body weight

The animals challenged with DSS had a 5% lower final body weight compared to the untreated animals (Figure 2). The final body weight of ZN and ZS groups was 23% higher than PF and ZT groups and 29% higher than the ZD group which had the lowest final body weight. Even though the ZT group had the same diet as the ZD group until the last 3 days of the study when it was changed to zinc-supplemented diet, the ZT rats had a 8% higher final body weight than the ZD rats.
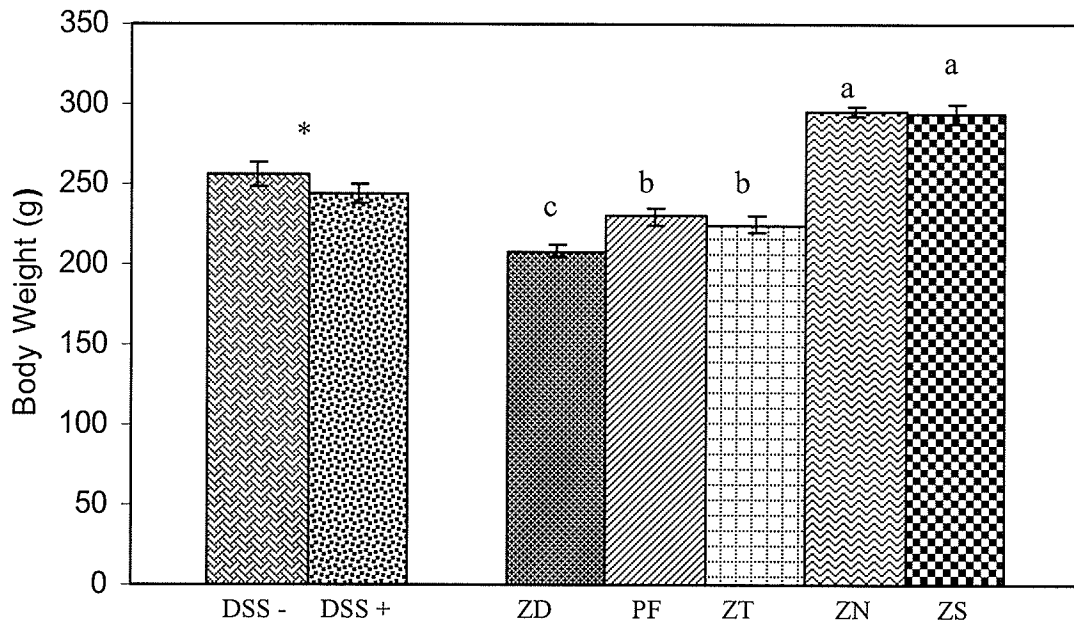
Figure 2 – Effects of DSS challenge and diet on the final body weight. There were significant main effects for both the DSS challenge (P< 0.003) and dietary treatment (P<0.0001), thus data was pooled to show the main effects. Statistical differences among means (P<0.05) are indicated by an asterisk (DSS effect) or different lower case letters (diet effect). Values are means ± SEM for n=40 (DSS challenge) and n=16 (dietary treatment). Abbreviations: untreated (DSS-) and dextran sodium sulfate treated (DSS+); zinc-deficient (ZD), pair-fed (PF), zinc-treated (ZT), zinc-normal (ZN) or zinc-supplemented (ZS) groups as described in the Methods.

Body and tail length

The DSS challenge had no significant effect on the body and tail length (P = 0.46, P = 0.63, respectively). The ZD and the ZT groups had a smaller body length than the PF, ZN and ZS groups (Figure 3a). The ZD, PF and ZT groups had a shorter tail length than the ZN and ZS groups (Figure 3b).

Feed intake

Total feed intake was reduced after DSS challenge (P<0.0001) and affected by diet before (P<0.0001) and after (P<0.0001) DSS challenge (Figure 4). Before the DSS challenge, ZN and ZS rats (474±10 and 468±6 g, respectively) consumed approximately 20% more feed than ZD, PF and ZT rats (380±10, 390±11 and 378±10 g, respectively; Figure 4a). After the DSS challenge, the DSS-treated rats consumed 26% less feed than the untreated rats (51±3 and 681±4, respectively; Figure 4b). As for the diet groups, the ZN and ZS rats along with the ZT rats (69±6, 72±5 and 69±8, respectively) consumed more feed that the ZD and PF rats (46±3, 41±2) during the challenge phase.

Water intake

Water intake corrected for body weight was not affected by DSS challenge during the last 4 days of the study (Figure 5). On the other hand, water intake was affected by diet. The PF rats had the lowest water intake compared to the ZD, ZT and ZN rats, while the ZS rats were not different from the other groups.
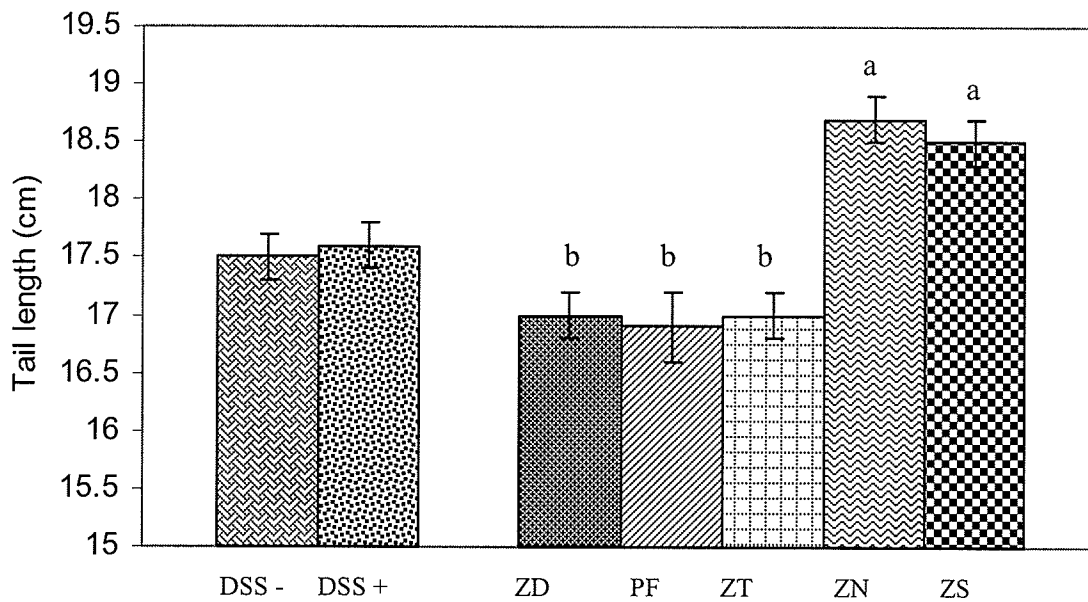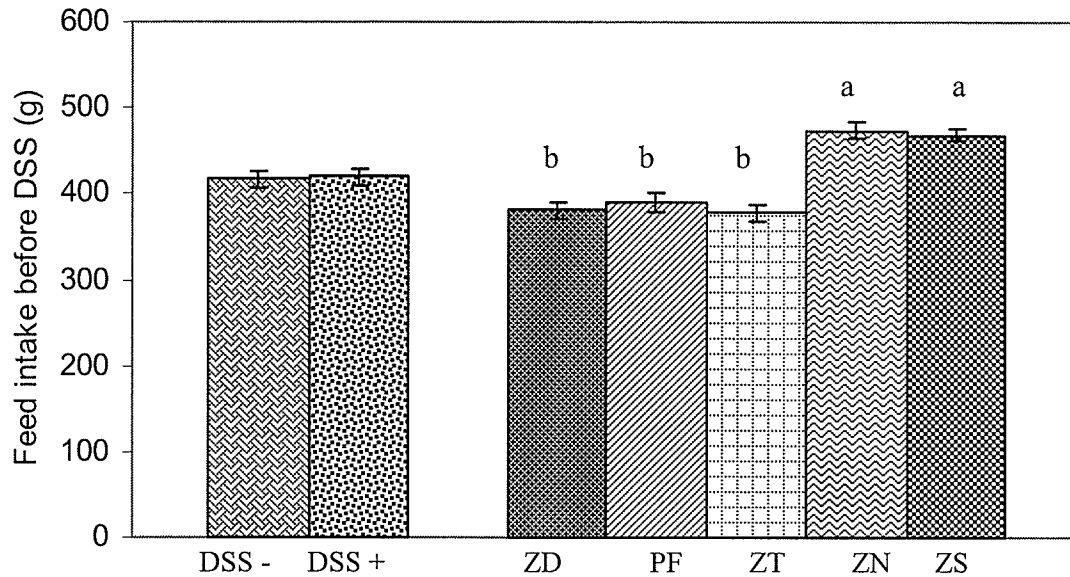
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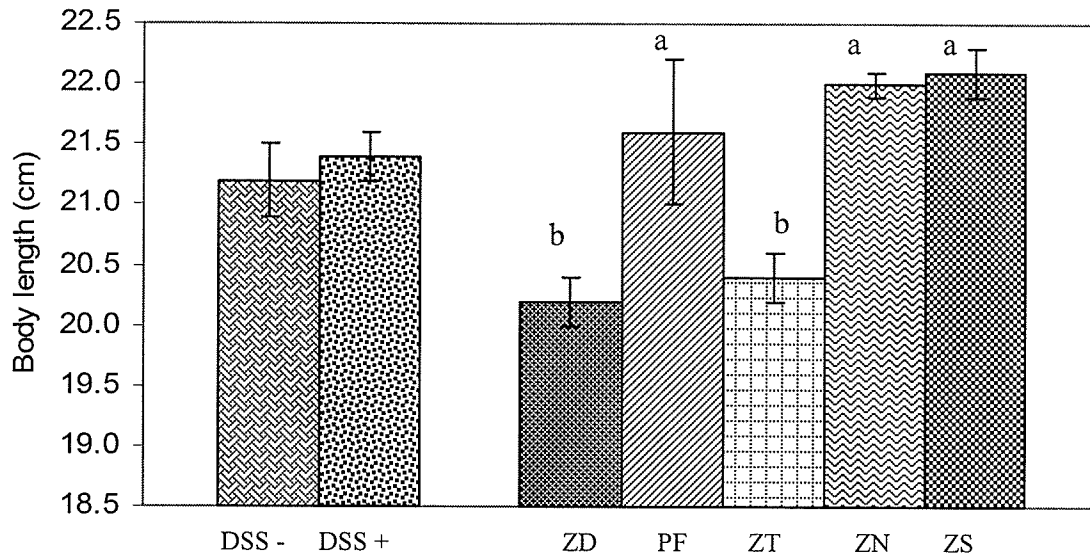
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Figure 3 – Effects of DSS challenge and diet on the (a) body and (b) tail length.
There was a significant main effect of dietary treatment (P<0.0001) but not DSS
challenge for both body and tail length. Statistical differences among means (P<0.05) are
indicated by different lower case letters (diet effect). Values are means ± SEM for n=40
(DSS challenge) and n=16 (dietary treatment).
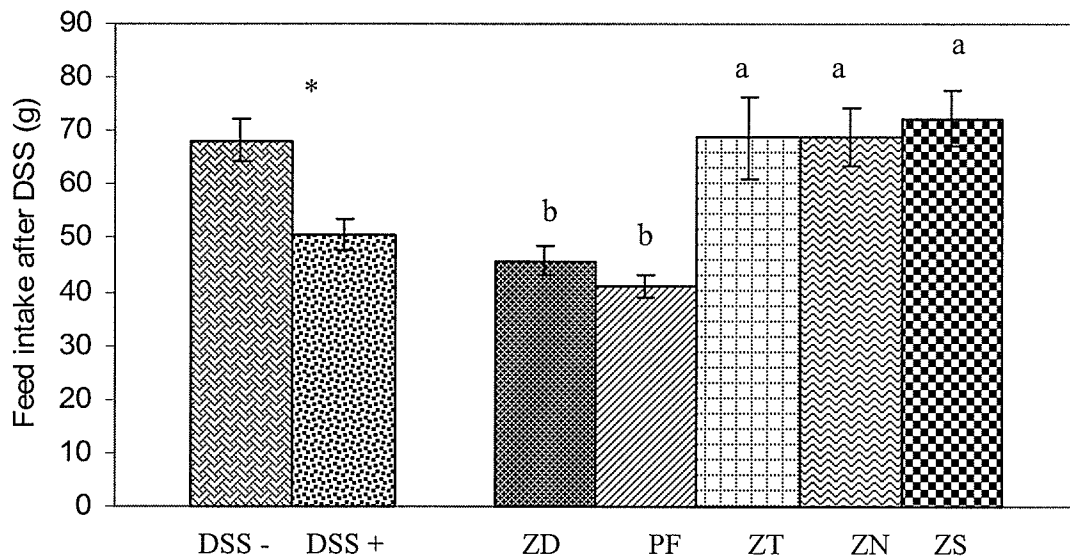
a)

b)

Figure 4 - Effects of DSS challenge and diet on feed intake (a) before and (b) after DSS challenge. There was a significant effect of significant effect of DSS (after challenge) on feed intake (P<0.0001) and of dietary treatment on feed intake before (P<0.0001) and after (P<0.0001) challenge. Statistical differences among means (P<0.05) are indicated by an asterisk (DSS effect) and by different lower case letters (diet effect). Values are means ± SEM for n=40 (DSS challenge) and n=16 (dietary treatment).
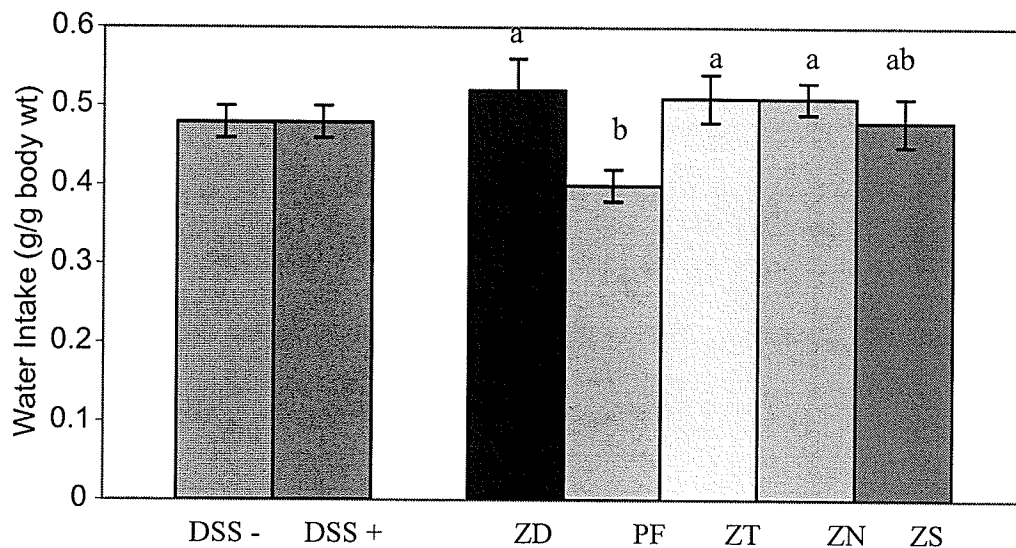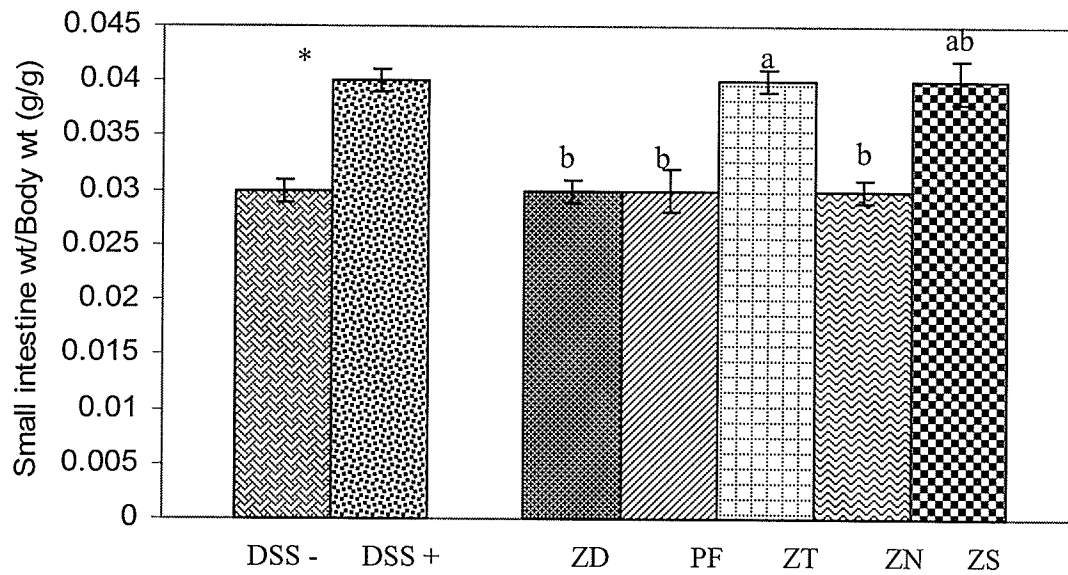
Figure 5 – Effect of DSS challenge and diet on water intake during DSS challenge.
There was a significant main effect of dietary treatment (P=0.05) on water intake
corrected for body weight but there was no main effect of DSS. Statistical differences
among means (P<0.05) are indicated by an asterisk (DSS effect) and by different lower
case letters (diet effect). Values are means ± SEM for n=40 (DSS challenge) and n=16
(dietary treatment).

Organ/body weight ratios

Small intestine weight and length: The DSS challenged rats had 1.2 fold greater

small intestine/body weight ratio (Figure 6a) but no difference in small intestine length

(Figure 6b) compared to the unchallenged rats. The small intestine/body weight ratio of

ZD, PF and ZN rats was not different. However, the ZT rats had a 1.3 fold higher small

intestine/body weight ratio than ZD, PF and ZN rats. The small intestine of ZN and the

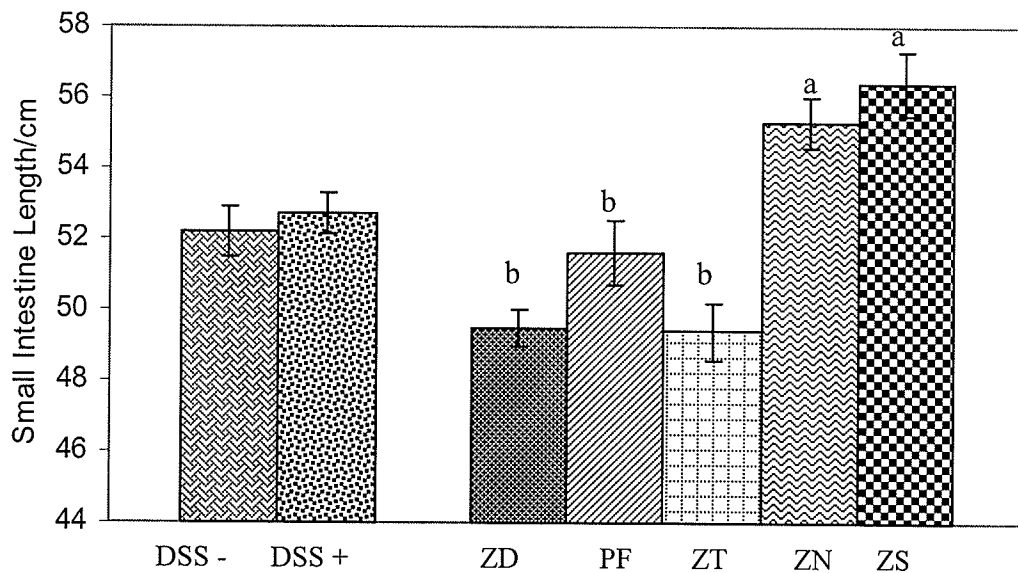ZS groups was longer than ZD, PF, and ZT rats.

a)

b)

Figure 6 - Effect of DSS challenge and diet on (a) small intestine weight/body weight ratio and (b) small intestine length. There were significant main effects of DSS challenge for small intestine/body weight ratio (P < 0.0001) and for dietary treatment on both small intestine weight and length (P = 0.0006 and P<0.0001, respectively). Statistical differences among means (P<0.05) are indicated by an asterisk (DSS effect) or different lower case letters (diet effect). Values are means ± SEM for n=40 (DSS challenge) and n=16 (dietary treatment).

Colon weight and length: The DSS challenged group had a 1.3 fold lower colon weight/body weight ratio than the untreated rats (Figure 7a). The ZD, PF and ZT groups had a higher colon weight/body weight ratio than ZN and ZS rats. However, colon length was less in the DSS challenged rats (10.7±0.3 cm) than the unchallenged rats (12.9±0.3 cm)(Figure 7b). The only difference in diet was seen between the ZD (10.8 ± 0.4 cm) and the ZS rats (12.5 ± 0.5 cm).

Thymus weight: DSS challenge and diet did not affect thymus weight/body weight ratio (Figure 8a).

Spleen weight: The spleen weight/body weight ratio was 7.4 % higher in the DSS-challenged group compared to the unchallenged rats (Figure 8b). Diet did not affect spleen wt/body wt ratio.

Liver weight: There was no effect of DSS challenge, but there was a diet effect on the liver weight/body weight ratio (Figure 9a). The ZD and PF rats had the lowest liver weight/body weight ratio compared to the other groups.

Adipose weight: The DSS challenged rats had a 15% less adipose tissue weight/body weight ratio than unchallenged rats (Figure 9b). The ZD and the ZT had the lowest adipose weight compared to PF, ZN and ZS rats.
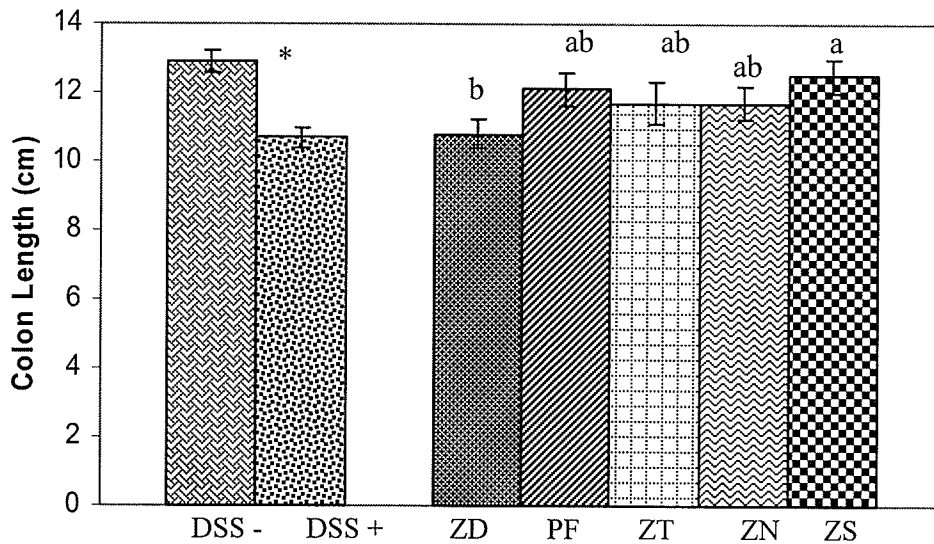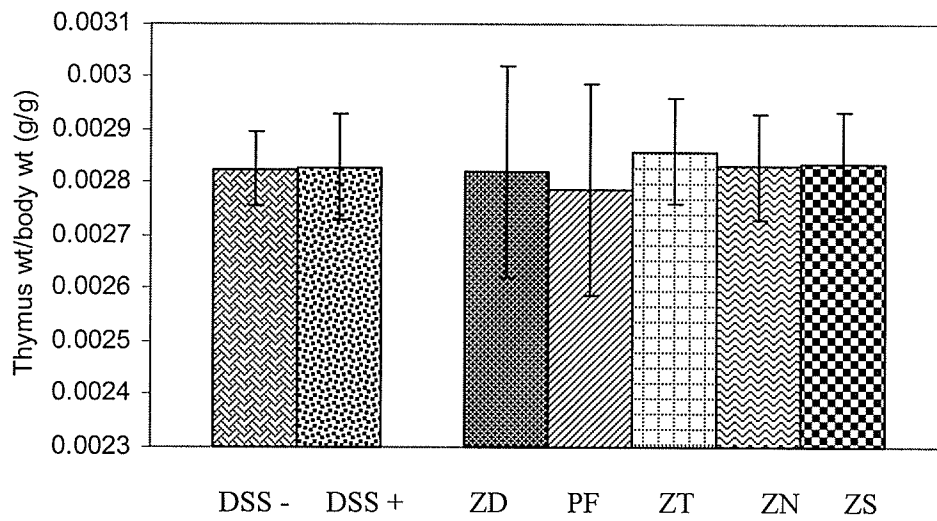
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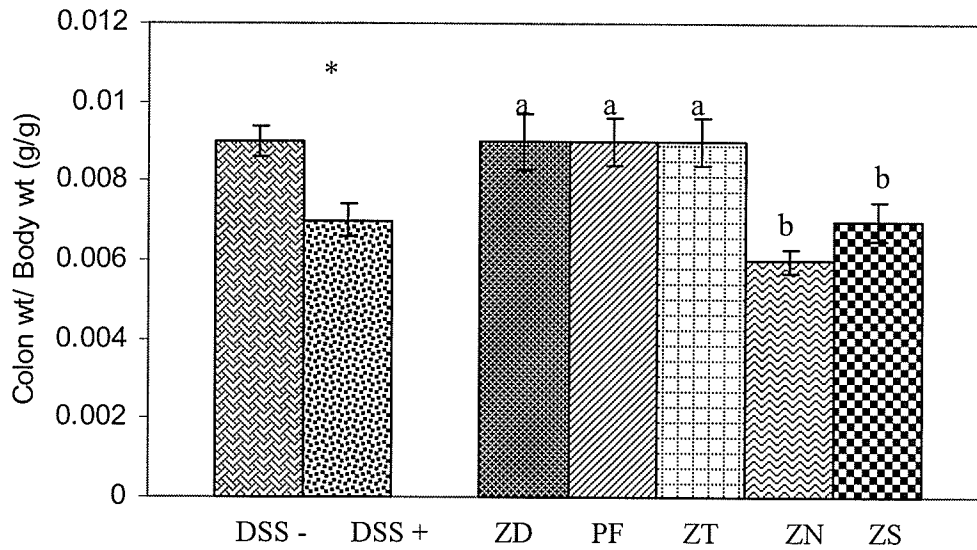
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Figure 7 – Effects of DSS challenge and diet on (a) colon weight/body weight ratio and (b) colon length. There were significant main effects of DSS challenge on colon weight/body weight (P = 0.0004) and dietary treatment on colon weight/body weight (P < 0.001). There were no significant differences regarding colon length. Statistical differences among means (P<0.05) are indicated by an asterisk (DSS effect) or different lower case letters (diet effect). Values are means ± SEM for n=40 (DSS challenge) and n=16 (dietary treatment).

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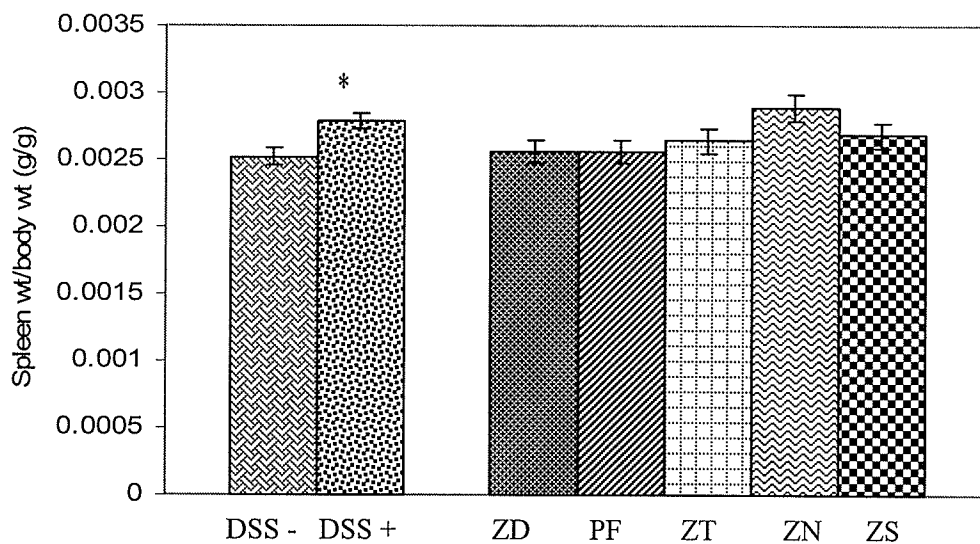
Figure 8 - Effects of DSS challenge and diet on (a) thymus weight/body weight ratio and (b) spleen weight/body weight ratio. There were no significant main effect of DSS challenge on thymus (P=0.999). There were significant main effects of DSS challenge on the spleen ratio (P=0.001) no dietary treatment significance on both thymus (P=0.998) and spleen (P= 0.08). Values are means ± SEM for n=40 (DSS challenge) and n=16 (dietary treatment).

a)

b)
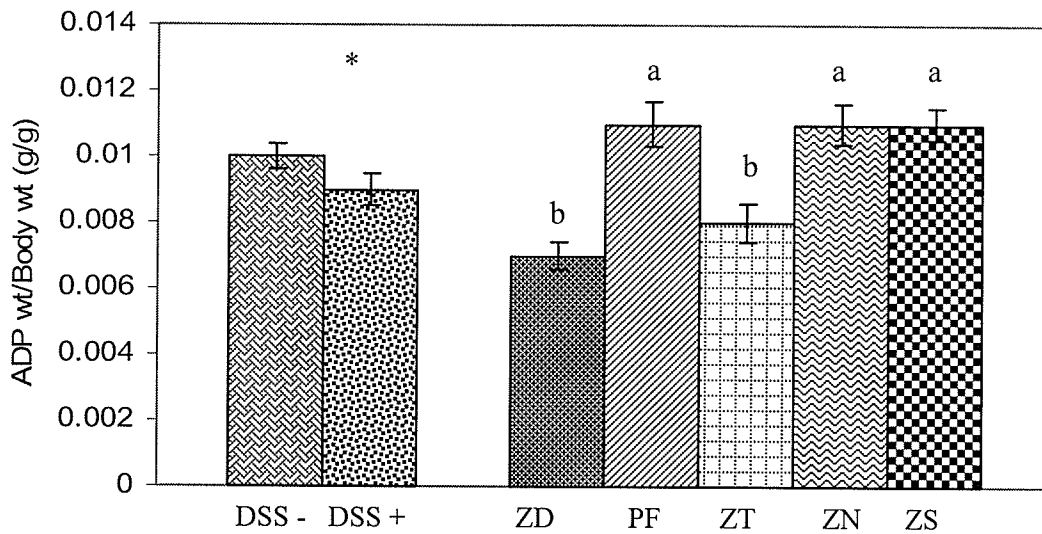
Figure 9 - Effects of DSS challenge and diet on (a) liver weight/body weight ratio (b) and adipose weight/body weight ratio. There were significant main effects of DSS challenge on adipose (P=0.031) and of dietary treatment on both liver (P<0.0001) and adipose (P<0.0001). Statistical differences among means (P<0.05) are indicated by an asterisk (DSS effect) or different lower case letters (diet effect). Values are means ± SEM for n=40 (DSS challenge) and n=16 (dietary treatment).
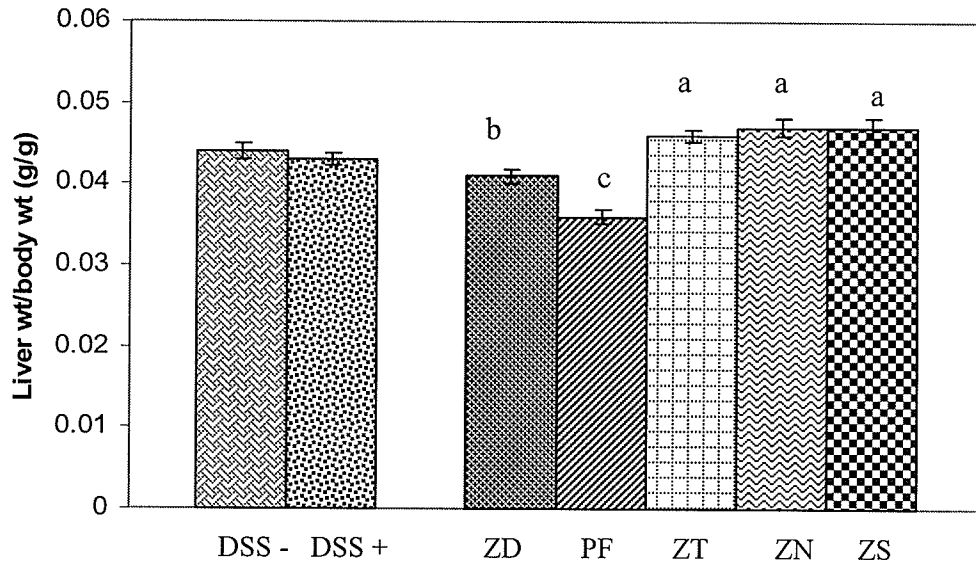
43

Trace Mineral Assessment

Serum Zn

In unchallenged rats, the ZD group had a 84% lower serum Zn concentration compared to PF and ZN rats, and the serum Zn of ZS rats was 69% higher than ZN (Figure 10). The 3 day Zn supplementation of ZT rats elevated serum Zn 7.8-fold compared to ZD rats and 1.3-fold compared to ZN rats.

With DSS challenge, ZD rats still had the lowest serum Zn and serum Zn of ZD was elevated compared to ZN. However, the elevation of serum Zn in the ZT+ group was attenuated compared to the ZT- group (28.7±2.6 and 37.9±4.0 umol/L, respectively), and the elevation of serum Zn in the ZS+ group was attenuated compared to the ZS- group (31.7±2.2 and 50.7±3.7 umol/L, respectively).
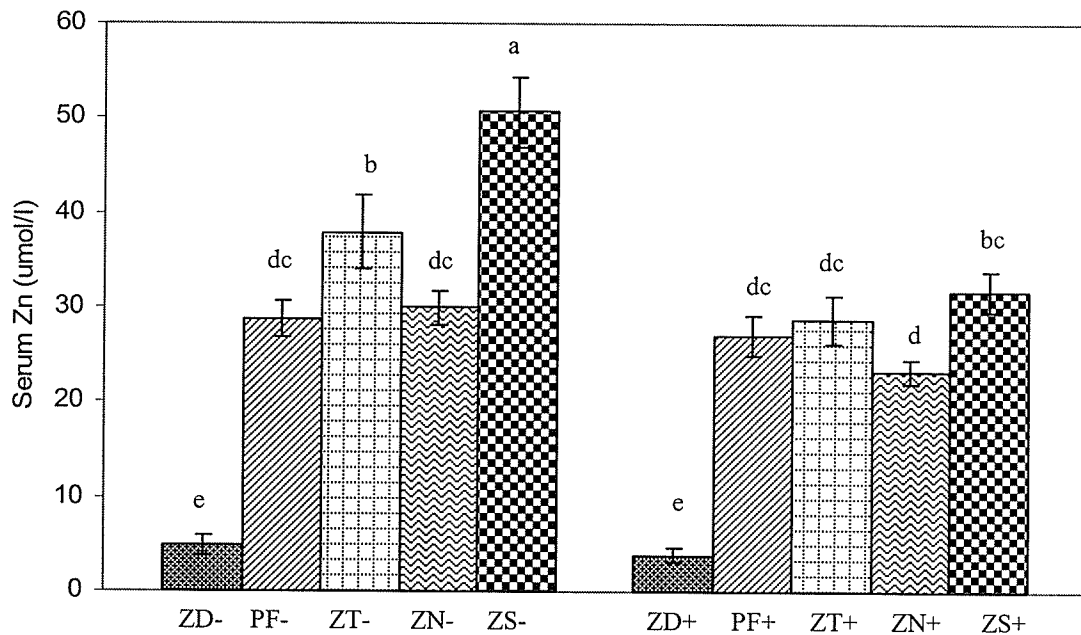

Figure 10 - Effects of DSS challenge and diet on serum Zn. There was a significant DSS x diet interaction (P = 0.00019). Statistical differences among means (P<0.05) are indicated by different lower case letters. Values are means ± SEM for n=10/group.

Femur Zn

DSS challenge did not alter femur Zn concentration (Figure 11a). The ZD group had the lowest femur Zn concentration (1.4 ± 0.1 mmol/g), the PF and ZN rats had almost the same femur Zn concentration (4.5 ± 0.1 and 4.6 ± 0.1 mmol/g, respectively), and ZS rats had the highest femur Zn concentration (7 ± 0.3 mmol/g). After 3 days of Zn supplementation, the ZT had a femur Zn concentration equivalent to the ZN group.

Liver Zn

Liver Zn concentration was not affected by DSS challenge (Figure 11b). The ZD and ZN had a 14% lower liver Zn concentration compared to PF and ZT while the ZS was not different from any of the other groups.

Liver Cu

DSS challenged rats had 1.2 fold lower liver Cu concentration than the unchallenged rats (Figure 12a). The PF rats had the highest liver Cu concentration while the ZS rats had the lowest. The ZD and ZN rats had a similar liver Cu concentration that was elevated compared to the ZT group.

Liver Fe

DSS challenge did not alter liver Fe concentration (Figure 12). Liver Fe was elevated 54% in ZD rats compared to PF, and was elevated 56% in PF compared to ZN. The ZN and ZS rats had similar liver Fe. The 3 days of Zn supplementation reduced liver Fe of the ZT group to a level equivalent to the PF group.

a)
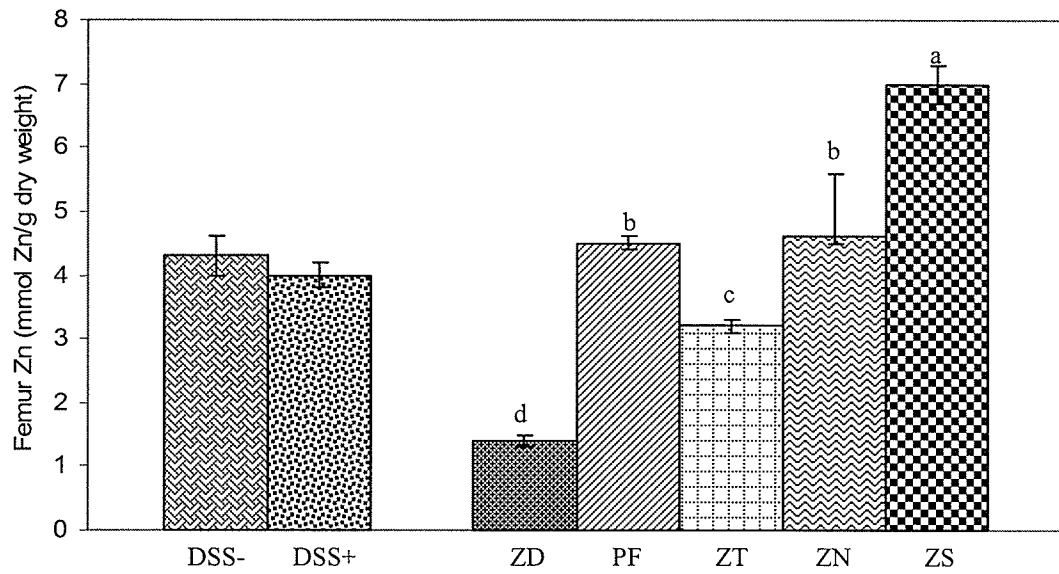
b)
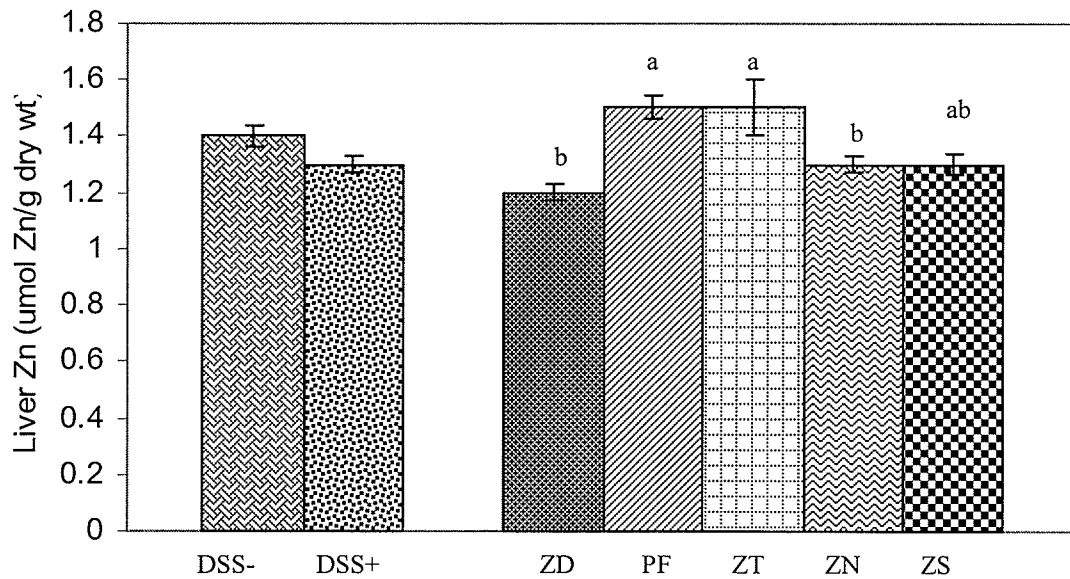
Figure 11 – Effects of DSS challenge and diet (a) femur Zn and (b) liver Zn.
There were significant main effects of diet on femur Zn (P<0.0001) and liver Zn (P = 0.002) but not DSS challenge. Statistical differences among means (P<0.05) are indicated by different lower case letters (diet effect). Values are means ± SEM for n=40 (DSS challenge) and n=16 (dietary treatment).

a)

46
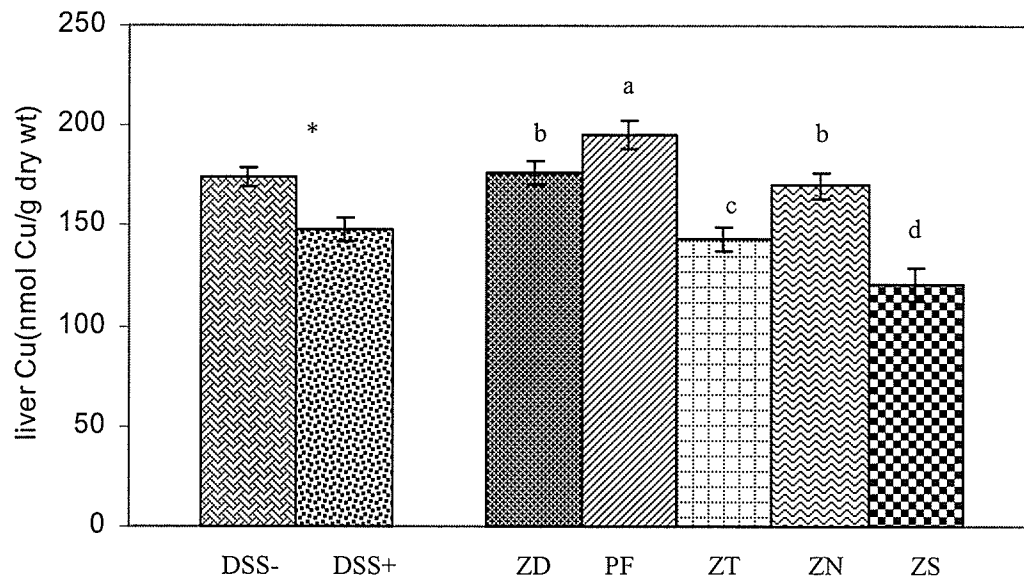
b)
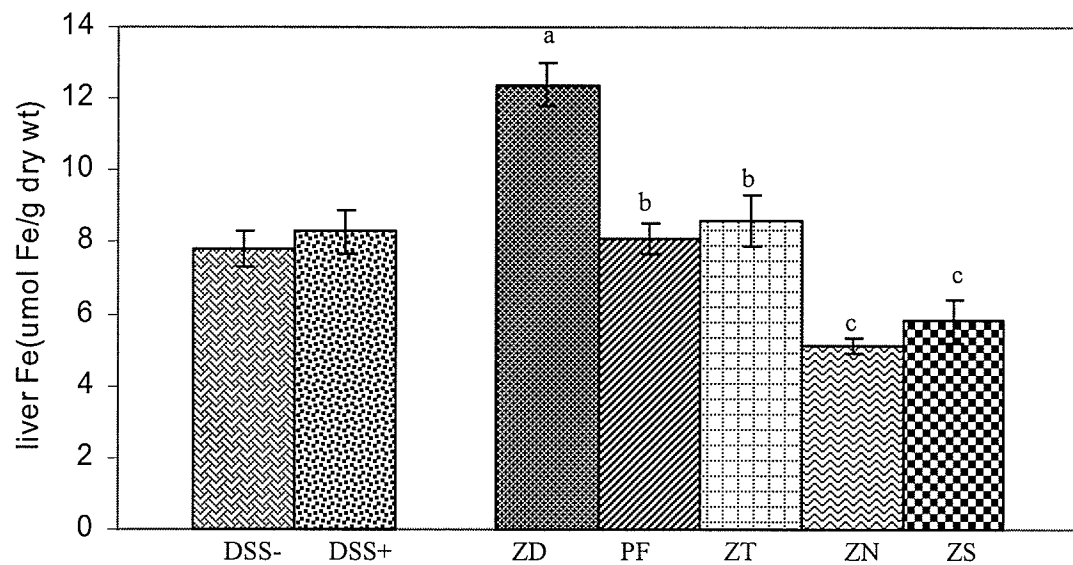
Figure 12 – Effects of DSS challenge and diet on (a) liver Cu and (b) liver Fe. There were significant main effects of DSS challenge on liver Cu (P < 0.0001) and of diet on liver Cu (P < 0.0001) and liver Fe (P<0.0001). Statistical differences among means (P<0.05) are indicated by an asterisk (DSS effect) or different lower case letters (diet effect). Values are means ± SEM for n=40 (DSS challenge) and n=16 (dietary treatment).

Complete blood count assessment (hematology)

Red blood cells (RBC)

The DSS-challenged rats had a 1.05 fold lower RBC count than the unchallenged rats (6.64 ± 0.15 and 6.94 ± 0.1 x 10^{12}/L, respectively)(Figure 13a). The ZD and PF rats had a higher RBC count than ZT, ZN and ZS groups.

*Hemoglobin (Hgb):* The DSS-challenged group had a 1.1 fold lower hemoglobin level than unchallenged group (138±3 and 146±2 g/L, respectively; Figure 13b). The ZD and PF rats had a higher hemoglobin level compared to the other groups.

Hematocrit (Hct): DSS challenge did not affect hematocrit (Figure 14a). The ZD rats had higher Hct levels than ZT and ZS (0.507 ± 0.013 versus 0.431 ± 0.015 and 0.450 ± 0.014, respectively). The hematocrit of ZD was not different from PF or ZN. The hematocrit of ZT was lower than ZD, PF and ZN.

Mean corpuscular volume (MCV): MCV was not altered by DSS challenge, however, there was a significant effect of diet (Figure 14b). ZD, PF and ZT rats had a lower MCV than the ZN and ZS rats.

Mean corpuscular hemoglobin (MCH): The DSS-challenged rats had lower MCH levels than the unchallenged rats (20.7 ± 0.1 and 21 ± 0.1 x10^9/L, respectively) (Figure 15a). ZD, PF, and ZT rats had lower MCH values than ZN and ZS rats).

Mean corpuscular hemoglobin concentration (MCHC): The DSS-challenged rats (299±2 x10^9/L) had a lower MCHC value than unchallenged rats (309±1 x10^9/L)(Figure 15b). ZN and ZS rats had lower MCHC levels (297±3 and 298±4 x10^9/L) than ZD, PF and ZT rats (309 ± 2, 307±3 and 306±2 x10^9/L, respectively).

a)
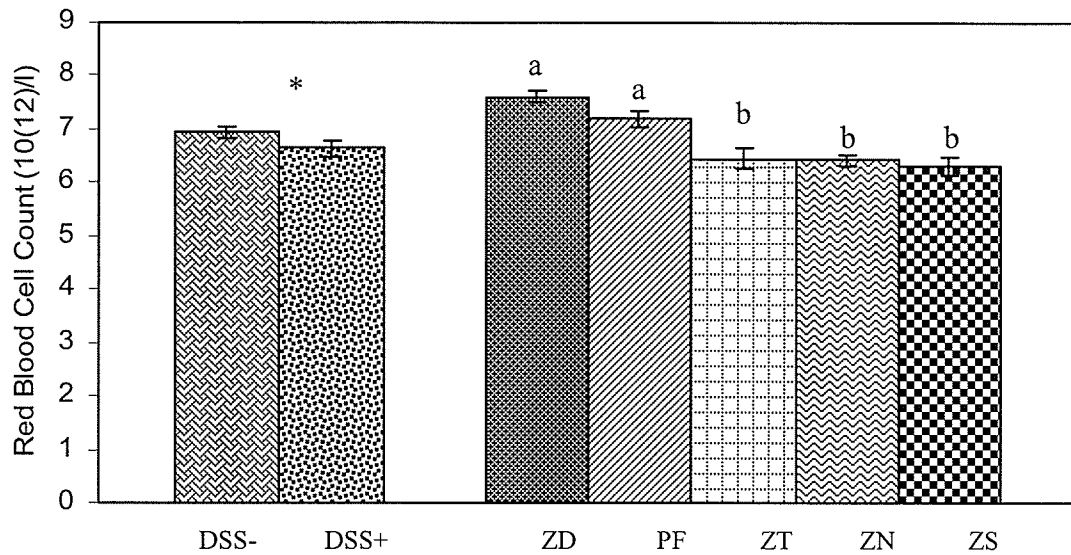
b)
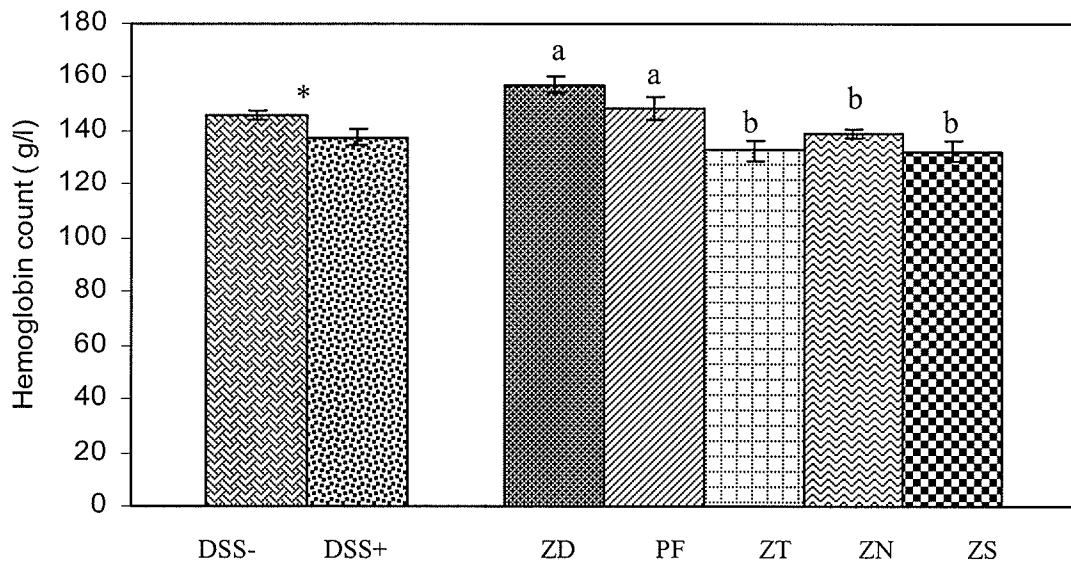
Figure 13 - Effect of Diet and DSS-Challenge on (a)red blood cell (RBC) count and (b) hemoglobin levels. There were significant main effects of DSS challenge on RBC count (P = 0.039) and Hgb effect (P = 0.007), and of diet on RBC count (P < 0.0001) and Hgb (P < 0.0001). Statistical differences among means (P<0.05) are indicated by an asterisk (DSS effect) or different lower case letters (diet effect). Values are means ± SEM for n=40 (DSS challenge) and n=16 (dietary treatment).

a)
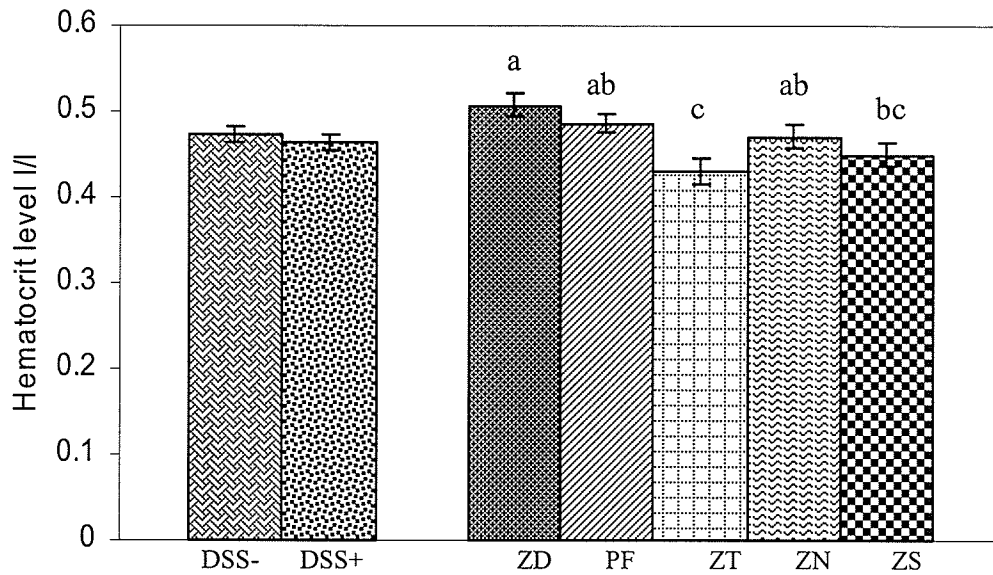
b)
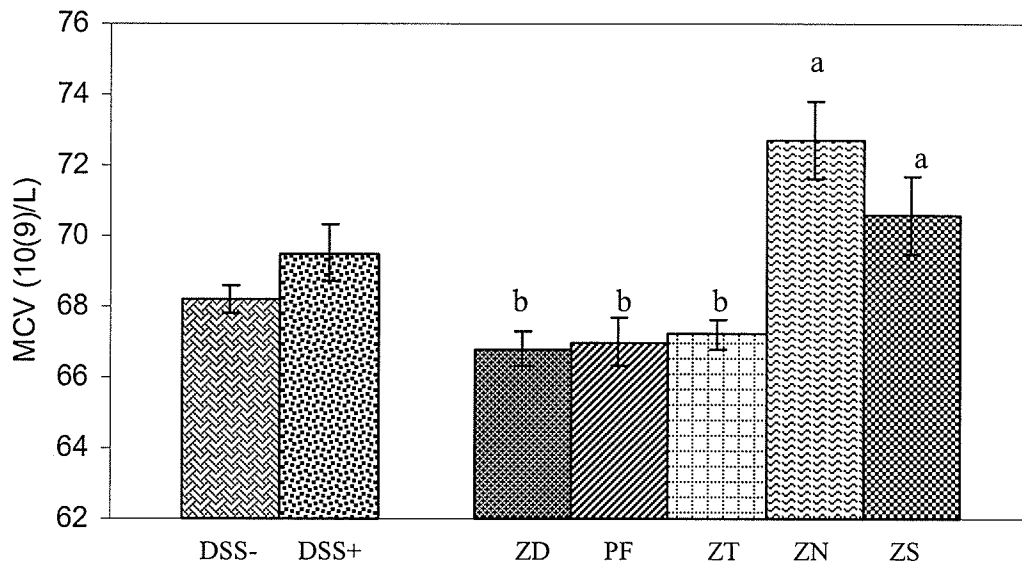
Figure 14 - Effects of DSS challenge and diet on (a) hematocrit (Hct) and (b) mean corpuscular volume (MCV). There were significant main effects of diet on Hct (P = 0.0009) and MCV (P< 0.0001). Statistical differences among means (P<0.05) are indicated by different lower case letters (diet effect). Values are means ± SEM for n=40 (DSS challenge) and n=16 (dietary treatment).

a)
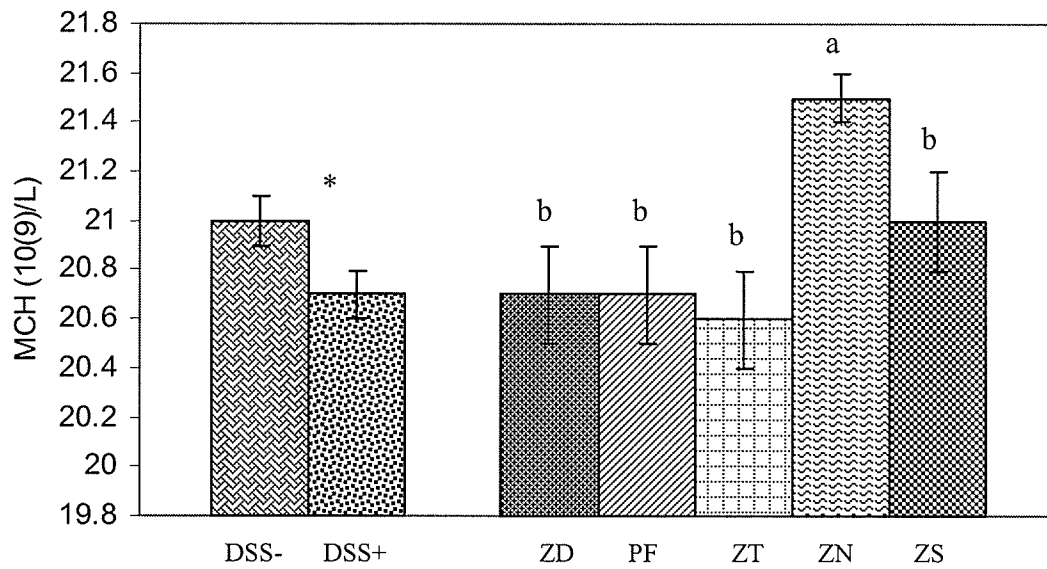
b)
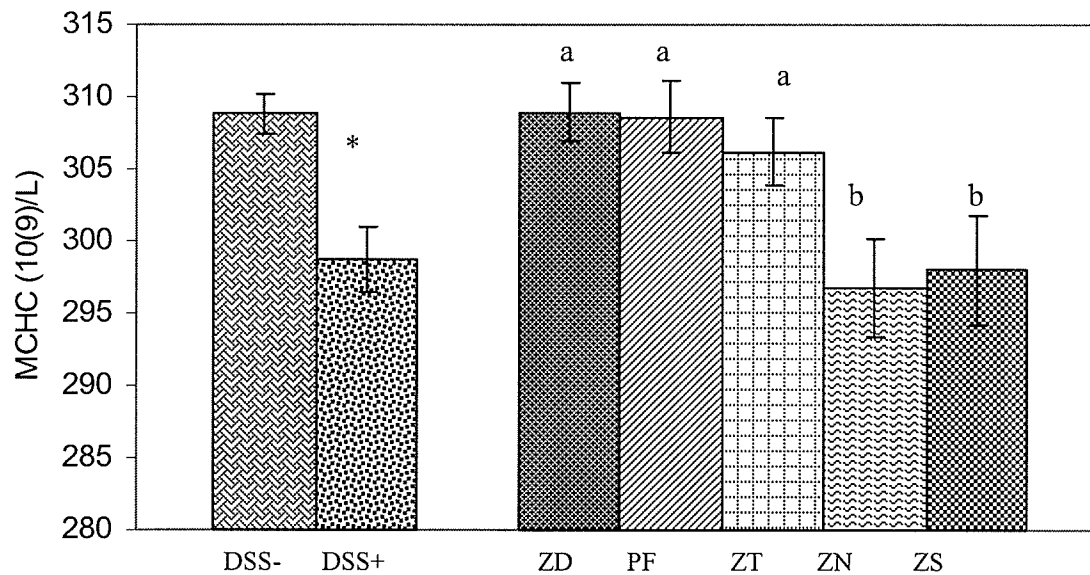
Figure 15 - Effects of DSS challenge on (a) mean corpuscular hemoglobin (MCH), and (b) mean corpuscular hemoglobin concentration (MCHC). There were significant main effects of DSS challenge on MCH (P = 0.03) and MCHC (P < 0.0001) and of diet on MCH (P=0.0009) and MCHC (P = 0.0007). Statistical differences among means (P<0.05) are indicated by an asterisk (DSS effect) or different lower case letters (diet effect). Values are means ± SEM for n=40 (DSS challenge) and n=16 (dietary treatment).

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White Blood Cell Count (WBC)

The DSS-challenged rats had a 1.9 fold higher WBC count than the untreated group (22.7 ± 1 and 12.7 ± 1 x10^9/L, respectively; Figure 16a). The WBC count was similar in the dietary groups except that ZS was higher than PF and ZT.

Lymphocyte Count: The DSS-challenged rats had a 1.6 fold higher lymphocyte count than the non-treated group (17.9 ± 1.2 and 11.1 ± 0.7, respectively; Figure 16b). Similar to the WBC counts, lymphocyte counts were elevated in ZS compared to PF and ZT. ZD and ZN were not different from any of the groups.

Neutrophils: The DSS-challenged rats had a higher neutrophil number than the unchallenged rats but there were no differences due to diet (Figure 17a).

Monocytes: DSS and diet did not alter number of monocytes (Figure 17b).

Platelet Count: There was a trend ($P = 0.06$) for a 7.7% lower platelet count in DSS-treated rats compared to unchallenged rats (Figure 18). There was a trend ($P=0.08$) for a reduced platelet count in the ZT group versus PF and ZS but not ZD and ZN.

a)
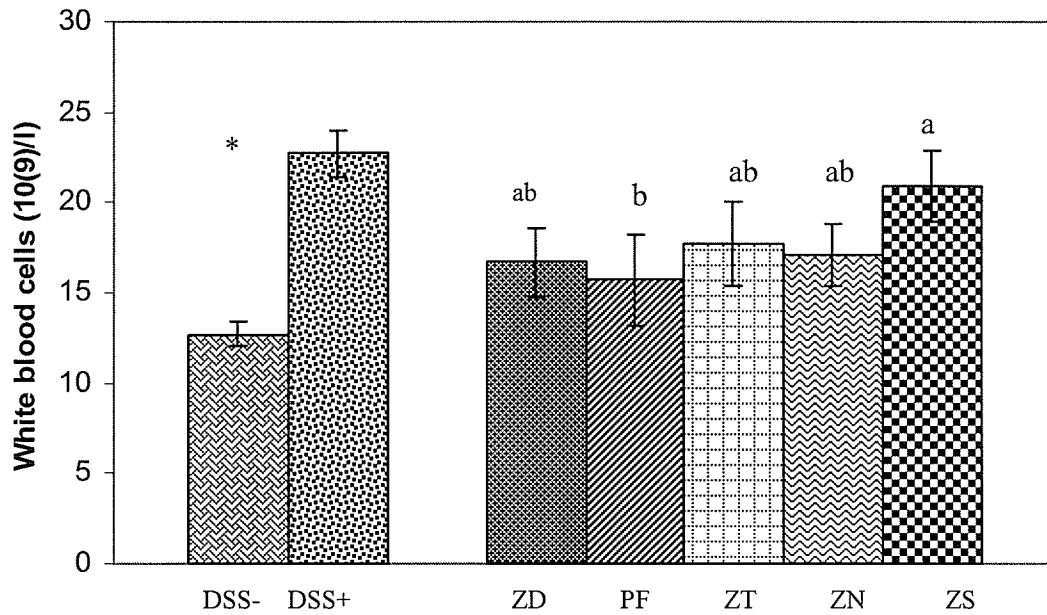
b)
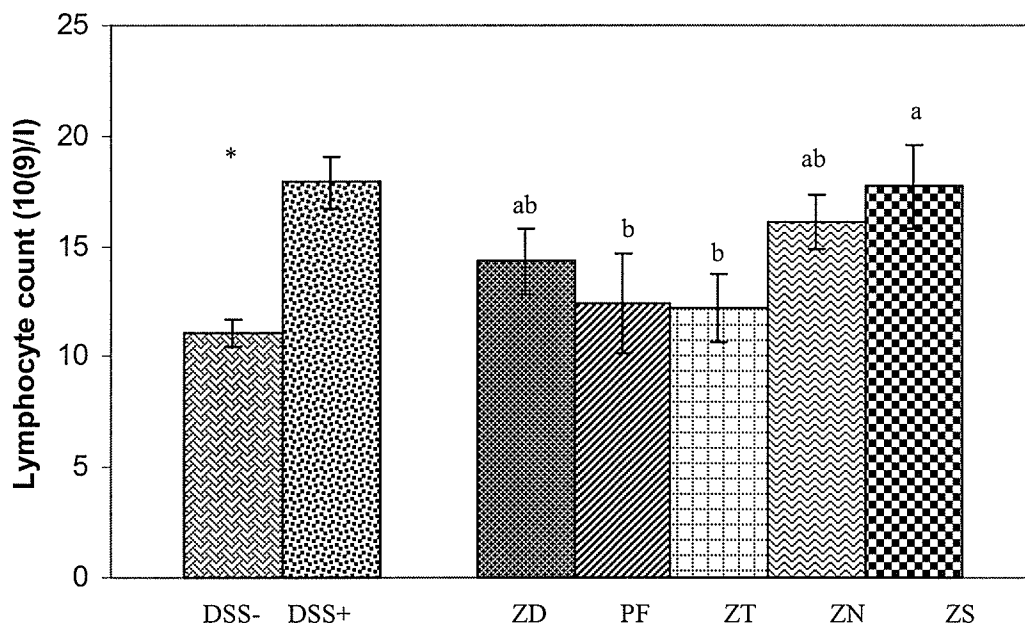
Figure 16 – Effects of DSS challenge and diet on (a) white blood cell (WBC) count and (b) lymphocyte count. There were significant main effects of DSS challenge on WBC (P < 0.0001) and lymphocyte count (P < 0.0001) and of diet on lymphocyte count (P = 0.035). Statistical differences among means (P < 0.05) are indicated by an asterisk (DSS effect) or different lower case letters (diet effect). Values are means ± SEM for n=40 (DSS challenge) and n=16 (dietary treatment).

a)
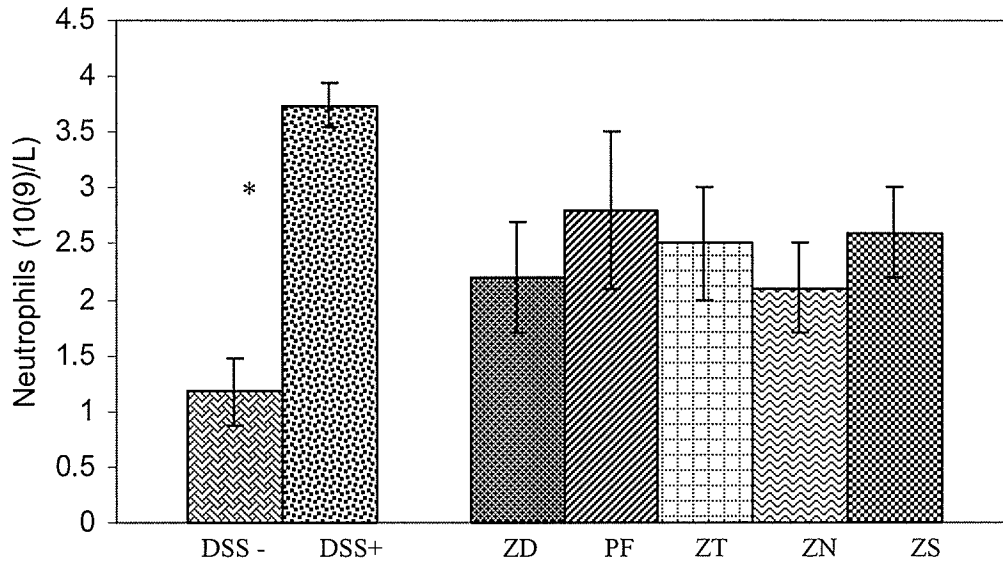
b)
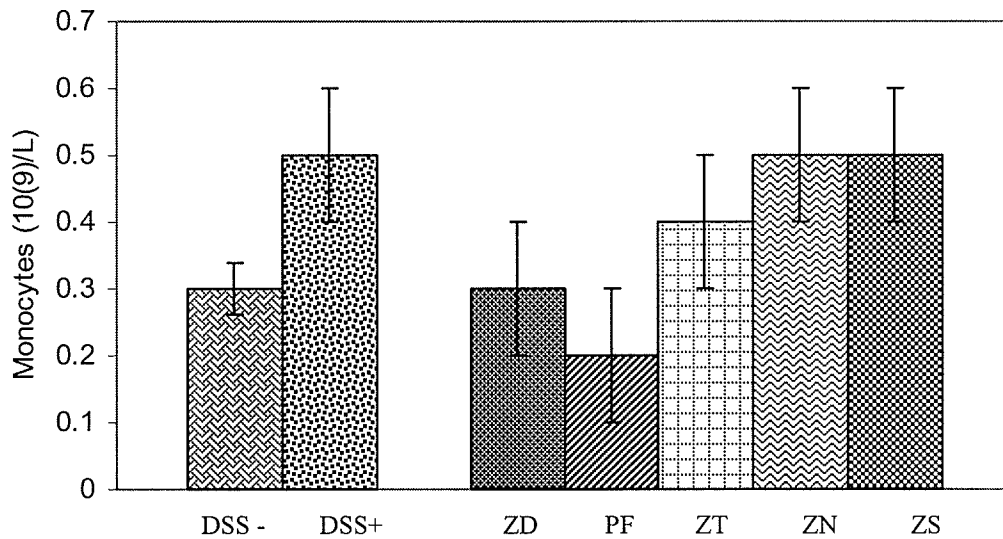
Figure 17 - Effects of DSS challenge and diet on (a) neutrophil count and (b) monocyte count. There was a significant main effect of DSS challenge on neutrophils (P < 0.0001). Statistical differences among means (P < 0.05) are indicated by an asterisk (DSS effect). Values are means ± SEM for n=40 (DSS challenge) and n=16 (dietary treatment).
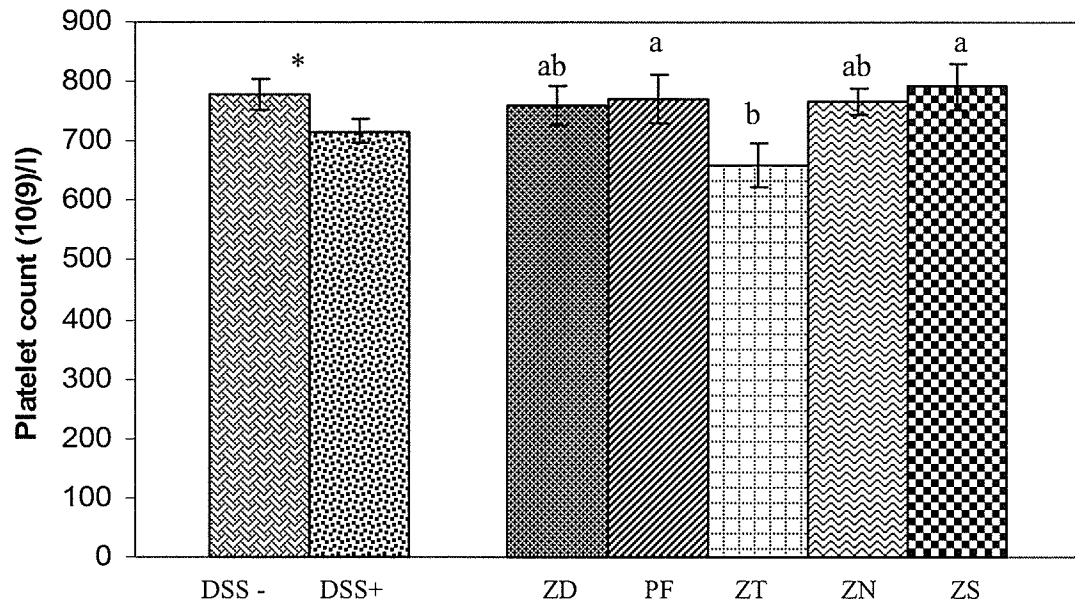
Figure 18 - Effect of DSS challenge and diet on platelet count. There was a trend for a significant main effect of DSS challenge (P=0.06) and diet (P=0.08). Trends for differences among means are indicated by an asterisk (DSS effect, P=0.06) or different lower case letters (diet effect, P=0.08). Values are means ± SEM for n=40 (DSS challenge) and n=16 (dietary treatment).

Inflammation Assessment

Rat history

The groups exposed to DSS challenge had diarrhea and bloody stool starting one day after giving the rats 5% DSS in the drinking water (Table 2). The severity of symptoms was similar among the groups. The PF and ZS groups had a greater incidence of severe symptoms on days 2 and 3. By day 4, all the rats had the same severity of bloody stool and diarrhea.

Table 2. Presence of bloody stool and diarrhea during DSS challenge.

Incidence of Complications[1]					
	ZD+	ZT+	ZN+	PF+	ZS+
Day 1					
Bloody stool	0	0	0	0	0
Diarrhea	0	0	0	0	0
Day 2					
Bloody stool	2 (25%)	2 (25%)	1 (12%)	3 (37%)	2 (25%)
Diarrhea	4 (50%)	4 (50%)	4 (50%)	2 (25%)	5 (62%)
Day 3					
Bloody stool	6(75%)	6 (75%)	6 (75%)	6(75%)	6 (75%)
Diarrhea	7 (87%)	7 (87%)	7 (87%)	8(100%)	8(100%)
Day 4					
Bloody stool	7 (87%)	8(100%)	8(100%)	8(100%)	8(100%)
Diarrhea	8(100%)	8(100%)	8(100%)	8(100%)	8(100%)

[1]The numbers indicate the number of rats that had the symptoms and the % indicates the % of the rats in each group with the symptoms. There were no significant differences among the DSS groups for bloody stool and diarrhea.

Serum haptoglobin

There was a significant DSS x diet interaction (P<0.0001) for serum haptoglobin. The ZN and ZS DSS-challenged rats had the highest haptoglobin levels, approximately 2.5-3 fold higher than all the other groups (Figure 19).
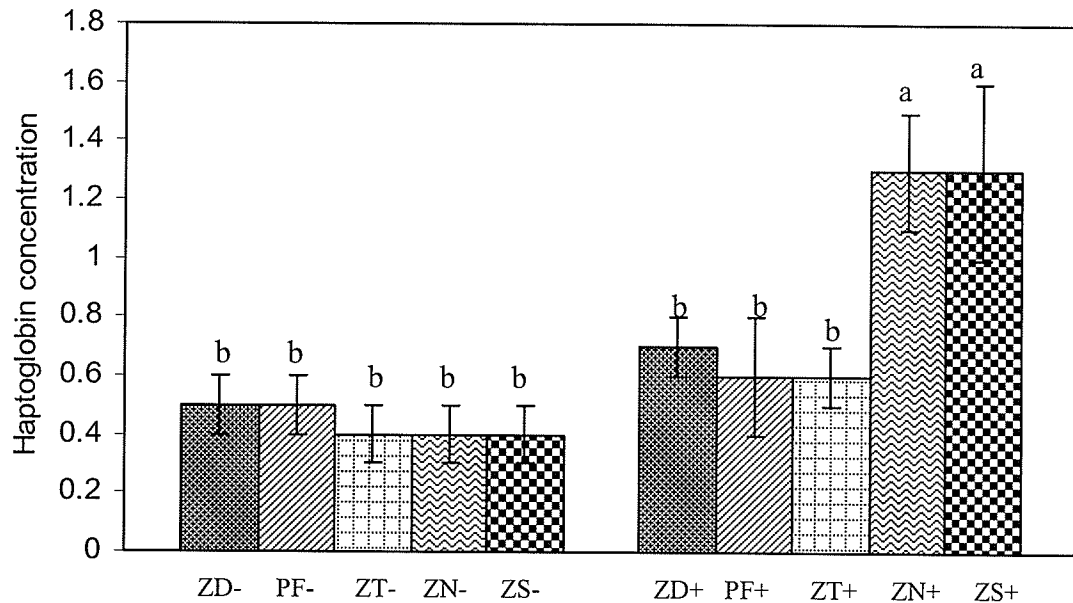
Figure 19 - Effect of diet and DSS on serum haptoglobin concentration. There was a significant diet x DSS interaction (P = 0.01). Statistical differences among means (P<0.05) are indicated by different lower case letters. Values are means ± SEM for n=8/group.

Histology

Small Intestine

There were no differences in the histology of the small intestine when comparing DSS-treated rats and the non-treated rats in each dietary group all slides seemed normal to us (Figure 24 a, b). However, we found that some of the untreated and treated rats had lymphocytic aggregates. In the untreated rats (1/8 ZD, 5/8 ZS rats) in the treated groups (1/8 ZD. 3/8 PF, 1/8 ZN, 1/8 ZS) (Figure 24 c, d)

Colon

Unexpectedly, the unchallenged rats had quiescent nonspecific chronic inflammation (5/8 in the ZD group, 4/8 in the ZT group, 7/8 in the ZN group, 5/8 in the PF group, 7/8 from the ZS group) represented by focal submucosal infiltration by mononuclear inflammatory cells, mainly lymphocytes, which sometimes extended to the deeper parts of the mucosa leading to atrophy of the overlying mucosa. Mucosal changes indicative of active inflammation, namely, significant neutrophilic and eosinophilic infiltration, crypt abscesses and ulceration were not seen (Figure 25 a, b).

The DSS-challenged cases showed focal mucosal changes of variable severity, consisting of edema, congestion (Figure 25 i, j) and significant infiltration of the lamina propria by neutrophils and eosinophils, in addition to mononuclear inflammatory cells (Figure 25 c, d). The inflammatory infiltrate in the mucosa extended to the submucosa in the more severe cases. Variable numbers of crypt abscesses (Figure 25 e, f white arrow) were also seen, appearing as dilated crypts lined by flattened epithelium devoid of goblet cells, and containing intraluminal neutrophilic aggregates. There was also variable focal

goblet cell depletion (Figure 25 e, f black arrow), as well as foci of crypt loss and/or

mucosal ulcers that varied in extent (Figure 25 g, h). The crypt epithelium adjoining the

ulceration often showed regenerative hyperplasia. The submucosa showed an extensive,

dense, predominantly lymphocytic mononuclear inflammatory cell infiltrate that focally

extended to the mucosa, a finding comparable to that noted in control and untreated

animals(Figure 25 c,d).

Table 3. Effects of DSS challenge and diet on degree of inflammation in the colon[1]

Inflammation	ZD+	ZT+	ZN+	PF+	ZS+
Mild	2	1	1	1	1
Moderate	2	1	2	0	0
Severe	4	6	5	7	7

[1]Values indicated number of rats per group with each level of inflammation.
Inflammation was rated as severe (mucosal ulceration), moderate (crypt abscess), mild
(mild active inflammation, loss of goblet cells, early mucosal changes).
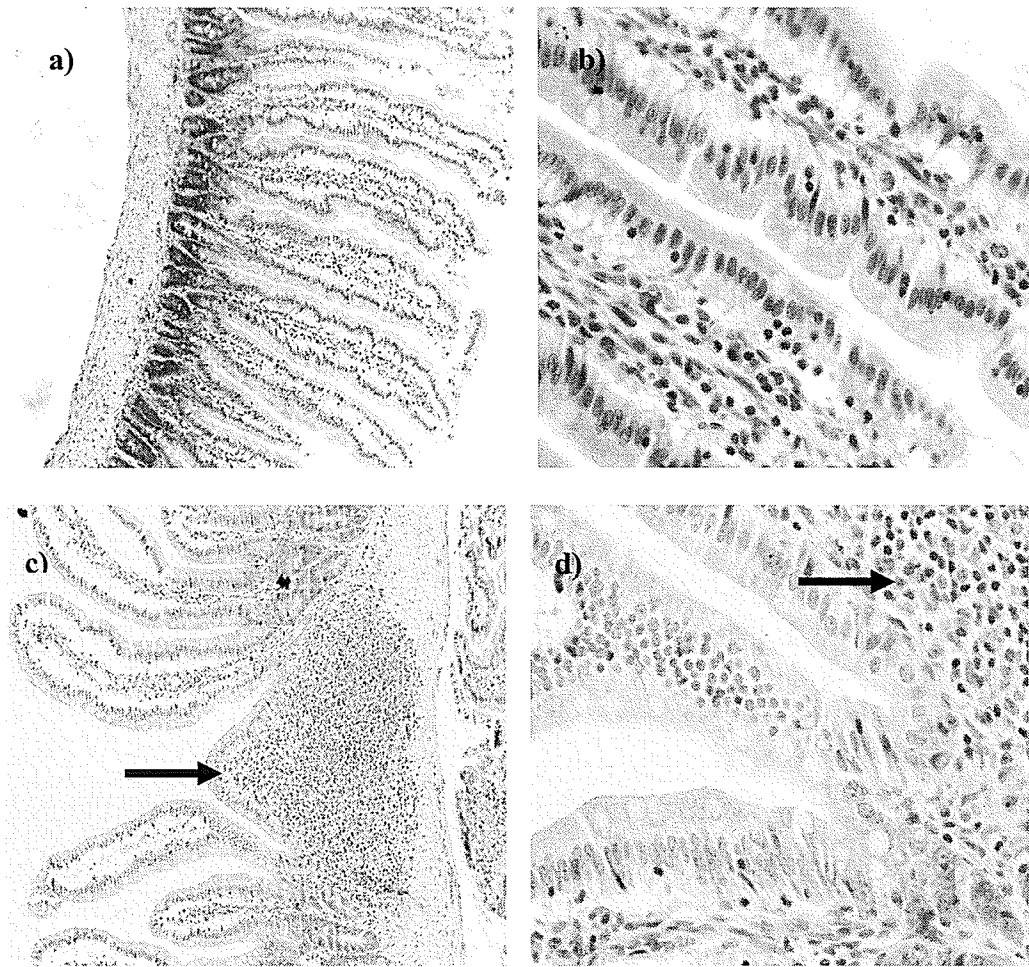
Figure 20. Representative histology sections of small intestine from DSS-challenged and unchallenged rats. Note lymphocytic aggregate (lymphoid follicle) (black arrow) in submucosa and mucosa in c & d; 10x magnification (a & c), 40x magnification (b&d).
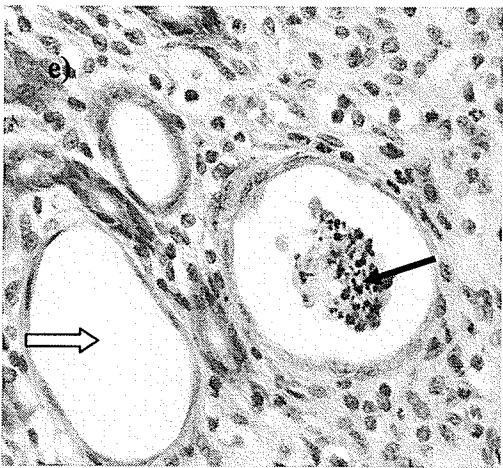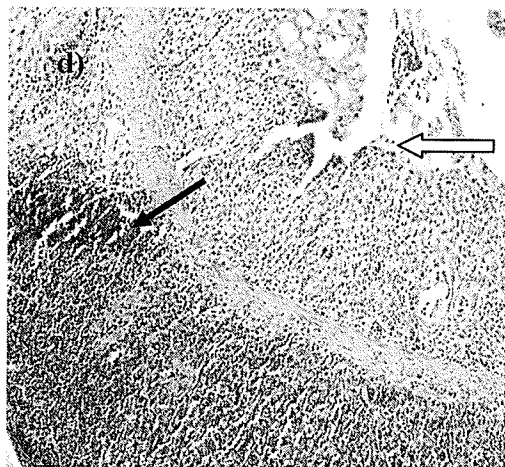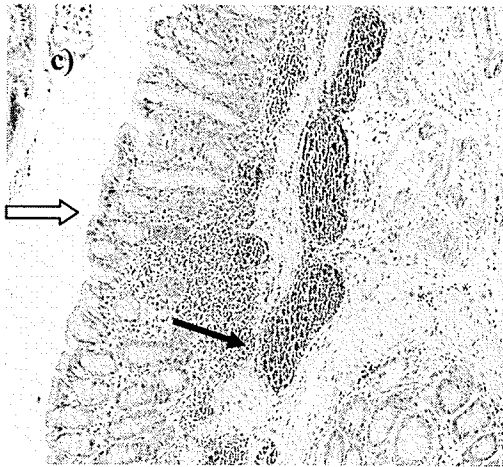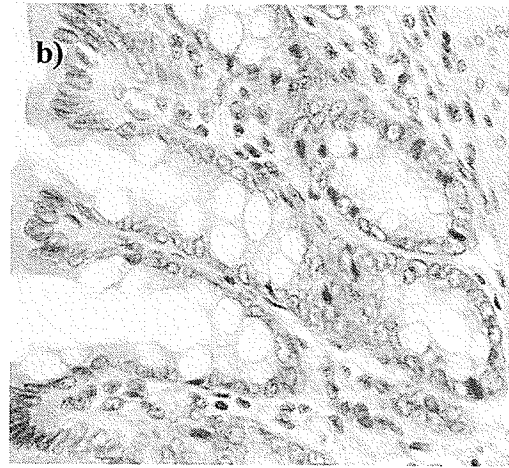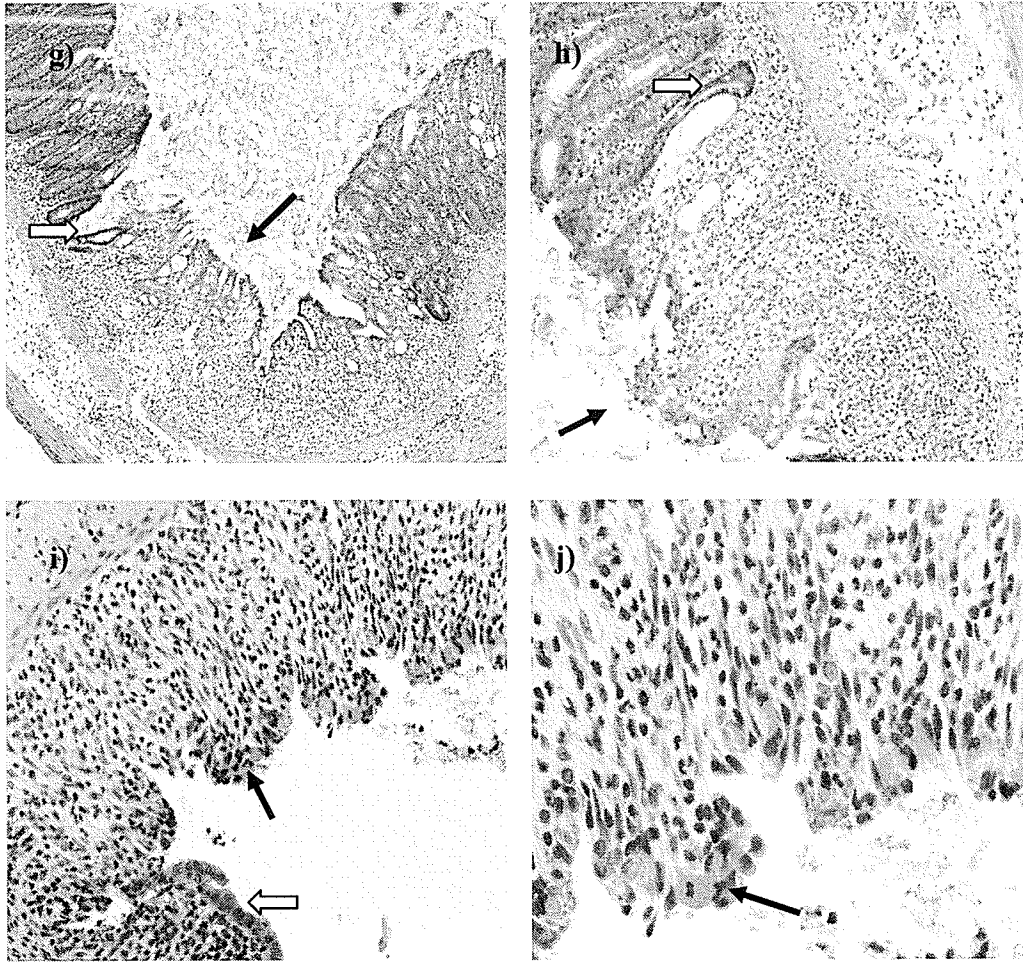
Figure 21. Representative histology sections of colon from DSS-challenged and unchallenged rats. H&E staining of the colon (a) 10x and (b) 40x magnification for normal colon tissue; (c) 10x and (d) 40x magnification showing lymphocytic aggregates in the submucosa extending to mucosa (black arrow) and loss of crypts (white arrow); (e) 10x and (f) 40x magnification showing crypt abscesses (white arrow) and goblet cell depletion (black arrow; dark blue cells are goblet cells); (g) 5x and (h) 10x magnification showing loss of crypts (black arrow), goblet cell depletion, infiltration of inflammatory cells, regenerative hyperplasia of crypt epithelium, and flattening of lining of crypt (white arrow); (i) 20x and (j) 40x magnification showing ulceration (white arrow) and congestion (black arrow).

Metallothionein

Immunohistochemical localization in small intestine

The results of immunohistochemical staining for MT in the rat small intestine are shown in Table 3. The staining was specific for MT because there was strong positive staining in the small intestine tissues from the zinc supplemented rats and no staining in the negative control tissues. Moderate to strong cytoplasmic and nuclear MT staining of Paneth cells (PC), surface epithelial cells (SEC), goblet cells (GC) and lamina propria (LP) was present in all groups with the exception of the ZD group which had no to very weak (one to two cells only) MT staining.

Table 4. Effects of DSS challenge and diet on immunolocalization and intensity of MT staining in small intestine.[1,2]

	ZD+	ZD-	ZT+	ZT-	ZN+	ZN-	PF+	PF-	ZS+	ZS-
PC	--	--	+++	+++	+++	+++	+++	+++	+++	+++
SEC	--	--	++	++	++	++	++	++	++	+++
GC	--	--	++	++	++	++	++	++	++	+++
LP	--	--	++	++	++	++	++	++	++	+++

[1]Scoring system: (--) none to very few cells, (+) mild, (++) moderate, (+++) high
[2]Abbreviations: PC=Paneth cells, SEC=surface epithelial cells, GC=goblet cells and LP=lamina propria.

The strongest and the most consistent MT immunostaining of Paneth cells, surface epithelial cells, goblet cells and lamina propria was seen in the unchallenged ZS

rats (Figure 18 I, j). There was strong MT immunostaining in Paneth cells and moderate immunostaining in surface epithelial cells, goblet cells and lamina propria in all other groups except the ZD rats which had very weak to no detectable MT immunostaining(Figure 18, 19).

Immunohistochemical localization in colon

The MT immunostaining of the colon was specific because the negative controls gave no staining and there was positive staining in the MT positive tissues (Figure 20 a,b). MT immunostaining was nondetectable in the unchallenged ZD group whereas it was very weak in the submucosa of the DSS-challenged ZD rats (Figure 20 and 21 a,b). All DSS-challenged rats had MT staining in the crypts and submucosa (Figure 21) while the non-challenged rats had MT immunostaining only in the crypts (Figure 20). The strongest crypt staining was seen in the unchallenged ZT rats and the unchallenged ZS rats (Figure 20). The strongest submucosal staining was seen in the DSS-challenged ZT, ZN, PF and ZS rats (Figure 21).

Table 5. Effects of DSS challenge and diet on immunolocalization and intensity of MT staining in the colon.[1]

	ZD+	ZD-	ZT+	ZT-	ZN+	ZN-	PF+	PF-	ZS+	ZS-
Submucosa	+	--	+++	--	+++	--	+++	--	+++	--
Crypt	--	--	+++	+++	+	++	++	++	++	+++

[1]Scoring system: (--) none to very weak, (+) mild, (++) moderate, (+++) high

a) ZD-

b) ZD-

c) ZT-

d) ZT-

e) PF-

f) PF-
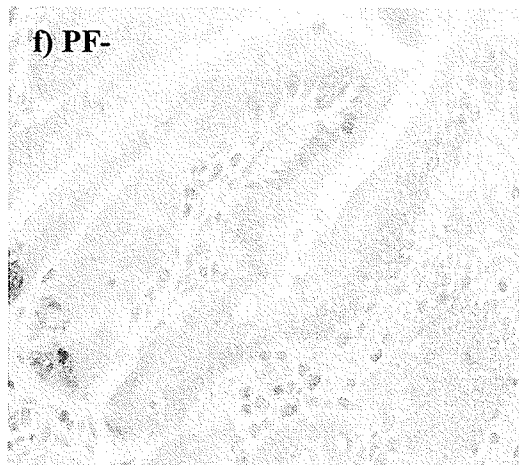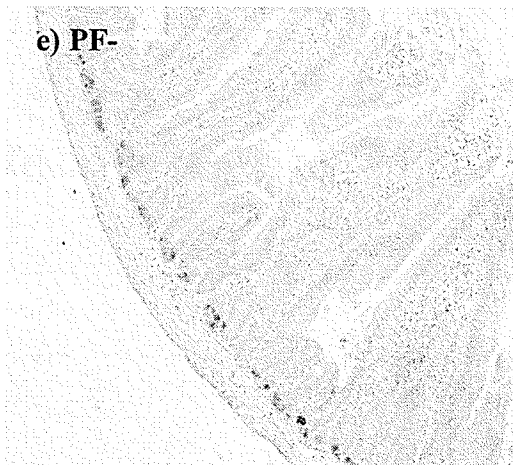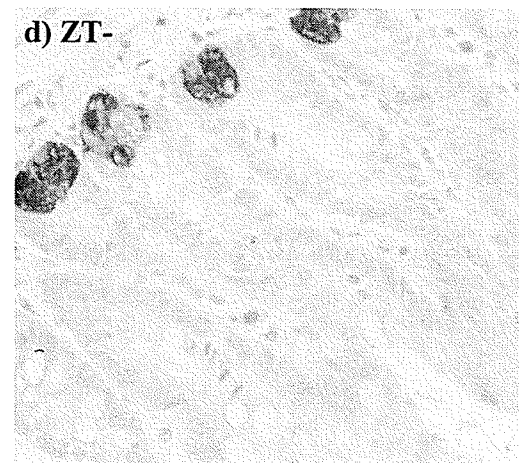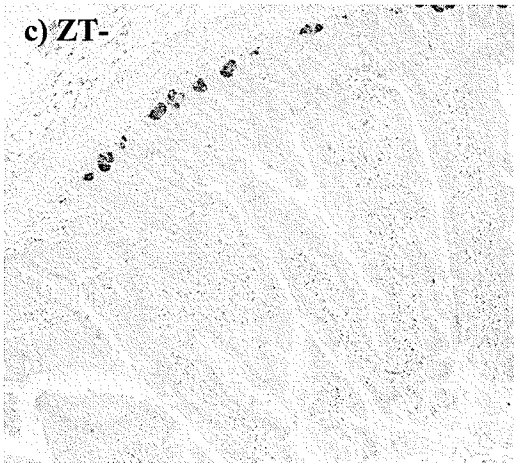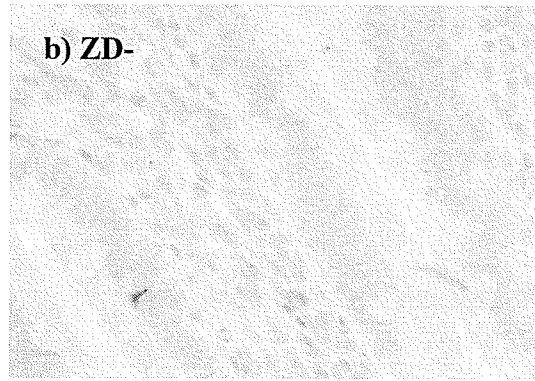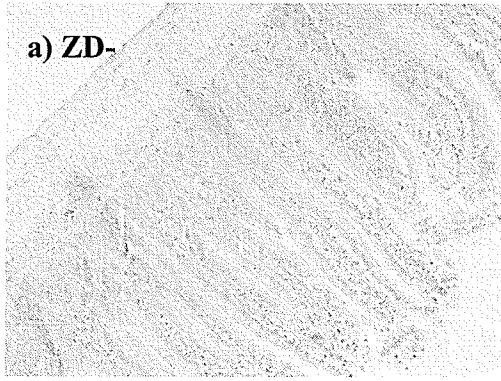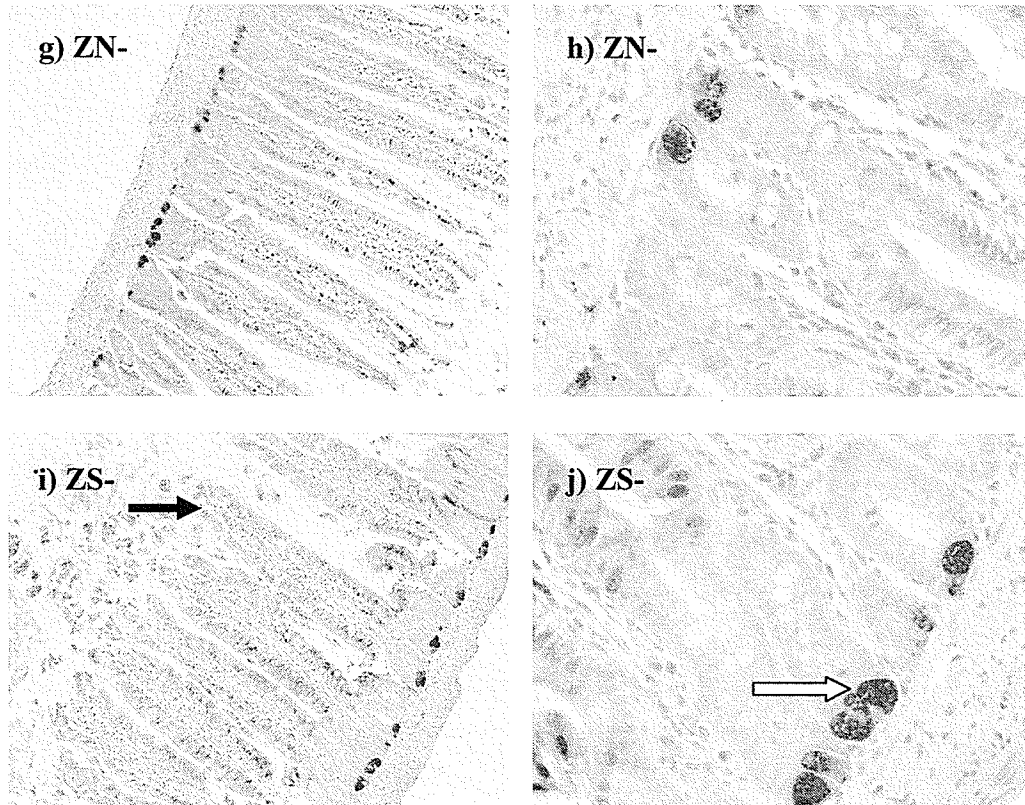
Figure 22. Effects of diet on immunohistochemical staining for MT in small intestine (mid-point) of rats not treated with DSS. (a) ZD- rats with no detectable MT staining (10x magnification), (b) ZD- rats (40x magnification), (c) ZT- rats with strong MT staining of Paneth cells (10x magnification), (d) ZT- rats (40x magnification), (e) PF- rats with MT staining of the Paneth cells and very few at the villi (10x magnification), (f) PF- rats (40x magnification), (g) ZN- rats with strong MT mainly at Paneth cells (10x magnification), (h) ZN- rats (40x magnification), (i) ZS- rats strong MT stain at the villi and Paneth cells (10x magnification), (j) ZS- rats (40x magnification).
Black arrow pointing at villi, white arrow pointing at Paneth cells.

a) ZD+

b) ZD+

c) ZT+

d) ZT+

e) PF+

f)PF+
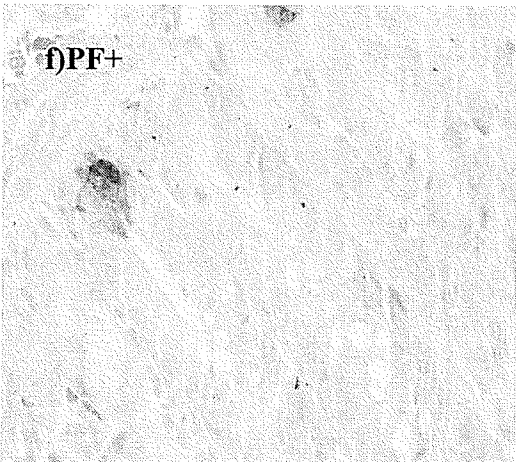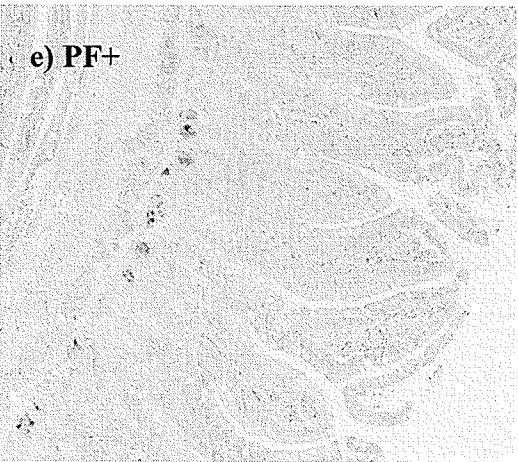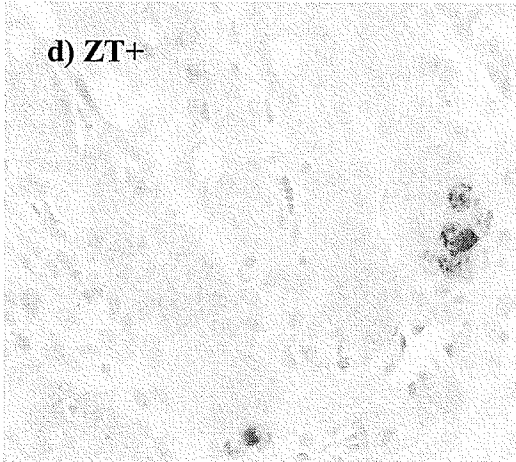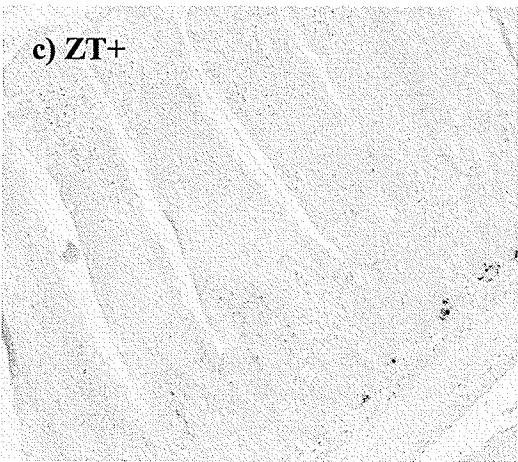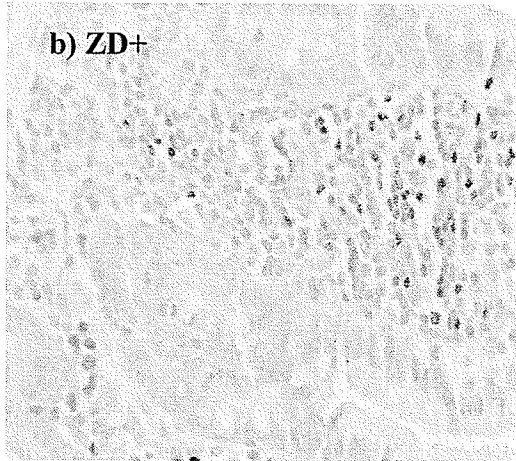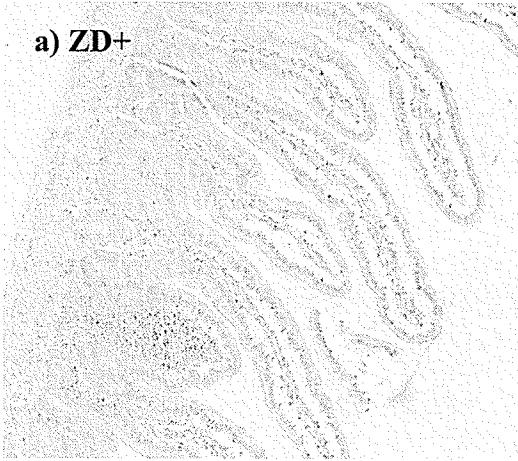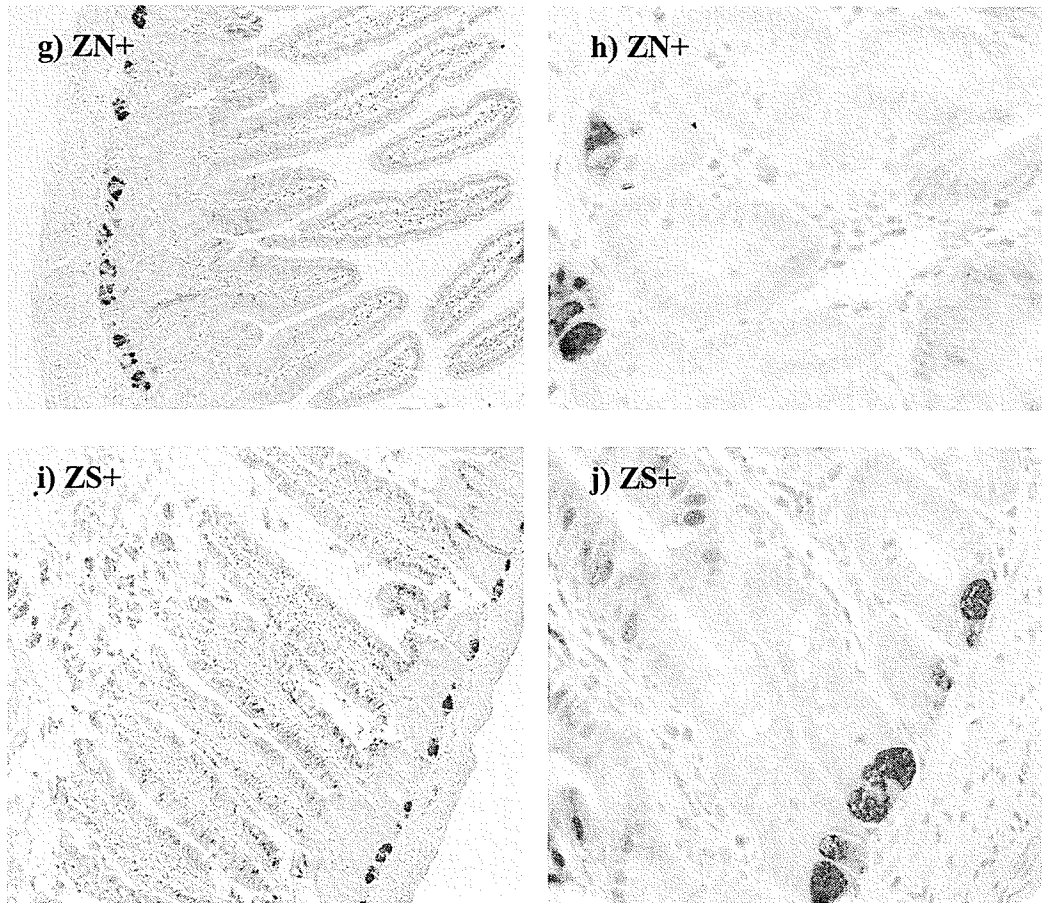
Figure 23. Effects of diet on immunohistochemical staining for MT in small intestine (mid-point) of rats treated with DSS. First two images show ZD+ rats with no MT stain, and the remaining images show positive staining. (a) ZD+ rats with no MT stain (10x magnification), (b) ZD+ rats (40x magnification), (c) ZT+ rats with MT staining at the villi and Paneth cells (10x magnification), (d) ZT+ rats (40x magnification), (e) PF+ rats with MT staining at villi and Paneth cells (10x magnification), (f) PF+ rats (40x magnification), (g) ZN+ rats with strong MT staining at villi and Paneth cells (10x magnification), (h) ZN+ rats (40x magnification), (i) ZS+ rats with strong MT staining at Paneth cells and villi (10x magnification), (j) ZS+ rats (40x magnification).

a) ZD-

b) ZD-

c) ZT-

d) ZT-

e) PF-

f) PF-
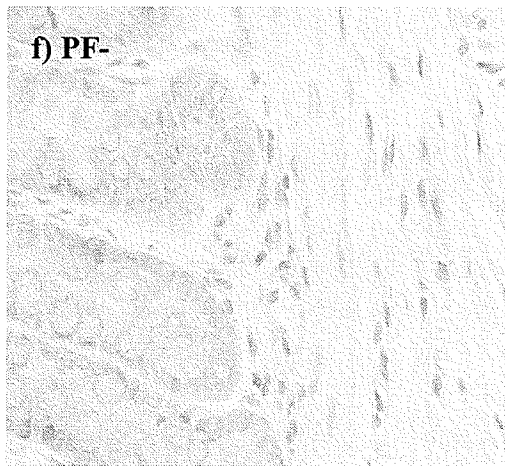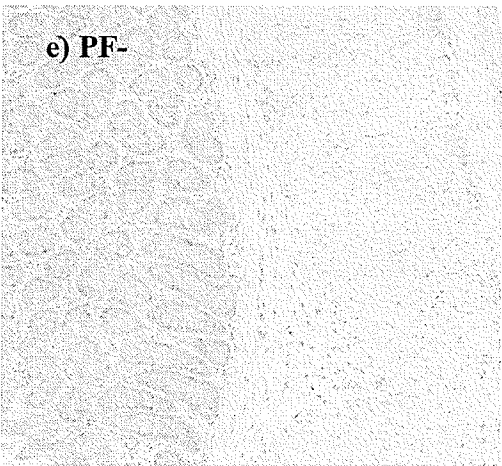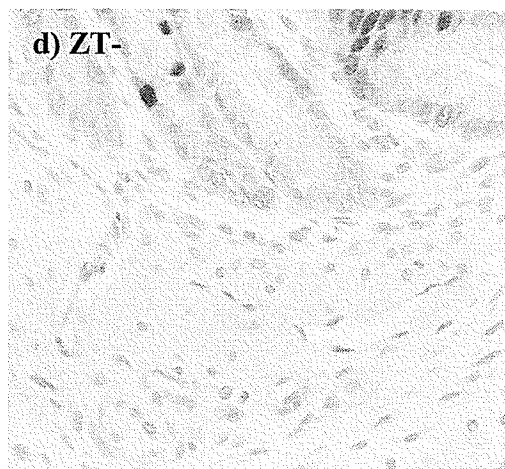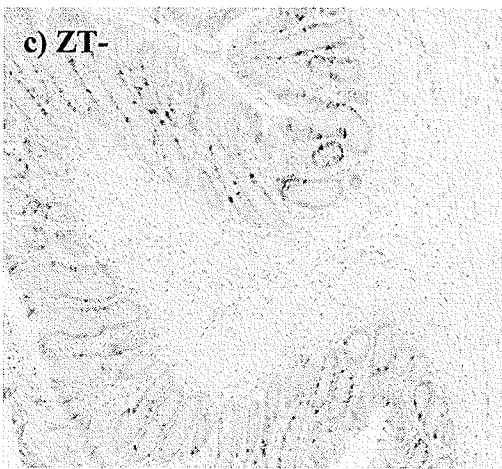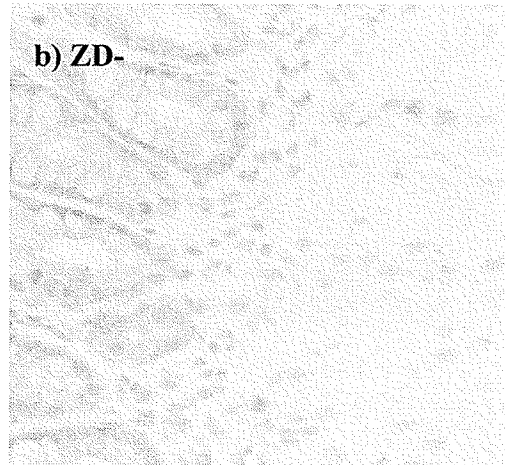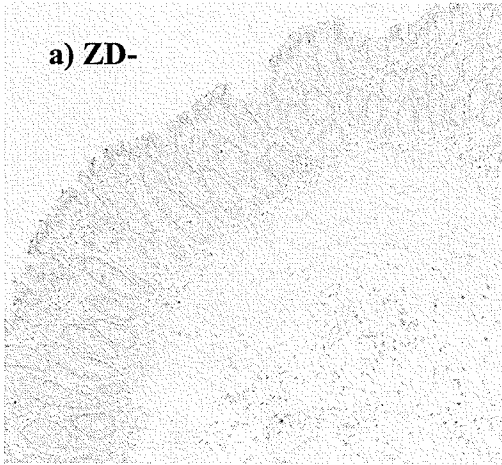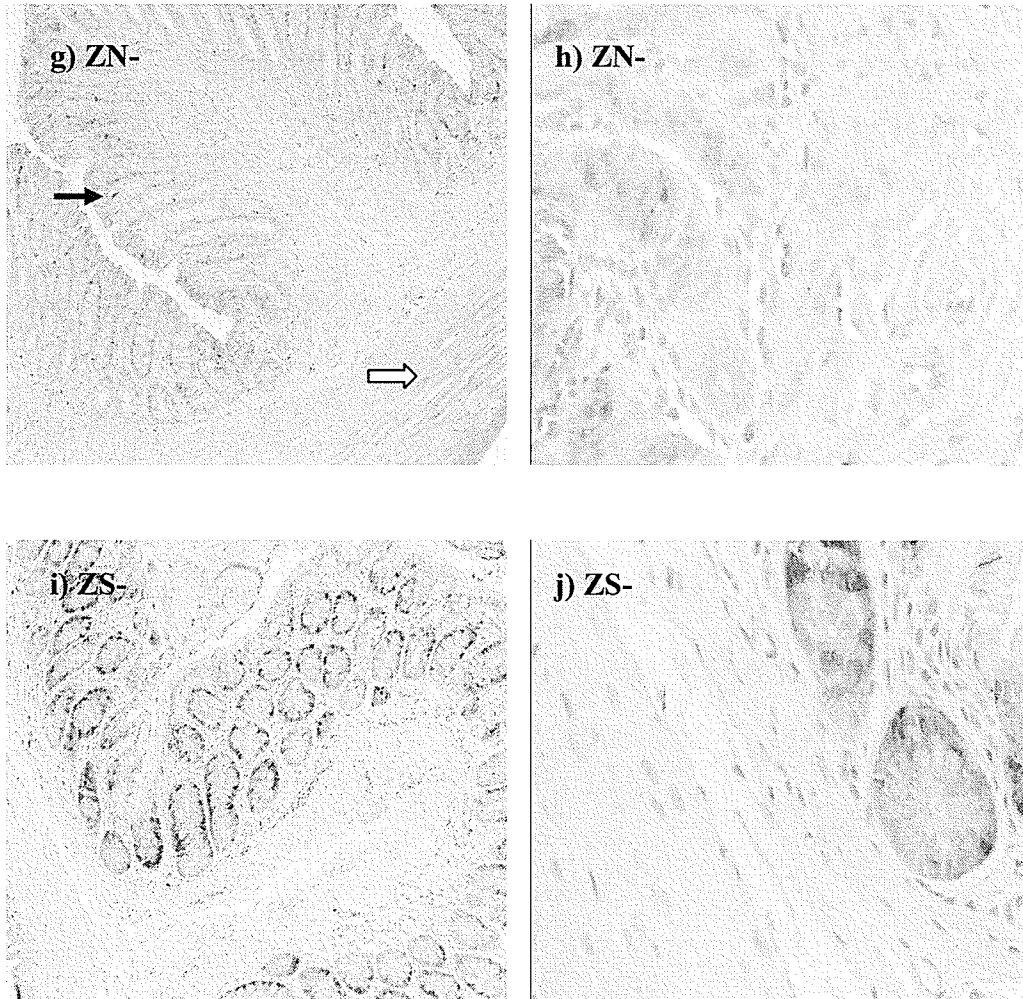
Figure 24. Effects of diet on immunohistochemical staining for MT in proximal colon of rats untreated with DSS. The first two images showing negative MT staining and the rest of the images show positive MT stain at the crypts of the colon. (a) ZD- rats with no MT staining (10x magnification), (b) ZD- rats (40x magnification), (c) ZT- rats with MT staining at the crypt (10x magnification), (d) ZT- rats (40x magnification), (e) PF- rats (10x magnification), (f) PF- (40x magnification), (g) ZN- (10x magnification), (h) ZN- (40x magnification), (i) ZS- (10x magnification), and (j) ZS- (40x magnification). Black arrow showing crypt of the colon, and white arrow showing the submucosa. Notice no staining in the submucosa of all the untreated rats.

a) ZD+

b) ZD+

c) ZT+

d) ZT+

e) PF+

f) PF+
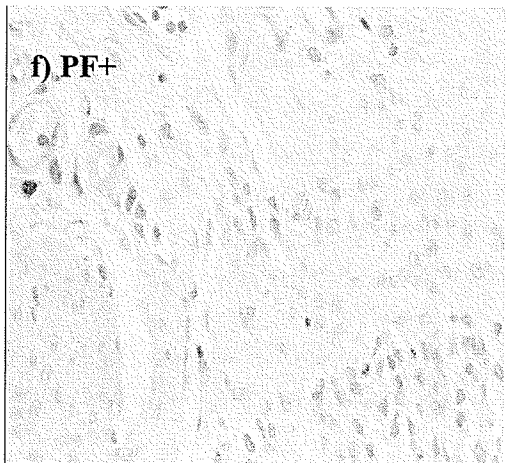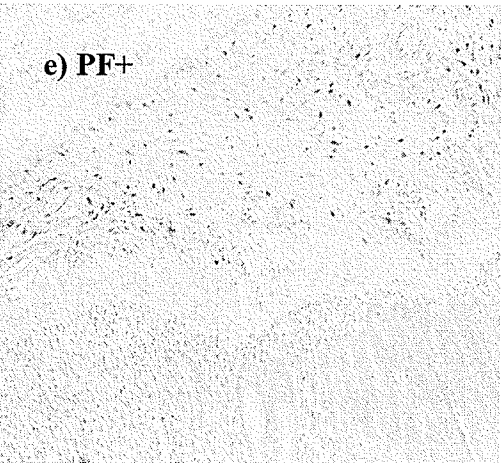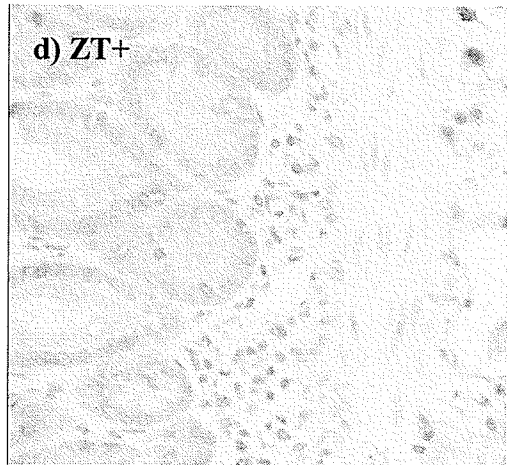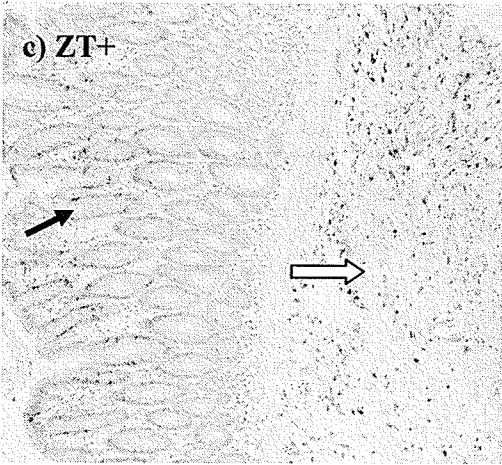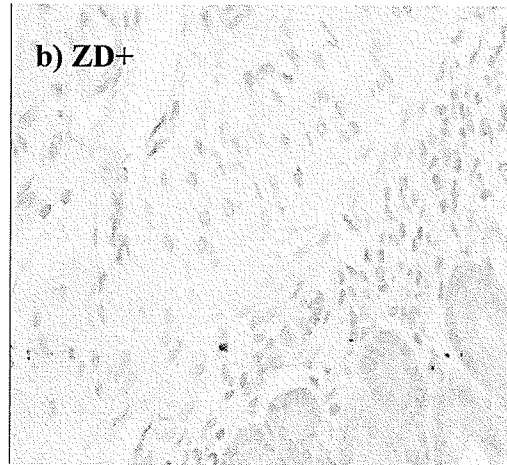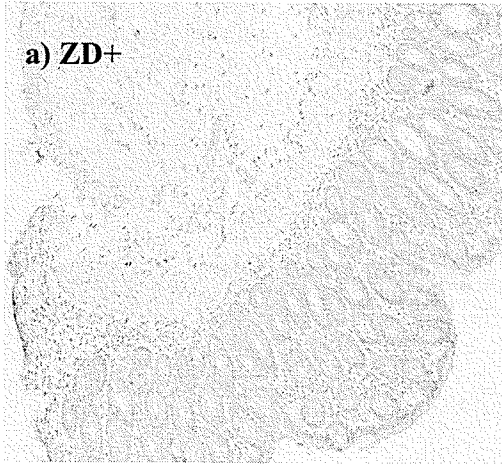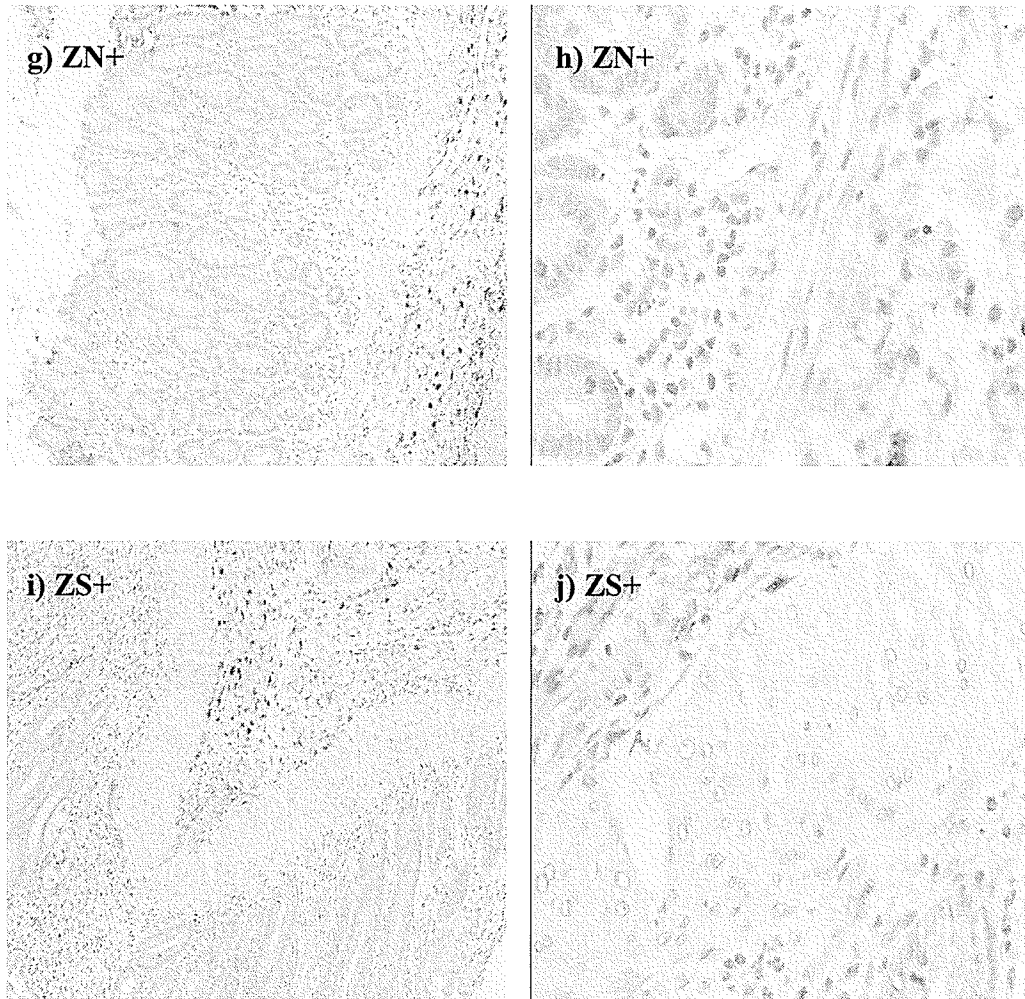
Figure 25. Effects of diet on immunohistochemical staining for MT in proximal colon of rats treated with DSS. Metallothionein negative staining in the first two images (a,b) while the rest of the images show positive MT staining at the submucosa and crypts. (a) ZD+ (10x magnification), (b) ZD+ (40x magnification), (c) ZT+ (10x magnification), (d) ZT+ (40x magnification), (e) PF+ (10x magnification), (f) PF+ (40x magnification), (g) ZN+ (10x magnification), (h) ZN+ (40x magnification), (i) ZS+ (10x magnification), (j) ZS+ (40x magnification). Black arrow indicates the crypt of the colon, and white arrow indicates the submucosa. There is staining in both the crypt and submucosa after the DSS challenge.

<u>Apoptosis</u>

Immunohistochemical staining of apoptosis (caspase-3) in small intestine

Apoptosis was graded per tissue section as the number of apoptotic-positive cells: 1-5 cells = low, 5-15 cells = moderate, > 15 cells severe apoptosis. The grading included the mucosa, submucosa and serosa of the small intestine (Figure 22).

The DSS-challenged ZD rats had the highest apoptotic cell number, while the ZS rats (DSS-challenged and unchallenged) had the lowest apoptotic cell number (Table 5). All the rest of the groups had moderate apoptosis. ZD+, ZT+ and PF+ had more apoptosis immunostaining than their respective unchallenged counterparts (Table 5).

Table 6. Effects of DSS challenge and diet on apoptosis (caspase-3) immunostaining in the small intestine.[1]

	ZD+	ZD-	ZT+	ZT-	ZN+	ZN-	PF+	PF-	ZS+	ZS-
apoptosis	+++	++	+++	++	++	++	+++	++	+	+

[1]Positive staining in 1-5 cells = (+), 5-15 cells = (++), > 15 (+++)

Immunohistochemical staining of apoptosis (caspase-3) in colon

Apoptosis was graded per tissue section as: 1-5 cells = low, 5-15 cells = moderate, > 15 cells severe apoptosis and the grading was completed on the submucosa and crypts of the colon (Figure 23)

ZS rats had the lowest apoptosis in all groups DSS challenge had no obvious effect regarding the ZS rats. All other groups had high apoptosis after the challenge however before the challenge it was moderate. Except for the ZN rats they remained moderate before and after the challenge (Table 5)

Table 7. Effects of DSS challenge and diet on apoptosis (caspase-3) immunostaining in the proximal colon.[1]

	ZD+	ZD-	ZT+	ZT-	ZN+	ZN-	PF+	PF-	ZS+	ZS-
apoptosis	++	++	++	+	+++	+	+++	++	++	+++

[1]Positive staining in 1-5 cells = (+), 5-15 cells = (++), > 15 (+++)

Apoptosis was elevated after the challenge in most of the groups. ZS rats had higher apoptosis before the challenge than after. ZD rats had moderate apoptosis in both before and after the challenge. ZN and PF rats had the highest apoptosis after the challenge. Untreated ZN and ZT had the lowest apoptosis.
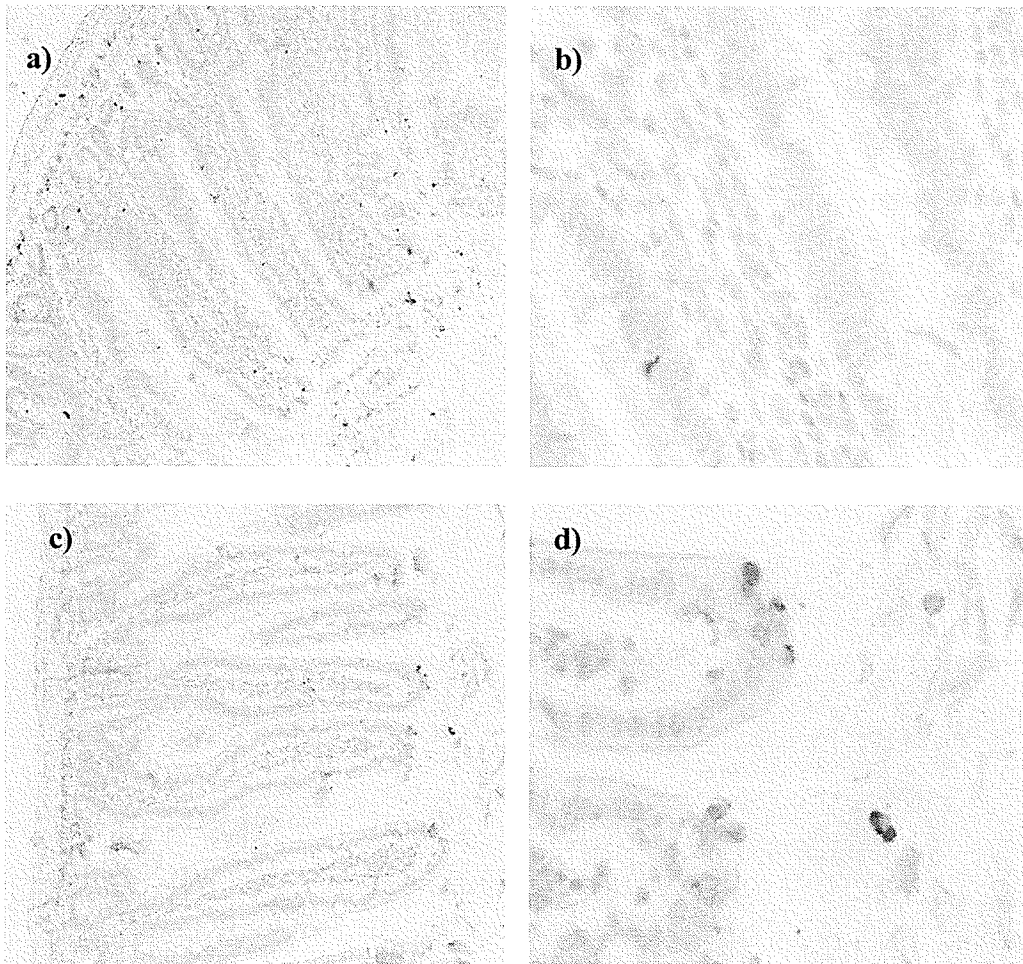
Figure 26. Representative images of immunohistochemical staining for apoptosis (caspase-3) in small intestine (mid section). The first two images show no apoptosis, and the last two images show moderate apoptosis. (a) negative control image (10x magnification), (b) negative control image (40x magnification), (c) positive sample image (10x magnification), and (d) positive sample image (40x magnification).
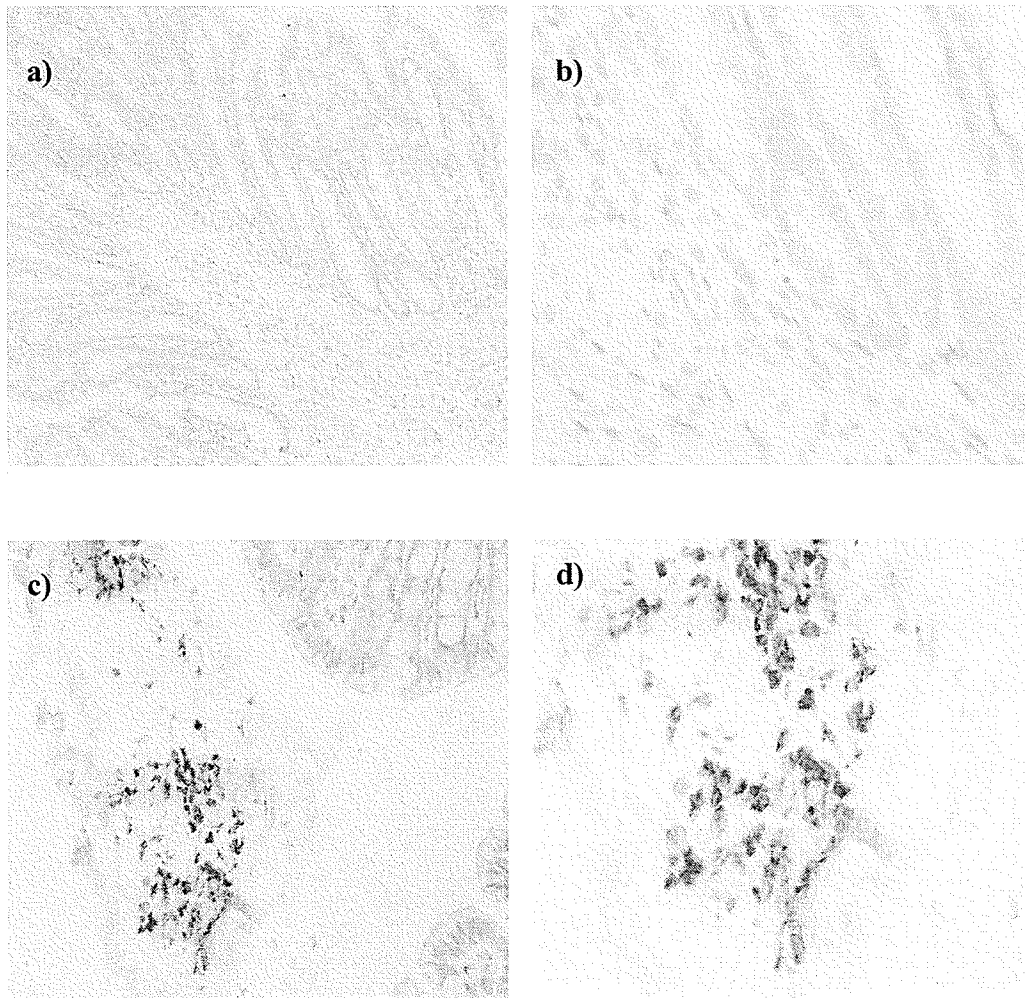
Figure 27. Representative images of immunohistochemical staining for apoptosis (caspase-3) in proximal colon. (a) negative control image (10x magnification), (b) negative control image (40x magnification), (c) positive sample image (10x magnification), and (d) positive sample image (40x magnification).

V. DISCUSSION

Growth Assessment

At the end of the experiment, the DSS treated rats had 5% lower **body weight** (Figure 2), 20% less feed intake after the DSS challenge (Figure 4b), more colonic inflammation based on histology (Figure 21) and presence of diarrhea (Table 2) compared to the untreated rats. Thus, the greater weight loss in the DSS treated rats could be due to loss of appetite, inflammation, and diarrhea causing dehydration. However, water intake was not affected by DSS consumption in the drinking water (Figure 5) and this was also reported by Geier et al (2007). The greater weight loss in DSS-treated rats could also be related to the blood loss due to bloody diarrhea (Table 2) since the rats challenged with DSS had a lower red blood cell count (Figure 13a) and lower hemoglobin level (Figure 13b) than the non-challenged groups. Fewer red blood cells and a lower concentration of hemoglobin could reduce the amount of oxygen transported to the cells to metabolize energy, thus, making it more difficult to maintain growth. In our study DSS, had no effect on **body or tail length** (Figures 3 a,b) which are indicators of longitudinal growth. This may be due to the short period of time (4 days) for the DSS treatment and this may not be enough time to produce measurable growth retardation in body or tail length. Other DSS studies have not reported body or tail length.

Other researchers have reported lower body weights after DSS challenge. Shimizu et al (2003) gave different concentrations of DSS (2%, 3% and 4%) in drinking water to 4 week old weanling rats. There were no differences in the body weight before DSS ingestion, but 7 days after the DSS challenge, the body weights were significantly lower. The body weights of the groups receiving 3% or 4% DSS were 29% lower than

the 2% DSS and control (0% DSS) rats. In another study by Geier et al (2007), 150 g

male Sprague Dawley rats were given 3% DSS in drinking water for 7 days. They found

that the total body weight gain and mean feed intake during the period of DSS ingestion

was 44% and 17% lower, respectively, in DSS treated rats compared to untreated controls.

In our study, the duration of the DSS challenge was 4 days because the amount of DSS

provided (5% DSS in drinking water) was higher than the previously mentioned studies.

In a pilot study, we found that the 7 week old rats could not survive for more than 5 days

with 5% DSS administered in their drinking water. We also tried 2% DSS in the drinking

water but after 8 days of treatment the rats had soft stool with a few blood spots and there

were no histological changes. Vicario et al (2005) reported that 100% of the young

Sprague Dawley rats in their study could not survive beyond day 6. To insure all the rats

in the present study would be exposed to the same number of days of DSS exposure and

considering that the deficient rats could be more susceptible to the DSS challenge, we

chose to terminate the rats at the fourth day of the treatment because the rats in the pilot

study deteriorated substantially after day 3 of DSS induction.

Dietary Zn intake also had an effect on the body weight, feed intake and

longitudinal growth of the rats. The ZD rats had the lowest body weight and they

weighed 29% less than the ZN and ZS groups (Figure 2). After changing the diet of the

ZD rats to the supplemented diet (300 mg Zn/kg diet) for 4 days, the feed intake of the

ZT group increased by 33% (Figure 4a) and the body weight of the ZT rats increased by

8% (Figure 2), indicating the importance of dietary Zn for appetite and weight gain.

During the whole study, the body weight of ZS rats did not differ from the ZN group.

Thus, the high amount of dietary Zn for a long term period (3 weeks) had no beneficial or

adverse effects on body weight. On the other hand, short term treatment (3 days) with high doses of Zn (300 mg Zn/kg) to rats with a Zn deficient status has beneficial effects on body weight gain (i.e. ZT group).

In our study, the ZD rats had 9.6% lower body weight than the PF (Figure 2) after 21 days. The ZD and PF rats had the same living conditions and consumed the same amount of diet with the only difference being the Zn level in the diet. The PF rats are energy deficient but have a better feed efficiency than the ZD, therefore, the PF weigh more.

A number of studies have investigated Zn deficiency and Zn repletion in weanling rats.. Szczurek et al (2001) fed 46-56 g male Sprague Dawley rats for 20 days, and reported that the ZD and PF rats had the same body weight and that they weighed 43-46% less than the control rats. Szczurek et al (2001) had a Zn repletion phase with normal dietary Zn levels (30 mg Zn/kg diet), and the body weight of the Zn replete group increased by 10%, and the PF replete group increased by 21% over 24 hrs. The feed intake of the Zn replete group increased 137% and the PF replete group increased 254% during the first 24 h of dietary Zn repletion.

Hosea et al (2004b) assigned 3 week old male Sprague Dawley rats to zinc-deficient (ZD, < 1 mg Zn/kg diet), control (CTL, 30 mg Zn/kg diet) or pair-fed (PF, provided control diet in the amount consumed by zinc-deficient rats) groups for 21 days. The ZD group consumed 52% less feed and weighed 49% less than the control group while the ZD rats weighed 14% less than PF rats. After repletion for 3 days with control diet, the feed intake was increased 88% in ZD and 123% in PF rats. After the three days of Zn repletion, the body weight of the ZD rats increased by 82% and the PF rats by 77%.

Sun et al (2006) gave 21 day old male Harlan Sprague Dawley rats a zinc-deficient (3.15 mg Zn/kg diet), zinc-adequate (35.9 mg Zn/kg diet), or zinc-supplemented diet (347.5 mg Zn/kg diet) for 6 weeks. Unlike our study, the rats fed the zinc-supplemented diet had a 2.6% higher body weight than the zinc-adequate rats. Similar to our study, the zinc-deficient rats had 48% lower body weight than the control group (zinc supplemented=331.4 ± 3.1, control= 323 ± 2.4, and zinc deficient =167.7 ± 3.4 g, respectively). The feed intake showed the same pattern (28.1 ± 1.5, 24 ± 1.4, and 17.3 ± 1.7 g/d) at the end of the six week experiment.

In our study, the **feed intake** of the ZD, PF and the ZT rats was the same before the DSS challenge (Figure 4a). The PF group received the same amount of feed as consumed by the ZD group, and the ZT rats were the same as ZD group before the DSS challenge. ZN and ZS both had higher feed intake than the other groups and there was no difference between ZN and ZS. After the DSS challenge the feed intake was changed such that the challenged group had a 1.3 fold lower feed intake than the unchallenged group. After the DSS challenge, the ZD and the PF continued to have lowest feed intake while the other groups had significantly higher feed intake. The most obvious change was the effect of dietary Zn on the ZT group as it increased the appetite and feed intake of the ZT rats even during the challenge with DSS.

There was no significant difference among the diet groups in regards to **water intake** corrected for body weight (Figure 4) except that the PF rats had the lowest water intake and we do not have an explanation for this observation. As for DSS challenge, water intake was not affected and this was also observed by Geier et al (2007) after 7 days treatment with 3% DSS.

ZD and ZT rats had a shorter **body length** and were not different from each other while the PF, ZN and ZS rats had the longest body length with no difference among those groups (Figure 3a). The **tail length** was shorter in ZD, PF, and ZT rats with no significant difference among them, and the ZN and ZS rats had a longer tail length than the other groups (Figure 3b). Similarly, Orbak et al (2007) reported that body length and tail length were retarded after 10-16 days of Zn deficiency in 24 day old Sprague Dawley rats. From this we can conclude that Zn has a significant effect on the longitudinal growth of the body and tail. The PF had a longer body length than ZD and the PF consumed the same amount of feed as ZD with the only difference from ZD being the presence of normal Zn levels; this indicates that Zn deficiency produces stunting malnutrition. Another finding is that Zn repletion did not change the body length of ZT compared to ZD (Figure 3a), and this could be due to the short (3 day) duration of the repletion.

The **small intestine weight/body weight** was increased in the DSS challenged rats and reached the highest levels in the ZT and ZS rats (Figure 6a). As for **small intestine length**, there was no significance between DSS challenged and non-challenged rats while ZN and ZS had the longest small intestine in comparison with the other groups (Figure 6b). We found that DSS had opposite effects on the organ/body weight ratios for the small intestine.

Two studies have reported that **colon weight/body weight** ratio was increased with DSS challenge and that **colon length** decreased in association with DSS colitis (Geier et al, 2007; Vowinkel et al 2004). In a study by Tsubouchi et al (2006), they found that the colon length of male Wistar rats treated with 2.5 % DSS for 7 days was decreased compared with the normal rats. The Tsubouchi study and the Vowinkel study

did not mention if the colon was emptied or not while in our study and the Geier study

the colon was emptied before weighing and measuring it. In the present study, a segment

of the colon was taken for histology to insure a good histology section and then the colon

was emptied, weighed and measured. The same was done for the small intestine.

In our study, the colon weight/body weight ratio was higher in the unchallenged

rats (Figure 7a) and the colon was shorter in the DSS-challenged rats (Figure 7b). We

found that lower Zn intake increased the colon weight/body weight ratio of the rats, and

higher Zn intake decreased it. Thus, the higher colon weight/body weight ratio in the

unchallenged rats which was equivalent in the dietary deficient groups (ZD, PF and ZT)

may represent a developmental delay associated with zinc and energy deficiencies.

DSS and diet had no significant effect on the **thymus weight/body weight**;

(Figure 8a). Like the study by Hosea et al (2004a), the ZD, energy-restricted rats (same

as PF rats in our study) and the control rats had no significant changes in the thymus

weight/body weight ratio. Even after the ZD group was repleted 3 days with Zn (30 mg

Zn/kg) there was still no change in thymus weight/body weight.

As for the spleen, DSS challenge elevated **spleen weight/body weight** ratio as

compared with the unchallenged rats (Figure 8b). This may be due to the response of the

immune system to the DSS challenge and production of more white blood cells (Figure

16a) and specifically lymphocytes (Figure 16b) and neutrophils (Figure 17a) to fight the

inflammation. To my knowledge, there are no published studies examining the effect of

DSS challenge in rats on the spleen weight. There were no significant differences in

spleen weight/body weight ratio among the diet groups. In contrast, Sun et al, (2006),

found a 23% increase in spleen weight/body weight in 21 day old male Harlan Sprague

Dawely rats given the supplemented diet (347.5 mg Zn/kg diet) for 6 weeks compared to the Zn deficient (3.15 mg Zn/kg diet) and Zn adequete (35.9 mg Zn/kg diet) groups.

In our study, the **liver weight/body** weight ratio was decreased in the ZD and PF rats compared to the ZT, ZN and ZS rats (Figure 9a). The previously mentioned study by Sun et al (2006) also found a lower liver weight/body weight ratio in the Zn deficient (6.7 %) and the Zn supplemented (4.9%) groups compared to their control rats. Szczurek et al (2001) reported that ZD and PF rats had lower liver weight than the control (4.13 ± 0.39, 3.95 ± 0.47, and 9.16 ± 0.52 g, respectively) including after repletion (5.31 ± 0.27 g) but these results were not expressed as liver weight/body weight ratio. Prescod et al (1998) found that the liver weight/body weight of ZD rats (1 mg Zn/kg diet) was not significantly different from the control rats (30 mg Zn/kg diet).

Adipose tissue/body weight was 15% lower in the DSS challenged rats (Figure 9b) which could be explained by the loss of energy and body weight in the challenged rats. Zn deficiency reduced the adipose weight/body weight and the 3 day Zn repletion of the ZT group was insufficient to restore adipose tissue. However, energy deficiency in the presence of Zn (i.e. PF group) was associated with maintenance of adipose stores.

Mineral analysis

As expected, ZD rats had the lowest **serum Zn** levels for both DSS treated and non-treated rats and there was no significant difference with DSS challenge (Figure 10). The 84% reduction of serum Zn in the ZD rats compared to PF and ZN rats demonstrated the severity of the Zn deficiency. Serum Zn of the PF rats was similar to ZN rats and was not altered by DSS challenge. The untreated ZS rats had the highest serum Zn levels but

the serum Zn was decreased by 37.5% in the ZS DSS-treated rats. Zn supplementation for 3 days elevated the serum Zn in the ZT group but DSS challenge decreased the serum Zn levels by 24.3% in the ZT rats. From this we can conclude that the presence of intestinal inflammation and diarrhea during DSS treatment affects Zn absorption, particularly at higher doses of Zn since this affect was not observed in ZD, PF or ZN rats. Al-Awadi et al (1998) fed rats normal levels of dietary Zn and challenged rats with acetic acid and TNBS (trinitrobenzenesulfonic acid) and there was no change in the serum Zn concentration.

On the other hand, **femur Zn** and **liver Zn** concentrations were not affected by DSS treatment (Figure 11a & 11b). As expected, the ZS rats had the highest femur Zn concentration while ZD rats had the lowest femur Zn. The 3 day supplementation with 300 mg Zn/kg produced a 56.2% increase in the femur Zn of the ZT rats but they did not recover to ZN levels. Unlike the femur Zn, the PF and the ZT rats had higher liver Zn compared to ZD and ZN. This could be due to developmental effects (PF) and rapid Zn accumulation in the liver (ZT). In a study done by Takashi et al (2004) they found that liver Zn was 24.1% lower in the ZD (0.6 μg/g diet) Sprague Dawley rats compared to control rats (41 μg/g diet) and that the femur Zn was 60% lower in the ZD than the control. Since the liver is involved in regulation of Zn, it is not necessarily an indicator of Zn deficiency (ZD group) or excess Zn consumption (ZS group) compared to serum or femur Zn.

Many studies have discussed the effect of other minerals on Zn absorption with Fe and Cu being the two main minerals affecting Zn absorption or vice versa. Large quantities of Zn could interfere with Cu bioavailability because the increased intake of Zn

will induce metallothionein (Cu-binding ligand) synthesis in the intestine and other tissues. This protein will trap Cu and make it unavailable for absorption (Shills, 1999). In our study, the DSS-challenged rats had 15% lower **liver Cu** than the unchallenged rats (Figure 12a). As will be described in the histology results, DSS challenge damaged the surface of the intestine (Figure 21) which could be a reason for decreased Cu absorption. The lower levels of liver Cu in the ZS rats support the hypothesis that higher dietary Zn levels reduce intestinal Cu absorption. As seen in Figure 12a, liver Cu was reduced in ZT versus ZN and it could be that the increased Zn in their new Zn supplemented diet produced a decrease in Cu absorption. PF rats had 11% higher liver Cu than the ZD and ZN rats. The PF rats had highest levels of liver Zn even though they had normal levels of serum Zn, and this could be due to the energy restriction and a developmental delay in reduction of liver Zn. In the previously mentioned study by Takashi et al (2004), the liver Cu was 42.9% lower in the ZD rats than their control.

Fe and Zn are two elements which have chemically similar absorption and transport mechanisms and therefore limit each other's bioavailability (Walker et al, 2004). In our study, **liver Fe** was not affected by DSS challenge (Figure 12b). The liver Fe was normal in the ZS rats compared to ZN indicating that Zn supplementation did not affect iron absorption. ZD rats, but not the PF rats, had the highest liver Fe indicating increased iron absorption in Zn deficiency. Liver Fe in PF was still higher than the ZN rats. The higher liver iron in the ZD and energy restricted rats may indicate that both of these conditions provide a greater chance for more iron to be absorbed. Likewise, the Takashi et al (2004) study showed a 61% increase in liver iron in the ZD rats compared to the control rats. The liver Fe of ZT rats was 31% lower than ZD rats due to the 3 days

supplementation with 300 mg Zn/kg which indicates that high dietary Zn reduces iron absorption. From this we conclude that supplementation with 300 mg Zn/kg did not adversely affect iron absorption, however, Zn deficiency may be associated with increased Fe absorption.

Hematology

Red blood cell count and **hemoglobin** levels after 4 days of DSS challenge were approximately 5% lower in the challenged rats than the unchallenged rats (Figures 13a,b) and this was likely due to the blood loss (bloody diarrhea) during the DSS challenge. However, the rats are not considered anemic because both values are within the hematological normal range according to the Canadian Council on Animal Care (Appendix 1). The results showed a trend towards descending levels and perhaps with longer exposure to DSS, the animals would have developed anemia. Furthermore, the **MCH** (Figure 15a; mean corpuscular hemoglobin) was reduced and the **MCHC** (Figure 15b; mean corpuscular hemoglobin concentration) was reduced in the DSS-challenged rats which indicates that the rats are hypochromic (hemoglobin concentration in the cell was low) compared to our normal rats. The **MCV** of the DSS-challenged rats was normal, therefore, the size of the red blood cell is considered normal (Figure 14b). However, **hematocrit** levels were not significantly altered after DSS challenge (Figure 14a). Anemia is defined as a hemoglobin concentration or red blood cell count or hematocrit level below the normal range (Turgeon, 2005). Our results showed lower RBC and hemoglobin if compared with the unchallenged rats, however, the results are normal according to the Canadian Council on Animal Care (Appendix 1).

Shimizu et al (2003) found that 8 week old male Wistar rats receiving 4% DSS for 7 days had lower hemoglobin levels (7.3 ± 1.8 g/dl) compared to untreated control rats (10.0 ± 1.2 g/dl). Vowenkle et al (2004) also found that the male mice (8-10 weeks old) that were given either 3% or 5% DSS in the drinking water for 7 days had anemia based on lower hematocrit levels than the untreated control rats (35 ± 10, 36 ± 8, and 46 ± 3% hematocrit, respectively). In our study, there was no significant difference in hematocrit between the challenged and the unchallenged rats (Figure 14a). All these studies compared to their control group, not to the normal values for healthy rats.

The red blood cell count and the hemoglobin levels were higher in the ZD and PF rats than the other groups (Figure 13a,b) which was also seen by Paterson et al (1986). They explained that severe restriction of dietary Zn depresses bone marrow Zn concentrations resulting in altered hematological development in young rats. Hematocrit levels were not significantly different except that the ZT rats had lower hematocrit levels than ZD, PF and ZN (Figure 14a). In contrast EL-Hendy et al (2001) found that Zn deficiency (3.8 mg Zn/kg diet) for 10 weeks produced a 17% decline in the hemoglobin levels of 1 month old albino rats compared to the control rats (38 mg Zn/kg diet). The authors postulated a reduction in the rates of red blood cell formation. They also found a 15.5% increase in the total leukocyte count in the Zn deficient rats compared to the control.

In our study, **white blood cell count, lymphocyte** count and **neutrophils** were significantly higher in the DSS challenged rats (Figure 16a,b and 17a) obviously due to inflammation. There were no differences among the diet groups except that ZS had elevated white blood cell counts and lymphocytes. **Platelets** were marginally lower in the

DSS challenged rats. ZT rats had the lowest platelet count (Figures 18). The Paterson et al (1986) study fed male Wistar rats ZD (<1.1 mg Zn/kg diet) or control diet (100 mg Zn/kg diet) as ad libitum-fed control or as PF controls. Both the Zn-deficient and pair-fed rats had 31% higher RBC and 14% higher Hb than the ad libitum-fed control rats and similar findings were observed in our study (Figure 13a,b). Paterson et al (1986) reported 20.3% higher Hct, 13.6% lower MCV, 20% lower MCH, and 7.1% lower MCHC in ZD rats compared to controls. Unlike our study, Paterson et al (1986) found no differences in platelet count and monocytes, but neutrophils were 81% higher in ZD compared to PF and control rats. Neutrophils were unchanged in our study (Figure 17a). The lymphocytes in the Paterson et al (1986) study were 47% lower in the Zn-deficient rats compared to control rats but lymphocytes were not different among these groups in our study although the values for lymphocyte counts were high in all groups compared with Canadian Council on Animal Care (Appendix 1).

Inflammation assessment

Although there has been extensive research to understand the etiology of IBD, it still remains poorly understood. It has been reported that DSS-induced colitis in rodent models presents the main features of human inflammatory bowel disease (Gaudio et al, 1999). There is a difference in susceptibility to DSS among animals. For example, guinea pigs develop colitis from DSS mainly in cecum (Hoshi et al, 1996). As for rats and mice, they develop the inflammation mainly in the colon (Jurjus et al, 2003).

In our study, the use of 5% DSS produced symptoms of inflammation, including reddish anus, diarrhea and weakness, from the second day of administration of DSS in the

drinking water. By the third day, all DSS-treated rats experienced severe diarrhea, bloody stool, swollen and reddish anus and extreme weakness (Table 2). All animals survived for 4 days until they were euthanized. The same findings were seen in a study done by Korkina et al, (2003). In their study, 200-220 g male Wistar rats were given 5% (40 kd M.W.) DSS in the drinking water for three days, and by day two, the rats had loose stool, reddish and swollen anus, slow movement and weakness. By day three, all animals had severe diarrhea, bleeding stools, severely inflamed anus and extreme weakness. The study by Orbak et al (2007) indicated that Zn deficiency (in the absence of DSS) produces diarrhea, but in our study, there was no diarrhea in the unchallenged ZD rats.

Haptoglobin is an acute phase protein and considered a systemic biomarker of inflammation (Larsson et al, 2006); it was used in our study to assess inflammation. Haptoglobin was one of the few parameters that had a statistical interaction between the diet and DSS challenge. We found that ZN and ZS challenged rats had the highest circulating haptoglobin levels while all the other groups had lower haptoglobin levels (Figure 19). The increase in haptoglobin levels of the ZN and ZS challenged rats could be due to the enhancement of the immune system leading to an increase of proteins known as acute phase proteins and which includes haptoglobin. Zn supplementation did not add extra benefits (eg. reduce haptoglobin) compared to adequate Zn. The lack of an increase in haptoglobin in DSS-challenged ZD and PF rats could be due to an impaired immune system as a result of Zn deficiency or energy deficiency in the ZD and PF rats, respectively. Two DSS studies have reported haptoglobin (Larsson et al, 2006; Ito et al 2006) and both found that DSS challenged mice had higher haptoglobin levels. There does not appear to be any published reports on dietary Zn and haptoglobin.

Histology

The use of 5% DSS (40 kDa) in the drinking water of the rats for 4 days produced mucosal changes of variable severity consisting of significant infiltration of white blood cells, crypt abscess, mucosal ulcers, goblet cell depletion, edema and congestion (Figures 21), but only in the colon. The small intestine of DSS-treated rats displayed no histological signs of inflammation (Figure 20) and this supports the view that DSS effects are more prominent in the colon than the small intestine (Gaudio et al, 1999; Vicario et al 2005. In our study, histology was conducted on intestinal samples from the mid point of the small intestine and the proximal colon, and this could be influencing our interpretation of the location of inflammation. The molecular weight of the DSS also affects the location of intestinal inflammation and this point requires more investigation in rats. When Vowinkel et al (2004) provided mice with 5% DSS of different molecular weights. The 40 kDa DSS produced severe disease mainly in the lower colon whereas the 5 kDa DSS produced more disease in the upper colon and the 500 kDa DSS-treated mice did not show signs of colitis.

In contrast to our hypothesis, dietary zinc deficiency did not exacerbate DSS-induced intestinal inflammation nor did acute (ZT group) or chronic (ZS group) supplementation attenuate intestinal inflammation. In the small intestine, all rats showed normal histology regardless of their diet. In the colon, the number of rats with severe ulcerations after DSS was greater in the ZS and PF groups while the ZD rats had the lowest number of rats with severe ulceration (Table 3). In the absence of a challenge, it has been reported that Zn deficiency promotes ulceration and inflammation in the small

intestine and that dietary Zn (70 mg Zn/kg diet) reverses Zn deficiency induced gut damage (Duff et al, 2002).

Both DSS challenged and the non-challenged rats had inflammatory cell infiltrates in the small intestine and colon, some of which extended from the submucosa to the mucosa (for example, Figure 20), and were likely Peyers patches which are observed in humans. However, most other studies report inflammatory cell infiltrates as a sign of inflammation and present only in DSS-challenged rodents (Erichsen et al, 2005). For example, when Erichsen et al (2005) gave forty, 12 week old male Wister rats 50 g/L DSS (MW 44000) in distilled water, and only the DSS-challenged rats had inflammatory infiltration. Kitajima et al (1999) provided 5% DSS (MW = 40,000) to female BALB/c mice in their drinking water for 7 days, and their findings showed loss of crypt and inflammatory infiltration in the lamina propria and submucosa of various cells. On the other hand, the normal control mice had no inflammatory cell infiltration. In another study by Tamaru et al (1993), untreated male Wistar rats (100-120 g) had no inflammatory changes, while the rats given 2% DSS (MW 54,000) for 2 to 4 weeks had various degrees of inflammatory cells and loss of surface epithelium. To my knowledge there is no study reporting abnormal inflammatory cell infiltration in untreated normal rats.

One explanation for the presence of the inflammatory cell infiltrate in the present study is that this region could be comparable to Peyer's patches in human small intestine. However, Peyer's patches in humans are only seen in the small intestine, and in our rats, the inflammatory cell infiltrate was present in both small intestine and colon. Another

reason could be that the inbred strain of the Sprague Dawley rats is more susceptible to inflammatory cell infiltrate.

Metallothionein

As expected, MT immunostaining in the small intestine was absent or very weak (one or two weakly stained cells) in the ZD rats, while all other groups had similar levels of strong MT immunostaining, with the highest levels in the untreated ZS rats (Table 4). Similarly, Szczurek et al (2001) also used immunohistochemistry to detect MT and they reported that Zn deficiency resulted in the absence of MT immunostaining in the small intestine of 46-56 gm male Sprague Dawley rats fed ZD diet for 16 days. All other groups, including the control, PF and Zn-repleted rats had strong MT immunostaining and higher levels of MT measured with a cadmium-binding assay. In our study, there was strong MT immunostaining in the small intestine after the 3 days repletion with 300 mg Zn/kg diet. Szczurek et al (2001) found that 24 hour repletion with 30 mg Zn/kg diet was sufficient for strong MT immunostaining in the small intestine of the Zn repleted group. Paski et al (2003) reported that ZD rats (3 mg Zn/kg diet for 6 weeks) had a 64% reduction in small intestinal MT compared with the control group (30 mg Zn/kg diet) while the ZS diet (115 mg Zn/kg diet) elevated MT by 13% compared with the control. The results of all three studies come to the same conclusion regarding Zn and MT whether MT is measured using the cadmium hemoglobin affinity assay (Szczurek et al, 2001; Paski et al, 2003) or immunohistochemistry (present study and Szczurek et al, 2001).

In our study, the effects of dietary Zn on MT were different in the colon compared to the small intestine. The untreated rats had no detectable MT immunostaining in the submucosa but the MT immunostaining was concentrated in the crypt of the colon (Figure 24). The ZS and ZT rats had the strongest MT immunostaining in the crypt of the colon (Table 5) whereas it was very weak (one to two weakly stained cells) in the crypt of ZD rats. To my knowledge there does not appear to be any study examining MT immunostaining in the colon.

In the small intestine, there was not much difference between DSS-treated and untreated rats regarding MT immunostaining except that the untreated ZS rats had the highest level of MT immunostaining compared to all other groups (Table 4). Given that the small intestine of DSS challenged rats had normal histology (Figures 22,23), it is logical that MT was relatively unchanged.

The intestinal inflammation in the colon (Figure 25) was accompanied by an elevation of MT in the colon after DSS challenge, with both submucosa and crypts showing positive staining for MT. The presence of MT in the colon of DSS-challenged rats varied with the amount of dietary Zn. The submucosa of the DSS-challenged rats showed strong MT immunostaining in all groups except the ZD rats, while the MT immunostaining in the crypt was strongest in ZT rats and ZS rats. PF and ZN had moderate and mild MT immunostaining, respectively, in the crypt of the colon. Three days repletion with ZN diet resulted in higher levels of MT immunostaining in ZT rats compared to ZD.

Di Leo et al (2001) analyzed MT of male Sprague Dawely rats in the colon and ileum using the Ag-hemoglobin method. Their rats were challenged with DNBS

(dinitrobenzene-sulphonic acid). Their results showed that Zn supplemented rats (155 mg Zn/kg diet) and the control rats had the same MT in the small intestine with and without DNBS treatment, however, in the colon, the treatment with DNBS resulted in a lower MT concentration. The 127 mg Zn/kg diet supplemented rats resulted in the highest MT concentrations in the small intestine and colon, and with DNBS challenge, MT concentration was reduced in both small intestine and colon and was lowest in the colon.

Few studies have examined MT immunohistochemically and specifically in models of inflammation. The results of the present study could be strengthened by assessing MT mRNA levels using Real Time PCR, or MT protein levels by Western blotting or the cadmium hemoglobin affinity assay.

We can conclude that Zn deficiency affects the presence of MT in the small intestine and the role of MT in Zn storage in the small intestine. The short term ZT had positive effects on the presence of strong MT immunostaining in the small intestine and colon which support other studies regarding Zn repletion and MT. ZS had favorable effects in the untreated small intestine. MT staining increased after the DSS-challenge in the colon and since the colon had the histological changes and ulceration and likely an increased cell turnover associated with the inflammation, the presence of MT could be to provide more Zn for cellular protection and cell synthesis.

Apoptosis

As hypothesized our results show a low level of apoptosis in the small intestine of the unchallenged ZS rats (Table 6) suggesting that Zn supplementation provides protection. The ZD rats had a higher level of apoptosis (more caspase-3 positive cells)

but only after the inflammatory challenge. Without the challenge, apoptosis (caspase-3) in the ZD rats was normal compared to the control rats. The PF and the ZT rats had the same apoptosis (caspase-3) as the ZD rats before and after inflammation. Energy restriction (PF rats) also increased apoptosis in the presence of intestinal inflammation (Table 6) and Zn repletion (ZT rats) for 3 days was not sufficient to decrease apoptosis during inflammation.

Others have demonstrated that apoptosis related factors (caspase-3 and Fas) are increased in mild dietary Zn deficiency (2 μg Zn/g diet), and the effects are most prominent in organs with elevated cell turnover and decreased Zn storage (Didier et al, 2004), but that study did not include inflammatory challenge. In our study, ZD rats had elevated apoptosis in small intestine after intestinal inflammation (Figure 26). To my knowledge, there are no other published studies investigating Zn, apoptosis and inflammation in the intestine.

In the colon of untreated rats, Zn deficiency (ZD), energy restriction (PF) and Zn supplementation elevated apoptosis compared to control (ZN). Surprisingly, DSS-induced inflammation elevated apoptosis in all groups except ZD and ZS rats, and the highest elevation of apoptosis was seen in the ZN challenged rats. Apoptosis in colon of ZS rats was reduced after the inflammatory challenge. This is in contrast to our hypothesis where ZD rats were expected to be more susceptible to apoptosis, however, we did see the expected decrease in apoptosis in ZS rats.

Diedier et al (2004) found that the lowest caspase-3 expression was in the colon compared to liver, jejunum and thymus. This was also observed in our study as the control (ZN) rats in the unchallenged state had a lower level of apoptosis in the colon

compared to the small intestine. More effort needs to be put towards the investigation of apoptosis in the colon and small intestine under inflammation. The use of quantitative methods for apoptosis and other apoptosis markers would be helpful in the interpretation of apoptosis.

VI. SUMMARY

Conclusions

Effects of DSS Challenge:

Administration of 5% DSS in the drinking water of growing rats for 4 days produced moderate to severe intestinal inflammation.

- DSS-treated rats had reduced body weights and feed intake. Organ to body weight ratios for spleen and small intestine were increased while colon and adipose body weight ratios were decreased.

- The red blood cell count and hemoglobin of DSS-challenged rats was lower than the untreated rats but still within the normal range for rats. However, the white blood cell, lymphocyte, and neutrophil counts were elevated in DSS-challenged rats compared to the normal range for rats.

- DSS-challenged rats had reduced liver Cu. Liver Fe, and liver and femur Zn were unchanged by DSS challenge.

- The rats developed diarrhea and bloody stool by the second day of consuming DSS in the drinking water. Water intake was not altered by DSS consumption.

- DSS was specific for the colon since no ulceration was seen in the small intestine based on histology. The histological sections of the colon had obvious ulceration of the crypt.

- In the colon, MT immunostaining was present in the crypt and submucosa of challenged rats but present only in the crypt of the untreated rats. DSS-induced inflammation increased MT immunostaining in the colon submucosa, while MT immunostaining remained steady in the small intestine after challenge with DSS.

• In small intestine, apoptosis (caspase-3 immunostaining) was higher in the ZD, ZT and PF DSS-treated rats. In the colon, apoptosis was elevated after DSS challenge in all the groups except ZD and ZS.

Effects of Dietary Zn:

Zn status was altered in the dietary groups as expected. However, overall, DSS-induced intestinal inflammation was not exacerbated by Zn deficiency (ZD) nor was it reduced by acute Zn treatment (ZT) or chronic Zn supplementation (ZS).

• ZD rats had lower feed intake, body weight, serum Zn and femur Zn. Three days of repletion with high doses of Zn increased body weight, feed intake and serum and femur Zn of ZT rats compared to ZD rats. ZN and ZS had similar feed intake and body weight but serum Zn and femur Zn were higher in ZS.

• ZD rats had elevated RBC and hemoglobin compared with ZN rats but these values were within the normal ranges for rats. White blood cells were not changed by dietary Zn.

• The highest liver Fe levels were seen in the ZD rats while the lowest liver Cu level was seen in the ZS rats.

• Zn supplementation, Zn treatment or Zn deficiency did not change incidence of diarrhea or bloody stool in DSS-challenged rats.

• Serum haptoglobin was elevated in ZS and ZN DSS-challenged rats but not in the other groups.

• The ZS and energy restricted rats (PF) had the most severe ulceration in the colon while the ZD rats had the mildest ulceration.

• ZD lowered the MT immunohistochemical staining while ZS and ZT elevated

MT immunohistochemical staining in small intestine and colon in both DSS-

challenged and unchallenged rats.

• ZD elevated apoptosis (caspase-3 immunostaining) and ZS lowered apoptosis

levels in small intestine of both DSS-challenged and unchallenged rats. There

was no consistent effect of Zn on apoptosis in the colon.

Strengths, Limitations, and Future Directions

- The use of a ZT group (Zn repletion of Zn-deficient rats after induction of

  inflammation) is strength and a unique feature of this study. This group mimics

  the practical situation whereby Zn treatment would not start until after diagnosis

  of inflammation. However, increasing the time of treatment could have helped in

  understanding more about the treatment with high doses of Zn, putting in mind

  that the concentration of DSS would need to be lower than 5% for the rats to

  survive longer after induction of the inflammation.

- The use of MT immunohistochemistry was a beneficial technique for

  understanding the position of the stained cells in the tissue; however, including a

  quantitative procedure for MT (e.g. Western blotting, cadmium-binding assay)

  would be an interesting comparison. The same applies on apoptosis, and other

  markers of apoptosis could be investigated.

- The use of CBC count was a strength in our study as it is a widely available

  simple test. This test helped us to determine if the rats were anemic or not from

  the red blood cell count. The white blood cell count helped us to understand the

  status of inflammation (present or absent), however, it would have given us a

better interpretation if we did a peripheral blood smear, and examined it microscopically for comparison with flow cytometry results.

- Liver zinc, copper and iron was an important part of our test, as they helped in understanding the status of those minerals in a vital organ. On the other hand, measuring the amount of those minerals in the small intestine and colon would have helped in our interpretations.

- The histology in our study was very important to assess the inflammation and ulceration. However, measurement of colon thickness to assess the degree of edema and thus inflammation could have been beneficial in our interpretations.

- Sprague Dawley rats are widely used animal in inflammatory bowel disease research; however, the use of germ free rats would allow determination of whether the inflammation was due to DSS or to the bacterial material traveling through intestine to bloodstream. DSS is an acute model of intestinal challenge whereas IBD is a chronic inflammatory disease. The use of genetic knockout or transgenic rodent models of IBD could be a chronic IBD model and could be used to investigate nutritional interventions such as dietary Zn.

- Haptoglobin was important to assess inflammation based on a circulating marker in blood. Measurement of tissue myeloperoxidase activity could help in understanding more about the inflammation in the tissues i.e. small intestine and colon.

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Appendix I

| Hematology	Rats
RBC (10^{12}/L)	5.4 – 8.5
HB (g/L)	115 – 160
PCV (L/L)	0.37 – 0.49
Platelates (10^9/L)	450 – 885
WBC (10^9/L)	4.0 – 10.2
Neutrophils (10^9/L)	1.3 – 3.6
Lymphocytes (10^9/L)	5.6 – 8.3
Blood volume (mL/kg)	50 - 65

The normal values may vary according to age, sex, breed and function of animals
Olfert et al (1993).

Appendix IIa
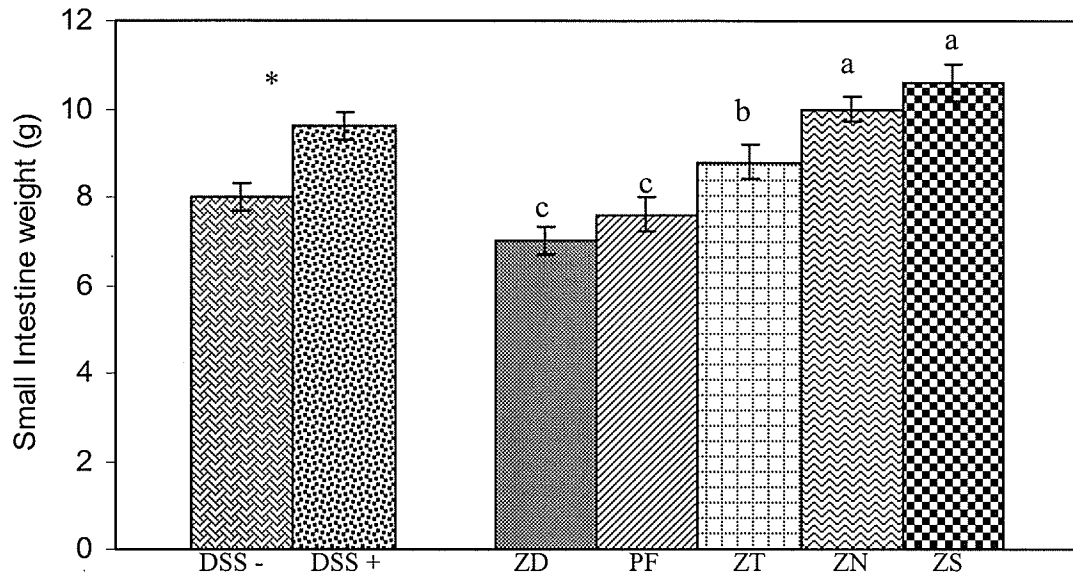
Effects of DSS challenge and diet on small intestine weight. There were significant main effects for both the DSS challenge (P< 0.0001) and dietary treatment (P<0.0001), thus data was pooled to show the main effects. Statistical differences among means (P<0.05) are indicated by an asterisk (DSS effect) or different lower case letters (diet effect). Values are means ± SEM for n=40 (DSS challenge) and n=16 (dietary treatment).
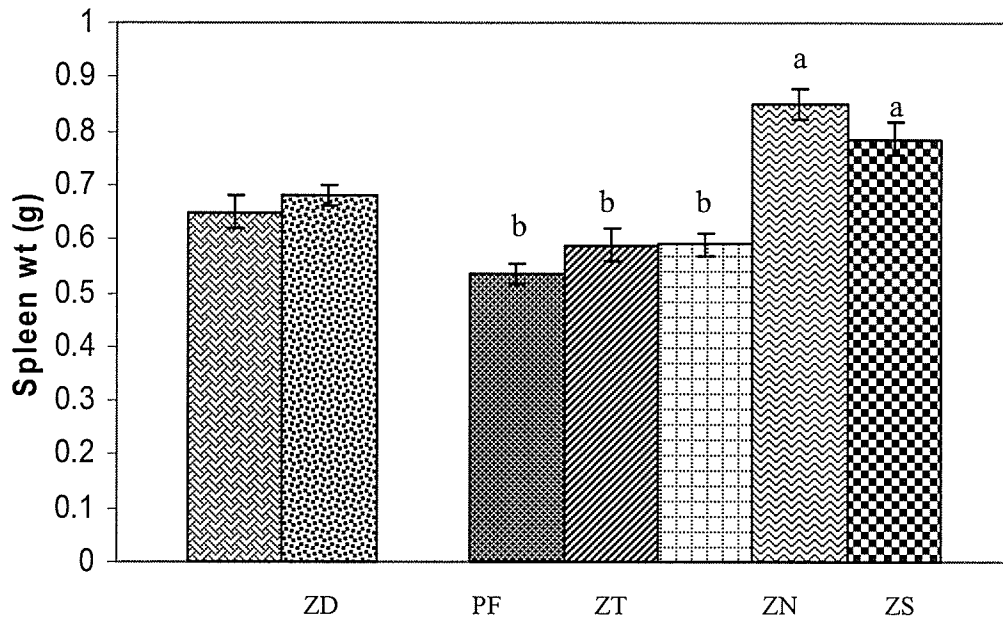
Appendix IIb
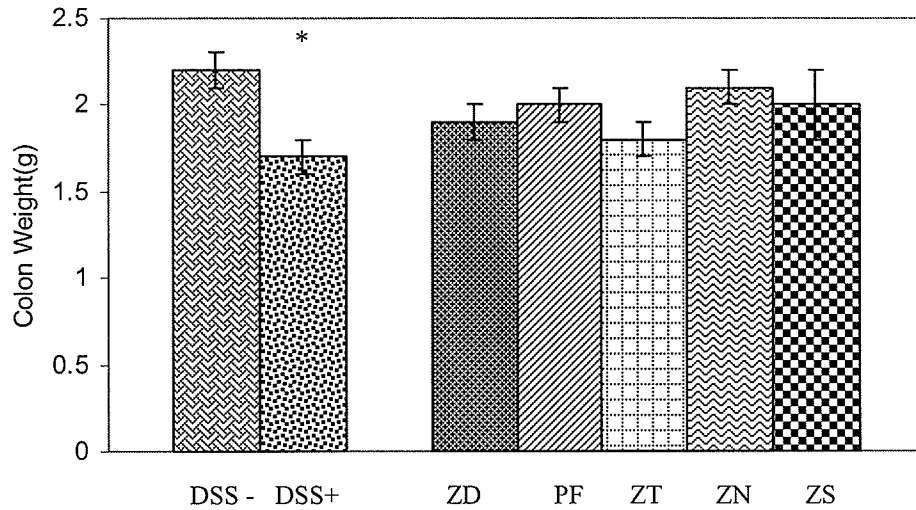
Effect of DSS challenge and diet on colon weight. There was a significant main effect of DSS challenge on colon wt (P<0.0001) but not diet. Statistical differences among means (P<0.05) are indicated by an asterisk (DSS effect). Values are means ± SEM for n=40 (DSS challenge) and n=16 (dietary treatment).
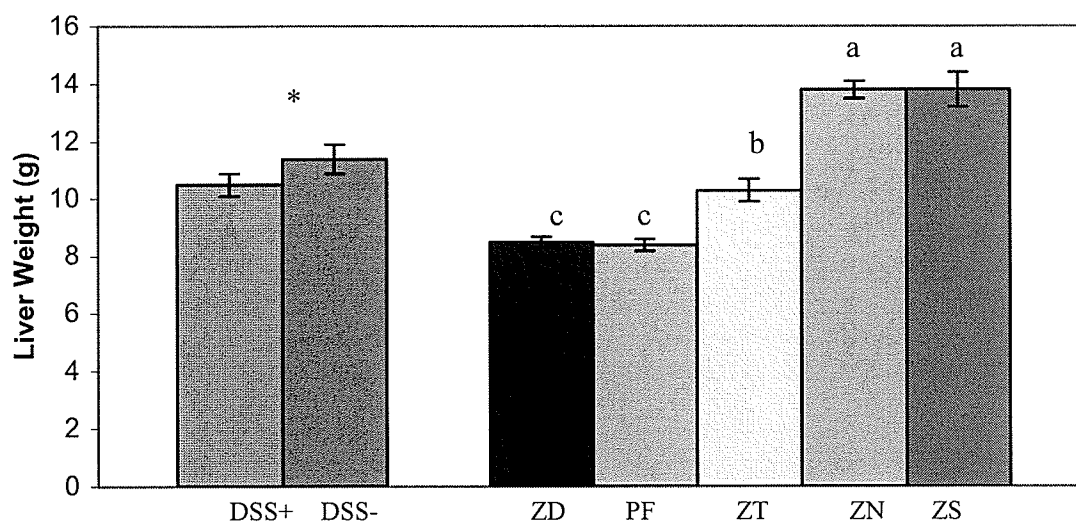
Appendix IIc
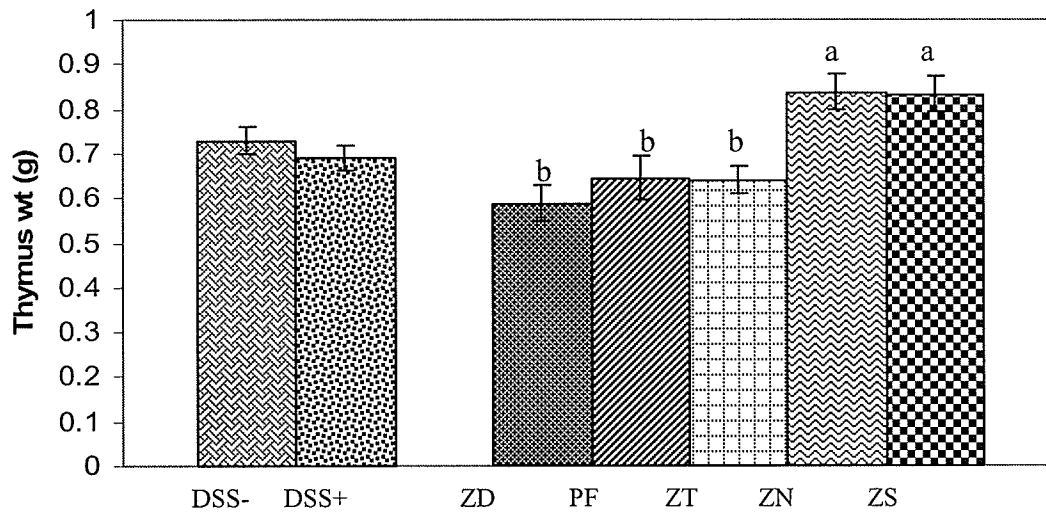
Effects of DSS challenge and diet on thymus weight. There was a significant main effect of diet (P=<0.0001) but not DSS challenge. Statistical differences among means (P<0.05) are indicated by different lower case letters (diet effect). Values are means ± SEM for n=40 (DSS challenge) and n=16 (dietary treatment).
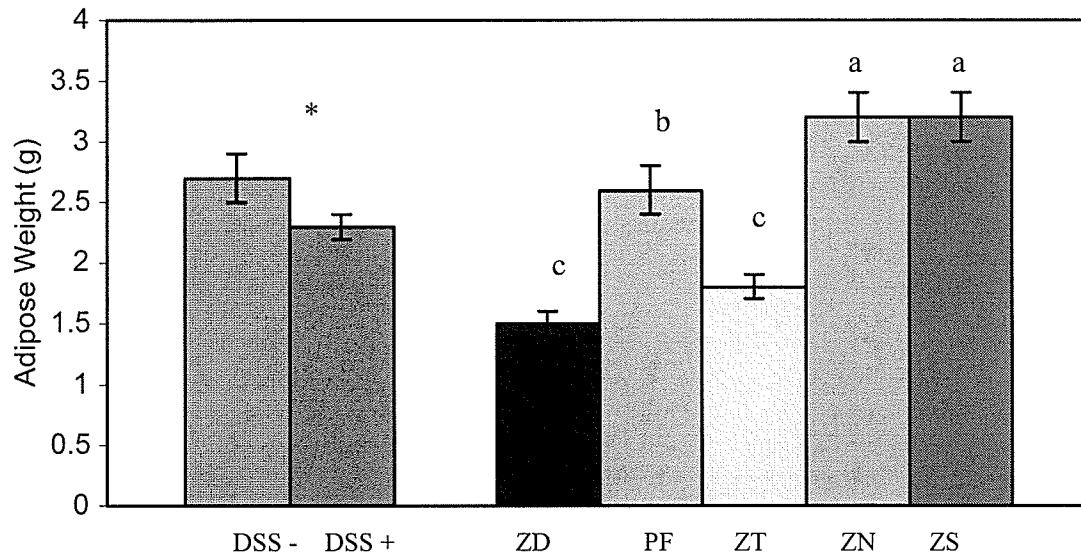
Appendix IId

Effect of DSS challenge and diet on spleen weight. There was a significant main effect of diet (P<0.0001) but not DSS challenge. Statistical differences among means (P<0.05) are indicated by different lower case letters (diet effect). Values are means ± SEM for n=40 (DSS challenge) and n=16 (dietary treatment).

Appendix IIe

Effects of DSS challenge and diet on liver weight. There were significant main effects of DSS (P=0.004) and diet (P<0.0001) on liver weight. Statistical differences among means (P<0.05) are indicated by an asterisk (DSS effect) or different lower case letters (diet effect). Values are means ± SEM for n=40 (DSS challenge) and n=16 (dietary treatment).

Appendix IIf

Effects of DSS challenge and diet on adipose weight. There were significant main effects of DSS challenge (P=0.005) and diet (P<0.0001) on adipose weight. Statistical differences among means (P<0.05) are indicated by an asterisk (DSS effect) or different lower case letters (diet effect). Values are means ± SEM for n=40 (DSS challenge) and n=16 (dietary treatment).