



**INTESTINAL ZINC AND METALLOTHIONEIN: ROLE IN CHEMOTHERAPY
INDUCED MUCOSITIS**

BY

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BSc (Hons)

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ABSTRACT

Intestinal zinc and metallothionein: role in chemotherapy-induced mucositis

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The aim of this thesis is to examine the regional localisation of zinc (Zn) and its intracellular binding protein, metallothionein, in determining susceptibility to damage and potential for repair in the gut. Zn and metallothionein concentrations in seven contiguous regions of the gastrointestinal tract were measured and their intracellular distribution demonstrated by specific antibody and fluorescence techniques. The effects of Zn supplementation with or without bovine whey growth factor extract (WGFE) on the severity of, and recovery from, methotrexate-induced intestinal mucositis were investigated.

Zn and metallothionein were co-localised in the base of crypt cells, mainly stem and Paneth cells most visibly in the ileum, using Zinquin fluorescence and immunohistochemistry, respectively (Chapter 2). This work was extended to examine the distribution gut Zn and metallothionein under different Zn feeding regimens (10, 100, 400 mg Zn/kg) in the rat (Chapter 3) and mouse (Chapter 4). Irrespective of dietary Zn content, Zn concentration was 20% higher in the ileum than other gut regions. Zn was 94% membrane-bound and 6% cytosolic. Metallothionein increased in all gut regions at dietary Zn at or above 100 mg/kg and was 40% higher in the ileum. The role of metallothionein in these findings was investigated using metallothionein-knockout mice (MT^{-/-}) (Chapter 4). Zn concentrations were higher in the distal gut at increasing Zn intakes in normal, but to a lesser extent in MT^{-/-} mice. Without metallothionein, there was little modification of regional gut Zn concentration in response to excess dietary Zn and poorer regulation of Zn. Glucagon administration stimulated gut as well as liver metallothionein, implicating it as a major component of the metallothionein response to fasting.

Intracellular intestinal Zn and metallothionein in response to methotrexate-induced intestinal damage was characterised in Chapter 5. Methotrexate (2.5 mg/kg) was administered subcutaneously for 3 d to induce gut mucositis. Intestinal damage was minimal in the ileum, moderate in the duodenum and most severe in the jejunum. Increase in gut metallothionein was greatest in the ileum, less in the jejunum and least in the duodenum. Plasma Zn decreased markedly (33%) after methotrexate at d 6, and then gradually recovered to control levels. Hepatic metallothionein progressively increased, reaching a maximum (5-fold) six days after the first methotrexate injection and rapidly decreased to control levels by d 7.

The effects of Zn and WGFE supplementation on methotrexate-induced gut damage were investigated in Chapter 6. Zn+WGFE supplementation resulted in an improvement on methotrexate-induced intestinal damage during the recovery phase. Zn, WGFE and Zn+WGFE supplementation to rats reduced intestinal permeability during the early phase and recovery phase of methotrexate-induced damage suggesting improved tight junction integrity during the early and recovery phase of bowel damage. A potential benefit of Zn and WGFE in the treatment of intestinal mucositis is thus indicated.

DECLARATION

This thesis contains no material that has been accepted for the award of any other degree or diploma in any institution. To the best of my knowledge and belief, this thesis contains no material that has been previously published or written by another person, except where due reference is made in the text.

I consent to the thesis being made available for photocopying and loan, if applicable, if it is accepted for award of the degree.

Cuong Duy Tran

Date... 21/2/2001

Signature

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CHAPTER 1

1.1 INTRODUCTION

Intestinal mucositis is a frequent toxic side effect and potentially severe complication of high-dose chemotherapy and abdominal radiotherapy, which may be manifested as erythema, desquamation, ulcer formation, and/or exudates (Wilkes, 1998). The frequency of severe mucositis is remarkably consistent among patients receiving aggressive conditioning regimens for bone marrow transplant, induction therapy for acute leukemia, continuous infusion therapy for treatment of breast or colon cancer and radiation and chemotherapy for tumours of the head and neck (Spijkervet and Sonis, 1998). All anti-cancer drugs have harmful side effects and each has its own spectrum of toxicity. All, however, have one common property in that they cannot distinguish between normal and malignant cells. Many drugs cause toxicity because of their preferential activity against rapidly proliferating cells, these include bone marrow, hair follicles, and gonads. The gastrointestinal epithelium has a rapid rate of cell turnover and appears to be at high risk for injury both as a result of the cytotoxic effects of chemotherapy and indirect effects of infectious agents.

Mucositis can be a serious problem resulting in significant morbidity in cancer patients receiving chemotherapy. Furthermore the incidence of mucositis is increasing, often in conjunction with chemotherapy dose-intensification. For example, mucositis is reported in approximately 40% of patients treated with 5-fluorouracil based regimens (Poureau-Schneider et al., 1992; Mahood et al., 1991; Rocke et al., 1993). As a result, oral and

intestinal mucositis is more frequently becoming the dose-limiting toxicity of intensive chemotherapy regimens.

While rarely life threatening, the discomfort associated with mucositis may impair communication, food intake, efficacy of therapy and sleep. The nutritional status of patients may be further compromised, resulting in anorexia, cachexia, dehydration, and malnutrition. There is also the negative impact on the patient's quality of life as a result of pain. Mucositis may result in treatment interruptions and dose reductions, and may affect a patient's compliance with other prescribed medications. In addition, complications related to mucositis may lead to costly hospitalisation and parenteral nutrition. A recent retrospective, case-controlled study of patients hospitalised for autologous bone marrow transplant for treatment of haematologic malignancies showed that mucositis adversely affected length of hospital stay leading to an additional 6 days at an average cost of US\$5000 per day (Reuscher et al., 1998).

Currently, no intervention exists that is completely successful at preventing mucositis, and the standard treatment for this complication of chemotherapy has been symptomatic relief with analgesics and nutritional support. The standard management of established mucositis involves dietary modification, and topical mucosal protectants.

The maintenance of adequate caloric intake in patients with mucositis can be a significant challenge. In an attempt to limit the degree of contact between the food and irritated mucosa, a diet requiring little or no chewing is recommended. In addition, the avoidance

of acidic, spicy, salty, coarse or dry food items may limit the discomfort associated with oral intake.

Topical mucosal coating agents such as sucralfate, magnesium hydroxide, and hydroxypropyl cellulose films have been used with varying success. There is no strong clinical evidence to support or refute the routine use of these common clinical approaches. Topical anaesthetics have been used to relieve the discomfort associated with mucositis using such agents as lidocaine and cocaine. However, these agents are poorly tolerated, short-acting and must be applied frequently, and the abuse potential for cocaine will likely prohibit its regular use. Topical capsaicin has been suggested to promote re-epithelialization (Watcher and Wheeland, 1989) and improvement in subjective mucositis pain (Berger et al., 1995) however, a larger clinical trial is required to evaluate its role in chemotherapy-induced mucositis.

In severe cases of mucositis, dietary modification, topical agents and supportive measures are inadequate to relieve the suffering experienced by patients. The use of systemic analgesia is required, however, its correct use is critical as it interferes with oral care, nutrition and may result in secondary infections (reviewed; Raber-Durlacher, 1999).

Therefore, trends to move away from conventional treatments of mucositis to alternative medicines with significantly less or no side effects have been substantial. However, most of the alternative treatments using physiological compounds, which produce fewer side effects and have not been proven to be more beneficial than conventional chemotherapy. An alternative approach is to reduce or prevent the adverse effects of chemotherapy. This

could be achieved by administration of agents prior to, during or after chemotherapy that can modify the severity of adverse effects, mainly gut mucositis in these cancer patients. Approaches to the prevention of mucositis induced by chemotherapy can be divided into three categories. First, the mucosal delivery and excretion of chemotherapeutic agents can be altered, secondly, attempts can be made to alter the potential for infectious or inflammatory complications. Finally, there have been attempts to modify the proliferative activity of the mucosal epithelia.

Cryotherapy, which involves the cooling of the oral mucosa with ice during administration of chemotherapy produces temporary vasoconstriction, thus reducing the delivery of chemotherapy to the mucosa. Studies (Mahood et al., 1991; Rocke et al., 1993) using cryotherapy have been shown to reduce mucositis by 50% in patients undergoing chemotherapy. Other agents such as allopurinol, propantheline, leucovorin and pilocarpine all have been suggested to have potential roles in the prevention of chemotherapy-induced mucositis (reviewed; Wilkes, 1998). However, the efficacy of these agents remains speculative and further randomised placebo-controlled trials are warranted.

It has been hypothesised that a reduction in the number of micro-organisms in the oropharynx and the small intestine may result in a decreased incidence of indirect mucositis. Oral antimicrobial agents have been studied in the prevention of chemotherapy-induced mucositis; however, these studies have provided conflicting data. Anti-microbial lozenges composed of polymyxin B, tobramycin and amphotericin B have been shown to be more effective in alleviating mucositis in patients receiving head and

neck irradiation (Spijkervet et al., 1990). These results are encouraging but require further confirmation.

Unfortunately, responses to currently available interventions are inconsistent. There is no preventative or therapeutic treatment that consistently prevents mucositis or reduces its severity or duration. It is well established that the rate of basal epithelial cell proliferation correlates with the susceptibility of mucosal tissue to the toxic effects of chemotherapy (Sonis et al., 1992). Thus agents that affect epithelial proliferation may potentially prevent chemotherapy-induced mucositis. Beta-carotene (Mills, 1988), and glutamine (Fox et al., 1988) have been suggested to have a beneficial role in the maintenance of mucosal integrity following cytotoxic chemotherapy. In addition, cytokines such as interleukin-1, interleukin-11, transforming growth factor- β , and epidermal growth factor appear to affect the proliferation of mucosal cells and may provide new approaches to the management of mucositis. Animal studies have demonstrated that interleukin-11 (Sonis et al., 1995) and transforming growth factor- β 3 (Sonis et al., 1994; Spijkervet and Sonis, 1998) have cytoprotective effects that significantly reduce the duration and severity of chemotherapy-induced mucositis.

Growth factors such as insulin-like growth factors and transforming growth factors are naturally present in bovine cheese whey and have been concentrated as a whey-derived growth factor extract (WGFE), and micronutrients, in particular zinc, have been demonstrated to possess anti-microbial activity. In addition these agents have been demonstrated to be effective growth promoting agents both in cultured cells and animal models, as well as acting as a mediator of the inflammatory immune response (reviews;

Regester and Belford, 1999; Walsh, 1994). The antimicrobial and healing properties of these nutrients could indicate that administration of zinc (Zn) and or growth factors might act to suppress the severity of mucositis. Consequently, we have characterised a rat model of chemotherapy-induced gut mucositis to measure the effectiveness of oral administration of Zn, growth factors or the combination in reversing the severity of mucosal damage.

This chapter will address the gastrointestinal side effects of chemotherapy and review the importance of Zn and its metal binding protein, metallothionein, in the protection and the repair of damaged and inflamed intestinal mucosa. The putative roles of a combination of Zn and growth factor supplementation for the treatment of chemotherapy-induced intestinal mucositis will also be explored.

1.1.1 Methotrexate and gut cytotoxicity

Methotrexate is the most widely used antimetabolite for the treatment of carcinoma. High doses of methotrexate (50-200 mg/kg) have anti-tumour activity in acute lymphocytic leukaemia, non-Hodgkin's lymphoma, osteosarcoma, choriocarcinoma, head and neck cancer and breast cancer (Jolivet et al., 1983). Administration of methotrexate has also been shown to be efficacious at a low dosage (25 mg or less, weekly) for several chronic inflammatory diseases, including rheumatoid arthritis and psoriasis (Egan and Sandborn, 1996).

Methotrexate exerts its chemotherapeutic effects by being highly toxic to rapidly dividing cells via inhibition of the enzyme dihydrofolate reductase with subsequent disturbance to folate metabolism, which is required for the synthesis of DNA and cell division (Jolivet et

al., 1983). Despite its usefulness as an anticancer drug, undesirable side effects occur particularly on tissues, which have a high rate of cell division and cell turnover, such as the gastrointestinal mucosa. The proliferating cells located in the crypts are most sensitive to the toxic effect of folate analogues, which prevent renewal of the epithelial surface (Chello et al., 1977). In addition, irreversible damage in mature, as well as in dividing cells, occurs through necrosis following administration of excessive methotrexate dosage (Achord, 1969).

The most common side effects of high-dose methotrexate chemotherapy include intestinal inflammation and/or ulceration (mucositis) of the gastrointestinal tract. Although not mediated exclusively by gut-associated damage, gastrointestinal toxicity is also characterised by oral ulcers, malaise, nausea, vomiting, anorexia and diarrhoea (Howarth et al., 1996). The histopathology of the gut associated with methotrexate cytotoxicity includes loss of crypts, reduced mitoses in the crypts, villus fusion and atrophy, gross capillary dilatation and a mixed cellular infiltrate (Taminiau et al., 1980; Vanderhoof et al., 1990). Other manifestations include appearance of cytoplasmic inclusion bodies, flattening of the intestinal crypt, metaplasia and desquamation of the epithelial lining and hemorrhagic ulcerations (Sandberg and Goldin, 1970).

Currently, there are no effective therapies for the treatment of chemotherapy-induced intestinal enteropathy. Attempts to alleviate the severity of methotrexate-induced cytotoxic injury and to improve the nutritional status of patients with cancer have not been adequate. Furthermore, targeting therapeutic regimens have been hindered by a lack of appropriate animal models. Howarth et al., (1996) in our laboratory, modified the

procedure of Vanderhoof et al., (1990) and produced experimental intestinal mucositis in rats by subcutaneously injecting with a high dose of the anti-neoplastic drug methotrexate on three consecutive days. This highly reproducible model of intestinal damage provides an ideal setting to investigate indices of cellular damage, mucosal protection and repair processes as well as monitoring the effectiveness of potential therapeutic agents.

Although numerous drugs have been used for the treatment of chemotherapy-induced intestinal mucositis, no uniformly effective agent has been developed (Verdi, 1993). Zn plays an integral role in the maintenance of a healthy gastrointestinal tract and may provide therapeutic benefits to assist in the recovery from gut damage. Furthermore, growth factors, specifically those in milk, enhance gut growth and repair and have been shown to alleviate the severity of gut mucositis (Read et al., 1992; Zumkeller, 1992; Howarth et al., 1996). Thus supplementation of diets with Zn, growth factors or combination of both may potentially reduce the toxicity induced by methotrexate and thereby the severity of intestinal damage.

1.2 BACKGROUND

1.2.1 ZINC

Zn is an essential trace element that is required for growth and cellular function in both humans and animals. This was evident in animal studies as early as the 1930's (Todd et al., 1934; Hove et al., 1937). However, It was not until the 1960's that the importance of Zn for human health was described (Prasad et al., 1963).

Zn plays an important role in human physiology due to its involvement in many enzymatic reactions and biochemical functions. Consequently, the importance of Zn and its nutritional role in human health has been well documented. Zn deficiency has been established as a component of many pathologies of the gastrointestinal tract, affecting men and women of all ages and all cultural and socio-economic classes (review; Wapnir, 2000). A wide variety of symptoms may occur, depending on the severity of Zn deficiency, ranging from growth retardation and neuropsychiatric abnormalities to impairment of immune function, delayed wound healing and alterations in biochemical Zn indices (reviewed; Walsh et al., 1994). Several gastrointestinal diseases can lead to malnutrition and consequently to Zn deficiency, posing the question as to whether Zn supplementation in these disorders would be beneficial.

1.2.1.1 The physiological roles of zinc

Zn is an essential intracellular cation necessary for the structural integrity and functional capacity of a wide range of macromolecules that are important for cell division, differentiation and growth (Chesters, 1991; Berg and Shi, 1996; MacDonald, 2000). In particular, Zn is structurally important in DNA polymerase, RNA polymerase, chromatin and a range of transcription factors (Table 1), as well as necessary for stabilising the DNA double helix and mRNA (Vallee and Falchuk, 1993). It also stabilises microtubule proteins and prevents their depolymerisation (Nickerson and Veldstra, 1972). Zn is a constituent of more than 300 metallo-enzyme reactions (Vallee and Auld, 1990; Sanstead, 1994) (Table 1), in which it maintains structural integrity or participates in the enzyme's catalytic activity (Vallee, 1988), as well as an inhibitor of enzymes.

Table 1. List of some of the enzymes, hormones and carrier and structural proteins that are dependent on zinc for their activity and function.

Enzymes	Hormones	Carrier and Structural Proteins
Aldolase	Insulin	Transferrin
Adenosine monophosphate aminohydrolase	Angiotensin-converting-enzyme	Albumin
DNA polymerase	Growth hormone	α_2 -Macroglobulin
RNA polymerase	Thymulin	Metallothionein
Deoxythymidine kinase	Steroid receptors	
Deoxy-RNT transferase		
5'-Nucleotidase		
Alcohol dehydrogenase		
Protein kinase C		
Superoxide dismutase		
Leucine aminopeptidase		
Reverse transcriptase		
tRNA synthetase		
Nucleoside polymerase		
Carboxypeptidase A		
Carboxypeptidase B		
Carboxypeptidase C		
Dipeptidase		
Collagenase		
Endopeptidases		
Glutathione-S-transferase		
Carbonic Anhydrase		
Glutamic dehydrogenase		
Malate dehydrogenase		
Renal dipeptidase		
D-lactate-cytochrome reductase		
Phospholipase C		
Glyceraldehyde-3-phosphate dehydrogenase		

Zn increases the affinity of haemoglobin for oxygen and favours the formation of oxyhaemoglobin (Kabat et al., 1979). It plays a central role in the immune system, and Zn deficiency has been shown to increase susceptibility to infectious disease (see review; Shankar and Prasad, 1998). Zn is crucial for normal development and function of immune cells including polymorphonuclear leukocytes (Singh et al., 1994), natural killer cells (Allen et al., 1983), lymphocytes and macrophages (Fraker et al., 1986). Recent evidence indicated that Zn also plays a modulating role in synaptic transmission by interacting with specific sites on ionotropic neurotransmitter receptor proteins (Westbrook and Mayer, 1987; Celentano et al., 1991; Xie and Smart, 1991). In conclusion, Zn plays a major role in numerous biological functions (Bettger and O'Dell, 1981). As a result, Zn deficiency is often associated with a decline in enzymatic and immunological reactions that may increase susceptibility to infection and predispose to a range of diseases.

1.2.1.2 Zinc supplementation

The physiological and pharmacological actions of Zn are of considerable potential therapeutic value, both as a topical and as an oral agent. Zn treatment has been used therapeutically in a range of disease states including Wilson's disease, peptic ulcers, oesophageal dysplasia and others (Table 2).

When Zn is administered via the oral route, it is reasonably well tolerated and essentially non-toxic (Fosmire, 1990). Only a few instances of acute Zn poisoning have been reported in humans, but only after large amounts have been consumed (4-8 g of Zn). The symptoms of Zn toxicity include nausea, vomiting, diarrhoea, fever and lethargy. Long-term exposure to high Zn intakes has been shown to result in interference with the

Table 2. List of illnesses and conditions in which oral zinc therapy has been shown to be beneficial.

Disease entities	Conditions/manifestations
Acne	Zinc deficiency
Acrodermatitis enteropathica	Cutaneous ulcers
Acute lymphoblastic leukemia	Hypogeusia (impaired taste acuity)
Alopecia areata (and related hair-loss conditions)	Hyposmia (impaired smell acuity)
Coryza (the common cold)	Impotence
Ehlers-Danlos syndrome	Infertility
Oesophageal dysplasia	Surgical wound healing
Furunculosis	
Hepatic encephalopathy	
Herpes simplex II (genital herpes)	
Leprosy	
Peptic disease (gastric and duodenal ulcers)	
Porphyria	
Primary biliary cirrhosis	
Prostatitis	
Rheumatoid arthritis	
Sickle cell anaemia	
Toxic optic neuropathy (tobacco-alcohol amblyopia)	
Wilson's disease	

metabolism of other trace elements, particularly copper (reviewed; Abdel-Mageed and Oehme, 1990).

A considerable body of data has shown the efficacy of Zn treatment in Wilson's disease (see reviews, Brewer, 1995; Hoogenraad, 1998). Wilson's disease is an autosomal, recessive, inborn error of metabolism leading to toxic accumulation of copper in tissues, in particular the liver, as well as the basal ganglia of the brain (Solomons et al., 1989). Oral Zn therapy is effective in controlling copper balance and blocks the intestinal absorption of copper (Yuzbasiyain-Gurkan et al., 1992). A regimen of Zn salt equivalent to 50 mg elemental Zn 3 times a day has been found to be most beneficial (Brewer et al., 1993).

Watanabe et al., (1995) demonstrated the importance of Zn in healing gastric ulcers. In their study rats were fed a Zn-replete or Zn-deficient diet for 6 weeks before gastric ulcers were induced by administration of acetic acid. The Zn-deficient animals had reduced cell proliferation in the crypts of the gastric mucosa by day 4, and had delayed ulcer healing compared to rats fed a control diet. A variety of novel Zn compounds have been found to protect against gastrointestinal disease in both animal and human studies (Solomons, 1988). Zn sulphate has been shown to progressively accelerate the healing of acetic acid-induced ulceration in rats after 15 days of treatment with 44 and 88 mg/kg Zn sulphate (Li, 1990). Rainsford and Whitehouse (1992) showed that a slow-release Zn complex, Zn monoglycerolate given orally was effective in preventing development of gastric lesions induced by non-steroidal anti-inflammatory drugs and ethanol.

Various novel Zn compounds have also been demonstrated to have anti-ulcerogenic action. These include ranitidine-Zn complex (Conchillo et al., 1995), Zn-cimetidine (Ito et al., 1995), polaprezinc, an insoluble Zn complex of L-carnosine (Furuta et al., 1995; Kato et al., 1997), Zn gluconate (Bandyopadhyay and Bandyopadhyay, 1997), and Zn sulphate (Troskot et al., 1997). Several mechanisms by which Zn might have an anti-ulcerogenic effect have been proposed. Li (1990), speculated that Zn ions may promote gastric ulcer healing by enhancing mucous formation to prevent acid from attacking the gastric mucosa. Additionally, Zn may decrease the acidity of the gastric secretion (Bandyopadhyay and Bandyopadhyay, 1997). Furthermore, Zn may stabilise membranes, promote wound healing by increasing cell proliferation or act as an antioxidant (reviewed; Walsh et al., 1994). Furuta et al., (1995) also showed that certain Zn complexes namely, Zn monoglycerolate and polaprezinc that have longer residence times in the stomach, are adhesive to the ulcerous sites and are more beneficial in healing and/or preventing gastric ulcers.

Zn appears to have a marked impact on infantile diarrhoea (see reviews; Hambidge, 1992; Folwaczny 1997; Black, 1998; Fuchs, 1998). Sachdev and co-workers (1990) demonstrated in a controlled randomised trial in 40 Zn deficient infants with persistent diarrhoea, that Zn supplementation improved their morbidity and Zn status. Sazawal et al., (1995) demonstrated that Zn supplementation (20 mg/day) significantly decreased the number of watery stools by 39% per day and number of days with watery stools by 21% in children with diarrhoeal illnesses in New Delhi, India. In subsequent studies, Sazawal and co-workers (1996) showed that among low socio-economic children in India Zn supplementation reduced the incidence of persistent diarrhoea by 73% in children with

low baseline plasma Zn (<7.65 $\mu\text{mol/L}$) and by 49% in children >11 months of age. Moreover, Sazawal et al., (1997) demonstrated that 6 months of Zn supplementation (10 mg/d) in children of low socio-economic status in urban India, decreased the incidence and prevalence of diarrhoea both in Zn deficient boys >11 months old (26% and 35%, respectively) and girls >11 month of age (17% and 19%, respectively) compared to untreated controls.

Another randomised, double-blind intervention trial on the effects of Zn supplementation on rural Guatemalan children (Ruel et al., 1997), revealed that the incidence of diarrhoea was reduced by 22%, with larger reductions in boys (39%) as well as a 67% reduction in the percentage of children who had one or more episodes of persistent diarrhoea. In a similar study conducted by Roy et al., (1997) in malnourished Bangladeshi children with acute diarrhoea, 20 mg of Zn per day for 2 weeks resulted in a reduction in stool output by 28% and duration by 14% compared to the placebo group. It has been proposed that Zn supplementation in diarrhoeal disease leads to accelerated regeneration of the intestinal mucosa, increased levels of brush border enzymes, enhanced cellular immunity and higher levels of secretory antibodies (Folwaczny, 1997). Similar conclusions were drawn by Bhutta et al., (1999) after meta-analysis of 7 “continuous” and 3 “short-course” Zn supplementations to assess the efficacy in the prevention of diarrhoea and pneumonia in children in developing countries.

1.2.1.3 Antioxidant role of zinc

Zn is also thought to have an antioxidant-like role but the amount necessary for protection *in vivo* is unclear. Nonetheless, Zn deficiency has been associated with an increased

production of free radical/reactive oxygen species or decreased destruction of free radicals.

Zn is protective against reactive oxygen species (ROS)-mediated damage, and has been shown to protect liposomes and micelles from iron-induced lipid peroxidation as well as cell membranes from haemolytic viruses and a range of cytotoxic agents (reviewed, Vallee and Falchuk, 1993). It is postulated that Zn induced protection against ROS-mediated damage occurs by two mechanisms of action, (1) induction of tissue metallothionein levels by Zn could produce cellular drug detoxification by binding the active agent to sulfhydryl residues on the protein and (2) Zn, as a cofactor of glutathione S-transferase activity, superoxide dismutase and other Zn dependent antioxidant enzymes (see review; Cho 1991) as well as maintaining the activity of antioxidant enzymes and therefore Zn supplementation may be beneficial in recovery from diseases.

Pharmacological doses of Zn have been shown to protect against pro-oxidant induced damage (review; Bray and Bettger, 1990). Padmaja and Ramamurthi (1997) showed that the activities of hepatic and brain antioxidant enzymes (glutathione peroxidase, glutathione reductase, glutathione-S-transferase, superoxide dismutase and catalase) in chick embryos were elevated 24 h after being treated with Zn on the 14th day of embryonic development. In other studies, oral Zn supplementation has been shown to prevent oxidant damage to the liver (Chvapil et al., 1973; Cagen and Klaasen, 1979), lung (Anttinen et al., 1985) and kidney (Fukino et al., 1986).

It is thought that oxygen-derived free radicals may contribute to intestinal damage in inflammatory bowel disease. Two copper and Zn containing proteins, metallothionein and superoxide dismutase, involved in the scavenging of free radicals were found to be decreased in the intestinal mucosa of patients with this disease (Mulder et al., 1994). The Zn concentration in the colon of rats with colitis induced by acetic acid or trinitrobenzene sulfonic acid was decreased by 21% and 28% respectively, compared to controls (Al-Awadi et al., 1998). Orally administered zinc-carnosine chelate compound (Z-103; zinc N-(3-aminopropionyl)-L-histidine) was found to inhibit gastric mucosa erosion after ischemia-reperfusion and lipid peroxidation (Yoshikawa et al., 1991a). In subsequent *in vitro* studies, these authors showed that Z-103 scavenged superoxide anion radicals, inhibited superoxide generation from polymorphonuclear leukocytes stimulated by opsonized zymosan, and also inhibited the generation of hydroxyl radicals (Yoshikawa et al., 1991). A recent study by Yoshikawa et al., (1997) showed that rats receiving Z-103 (30 mg/kg) and Zn sulphate (30 mg/kg) for 1 week had a reduced inflammatory response induced by 2,4,6-trinitrobenzene sulfonic acid (120 mg/ml) as evaluated macro- and microscopically.

1.2.1.4 The gastrointestinal tract and zinc

The nutritional importance of Zn is well established and the gastrointestinal tract has been recognised as pivotal in the maintenance of Zn homeostasis. The epithelium of the intestinal mucosa undergoes continuous renewal and is in constant need of Zn. Whole body Zn homeostasis is believed to be regulated by absorptive and secretory processes in the small intestine, with only a very small proportion of Zn is normally excreted by the kidneys, (Cousins 1985). The general consensus is that Zn absorption primarily occurs in

the upper small intestine (see reviews; Lonnerdal, 1989; Reyes, 1996; Krebs, 2000). However, the contribution of the ileum may be important due to the long residence time of the digesta. The contribution of the large intestine in nutrient absorption appears to be minimal. The mechanisms of absorption in the small intestine are poorly understood but are believed to occur by both passive and facilitated processes.

1.2.1.5 Zinc carriers

The understanding of mechanisms controlling Zn absorption at the molecular level has advanced recently with the cloning of 4 putative Zn transporters (Huang and Gitscher, 1997; Palmiter et al., 1996; Palmiter et al., 1996a; Palmiter and Findley, 1995). Palmiter and Findley (1995) encoded a cDNA Zn transporter (ZnT-1), isolated from a rat kidney cDNA expression library by complementation of a mutated, zinc-sensitive BHK cell line. The authors proposed that ZnT-1, a plasma membrane protein, transports Zn out of cells and that its absence accounts for the increased sensitivity of mutant cells to Zn toxicity. ZnT-1 in particular, has been located in the basolateral membrane of the small intestine, its mRNA have been shown to be increased by high dietary Zn and it appears to be essential for Zn efflux from the enterocytes (McMahon and Cousins, 1998). Unlike ZnT-1, ZnT-2 appears localised on vesicle membrane and transports Zn into vesicles to lower the cytoplasmic Zn concentration (Palmiter et al., 1996). ZnT-3 is most abundant in the hippocampus and cerebral cortex in the mouse and is proposed to facilitate the accumulation of Zn in synaptic vesicles (Palmiter et al., 1996a), whereas the ZnT-4 gene confers resistance to a zinc-sensitive yeast strain and is expressed in the mammary epithelia and brain (Huang and Gitschier, 1997). Transporters controlling the influx of Zn, however, remain elusive. Zn transporters have also been described in yeast, which

have two separate systems for Zn uptake. The first Zn transporter, ZRT1, is proposed to have high affinity for Zn and is induced in zinc-deficient yeast (Zhao and Eide, 1996) and the second, ZRT2 has low affinity for Zn and is not highly regulated by Zn status (Zhao and Eide, 1996a). In addition, Zn transporter genes from plants have been cloned, the ZIP1, ZIP2, ZIP3 and ZIP4 genes from *Arabidopsis thaliana* (Grotz et al., 1998). The human equivalent to these proteins hZIP1, hZIP2, and hZIP3 have been isolated and intensive research is being carried out to determine their role in Zn transport (Gaither and Eide, 2000).

Several intracellular ligands have been proposed to play a pivotal role in the transfer of Zn flux across the enterocytes and these include metallothionein and cysteine-rich intestinal protein (CRIP) (Hempe and Cousins, 1991; Hempe and Cousins, 1992). CRIP, an 8.6 kDa polypeptide with 77 amino acids, of which seven are cysteine and which binds to Zn, was first isolated and identified from a rat intestinal cDNA library by Birkenmeier and Gordon (1986). However, Fleet et al., (1993) argued that CRIP did not act as an intracellular Zn ferry since in their studies, increased Zn transport occurred concurrently with increased metallothionein gene expression, and decreased CRIP mRNA levels. In addition, recent studies with transgenic mice that over-express CRIP have shown that CRIP is involved in erythroid differentiation making it less likely that CRIP is truly a Zn carrier protein in the intestine.

The mucosal epithelial cell not only modulates Zn absorptive and secretory processes but also maintains an adequate intracellular Zn pool by binding to intracellular ligands. In the mucosal cell, the greatest proportion of Zn is avidly bound to high molecular weight

proteins (poorly exchangeable) and under physiological conditions little dissociation occurs (Henkin, 1974; Cousins, 1985). Only a very small proportion of the total Zn is exchangeable and this is found in the low-molecular weight fraction, the largest pool being Zn-bound metallothionein.

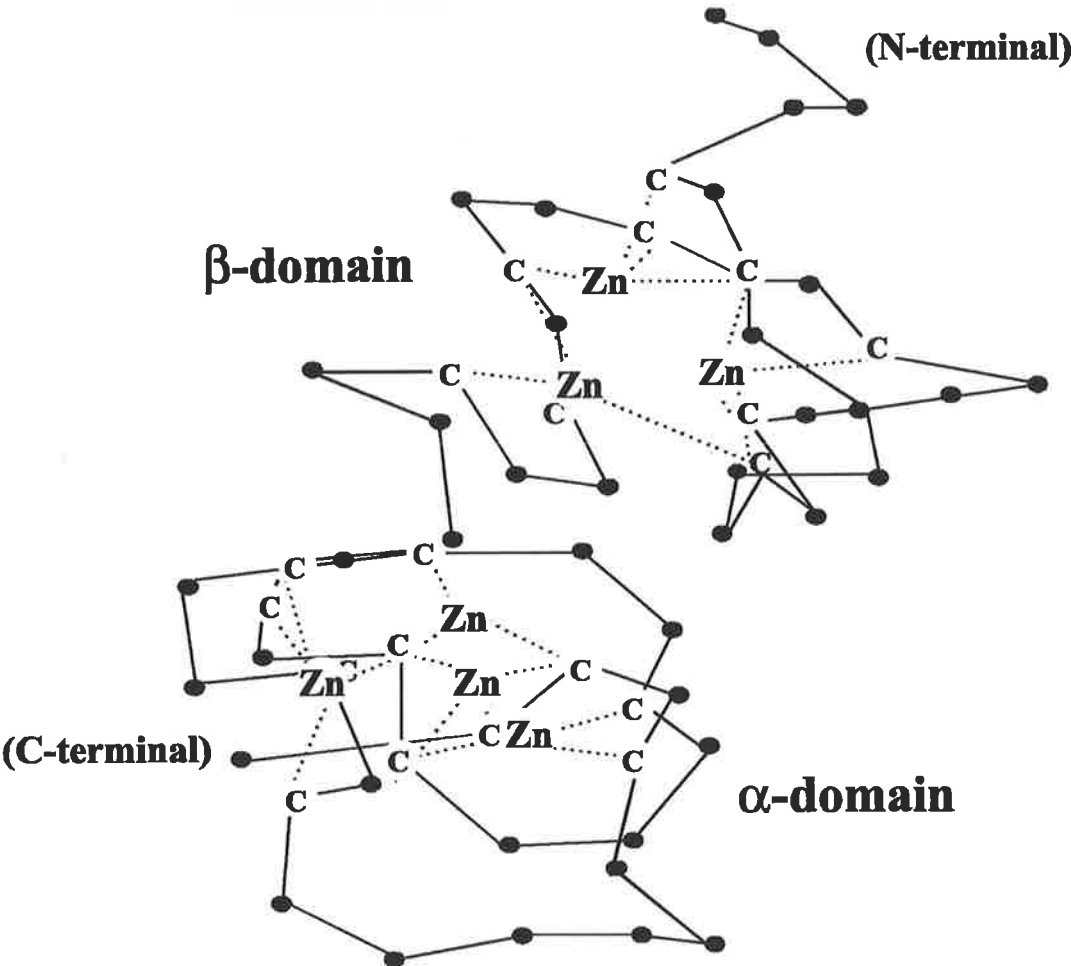
1.2.2 METALLOTHIONEIN

Metallothionein is a low molecular weight (MW 6700) cytosolic protein composed of 62 amino acids, 20 of which are cysteine residues (Vallee and Falchuk, 1993). Metallothionein potentially binds 7 atoms of Zn per molecule. The tertiary structure of metallothionein contains two domains; the α -domain of the C-terminal contains 4 Zn atoms and 11 cysteine molecules while the N-terminal β -domain contains 3 Zn and 9 cysteine residues (Fig. 1) (Vallee and Auld, 1990; Vallee and Falchuk, 1993). While metallothionein is thermodynamically stable, the mercaptile bonds are kinetically labile. This allows a constant intramolecular metal exchange by the cleavage and reformation of the cysteine-metal bonds, which is more pronounced in the β -domain (Otvos et al., 1993).

1.2.2.1 Isoforms of metallothionein

In humans and rodents, four metallothionein isoforms have been identified, termed metallothionein-I to -IV. Metallothionein-I and -II are inducible isoforms, which are found in nearly all tissues but in particular the liver, pancreas, gut and kidneys. Metallothionein-I and -II isoforms differ slightly in amino acid composition and are separable by DEAE ion-exchange chromatography or gel-permeation high-performance liquid chromatography (HPLC) (Cousins, 1985). The more recently discovered isoforms, metallothionein-III and -IV, have a more restricted expression, normally in the brain and

Figure 1. Metallothionein. The molecule is composed of two domain. Each zinc is tetrahedrally co-ordinated to four thiolate bonds.



in the keratinising epithelia, respectively (reviewed by Hidalgo and Carrasco, 1998). Metallothionein -III and -IV are distinguished from the other two isoforms by the insertion of a threonine or a glutamate at position 5, respectively (Palmiter et al., 1992; Quaife et al., 1994). All four metallothionein isoforms share a substantial homogeneity regarding their heavy metal binding properties, hence they may have similar physiological roles. However, the different patterns of expression suggest that the metallothionein isoforms may have specific functions (Quaife et al., 1994; Carrasco et al., 1998).

1.2.2.2 Induction of metallothionein

Piscator (1964) was the first to demonstrate that metallothionein is an inducible protein in certain tissues and a large number of physiological and pathological agents have now been found to induce metallothionein synthesis *in vivo*. Metallothionein is often considered a “stress” protein, expressed in many tissues and can be induced in the liver by cytokines (TNF- α Interleukin-1 and -6), catecholamines, glucocorticoids and various metals (Bremner and Beattie, 1990; Moffat and Denizeau, 1997), as well as physical and chemical stressors (Table 3), (Kagi, 1991). Cadmium and Zn are potent inducers of metallothionein in liver, pancreas, kidney, intestine, lungs, heart, stomach and spleen (Onosaka and Cherian, 1981; Onosaka and Cherian, 1982). Other metals vary in their ability to induce metallothionein and some may work indirectly by exchanging and releasing cadmium and Zn from other intracellular ligands. A range of drugs and toxins (Table 3) also induce metallothionein, either indirectly via an inflammatory response, or by an oxidant response element on the metallothionein promoter (Dalton et al., 1994; Palmiter, 1994).

Table 3. Inducers of metallothionein; heavy metals, hormones, cytokines, growth factors, drugs, oxidative stress and physical stress.

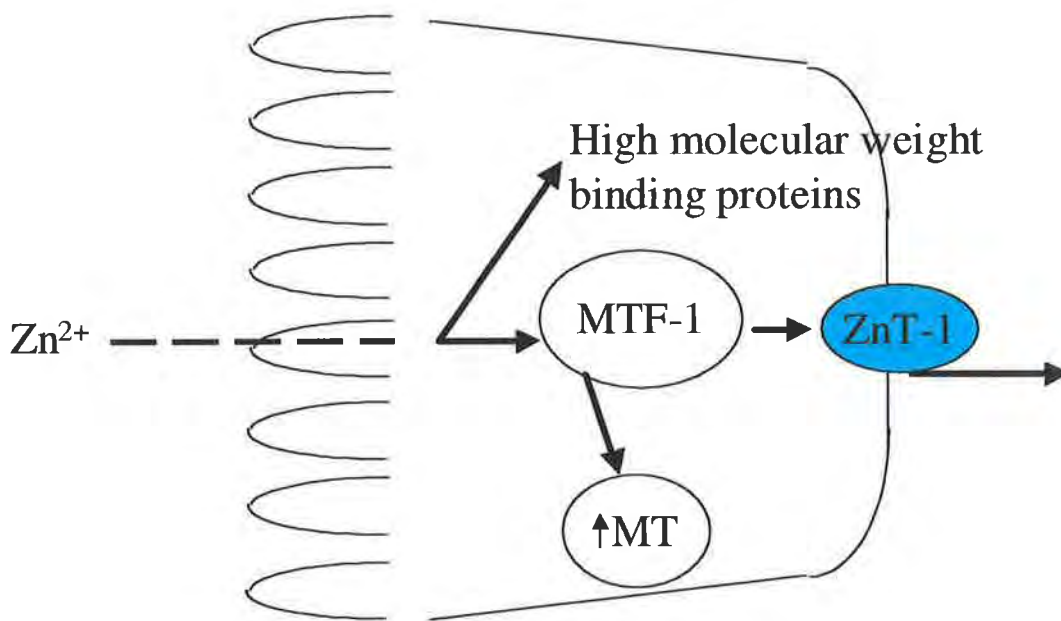
Heavy metals	Hormones/ Cytokines/ Growth factors	Drugs	Physical stress	Oxidative stress
Cadmium	Glucocorticoid	Acetaminophen	UV radiation	tert-Butyl hydroperoxide
Zinc	Glucagon	Adriamycin	X radiation	Menadione
Copper	Leukaemia	cis-Platinum	Cold	Hydrogen peroxide
Bismuth	inhibiting factor		temperature	High-oxygen tension
Mercury	Tumour necrosis factor- α	Penicillamine	Exercise	Alcohol
Gold	Interferon- γ	Streptozotocine		
Cobalt	Interferon- α			
Nickel	Interleukin-1			
Arsenic	Interleukin-6			
Lead	Interleukin-11			
Iron	Progesterone			
Chromium				
Manganese				

Transcription of metallothionein gene activation via heavy metals is mediated through the metal regulating elements (MRE), present in multiple copies in the promoter region of the metallothionein gene that serve to amplify the transcription of the metallothionein mRNA (Palmiter, 1987; Koizumi, 1999). The transcription factor (MTF-I) acts as an intracellular Zn sensor and in the presence of Zn binds to the MRE causing transcription of the metallothionein gene. Other metal ion inducers may work indirectly by releasing Zn from intracellular ligands (Koizumi, 1999). Interestingly, Langmade et al., (2000) has shown that metallothionein and ZnT-1 (the Zn efflux protein) are co-ordinately regulated by the MTF-I in enterocytes in response to dietary Zn. In this regard, it is proposed that dietary Zn excess raises the intracellular Zn that is sensed by MTF-I and this in turn stimulates efflux of Zn from the enterocyte via ZnT-1 as well as increased MT expression that sequesters Zn and prevents free Zn levels from becoming toxic to the cell (Fig.2).

1.2.2.3 Metallothionein induction by dietary zinc in liver and intestine

When the dietary supply of Zn is sufficiently elevated, induction of metallothionein occurs primarily in the liver and intestine, and is followed by increased accumulation and/or redistribution of the metal in both tissues (Richard and Cousins, 1976a). Cousins (1985) showed that rats with low hepatic and intestinal metallothionein levels (0.4 and 1.4 $\mu\text{g Zn/g}$, respectively), consuming a Zn deficient diet (<1 mg zinc/kg) for one day decreased metallothionein to only trace levels. However, repletion with a semi-purified diet containing 150 mg Zn/kg increased metallothionein-bound Zn in both organs (10.7 and 12.5 $\mu\text{g zinc/g}$, respectively).

Figure 2. Zinc homeostasis is regulated by the expression of the ZnT1 that is induced by dietary zinc and this regulation is dependent of the transcription factor MTF-1. MTF-1 regulates the expression of the ZnT1 gene, cells exposed to excess zinc results in the increased expression of the metallothionein gene, which encodes the major intracellular zinc storage protein, and the expression of ZnT1 which effluxes the metal from the cell. On the other hand, under conditions of zinc depletion, metallothionein is degraded to provide a biologically active labile pool of zinc and the efflux of zinc via ZnT1 is attenuated leading to the conservation of this metal in the cell.



Olafson (1983) showed a 25% increase in intestinal metallothionein in Zn-deficient mice two weeks after supplementation with 300 mg Zn/kg. Four-fold inductions have been achieved in rats (Menard et al., 1981), such studies, however, compare the increase in metallothionein following supplemental Zn with the trace levels of metallothionein caused by Zn depletion. There have been few reports of intestinal metallothionein induction at adequate or excess Zn levels. In experiments with rats fed diets containing 60 or 350 mg Zn/kg, intestinal metallothionein was found to be increased by 44% after 3 weeks on the high Zn diet (Reeves et al., 1996). In another study (Blalock et al., 1988), metallothionein mRNA expression was enhanced sixfold in the small intestine of rats supplemented with Zn (180 mg Zn/kg) in their diets.

Food deprivation has been shown to increase liver metallothionein in rodents (Bremner and Davies, 1975; Shimizu and Morita, 1990; Kramer et al., 1993), but the effect of fasting on intestinal metallothionein is unclear. In a temporal study in rats, liver metallothionein was only slightly increased 36 hr after fasting but there was no change in intestinal metallothionein (Kramer et al., 1993). In another study, mucosal metallothionein was increased by 45% after an overnight fast (Hoadley et al., 1988).

Although, the mechanism of induction of gut metallothionein after fasting is unclear, much more is known about fasting and the induction of liver metallothionein. Failla and Cousins (1978) demonstrated that metallothionein induction by stress and fasting was related to hormonal control and that glucocorticoids were capable of enhancing Zn uptake and metallothionein induction in primary cultures of rat hepatocytes. Fasting is associated with a lowered blood glucose, gluconeogenesis and glycogenolysis and is affected by the

hormones glucagon, insulin, catecholamines, vasopressin, angiotensin II and glucocorticoids (Kramner et al., 1993). It is thought that glucagon is a potential inducer of intestinal metallothionein, as it has been found to be a stimulator of hepatic metallothionein (Etzel and Cousins, 1981; Coyle et al., 1993) in cell culture experiments.

1.2.2.4 Functions of metallothionein

Although metallothionein was discovered over 40 years ago, its primary physiological role remains elusive. Metallothionein has been ascribed a broad range of functions including regulation of Zn homeostasis, detoxification of heavy metals (Lonnerdal et al., 1984; Hogstrand and Haux, 1991), protection against free radical ions, and a provider of Zn for metalloenzymes and Zn finger motifs of DNA-binding transcription factors (Kagi and Schaffer, 1988). Studies have shown that the expression of hepatic metallothionein during embryogenesis (1000 µg/g tissue) in rats is markedly higher compared to the levels found in adults (25 µg/g tissue) with a concurrent Zn accumulation (Panemangalore et al., 1983; Templeton et al., 1985). This indicates that metallothionein is also important in embryonic development, where it may be associated with the changes in Zn requirements of proliferating and differentiating tissues (Kern et al., 1981).

Metallothionein may have a protective role in the small intestine in inflammatory disease by sequestering ROS (Bremner and Beattie, 1990; Templeton and Cherian 1991). Metallothionein concentrations have been reported to be low in intestinal mucosa of inflammatory bowel disease patients and oral Zn supplementation has been shown to increase metallothionein levels in both inflamed and non-inflamed regions compared to unsupplemented controls (Mulder et al., 1994). The induction of intestinal

metallothionein may be important in relation to ameliorating the severity of intestinal diseases and may complement the effects of Zn. Miura et al., (1997) showed that metallothionein protected DNA from oxidative attack by sequestering hydroxyl radicals. In these studies, it was reported that the scavenging activity of metallothionein was 50 times greater than glutathione, the main intracellular antioxidant molecule in the cell. Furthermore, Hussain et al., (1996) suggested that metallothionein-II has a 6 fold higher capacity to scavenge superoxide radicals than metallothionein-I.

Metallothionein can function as an expendable target for oxidants due to its highly enriched cysteine residue structure. These cysteine clusters are quite labile and freely exchange native metals with electrophiles. This was verified with intact cells by Quesada and co-workers (1996) who demonstrated that the sulfhydryl groups of metallothionein isolated from HL-60 cells were oxidised by H₂O₂. It is interesting to speculate whether or not a metallothionein reductase system may exist. The unique characteristics of the Zn clusters of metallothionein were recently recognised to react through a redox mechanism in which the oxidation of the cysteine ligands of Zn result in release of the metal (Maret and Vallee, 1998).

Redox control of the Zn content of metallothionein also opens a new functional aspect for metallothionein. Mobilization of Zn from metallothionein by an oxidative reaction may either constitute a general pathway by which Zn is distributed in the cell or it may be restricted to conditions of stress where Zn is needed in antioxidant defense systems including enzymatic repair functions.

1.2.3 METALLOTHIONEIN TRANSGENIC AND KNOCK-OUT ANIMAL MODELS

The main advancement in determining the importance of metallothionein has come from transgenic animal research (review; Klaassen and Liu, 1998). Transgenic animals are now available that over-express metallothionein (Palmiter et al., 1993) or lack metallothionein by deleting or inactivating the metallothionein gene (Michalska and Choo, 1993).

1.2.3.1 Transgenic mice over-expressing metallothionein

Dalton et al., (1996) showed that transgenic mice that over-express metallothionein-I accumulated more metallothionein-bound Zn in major organs compared to control mice and suggested that the larger pool of Zn-metallothionein provides an important source of labile Zn during periods of Zn deficiency. In addition, Liu et al., (1995) showed that metallothionein-I transgenic mice have 10 to 20-fold higher liver metallothionein concentrations, and these levels are sufficient to protect against cadmium-induced lethality and hepatotoxicity.

1.2.3.2 Transgenic mice deficient in metallothionein-I and -II expression

A colony of the metallothionein-knockout mouse derived by Michalska and Choo (1993) (Murdoch Institute, Royal Children's Hospital, Melbourne, Vic., Australia), in which there is a null mutation at the metallothionein-I and -II gene loci (the primary MT isoforms and the only isoforms found in the mouse gut) has been established in the IMVS laboratory. These mice reproduce and develop normally when the animals are maintained in a nutritionally adequate, low stress, disease free environment. Beattie et al., (1998), however, showed that the Michalska and Choo older metallothionein-null mice had a

predisposition to be larger than most strains of mice, with increased fat accretion, elevated obese gene expression and high plasma leptin levels.

Coyle et al., (1995) showed the importance of metallothionein for hepatic zinc accumulation by demonstrating that hepatic Zn accumulation in cultured hepatocytes prepared from metallothionein-null mice was only 60% of the control cells. In addition, 16 hr after intraperitoneal injection of 5 μ g/g Zn, hepatic cytosolic Zn concentration doubled in control mice whereas only a 15% increase was detected in metallothionein-null mice, suggesting that little if any accumulation of Zn occurred within the liver when metallothionein is unavailable for its sequestration. Rofe et al., (1996) investigated the effects of Zn accumulation during infection or inflammation in metallothionein-null mice. The authors showed that 16 hr after intraperitoneal injection of endotoxin (1 mg/kg LPS), hypozincemia was pronounced in normal mice, and hepatic Zn and metallothionein was increased by 36% and 10-fold, respectively. However, plasma Zn and hepatic Zn were unchanged in metallothionein-null mice (Philcox et al., 1995). Similar findings were obtained after LPS injection (5 mg/kg), suggesting that metallothionein has a role in maintaining whole body Zn homeostasis. Metallothionein-null mice have also been shown to be sensitive to a variety of stimuli. For example metallothionein-null mice are more susceptible than control mice to paracetamol toxicity (Rofe et al., 1998), cisplatin-induced nephrotoxicity (Sato et al., 1997; Liu et al., 1998), as well as being more sensitive to dietary Zn depletion and Zn toxicity (Kelly et al., 1996).

Coyle et al., (1999) demonstrated that Zn absorption was not significantly different between normal and metallothionein-null mice when fed a Zn-replete diet, but absorption

was directly proportional to the oral dose of aqueous $^{65}\text{ZnSO}_4$. However, normal mice had greater Zn absorption from an egg-white diet than did metallothionein null mice, indicating that gut metallothionein confers an absorptive advantage, but only when Zn is incorporated into solid food and at low Zn concentrations in solution when mice are made Zn deficient (Coyle et al., 2000). In another study, Rofe et al., (1999) examined the role of the pancreas in intestinal Zn secretion in metallothionein-null mice. The authors showed that ^{65}Zn levels were significantly higher in the liver and pancreas of normal mice, whereas in metallothionein-null mice, ^{65}Zn levels were significantly higher in muscle, skin and most of the gastrointestinal tract other than the stomach and upper small intestine. The retention of ^{65}Zn in the pancreas after fasting in metallothionein-null mice was half that of normal mice and metallothionein-null mice also had significantly lower Zn concentrations in the pancreas. They concluded that mice lacking metallothionein-I and -II lose more endogenous Zn into the gut because of a relative failure of the pancreas to retain Zn. In addition, increased Zn secretion via the small intestinal mucosa may also contribute to intestinal Zn loss in the metallothionein-null mice.

Andrews and Geiser (1999) demonstrated the importance of metallothionein in Zn homeostasis in pregnant metallothionein-null mice fed a Zn deficient diet. The authors found that severe dietary Zn deficiency during periods of pregnancy was embryotoxic and teratogenic and the majority of embryos died by mid-gestation. 53% of the surviving embryos in the metallothionein-null mice were morphologically abnormal compared to only 32% of the embryos in the control mice. These results demonstrate that the expression of the metallothionein-I and -II genes in pregnant females improves reproductive success during maternal dietary Zn deficiency. On the other hand, Carey et

al., (2000) demonstrated that the incidence of fetal dysmorphology from alcohol exposure was greatly increased in normal mice given alcohol on d 8 of gestation than in metallothionein-null mice. These findings link the teratogenic effects of alcohol to the alcohol-induced rise in maternal metallothionein and the consequent fall in plasma Zn, which limits the fetal Zn supply.

The metallothionein knock-out mouse enables the examination of the relationship between metallothionein and the effects of dietary Zn supplementation. Key questions as to whether metallothionein has a dominant role in controlling Zn availability within the damaged or inflamed mucosa and whether metallothionein is important in protection and repair can now be answered.

1.2.4 GROWTH FACTORS AND THE GUT

Growth factors are essential for mammalian embryogenesis, mitosis and cell differentiation and normal cell regeneration (Schultz et al., 1994). Several epidermal growth factors (EGF) and transforming growth factor (TGF)- α and other luminal growth factors have been identified in the gut (Murphy, 1998), these agents may act in regulating mucosal growth and thus may play a key role in the development and maintenance of epithelial integrity (review; Goodlad and Wright, 1995; Seare and Playford, 1998; Murphy, 1998). Exogenously administered growth factors, particularly epidermal growth factor (EGF) have been found to be beneficial for gut regeneration following injury by radiotherapy (McKenna et al., 1994), chemotherapy (Sonis et al., 1992; Sonis et al., 1994) and other disease conditions (Mustoe et al., 1990; Read et al., 1992). In addition, several growth factors have been shown to promote ulcer healing (review; Szabo et al., 1995).

There have been several reports of the efficacy of exogenously administered growth factors in the treatment of experimental colitis. These growth factors have included EGF (Luck and Bass, 1993; Procaccino et al., 1994), platelet-derived growth factor (Sandor et al., 1995), keratinocyte growth factor (Zeeh et al., 1996) and transforming growth factor- β (TGF- β) (Murthy et al., 1992) and insulin-like growth factor- I (IGF-I) (Howarth et al., 1998a).

Only a few studies have investigated the efficacy of growth factors in acceleration of gut repair after both chemotherapy-induced intestinal damage and mucosal damage elicited using other agents. These have mainly used animal models and have almost exclusively used trinitrobenzene sulfonic acid and dextran sodium sulphate to induce inflammatory bowel disease (Dykens and Baginski, 1998; Rogler and Andus, 1998). However, the methotrexate model of small intestinal damage, which shares some clinical and histopathologic features with inflammatory bowel disease, has been used less frequently. In addition, there have been few investigations into the trophic effects of growth factors in this model.

1.2.4.1 Epidermal growth factors

EGF is known to exert a mitogenic effect in epithelial tissues, including the gastrointestinal tract (Jansen et al., 1997). EGF is a polypeptide containing 53 amino acids and has been detected in the salivary glands, Brunner's glands and the pancreas (Gresik et al., 1979; Jaworek and Konturek, 1990). Olsen et al., (1984) reported that removal of submandibular glands resulted in a marked reduction (90%) in the gastric content of EGF. EGF receptors have been demonstrated to be located on the baso-lateral

surface of the epithelial cells, thus EGF given lumenally might be useful in cytoprotection and in stimulating repair and regeneration in the gut (Wong and Wright, 1999).

It has also been shown that oral administration of synthetic human EGF accelerates the healing of chronic duodenal ulcerations induced by cysteamine in rats (Kirkegaard et al., 1983). It is postulated that EGF acts directly on the duodenal mucosa by promotion of mucosal growth and the stimulation of re-epithelialization. Jansen et al., (1997), demonstrated that mice continuously infused with 1.6 nmol/kg/h EGF for 1, 3 and 7 d resulted in increased depth of crypts in the duodenum after 1 d, followed by increased villus height after 3 and 7 d, whereas in the jejunum and ileum villus, the height increased after 7 d. The effect of oral EGF on recovery of methotrexate-induced bowel damage was also investigated, rats fed a liquid diet supplemented with EGF at 0, 1, 10 or 20 times the estimated daily intake from human milk showed a significant increase in mucosal disaccharidase and leucine aminopeptidase activity by d 6 at the 1 and 10 times human milk EGF levels. It was proposed that the induction of these enzymes may aid repair of the intestinal mucosa (Petschow et al., 1993). Hirano et al., (1995) postulated that EGFs may enhance intestinal repair following methotrexate treatment in rats by increasing the ornithine decarboxylase activity in the methotrexate-damaged mucosa.

1.2.4.2 Transforming growth factors

There is mounting evidence to suggest that transforming growth factor (TGF) - α produced by the gastric mucosa is a critical mediator of gastric mucosal homeostasis (Coffey et al., 1995). TGF- α inhibits gastric acid secretion and appears to be important in epithelial repair (Scheiman et al., 1997), in stimulating mucosal restitution after injury (cell

migration and proliferation) and augmenting gastric mucin levels (Coffey et al., 1995). In studies with genetically engineered mice, TGF- α knock-out mice had increased susceptibility to dextran sodium sulphate induced colitis while mice over-expressing TGF- α had only 9% of the entire colonic mucosa destroyed compared to 35%-39% in controls (Egger et al., 1998). In addition, mucin and bromodeoxyuridine staining were markedly enhanced in the colon of TGF- α over-expressing mice, suggesting increased mucosal protection and regeneration.

TGF- β is a polypeptide with multiple physiological functions and its isoforms have important roles in control of the cell cycle, in regulation of cell-cell interactions and in growth and development (Kloen et al., 1994). Mustoe et al., (1987) demonstrated that a single application of TGF- β was sufficient to accelerate healing in a full-thickness rat skin incision model. In later studies, a single dose of TGF- β was found to enhance gastric healing by 50% on d 7 after injury, and result in an acceleration of wound breaking strength by d 4 (Mustoe et al., 1990).

1.2.4.3 Insulin-like growth factors

IGFs are single chain peptides of 7.5 kDa, composed of 70 and 67 amino acid residues for IGF-I and IGF-II, respectively (Thissen et al., 1994) and these isoforms are structurally related to insulin. IGFs are secreted by most organs and exert biological effects on most cell types. Although the gut expresses IGF-I, there is little known in regard to mitogenic responses in epithelial cells of the gastrointestinal tract.

Several studies in animal models of gut adaptation have demonstrated that exogenously delivered IGF peptides selectively stimulate gastrointestinal growth (Lemmey et al., 1991; Read et al., 1992). Read et al., (1992) have shown that the gut of rats treated with dexamethasone is very sensitive to the growth-promoting actions of IGF-I *in vivo*. Subcutaneous infusion of IGF-I for 7 d increased absolute gut mass by 60% and fractional gut weight by 32% compared to rats treated with dexamethasone alone. The short term effects of IGF-I (2.5 mg/kg/day) administration on intestinal proliferation were investigated by Steeb et al., (1995), in a study which showed that within 3 d of treatment, tritiated thymidine labelling indices were increased up to 14% in the duodenum and ileum of rats compared to untreated controls. In a study by Chen et al., (1995) rats receiving total parenteral nutrition with IGF-I (4 mg/kg/day) for 3 d demonstrated increased body weight, plasma IGF-I, and increased gut mucosal weights. Histologic and biochemical analyses showed greater villus height and crypt depth in the jejunum as well as higher mucosal DNA and protein content compared to control rats. In addition, subcutaneous administration of IGF-I to normal or partially gut-resected (Lemmey et al., 1991) rats resulted in an increased weight gain, suggesting that IGF-I promotes growth.

1.2.4.4 Insulin-like growth factor-I and zinc

IGFs have been reported to be an important growth factor in the regulation of gastrointestinal growth and function, however, whether these properties may be enhanced in the presence of Zn is not well defined. Tarnow et al., (1994) measured IGF-I mRNA concentrations in the wounds of pigs treated with or without topical Zn. The authors found that IGF-I concentrations were 50% higher in wounds treated with Zn oxide compared with control wounds on days 3-4 and that Zn oxide also increased the healing

rate of wounds. The authors postulated that the increased gene expression of IGF-I may be one of the mechanisms by which topical Zn oxide enhances wound healing. Furthermore, Ninh et al., (1996) reported that Zn supplementation stimulated growth and increased plasma IGF-I in growth retarded Vietnamese children. It is possible that the increased plasma IGF-I concentrations, as a result of Zn supplementation, may further facilitate gut function and improve Zn absorption. Howarth et al., (1998) found that administration of IGF-I to rats with methotrexate-induced intestinal mucositis, stimulated regrowth of the damaged intestine, particularly the ileum, with 22%, 32% and 29% increases in small intestinal weight, ileal villus height and ileal crypt depth, respectively. All gastrointestinal regions responded to IGF-I treatment with the small intestine being most responsive.

1.2.4.5 Whey growth factor extract

Whey, the yellow-green liquid that separates from the curd during manufacture of cheese and casein, has long been considered by the dairy industry to be a waste product. The disposal component of whey consists of two fractions. Firstly, whey contains 100% of the total milk carbohydrate (lactose) and second, whey contains about 20% of the total milk protein (Smithers et al., 1996).

Werner (1981) showed that whey protein is the most effective in meeting the body's energy and amino acid requirements, and in this respect is superior to other protein sources, notably egg, beef, soy and casein. In addition to nutrient value, these whey proteins exhibit biological activity involved in the maintenance, repair and proliferation of cells, such actions are ascribed to a category of milk constituents such as growth factors.

Milk of different species contains a rich composite of cell growth factors and bioactive proteins. Unconcentrated milk or whey alone cannot sustain long-term culture of mammalian cells. Thus purification strategies including, ion exchange chromatography, have been considered to concentrate the bioactive proteins in unprocessed bovine milk and whey. Subsequently, purification of cheddar whey by cation exchange chromatography yielded a fraction containing less than 1% of the whey protein (Francis et al., 1995). Subsequent studies of whey growth factor extract (WGFE) have demonstrated that the extract contained potent mitogens (Belford et al., 1995; Rogers et al., 1995).

Further identification studies of these mitogens responsible for biological activity of the cation exchange-derived extract are needed, however, the concentration of several known growth factors have been measured. The extract contains a variety of growth factors, including high concentrations of insulin-like growth factors-I and -II (IGF-I and IGF-II), acidic and basic fibroblast growth factors (aFGF and bFGF) and platelet-derived growth factors (PDGF), as well as transforming growth factor- β (TGF- β)(Francis et al., 1995). There is also other, as yet unidentified, growth factor activity in WGFE, and a sizable non-growth factor component that could be efficacious in the intestine (Belford et al., 1997).

Bovine WGFE is a rich source of growth factors that has been considered recently for its potential clinical use in preventing and treating tissue damage. WGFE has been shown to be effective in promoting growth of mammalian cells *in vitro* (Belford et al., 1995; Francis et al., 1995). Ulceration, inflammation, and secondary infection of the gastrointestinal mucosa are dose-limiting side effects of chemotherapy treatment, referred to as mucositis.

WGFE contains a variety of antibacterial factors, cytokines, and growth factors that might provide effective therapy against mucositis (review; Regester and Belford, 1999). Biologically active factors have been considered for their efficacy in preventing and/or treating oral and gut mucositis. Administration of individual growth factors such as IGFs, TGFs and PDGFs, which WGFE contains, has resulted in beneficial effects.

1.2.4.6 Combinations of growth factors and zinc

While it can be demonstrated that individual growth factors protect the gastrointestinal mucosa, it is not known whether combinations of growth factors would give further protection or aid in the repair processes of the damaged gut. An array of growth factors has recently been extracted from bovine cheese whey obtained as a by-product of the cheese making process (Francis et al., 1995; Rogers et al., 1995). The growth factors contained in the whey extract include TGF- β , IGF-I, acidic and basic fibroblast growth factors, as well as lactoperoxidase and immunoglobulin as the most abundant proteins (Francis et al., 1995).

Howarth et al., (1996) investigated the efficacy of a growth factor extract derived from cheese whey in a rat model of methotrexate-induced intestinal mucositis. Oral administration of whey extract for 5 days increased the villus surface length indices in the jejunum and ileum by 52% and 56% respectively compared with animals receiving a control diet. The therapeutic potential of combinations of whey extract and Zn supplementation warrant investigation as these might be expected to act synergistically in the protection and repair of gastrointestinal damage.

1.2.5 INTESTINAL PERMEABILITY

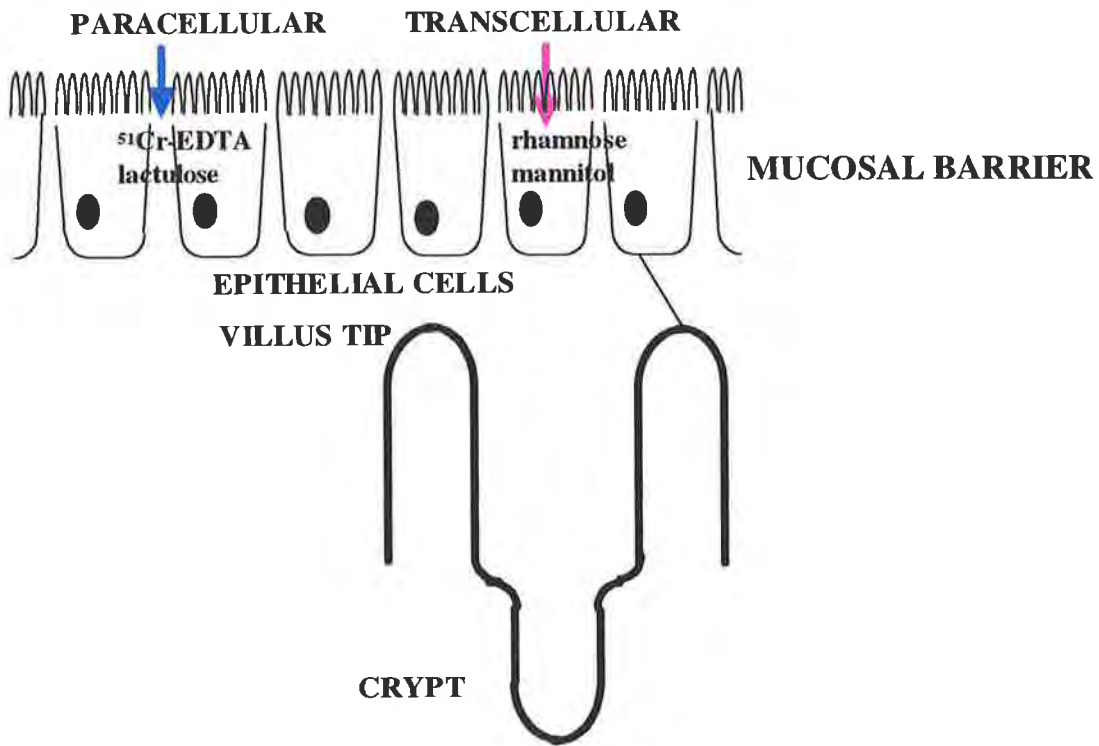
A primary function of the intestinal wall is to separate the internal environment of the body from the infectious, immunogenic and toxic chemicals that pass through the gut lumen. These agents may affect gut function in particular altering the uptake of inert molecules that normally are not absorbed and remain in the lumen. Pathologic increases in intestinal permeability to hydrophilic macromolecules have been identified in various clinical conditions (see review, Unno and Fink, 1998). Altered mucosal barrier function as indicated by intestinal permeability, may be assessed non-invasively by measuring the ratio of urinary excretion of intestinal permeation of a large molecule with that of a smaller molecule after oral administration of these test substances. These two molecules follow different routes of intestinal permeation; the large molecules are assumed to permeate paracellularly ($^{51}\text{Cr-EDTA}$, lactulose), and the smaller molecules are assumed to permeate transcellularly, such as rhamnose, mannitol (van Nieuwenhoven et al., 1999) (Fig. 3).

Damage to the intestinal mucosa resulting from toxins, drugs or disease states, can be detected early by increases in gut permeability. Patients with chronic inflammatory bowel disease have a defective epithelial barrier function manifested by an increase in intestinal permeability (reviewed; Ma, 1997; Miki et al., 1998).

1.2.5.1 Zinc, growth factors and intestinal permeability

The beneficial role of Zn in function of the epithelial barrier has been shown both *in vitro* (Hennig et al., 1992; Hennig et al., 1993) and *in vivo* (Roy et al., 1992; Alam et al., 1994). In culture experiments, Hennig et al., (1992) showed that Zn deficiency caused partial

Figure 3. The mucosal barrier is made up of epithelial cells. The two routes that molecules can travel through the mucosal barrier is via either; the paracellular pathway ($^{51}\text{Cr-EDTA}$, lactulose), between the epithelial cells (blue arrow) or the transcellular pathway (rhamnose, mannitol), through the epithelial cells (red arrow).



disruption of the endothelial barrier. They also demonstrated that adding Zn to the normal culture medium, prevented the disruption of the endothelial barrier induced by TNF- α (Hennig et al., 1993). In human trials, increased intestinal permeability in children with diarrhoea was significantly reduced by Zn supplementation (Roy et al., 1992; Alam et al., 1994). Roy et al., (1992) demonstrated that supplementation with 5 mg Zn/kg body wt/day for 2 weeks in children suffering acute and persistent diarrhoea, resulted in a reduction in intestinal permeability. Bates et al., (1993) also showed that Zn supplementation improved intestinal permeability in young rural Gambian children thought to be deprived of Zn. In addition, supplementing children with Zn acetate 15 mg Zn/kg/day for a month during bouts of acute shigellosis improved intestinal permeability compared to untreated children, suggesting a resolution of mucosal damage, (Alam et al., 1994). Rodriguez et al., (1996) also showed that high dietary Zn intake (1800 ppm Zn) for 3 weeks had beneficial effects on epithelial barrier function and reduced intestinal permeability in guinea pigs. The efficacy of Zn supplementation in improving intestinal permeability suggests a specific role for Zn in maintaining intestinal structure and function. Further investigations are warranted to determine the potential additive effect of growth factors and Zn on changes in intestinal permeability following induction of mucosal damage.

1.2.5.2 Intestinal permeability test a predictor of gut diseases

The clinical significance of gut barrier dysfunction remains to be delineated, although it seems likely that alterations in gut epithelial permeability play a causative role in a number of conditions. The intestinal barrier function has implications for the aetiology and pathogenesis of various intestinal and systemic diseases and intestinal permeability

may be used in screening for small intestinal disease, assessing the response to treatment and predicting the prognosis (Uil et al., 1997).

1.2.5.3 ⁵¹Cr-EDTA intestinal permeability test

The ratio of excretion of two sugars following different absorptive pathways provides a more sensitive test than measurement of a single molecule. However, Bjarnason et al., (1983) have used ⁵¹Cr-EDTA alone as a radio-labelled probe for testing the permeability of the larger intercellular channels. It can be rapidly and simply quantified in the urine and is claimed to be more sensitive than sugar probes in detecting mucosal damage. ⁵¹Cr-EDTA is a physiologically and biochemically inert substance. Although test results for ⁵¹Cr-EDTA are reliable, the radioactivity of the probe makes it unattractive for repeated use, especially in humans.

1.2.5.4 Dual-sugar intestinal permeability test

The differential intestinal permeability for two sugars, a disaccharide and a monosaccharide, is determined and results are expressed as the disaccharide/monosaccharide ratio in urine. The most frequently used combinations are lactulose/mannitol, lactulose/rhamnose and cellobiose/mannitol.

The advantage of differential sugar absorption tests is that all variable intestinal and extra intestinal factors affect both molecules similarly and are cancelled by expressing the results as a ratio. The test is simple, non-invasive and sensitive for mucosal damage (Uil et al., 1996). However, proper storage of the test solution and the urine specimens is recommended, because of the possible degradation of the sugars.

1.3 SUMMARY

Zn is an essential nutrient for humans, plants, and micro-organisms. The increasing awareness of the biological role of Zn and possible therapeutic effects in various diseases has focused interest on Zn supplementation as a potential adjuvant therapy. Oral Zn therapy has been shown to be beneficial in a variety of diseases and conditions. The usefulness of Zn supplementation in gastrointestinal disease, specifically intestinal mucositis induced by chemotherapy drugs, has received little attention. The availability of Zn for repair of the intestinal lining appears to be modulated by the Zn binding protein, metallothionein, possibly by facilitating dietary Zn absorption and by providing an exchangeable pool of Zn for cell division and repair. In addition, the antioxidant properties of metallothionein could also help protect the small intestine against inflammatory disease (reviews; Hogstarnd and Haux, 1991; Moffat and Denizeau, 1997).

Zn compounds, used in low doses as dietary additives or when used at pharmacological doses may be beneficial in the protection and recovery from gastrointestinal diseases. However, no detailed studies on the characterisation of the regional distribution of Zn along the gut in response to increasing concentrations of Zn in the diet, ranging from marginal to excess have been reported. In addition, the concentrations of metallothionein associated with these intakes of Zn and localisation of Zn and metallothionein in the gut wall requires investigation.

Further investigation is also warranted to determine the benefits of Zn and growth factor supplementation and their roles in epithelial growth and repair after gastrointestinal

damage. In this regard the experimental model of methotrexate-induced small intestinal damage will provide useful insight into the potential therapeutic role of Zn. In addition, the role of metallothionein in the regulation of Zn and its possible protective effect against intestinal inflammatory diseases can be elucidated using metallothionein knock-out mice.

1.4 AIMS OF THE THESIS

This thesis seeks to understand the role and function of Zn and its binding protein, metallothionein in limiting damage, enhancing repair and promoting growth of the intestine. The thesis further describes the levels of Zn and metallothionein in the gut, which may affect the repair process of the gut epithelium. The aim of this thesis is to investigate the effects of Zn supplementation and its use in combination with a bovine whey growth factor on the severity of methotrexate-induced intestinal damage, as assessed histologically and by effects on intestinal permeability. It is hoped that this work will facilitate the development of more effective nutritional and adjunctive therapies for the treatment of intestinal disease.

The specific aims are;

1. To determine the localisation of Zn and metallothionein in the wall of small intestine.
2. (a) To determine the concentration of Zn and metallothionein along the gastrointestinal tract in rats fed diets containing varying amounts of Zn.
(b) To determine the concentration of Zn along the gastrointestinal tract in metallothionein-deficient mice fed diets containing varying amounts of Zn.
3. To characterise the changes in intestinal Zn and metallothionein concentrations following methotrexate-induced intestinal damage in rats.

4. To establish the potential adequacy of oral Zn therapies and their combination with a growth factor extract on intestinal permeability, mucosal repair and protection against chemotherapy-induced mucositis.

CHAPTER 2

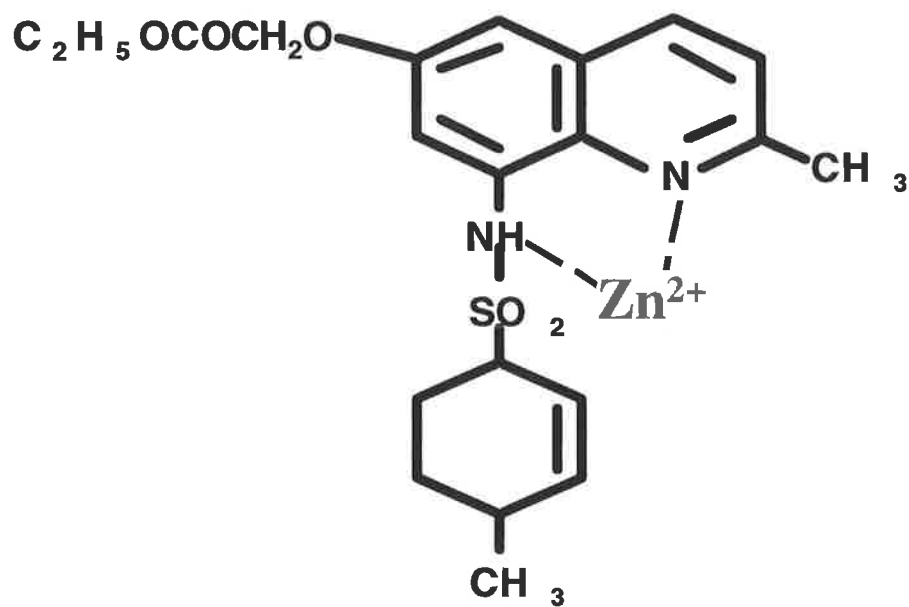
Localisation of Zinc and Metallothionein in the Small Intestine of Rats Fed a Standard Zinc-Containing Diet

INTRODUCTION

The histochemical staining techniques used most frequently for zinc (Zn) detection have been dithizone or sulfide-silver techniques. Dithizone has been used extensively to stain chelatable pools of Zn in various tissues, including the pancreas and intestine. However, the technique is relatively insensitive, not readily quantifiable, and poorly suited for demonstration of the subcellular distribution of Zn. Recently, the measurement and visualisation of pools of intracellular Zn has been made feasible by the introduction of sensitive fluorescence techniques. A new Zn-specific fluorophore has been used to detect labile Zn in cells and tissue sections of brain, heart and others (Frederickson and Danscher, 1990; Zalewski et al., 1993; Zalewski et al., 1994). The fluorophore is called Zinquin or Toluenesulphonamidoquinoline [N-(6-methoxy-8-quinolyl)-p-toluenesulphoamide] (Fig. 1).

Zinquin is a compound that fluoresces specifically on contact with Zn and has been used as a histochemical stain for Zn. In this chapter Zinquin has been used to study the localisation of Zn in metabolically active cells. Zinquin has been used to localise Zn and/or metallothionein-bound Zn in pancreatic islet cells (Zalewski et al., 1994), hepatocytes (Coyle et al., 1994; Brand and Kleineke, 1996) and mouse spermatozoa

Figure 1. Molecular structure of Toluenesulphonamidoquinoline [N-(6-methoxy-8-quinolyl)-p-toluenesulphoamide] or commonly known as Zinquin with a zinc atom attached.



(Zalewski et al., 1996). Others have used Zinquin to localise Zn transporters ZnT-1 in gerbil neurones (Tsuda et al., 1997) and ZnT-2 in vesicles of the BHK cell line (Palmiter et al., 1996).

The localisation of metallothionein is also important as previous evidence has suggested that cytoplasmic metallothionein is more protective against metals and oxidants than nuclear metallothionein (Lazo et al., 1998). Localisation of metallothionein in both the nucleus and cytoplasm has been described in mammalian hepatocytes during development (Panemangalore et al., 1983) and also in certain tumour cells (Lazo et al., 1998). Immunohistochemical localisation of metallothionein in various tissues, including liver, kidney, lung and testis has been reported, however, there is limited information regarding the localisation of Zn and metallothionein within the intestinal epithelium. The localisation of Zn and metallothionein in specific cell types may indicate its possible role and function within the intestine. Thus, the aim of this chapter is to determine the localisation of Zn and metallothionein in the small intestinal epithelium using Zinquin and metallothionein-antibody in rats fed a standard Zn containing (100 mg/kg) diet.

MATERIALS AND METHODS

Animals and diets

Rats

Male Sprague Dawley rats weighing 250 ± 12 g were housed on sawdust in plastic cages in an animal care facility of the Women's and Children's Hospital maintained at 25°C with a 12 hr light/dark cycle and fed commercial pelleted diet (Ridley Agriproducts Pty Ltd, Murray Bridge, SA, Australia).

Diets

During experimental protocols animals were fed a casein-based diet (Howarth et al., 1996) for 7 days supplemented with ZnCl₂ to a standard diet containing 100 mg Zn/kg. The composition of the purified diet is shown in Table 1, diets containing additional Zn were obtained by supplementing the purified diet with an appropriate quantity of ZnCl₂. The protocol followed the Australian Code of Practice for the care and Use of Animals for Scientific purpose and was approved by the Animal Care and Ethics Committee of the Women's and Children's Hospital, Adelaide and the University of Adelaide.

Tissue collections

Animals were weighed, asphyxiated by CO₂ overdose and blood was withdrawn by cardiac puncture. The animals were then killed by cervical dislocation. The liver and gut were excised and the mesentery removed. The gut was separated into stomach, small intestine, caecum and colon. The small intestine was further divided into the duodenum; from the gastro-duodenal junction to the ligament of Treitz, and the remainder divided into three segments of equal length comprising the jejunum, jejunum/ileum and ileum. The large intestine was divided equally into proximal and distal colon. The stomach and caecum were cut open and their contents removed by washing in normal saline, blotted dry and stored at -70⁰C before measurement of Zn and metallothionein. The contents of the small and large intestinal segments were flushed thoroughly with saline and the first 4-6 cm of each segment stored as above. The contents of the small intestinal segments were flushed thoroughly with saline and a 2 cm segment from each region of the small intestine was fixed in methacarn for 2 h, then transferred to 70% alcohol and embedded in paraffin-wax for assessment of metallothionein localisation. An adjacent 2 cm segment of each

Table 1. The composition of the purified casein-based diet (Howarth et al., 1996), which have been used throughout the studies of this thesis.

Basal component (g/kg diet)	Mineral profile (g/kg diet)	Vitamin profile (mg/kg diet)
cornflour starch; 514	KH_2PO_4 ; 17.155	thiamine HCl; 70
casein; 180	CaCO_3 ; 14.645	riboflavin; 30
sucrose; 152	NaCl ; 12.530	niacin (nicotinic acid); 50
wheat bran; 50	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 4.99	pantothenic acid; 150
peanut oil; 50	$\text{FeC}_6\text{H}_5\text{O}_7 \cdot 5\text{H}_2\text{O}$; 0.296	pyridoxal HCl; 15
D,L-methionine; 2.5	CaPO_4 ; 0.170	hydroxycobalamin; 0.02
choline chloride; 1	$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$; 0.080	inositol; 400
cod-liver oil; 4.4	CuSO_4 ; 0.123	ρ -aminobenzoic acid; 50
	KI; 0.00025	folic acid; 10
	$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$; 0.00125	biotin; 0.4
	Na_2SeO_3 ; 0.00005	glucose; 225

region of the small intestine was embedded in Tissue-Tek OCT compound (Sakura Finetek, CA., U.S.A.) and snap frozen in liquid nitrogen and used for Zinquin staining.

Detection of zinc with Zinquin

Frozen sections (8 μm) of the duodenum, jejunum and ileum were labelled with Zinquin for determination of Zn localisation as described by Zalewski et al., (1994) with minor changes to incubation time, temperature and fixation of tissue sections. Briefly, sections were immersed in chilled acetone for 5 min and washed thoroughly with Phosphate Buffered Saline (PBS) then incubated with 50 μM Zinquin (Luminis Pty. Ltd., Adelaide, SA, Australia) for 20 min at room temperature. Slides were washed again with PBS, a coverslip added and Zinquin fluorescence was visualised using the 10 \times objective of a BH-2 fluorescence microscope equipped with a UV B dichroic mirror (Olympus Optical Co. Ltd. Tokyo, Japan) with single excitation and emission spectral peaks at wavelengths of 370 nm and 490 nm, respectively.

For video image analysis, images of the specimen were captured on a colour video camera (TK-1280E, JVC) and fluorescence was quantified using a Video Pro 32 Colour Image Analysis System (Leading Edge Pty Ltd, Adelaide, Australia). Mean fluorescence intensity of individual fields was computed with the Video Pro program by outlining the perimeters of at least 15 well oriented crypts and villi, measuring the average fluorescence intensity of the outlined areas and subtracting background illumination (Zalewski et al., 1994).

Immunohistochemical detection of metallothionein

Paraffin-wax embedded sections of intestine was stained for metallothionein as described by Cherian and Banerjee (1991). Briefly 2 μm sections were treated with 3% H_2O_2 in 100% methanol for 30 min to eliminate endogenous peroxidase. Non-specific binding was then inactivated with 10% normal rabbit serum for 60 min. Sections were then incubated overnight at 4°C with a monoclonal mouse anti-horse antibody to metallothionein (1:1000 in PBS) and negative controls were incubated with PBS. Slides were then incubated with biotinylated rabbit anti-mouse immunoglobulin (1:200) for 60 min and peroxidase-conjugated avidin-biotin complex (1:100) for 30 min. Finally, 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich Pty. Ltd., Castle Hill, Australia) chromogen (1 mg/ml) was incubated with 2% H_2O_2 until a brown colour was detected and then washed and counterstained with Harris haematoxylin (1:8) for 30 sec. Unless stated, all reagents were purchased from Dako A/S Denmark.

Statistical analysis

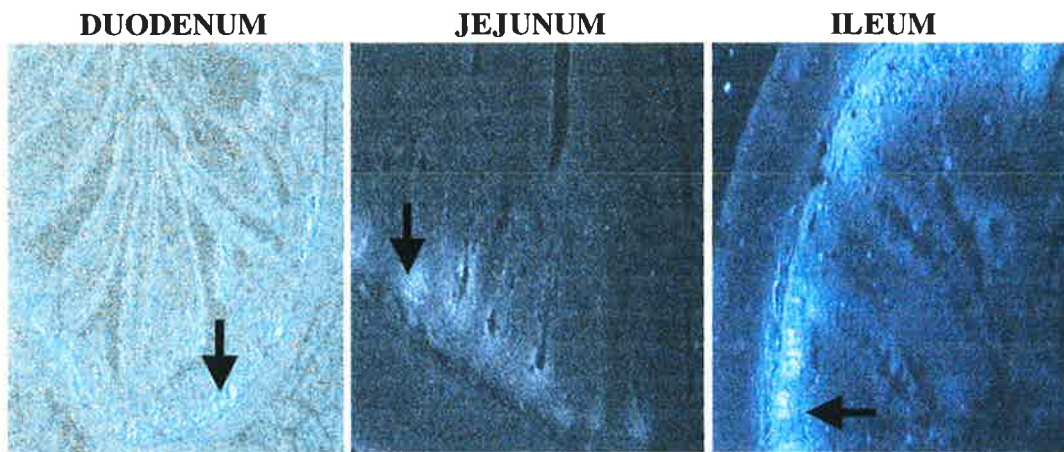
Data are expressed as mean \pm standard error of the mean (SEM). Results were analysed using ANOVA. When interactions were significant, the unpaired Student's t-test with unequal variance was used to determine statistical significance between specific means. Differences were considered significant at $p < 0.05$.

RESULTS

Zinquin fluorescence in the small intestine

Frozen sections (8 μm) of the small intestine of rats fed a standard Zn diet were stained with Zinquin to determine the localisation of Zn in the intestinal epithelium (Fig. 2).

Figure 2. Immunohistochemical localisation of zinc using Zinquin in frozen sections of the small intestine in rats fed a standard (100 mg/kg) zinc diet for 7 d. Zinquin fluorescence was localised to the base of the crypts in all segments of the small bowel, indicated by the arrows, with increasing intensity of fluorescence in the ileum (100X magnification).



Zinquin fluorescence was observed mainly in the base of the crypts in all sections of the small intestine but was most marked in the ileum. A small but scattered Zinquin fluorescence was apparent along the villus particularly in the duodenum. There was no fluorescence associated with the muscularis externa layer. Using a video image analysis system, the greatest intensity fluorescence was visible in the crypt regions of the small intestine, with marked intensity in the ileal crypts (Fig. 3). There was no difference in Zinquin fluorescence intensity within the small intestine either in the upper or lower villus regions (Fig. 3).

Metallothionein localisation in the small intestine

Localisation of immunoreactive metallothionein was determined in paraffin-wax embedded sections of the small intestine sourced from rats fed Zn100 (Fig. 4). Metallothionein staining was primarily detected in the base of the crypts (in the most basal 6-10 crypt cells) along the small intestine with increasing frequency of stained crypts and cells moving distally. No metallothionein staining was detected in either the villus or the muscularis regions. Under high magnification (X1000) staining was evident within the cytoplasm and nucleus of all cells at the base of the crypts including stem and Paneth cells the latter comprising almost half of the basal cells in the ileal crypts.

DISCUSSION

In this chapter, immunofluorescence and immunoperoxidase staining techniques were used to determine the localisation of Zn and metallothionein, respectively, in small intestinal tissue sections of rats fed a standard Zn-containing (100 mg Zn/kg) diet. The results showed that Zn and its metal binding protein, metallothionein were co-localised in

Figure 3. Zinquin fluorescence intensity was quantified using the Video Pro Image Analysis System of crypts and villus 1 (upper villus region) and villus 2 (lower villus region) along the small intestine of rats fed a standard zinc diet (100 mg/kg) for 7 d. * Significantly ($p < 0.05$) different compared to the duodenal and jejunal crypts.

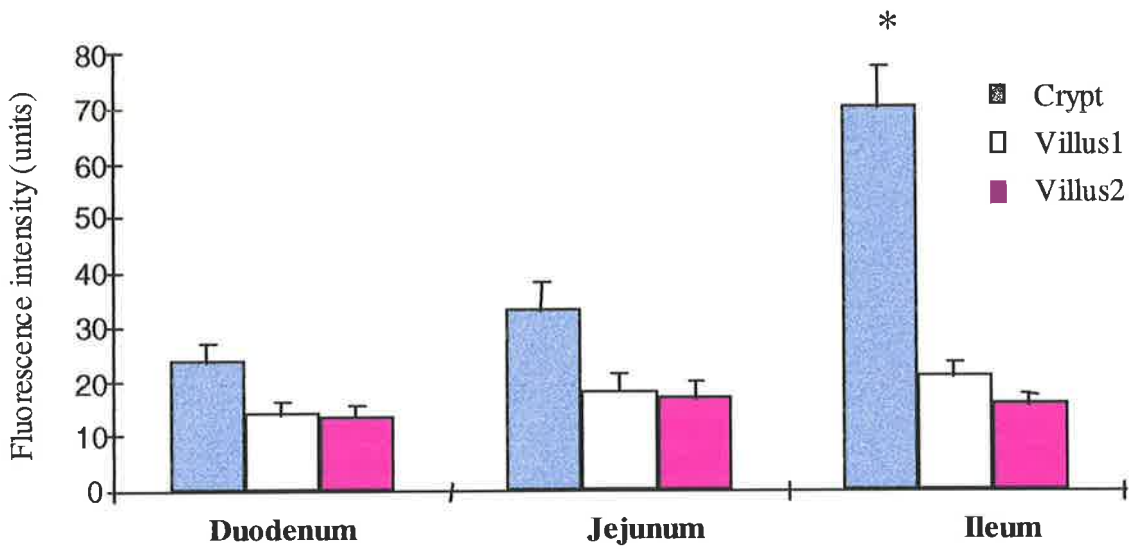
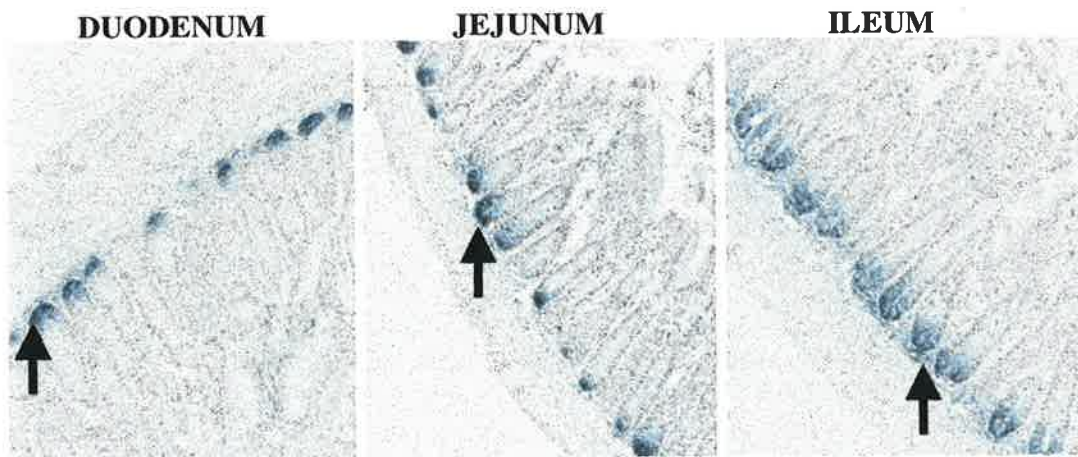


Figure 4. Localisation of metallothionein in methacarn fixed sections of the small intestine in rats fed a standard (100 mg/kg) zinc diet for 7 d. Metallothionein was localised to the base of the crypts in all segments of the small bowel, indicated by the arrows, with increased staining in the distal small intestine (100X magnification).



the basal cells of the small intestinal crypts. Intense co-staining for Zn and metallothionein using Zinquin and an anti-metallothionein antibody, respectively, was detected in the cytoplasm of the basal small intestinal crypt cells. In addition, the nuclei of these cells were stained for metallothionein. The co-staining of Zn and metallothionein to the base of the small intestinal crypts was consistent with the regions of the stem and Paneth cells.

Early studies in rats have shown histochemical localisation of Zn within Paneth cell granules, using both dithizone and sulfide-silver techniques (Elmes, 1976; Brunk et al., 1968). Other studies (Dinsdale, 1984) have demonstrated localisation of Zn in the granules of Paneth cells from the ileum of adult rats by quantitative electron probe X-ray microanalysis. In the present study, we have used Zinquin, the first in a series of Zn-specific intracellular fluorophores, to determine the localisation of Zn in frozen tissue sections with greater sensitivity than conventional methods.

Immunohistochemistry performed on small intestinal sections confirmed high ileal Zn and metallothionein concentrations after feeding dietary Zn in the normal range, with Zinquin fluorescence and metallothionein-antibody staining co-localised to the base of the crypts, predominantly in the ileum. Zinquin fluorescence and metallothionein antibody staining were localised to the base of the crypts consistent with the regions of stem and Paneth cells. This provides strong circumstantial evidence of Zn and metallothionein localisation within stem and Paneth cells, which comprise over more than 50% of the basal 6-10 cells of the crypts, although immuno electron microscopy would be required to confirm the presence of Zn and metallothionein within individual cells.

Coyle et al. (1994) incubated Zinquin with a cytosolic fraction of liver proteins and fluorescence was obtained with free, metallothionein-incorporated and protein bound Zn. This would suggest that a large proportion of the Zinquin fluorescence in the present study may have represented Zn bound to metallothionein or other Zn containing proteins. The chemistry of Zinquin suggests that there is a free orbital for Zn to bind to another ligand other than Zinquin. This means that providing there is no molecular hindrance, Zinquin will bind to all proteins containing Zn. Although there was no visible Zinquin fluorescence in the nucleus, it is probable that Zinquin does not transfer into the nucleus as the negatively charged species formed by cleavage of the parent compound by cytoplasmic esterases could remain confined to the cytoplasm (Zalewski et al., 1994).

Cysteine-rich intestinal protein (CRIP) has a double Zn-finger motif as the defining feature and the protein is abundantly expressed in the intestine. Fernandes et al., (1997) reported that using immunohistochemical and electron microscopy studies revealed particularly high abundance of CRIP in the cytoplasmic granules of Paneth cells of the intestine. The localization of CRIP in Paneth cells and its presence in mononuclear cells suggests that CRIP may be involved in host defense mechanisms and/or tissue differentiation/remodelling processes common to these cell types. In addition, this may explain the Zinquin fluorescence seen in the intestine as a result of binding to CRIP.

Paneth cells also secrete apical granules that contain antimicrobial peptides, including alpha-defensins, termed cryptdins (Ouellette et al., 2000). 17 cryptdin isoforms have been characterised from a cDNA library generated from a single jejunal crypt. Six cryptdin

cDNAs correspond to known peptides, and the remainder predict 11 novel Paneth cell defensins (Ouellette et al., 1994). Of the known Paneth cell alpha-defensins, the cryptdin-4 gene is unique, because it is inactive in the duodenum and expressed at maximal levels in the distal small bowel. This was demonstrated by Ouellette et al., (1999), who showed that with a cryptdin-4-specific antibody, immunohistochemical staining of ileal Paneth cells was strong and specific for cytoplasmic granules. This may further suggest an association with the intense Zinquin fluorescence seen in the ileum, however, no Zn fingers or motifs have yet been identified on the structure of cryptdin.

Paneth cells are found with increasing frequency at the base of the crypts of Lieberkuhn in a proximal to distal gradient in the small intestine of most mammals, (Erlandsen and Chase, 1972). This could explain the increased Zn and metallothionein staining evident in the ileum. Paneth cells are pyramidal-shaped and the apex of these cells, borders the crypt lumen and contains two types of secretory granules. The most numerous granule type is characterised by a homogeneous granular matrix of variable density while the other type contains striated rod-shaped bars surrounded by irregular threads of flocculent material (Sawada et al., 1994). In addition to secretory granules, numerous small vesicles frequently containing clumps of flocculent material were observed in the apical cytoplasm.

The actual role(s) of Paneth cells remains unknown. Metallothionein has been shown to donate Zn to other protein ligands (Jacob et al., 1998) and may have importance in Paneth cells by activating Zn requiring enzymes for anti-bacterial activity. Other studies have found, however, that cadmium (Danielson et al., 1982) and copper (Mullins and

Fuentealba 1998) when given in excess amounts result in both metals being sequestered by metallothionein in Paneth cells, indicating that they may also be involved in secretion of potential toxic metals (Sawada et al., 1994). In addition, a wide variety of functions have been attributed to Paneth cells such as the secretion of digestive enzymes, (Sawada et al., 1993) and the production of a trophic factor related to the rapid turnover rate of the crypt cell population, (Erlandsen and Chase, 1972). Furthermore, Paneth cells have been proposed to regulate intestinal flora, either by phagocytic activity or by secretion of lysozymes-like enzymes and immunoglobulins found in their cytoplasmic granules, (Sawada et al., 1994).

The presence of metallothionein in the Paneth cells of the small intestine at normal dietary zinc intake suggests that metallothionein may be required to protect against ionised zinc or free radicals produced by bacteria in the crypts, or to provide a source of zinc that is exchangeable with other proteins. Immunohistochemical studies of metallothionein have revealed that the protein is induced in various tissues (Danielson et al., 1982; Tsujikawa et al., 1991; Goyer et al., 1992; Suzuki et al., 1992; Douglas-Jones et al., 1995), and found predominantly in the cytoplasm. However others have reported its presence in the nucleus (Cherian and Banerjee, 1991). It has been suggested that metallothionein's role in the nucleus is that it inhibits oxidation of DNA by hydroxyl radicals (Thornalley and Vasak, 1985; Chubatsu and Meneghini, 1993).

Nishimura et al., (1989) found a considerable change in the localisation of metallothionein in the small intestine during development in rats and also suggested a possible involvement of metallothionein in cell proliferation and differentiation, since a marked

increase in metallothionein was observed at the time when active development and differentiation occurred during the perinatal period. The authors also found only weak immunostaining of metallothionein present in goblet cells in older rats compared to strong staining of goblet cells in young rats. Zn in the intestinal mucosa of adult rats has thus been shown to be almost entirely restricted to the granules of Paneth cells, but the importance of these cells in the elimination of Zn and other metals remains to be evaluated.

In conclusion we have demonstrated that Zn and metallothionein are co-localised in the crypt regions consistent with the localisation of stem and Paneth cells. The increased staining evident in the ileum may be a result of increased numbers of Paneth cells and other Zn-binding protein such as CRIP or other possible Zn-containing ligands including cryptdins.

CHAPTER 3

Regional Distribution of Gut Zinc and Metallothionein in Fasted and Non-fasted Rats and When Fed Diets Differing in Zinc Content

INTRODUCTION

Zinc (Zn) has been shown to be protective and enhances epithelial repair in gut diseases (review; Ziegler et al., 1999) and metallothionein has been implicated to be therapeutically beneficial in a variety of disease states (Sturniolo et al., 1999; Jasani and Schmid, 1997; Bremner and Beattie, 1995). The epithelial lining of the gastrointestinal tract is sensitive to damage since it has a high proliferative rate and certain regions of the gastrointestinal tract are more susceptible to damage than others. One explanation may be that different regions of the gut may have varying concentrations of Zn and metallothionein, which predispose certain gut regions to damage.

Conflicting findings have been reported for the effect of fasting on intestinal metallothionein. Studies in rats have shown that liver metallothionein was only slightly elevated 36 h after fasting, whilst intestinal metallothionein was unaltered (Kramer et al., 1993). In another study, mucosal metallothionein was increased by 45% after an overnight fast (Hoadley et al., 1988). Richards and Cousins (1976a) have found that metallothionein increased 10-fold in rats fed either Zn-adequate or Zn-deficient diets immediately prior to a fast. Hence, the effect of fasting on the expression of intestinal metallothionein warrants further investigation.

Little is known about the concentrations and distribution of intestinal Zn and metallothionein along the gastrointestinal tract of rodents consuming a standard Zn diet nor in response to increasing dietary Zn. Only a few studies (Richards and Cousins 1976a; Menard et al., 1981; Flanagan et al., 1983; Olfason et al., 1983) have investigated Zn and/or metallothionein changes in either isolated regions or whole small intestine in response to dietary or injected Zn. Olfason (1983) reported that the highest concentrations of metallothionein were found in the proximal small intestine, diminishing towards the colon of mice, but did not investigate the effect of dietary Zn on the regional distribution of metallothionein.

Thus the aim of this chapter is to determine the changes in concentration of Zn and metallothionein in response to feeding differing levels of dietary Zn, in all regions of the gastrointestinal tract and compared with the effects of fasting.

MATERIALS AND METHODS

Animals and diets

Rats were fed for 7 d with a casein-based diet (see Chapter 2) supplemented with ZnCl₂ to 10 (low; Zn10), 100 (normal; Zn100), 400 (high; Zn400) or 1000 (excess; Zn1000) mg Zn/kg before being sacrificed. Rats fed the Zn10 and Zn100 were either being sacrificed or fasted for an additional 20 h then sacrificed (n=8 per dietary group).

Tissue collections

Gut tissues and liver were collected as previously described (Chapter 2).

Membrane-bound and intracellular zinc

The washed small intestinal segments were blotted dry, opened along the horizontal axis and the mucosal lining of each segment scraped from the serosa with a glass microscope slide. The mucosal scrapings were diluted 1:5 (w/v) with cold homogenate buffer (10 mM Tris-HCl, pH 8.2) and homogenised using a motor-driven Potter/Elvehjem homogeniser. The samples were then centrifuged at $14000 \times g$ for 10 min, the supernatant was removed, weighed and stored at -20°C . The pellet was weighed, dried overnight at 80°C and then digested with concentrated nitric acid for another 24 h. Zn concentrations were determined on the supernatant, to determine cytosolic Zn, and on the pellet to determine Zn bound to membrane and structural proteins by the methods described below.

Total zinc and metallothionein in gut wall segments and liver

Gut wall segments and liver were diluted 1:5 with cold homogenate buffer and homogenised using an Ultra Turrax homogeniser (Janke & Kunkel, Staufen, Germany). A portion of the sample was then boiled and centrifuged at $14000 \times g$ for 3 min. The supernatant was analysed for metallothionein using a ^{109}Cd /haem affinity assay (Eaton and Toal 1982). The remainder of the homogenate was weighed, dried overnight at 80°C and then digested with concentrated nitric acid for another 24 h (Aristar, BDH Laboratory Supplies, Poole, England). Zn in plasma samples, cytosolic preparations and dried gut and liver homogenates subjected to nitric acid digestion was analysed by Atomic Absorption Spectrophotometry using a Perkin-Elmer 3030 (überlingen, Germany).

Statistical analysis

Data were analysed as previously described (Chapter 2).

RESULTS

Gut zinc

Zn distribution in various regions of the gastrointestinal tract was assessed after feeding four diets of differing Zn content (see Table 1). Zn concentrations were 20% higher in the ileum compared to all other gut regions of rats fed low, normal or high Zn diets. Rats fed the Zn100 diet had significantly increased Zn concentrations in the duodenum (10%), ileum (17%), caecum (19%) and the large intestine (30%) compared to rats fed the Zn10 diet. Feeding rats the Zn400 diet caused a significant increase ($p < 0.05$) in Zn in most gut segments above those fed Zn10 and Zn100 diets. In addition, the Zn content of all gut segments was significantly higher in rats fed Zn1000 with average increases of 173%, 136% and 62% above the Zn10, Zn100 and Zn400 diets, respectively (Table 1). Rats fed the Zn10 or Zn100 diet for the 7 d showed no difference in gut Zn compared to the animals fasted for an additional 20 h.

Gut metallothionein

Rats fed the Zn10 and Zn100 diets had 40% higher metallothionein concentrations in the ileum compared to other gut segments (Table 2). However, metallothionein concentrations increased (by 100%) in rats fed the Zn400 diet in most regions of the gut compared to the Zn10 and Zn100 diets and these levels were almost doubled again in the ileum, caecum and large intestine in rats fed the Zn1000 diet.

Deprivation of food caused a significant ($p < 0.001$) increase in gut metallothionein compared to rats fed the Zn10 diet and the Zn100 diet (Table 3). The percentage of gut metallothionein increased for individual regions of rats fed the Zn10 diet and then fasted

Table 1. Zinc (nmol/g wet weight) concentration in segments of the gut wall in rats fed diets of increasing zinc composition.

	Dietary zinc (mg zinc/kg)			
	10	100	400	1000
Stomach	379 ± 18	413 ± 30	530 ± 43 ^{ab}	1226 ± 78 ^{abc}
Duodenum	342 ± 6	377 ± 13 ^a	564 ± 58 ^{ab}	966 ± 48 ^{abc}
Jejunum	397 ± 13	430 ± 17	643 ± 47 ^{ab}	1095 ± 126 ^{abc}
Jejunum/Ileum	410 ± 17	459 ± 35	685 ± 42 ^{ab}	983 ± 91 ^{abc}
Ileum	540 ± 20*	634 ± 29* ^a	879 ± 60* ^{ab}	1098 ± 63 ^{abc}
Caecum	329 ± 8	391 ± 22 ^a	740 ± 50 ^{ab}	1210 ± 115 ^{abc}
Proximal Colon	394 ± 19	486 ± 35 ^a	778 ± 84 ^{ab}	996 ± 104 ^{ab}
Distal Colon	383 ± 21	524 ± 60 ^a	577 ± 42 ^a	922 ± 86 ^{abc}

Data are expressed as mean ± sem (n=8). For each tissue, significance versus the 10, 100 and 400 mg zinc/kg diets is indicated by a, b and c, respectively. * Significantly (p<0.05) different compared with other regions within a dietary group.

Table 2. Gut metallothionein (nmol Cd bound/g wet weight) concentration in rats fed diets of increasing zinc composition.

	Dietary zinc (mg zinc/kg)			
	10	100	400	1000
Stomach	5.4 ± 0.2	4.3 ± 0.2	6.3 ± 0.6 ^b	7.7 ± 0.6 ^{ab}
Duodenum	6.8 ± 0.3	5.5 ± 0.4	18.8 ± 4.5 ^{*ab}	27.7 ± 4.0 ^{*ab}
Jejunum	4.7 ± 0.1	4.6 ± 0.3	9.6 ± 0.8 ^{ab}	13.8 ± 2.1 ^{ab}
Jejunum/Ileum	6.0 ± 0.5	5.3 ± 0.9	12.6 ± 0.9 ^{ab}	19.2 ± 3.3 ^{ab}
Ileum	9.0 ± 0.4 [*]	8.4 ± 0.6 [*]	12.5 ± 1.2 ^{ab}	19.8 ± 2.6 ^{abc}
Caecum	4.4 ± 0.2	3.8 ± 0.3	7.5 ± 0.9 ^{ab}	11.3 ± 1.3 ^{abc}
Proximal Colon	6.0 ± 0.5	6.2 ± 0.7	7.3 ± 0.6	18.5 ± 2.8 ^{abc}
Distal Colon	3.6 ± 0.5	3.7 ± 0.5	6.1 ± 1.0 ^{ab}	16.1 ± 2.6 ^{abc}

Data are expressed as mean ± sem (n=8). For each tissue, significance versus the 10, 100 and 400 mg zinc/kg diets is indicated by a, b and c, respectively. * Significantly (p<0.05) different compared with other regions within a dietary group.

Table 3. Gut metallothionein (nmol Cd bound/g wet weight) concentration in rats fed diets of increasing zinc composition and fasted for an additional 20 h.

	Dietary zinc (mg zinc/kg)			
	Fed		Fasted	
	10	100	10	100
Stomach	5.4 ± 0.2	4.3 ± 0.2	6.9 ± 0.6	6.8 ± 0.4
Duodenum	6.8 ± 0.3	5.5 ± 0.4	12.3 ± 0.9*	14.6 ± 1.6*
Jejunum	4.7 ± 0.1	4.6 ± 0.3	12.9 ± 1.0*	14.8 ± 1.2*
Jejunum/Ileum	6.0 ± 0.5	5.3 ± 0.9	15.1 ± 1.5*	17.0 ± 1.9*
Ileum	9.0 ± 0.4	8.4 ± 0.6	18.6 ± 1.3*	16.7 ± 1.5*
Caecum	4.4 ± 0.2	3.8 ± 0.3	10.1 ± 1.3*	9.8 ± 1.2*
Proximal Colon	6.0 ± 0.5	6.2 ± 0.7	11.1 ± 2.3*	12.9 ± 1.3*
Distal Colon	3.6 ± 0.5	3.7 ± 0.5	9.9 ± 0.6*	8.4 ± 1.1*

Data are expressed as mean ± sem (n=8). For each tissue, * Significantly (p<0.05) different compared to feeding the same diet.

are; duodenum (1.8-fold), jejunum (2.7-fold), jejunum/ileum (2.5-fold), ileum (2.1-fold), caecum (2.3-fold), proximal colon (1.9-fold) and distal colon (2.7-fold) compared to fed animals. Whereas rats fed the Zn100 diet and then fasted, the individual regional increases are: duodenum (2.7-fold), jejunum (3.2-fold), jejunum/ileum (3.2-fold), ileum (2.1-fold), caecum (2.5-fold), proximal colon (2.1-fold) and distal colon (2.3-fold) compared to fed animals. However, the stomach was unaffected by fasting after consuming either diet.

Membrane-bound and intracellular zinc

The total concentration of Zn in the membrane-bound fraction was 72% higher in rats fed the Zn1000 diet than those fed the Zn100 diet (Table 4). However, approximately 94% of the total Zn in mucosal scrapings was associated with the membrane bound fraction in rats regardless of dietary Zn, indicating that the membrane-bound Zn increased in proportion to the concentration of Zn in the diet. In addition, for a given diet, Zn concentrations were unchanged in the membrane-bound fraction in the segments along the small intestine. Differences were observed in the Zn concentrations of the cytosolic fraction, with values in the duodenum higher than those in the other segments for both diets. Rats fed the Zn1000 diet generally had double the cytosolic Zn levels than those fed the Zn100 diet (Table 4).

Hepatic zinc and metallothionein

Liver Zn and metallothionein concentrations were unaffected by dietary Zn (Table 5). Plasma Zn increased in proportion with dietary Zn [$y = 0.0074x + 14.576$; $r = 0.961$, where y = plasma Zn ($\mu\text{mol/L}$) and x = dietary Zn (mg/kg)]. Liver Zn was

Table 4. Membrane-bound and cytosolic zinc (nmol/g wet weight) concentrations of small intestinal mucosal scraping of rats fed 100 or 1000 mg zinc/kg diets.

	Dietary zinc (mg zinc/kg)			
	100		1000	
	Membrane-bound	Cytosolic	Membrane-bound	Cytosolic
Duodenum	697 ± 47	86 ± 5*	1121 ± 184 ^a	181 ± 29* ^a
Jejunum	580 ± 44	32 ± 2	1104 ± 237 ^a	39 ± 11
Jejunum/Ileum	622 ± 57	19 ± 2	1106 ± 155 ^a	36 ± 4 ^a
Ileum	755 ± 25	41 ± 3	1199 ± 192 ^a	93 ± 18 ^a

Data are expressed as mean ± sem (n=4). ^a Significantly (p<0.05) different from 100 mg zinc/kg diet counterpart. * Significantly (p<0.05) different from other gut segments within each dietary group.

Table 5. Liver zinc (nmol/g wet weight), metallothionein (nmol Cd bound/g wet weight) and plasma zinc ($\mu\text{mol/L}$) concentrations in rats fed diets of increasing zinc composition.

	Dietary zinc (mg zinc/kg)			
	10	100	400	1000
Liver zinc	287 \pm 14	295 \pm 15	310 \pm 5	353 \pm 32
Liver metallothionein	7.1 \pm 1.3	5.1 \pm 0.8	7.0 \pm 0.8	8.8 \pm 1.0
Plasma zinc	13.5 \pm 1.0	16.5 \pm 0.6 ^a	-	21.9 \pm 0.6 ^b

Data are expressed as mean \pm sem (n= 4-8). ^a Significantly (p<0.05) different from the 10mg zinc/kg diet. ^b Significantly (p<0.05) different from the 10 and 100 mg zinc/kg diet.

significantly increased by 50% in fasted animals prior to feeding either the Zn10 or the Zn100 diets compared to fed rats. Fasting, prior to feeding the Zn10 and Zn100 diets showed a 4.7 fold and 7.7 fold induction, respectively, in liver metallothionein compared with feeding and no difference between the two fasted groups (Table 6). The magnitude of metallothionein induction due to fasting was more prominent than that in the gut. Plasma Zn did not alter irrespective of feeding diets, between feeding and fasting or starvation alone (data not shown).

DISCUSSION

Dietary Zn supplementation has been shown to be beneficial in protection and recovery from several diarrhoeal and inflammatory intestinal diseases. Local Zn levels, either intracellular or attached to the mucosal cell surface, presumably reduce the susceptibility to damage, and improve the rate of mucosal repair and restitution. Metallothionein is a marker of Zn status within the mucosal cell and is induced by excessive amounts of dietary Zn. Metallothionein has been shown to donate Zn to other proteins (Jacob et al., 1998) and as such may provide an intracellular source of Zn for tissue growth and repair. There have been few reports in which the distributions of Zn and metallothionein in the gut have been determined in relation to increasing dietary Zn. In experiments in which rats were fed a normal chow diet, the distribution of intestinal metallothionein was found to be highest in the duodenum and decreased towards the colon (Olfason 1983).

The differences in gut metallothionein between rats fed the Zn100, Zn400 and Zn1000 diets were very small in comparison to the corresponding gut Zn increases, one to two orders of magnitude less than the differences in distal gut Zn concentrations between rats

Table 6. Liver zinc (nmol/g wet weight) and metallothionein (nmol Cd bound/g wet weight) and concentrations in rats fed diets of increasing zinc composition and fasted for additional 20 h.

	Dietary zinc (mg zinc/kg)			
	Fed		Fasted	
	10	100	10	100
Liver zinc	287 ± 14	295 ± 15	438 ± 42*	429 ± 23*
Liver metallothionein	7.1 ± 1.3	5.1 ± 0.8	40.8 ± 6.8*	45.1 ± 6.8*
Plasma zinc	13.5 ± 0.9	15.3 ± 0.3	16.1 ± 0.5	15.2 ± 0.6

Data are expressed as mean ± sem (n= 4-8). * Significantly (p<0.05) different compared to their fed counterpart.

fed similar diets, far too low to account for the observed differences in Zn predicted by simple stoichiometric binding of Zn by metallothionein (7 Zn : 1 metallothionein). Elevated Zn concentrations in the caecum and colon could be expected because of progressive dehydration of the contents and much reduced flow of luminal contents. The action of metallothionein appears to be governed more by its dynamic state than by static concentrations, presumably by increased metallothionein turnover and transfer of Zn to other mucosal protein ligands. The situation in the gut contrasts with that in the liver, where incorporation into metallothionein is responsible for virtually all of the hepatic Zn uptake in the rat (Coyle et al., 1995; Philcox et al., 1995).

This chapter has revealed that regional levels of Zn in the gut were similar when rats were fed 10-100 mg Zn/kg diet. Feeding 400 or 1000 mg Zn/kg diet, however, resulted in markedly higher levels of Zn associated with all regions of the gut. In addition, determined the ratio of Zn bound to membranes and structural proteins, compared with soluble Zn ligands located in the cytosol, in mucosal scrapings derived from rats fed diets of 100 and 1000 mg Zn/kg. The Zn concentrations in the mucosal scrapings were higher than their counterparts in the total gut wall indicating that the non-mucosal component contributes less to the total Zn concentration than the mucosa. The non-mucosal Zn component of the total Zn was much smaller in rats fed the 1000 mg Zn/kg diet compared to the 100 mg Zn/kg diet indicating that rats consuming the 1000 mg Zn/kg diet, had a much larger proportion of Zn bound to the mucosal fraction. Membrane-bound Zn increased by 71% and the cytosolic fraction by 96% in rats fed the 1000 mg Zn/kg diet compared with those fed the 100 mg Zn/kg diet. The greatest proportion (94%) of Zn in the mucosal scrapings was associated with the membrane-bound fraction regardless of the

dietary Zn level. There was no significant difference in Zn concentrations in mucosal scrapings of specific small intestinal regions within each diet. However, it should be emphasized that the ileum in fact possesses 26% more Zn than the jejunum and jejunum/ileum segments.

This trend agrees with the results for the total gut wall Zn concentrations. The lack of statistical significance of Zn concentrations seen in mucosal scrapings resides in the greater variability and smaller *n* values. Although we can not exclude changes in Zn content in the muscularis layer along the gut, this would seem unlikely from other studies (Rojas et al., 1995). Possibly Zn in the membrane-bound fraction is associated with the glycocalyx, a mucous layer of polysaccharides covering the intestinal mucosa lining, or with the brush border membrane proteins and a minor proportion associated with membranes of cellular organelles.

The regional distribution of intestinal metallothionein was similar in rats fed the 10 and 100 mg Zn/kg diets but increased markedly in nearly all gut segments in rats fed the 100 and 1000 mg Zn/kg diets. The concentration of metallothionein was too low to account for the differences in cell associated Zn as predicted by simple stoichiometric binding of Zn by metallothionein. As the greatest proportion of the Zn was membrane-bound, the cytosolic Zn represented only a small proportion (approximately 6%) of the cell-associated Zn that was available to induce metallothionein. Thus, metallothionein synthesis in the mucosal cell appears to be a marker of the fraction of cell-associated Zn, which becomes internalised at greater than normal dietary Zn concentrations.

Although plasma Zn increased in direct proportion to dietary Zn concentration, indicating that more Zn is absorbed at the higher dietary intakes, this rise was relatively small, and had little effect on hepatic Zn or metallothionein levels. In studies in which ⁶⁵Zn was mixed in a meal containing 100 mg Zn/kg, only 3% of the administered dose was absorbed after 4 h, 2% was found in the stomach, 14% in the small intestine and 81% in the caecum and colon. However, when mice were gavaged with ⁶⁵Zn in solution, up to 17% of the administered dose was absorbed (Coyle et al., 1999).

The changes in Zn and metallothionein levels in response to an overnight fast prior to feeding a low to normal Zn diet in both the liver and gut yielded different responses. Fasting did not alter gut Zn concentrations irrespective of prior feeding of the Zn10 or Zn100 diets. Similar findings were observed by Quarterman and Morrison (1981) where they showed that fasting did not affect duodenal Zn concentrations. Furthermore, short-term starvation did not change plasma Zn levels, this finding is consistent with other experiments in the rat (Sato et al., 1984; Hidalgo et al., 1990). However, this differs from previous results for turkey poults (Richards et al., 1987) and humans (Spencer et al., 1985), which showed a decline or an increase in serum Zn. Differing results between these studies are likely to be due to effects of species, age, prior Zn status and circadian variation (Richards et al., 1987). Spencer et al., (1985) reported that in starvation, serum Zn concentrations are supplemented from muscle proteins, which are catabolized to provide gluconeogenic substrates and the released Zn keeps serum Zn unchanged or augmented and excess Zn is excreted in the urine.

The induction of gut metallothionein after fasting has not been documented in detail. Our study showed that fasted rats fed Zn10 and Zn100 showed a 1.3- and 1.7-fold increase respectively, in agreement with data of Bremner and Davies (1975) and Hoadley et al. (1988). However in the liver, fasting showed a marked increase in hepatic Zn (47.5%) and metallothionein (6.2-fold) compared to feeding both Zn diets. This was supported by Richards and Cousins (1976a) where a 10-fold induction of metallothionein was seen after fasting and Bremner and Davies (1975) also showed an increase in liver Zn associated with food restriction.

The findings in this chapter indicate that metallothionein acts as a marker of excess Zn in the cell. Interestingly, metallothionein levels were induced by dietary Zn to similar levels in all regions of the gut from stomach through to colon implicating a common Zn signal in the induction of metallothionein along the gut. This signal may be elicited via plasma Zn since luminal Zn would be expected to concentrate distally and cause higher metallothionein concentrations in the colon.

In conclusion, these findings indicate that as rats are fed increasing amounts of Zn, an increasing proportion of Zn attaches to the luminal surface of the gut epithelium, from stomach to colon. When dietary Zn becomes high or excessive (greater than >100 mg Zn/kg) however, more Zn is internalised, more is absorbed, and more accumulates within the cytosol as it becomes sequestered by newly synthesised metallothionein. Membrane-bound Zn, and the fraction loosely attached to metallothionein in the cytosol, may be important sources of Zn for defence and repair of the gut epithelium. Zn which is loosely attached to the outside surface of the mucosal cell may be important for immune defence

at the mucosal cell surface, integrity of the glycocalyx and for the function of Zn containing enzymes in the gut lumen, whereas that bound to metallothionein may provide Zn for mucosal cell function, differentiation, growth and repair. The characterisation of regional concentrations of both Zn and metallothionein along the gut and the induction of metallothionein will provide useful information to indicate the potential adequacy of oral Zn therapies particularly those used to improve mucosal repair in specific gut related diseases.

CHAPTER 4

Distribution of Gut Zinc and Metallothionein in Normal and Metallothionein Knock-out Mice After Fasting or Feeding Diets of Varying Zinc Content

INTRODUCTION

Defining the exact role of metallothionein has proven difficult, one potentially powerful approach to this problem is to create mutants that under-express or over-produce metallothionein. Developments in gene transfer technology have made this possible. The generation of mice lacking expression of metallothionein (Michalska and Choo, 1993; Masters et al., 1994) has enabled more rigorous investigation of the role metallothionein plays in protection against heavy metal toxicity (Yanagiya et al., 1999; Yoshida et al., 1999), oxidative damage (Rojas and Klaassen, 1999) and anticancer drug-induced damage (Liu et al., 1998; Kondo et al., 1995). In addition, metallothionein has been demonstrated to be essential for wound repair (Penkowa et al., 1999) and involved in the proliferative processes (Hanada et al., 1998).

The effect of zinc (Zn) deficiency on cell division has been reported to be most obvious in rapidly proliferating tissues (reviewed; Prasad, 1995). Southon and co-workers (1985) demonstrated that Zn deficiency in the rat was associated with a reduction in the crypt to villus ratio, and a lower rate of crypt cell division in the jejunum, as well as the colonocytes in the large bowel of rats (Lawson et al., 1988). This resulted in a substantial decrease in the net influx of new cells into the villi of the Zn-deficient animals compared

with controls. In addition to the reduction of intestinal enzyme activities, protein synthesis and somatic growth are also inhibited (Prasad et al., 1978; Gebhard et al., 1983). These effects could contribute to reduce cellular protection of the gastrointestinal tract. The effects of Zn on the gastrointestinal tract and its role in disorders of the gut have been limited.

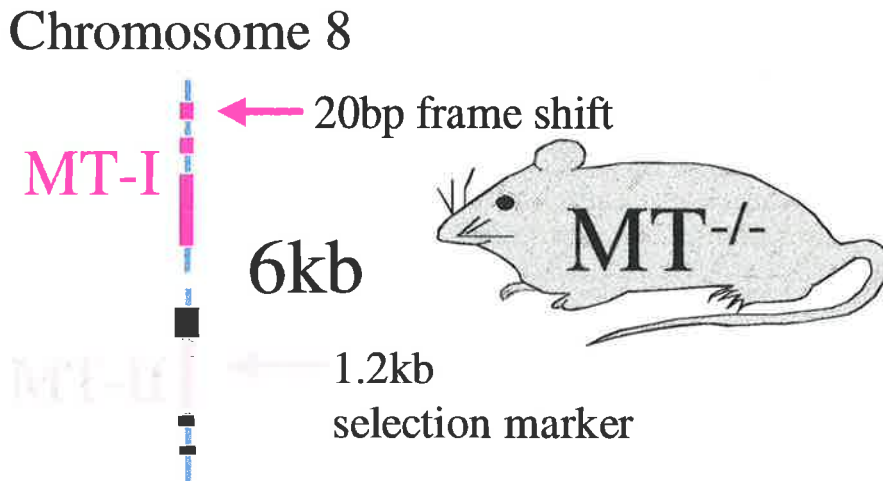
Little is known about whether local endogenous intestinal Zn and metallothionein levels may be sufficient in protecting and/or preventing the gut mucosa from damage or that elevated levels are required. However, in order to elucidate a protective role for Zn and metallothionein in gut related disease, it is necessary to characterise the endogenous levels of intestinal metallothionein and Zn. This chapter describes the Zn and metallothionein levels and their distribution along the gastrointestinal tract in both normal and metallothionein-null mice in response to feeding a low, normal and high Zn concentration in their diets and whether fasting influences the expression or distribution of Zn and metallothionein in the gut.

MATERIALS AND METHODS

Animals and diets

Control (MT^{+/+}) and metallothionein-null (MT^{-/-}) mice were F3 derivatives of the interbreeding of normal C57Bl/6 mice (Animal Resources Centre, Canning Vale, Western Australia) and MT^{-/-} (mixed genetic background of OLA129 and C57BL6 strains) mice (Michalska and Choo 1993) (Fig 1). Female mice were housed on sawdust in plastic cages in an animal care facility of the Institute of Medical and Veterinary Science maintained at 22°C with a 14 h light/10 h dark cycle and fed commercial pelleted diet.

Figure 1. Metallothionein-null mice were produced by Michalska and Choo (1993). This was achieved via incorporating a 20bp frame shift at the metallothionein-I gene resulting in the gene being able to be transcribed to mRNA but is not translated. Furthermore, a 1.2kb selection marker was placed at the metallothionein-II gene and this gene cannot be transcribed to mRNA thereby making them unable to express the metallothionein-I and II genes.



Female MT^{+/+} and MT^{-/-} mice (n=8) on reaching 26-28 g were fed a purified diet (see Chapter 2) supplemented with ZnCl₂ to 10 (low, Zn10), 150 (normal, Zn150) or 400 (high, Zn400) mg Zn/kg for 7 d before either being sacrificed or fasted for an additional 20 h and then sacrificed (n=4 per dietary group).

Tissue collection

Gut tissues and liver were collected similarly to the rat studies as previously described in Chapter 2.

Glucagon administration

In another study, 12 female C57Bl/6 mice fed a commercial diet (Ridley Agriproducts Pty Ltd, Murray Bridge, SA, Australia) were separated into two equal groups and given intraperitoneal injections of physiological saline or glucagon (LgucaGen Hypokit, Novo Nordisk Pharmaceuticals Pty Ltd, North Rocks, NSW, Australia). Three 20 µl injections of glucagon (2 mg/kg) or saline were given at 90 min intervals and mice were killed 9 h after the first injection. The stomach, three equal lengths of the small intestine, caecum, colon, and the liver were analysed for metallothionein concentrations.

Metallothionein and zinc analysis

Zn and metallothionein concentrations were analysed similar to the rat studies as previously described in Chapter 3.

Statistical analysis

All data presented in the tables in the results section were analysed by repeated measures of ANOVA using S-PLUS language, (Becker et al. 1988, Venables and Ripley 1994). The results presented are from analysis of the raw data, corresponding analyses on the log scale produced essentially the same results. Treatments differ significantly at $p < 0.05$ if their difference exceeds the appropriate “least significant difference” (LSD), generally given here by twice the standard error of a difference.

RESULTS

Gut zinc

Zn concentrations in $MT^{+/+}$ fed Zn10 diet were similar in each segment of the gastrointestinal tract sampled (Table 1). $MT^{-/-}$ mice had slightly higher values in all regions, significantly higher (45%) in the jejunum/ileum. Compared to the Zn10 diet, $MT^{+/+}$ mice fed a Zn150 diet had increased Zn concentrations in the jejunum, jejunum/ileum, ileum and proximal colon. The Zn concentrations in the $MT^{-/-}$ mice were unchanged with this diet. In $MT^{+/+}$ mice Zn concentrations were further increased by 58%, 102% and 94% in the distal half of the small intestine, caecum and colon, respectively, by feeding the Zn400 diet. The $MT^{-/-}$ mice had much lower increments of 40%, 59% and 12% in the corresponding gut segments. In general, in all mice fasted for 20 h, irrespective of the Zn content of the previous diet, gut Zn concentrations were similar to those fed the Zn10 diet.

Table 1. Zinc concentrations (nmol/g wet weight) in segments of the gut wall of MT^{+/+} (normal) and MT^{-/-} (metallothionein-null) mice fed diets of different zinc content. MT^{+/+} and MT^{-/-} mice (n=8/group) were fed diets containing 10, 150 or 400 mg zinc/kg diet for 7 d before either being sacrificed or fasted for a further 20 h.

Tissue	MT genotype	Fed			Fasted		
		10	150	400	10	150	400
Stomach	+/+	428	439	426	441	400	449
	-/-	539	390	511	469	377	385
Duodenum	+/+	422	322	479	408	466	477
	-/-	509	390	487	444	370	408
Jejunum	+/+	423	645 ^a	654 ^a	432	591 ^a	467
	-/-	507	509	624	484	408 ^c	480
Jejunum/Ileum	+/+	394	684 ^a	1048 ^{ab}	416	505	523
	-/-	572 ^c	522 ^c	601 ^c	451	369	478
Ileum	+/+	430	595 ^a	973 ^{ab}	474	468	463
	-/-	529	470	785 ^{abc}	403	357	422
Caecum	+/+	453	543	1095 ^{ab}	434	386	460
	-/-	494	499	795 ^{abc}	432	356	421
Proximal Colon	+/+	433	598 ^a	765 ^{ab}	461	431	633 ^a
	-/-	519	635	797 ^a	498	399	482
Distal Colon	+/+	581	669	1691 ^{ab}	574	430	548
	-/-	599	636	632 ^c	580	360 ^a	504

within mice set: LSD = 154, between mice sets: LSD = 163

Shown are the means (n=4) with the least significant difference (LSD). There are two values for the LSD between treatment means, according to whether the treatment means concerned correspond to the same set of mice (same genotype, down column) or different sets of mice (across columns). When the difference between two means is greater than the appropriate LSD the results are statistically significant at p<0.05. ^a Statistically significant from fed 10 mg zinc/kg diet. ^b Statistically significant from the fed 10 and 150 mg zinc/kg diets. ^c Statistically significant from MT^{+/+} counterpart.

Liver zinc and metallothionein

Dietary Zn did not significantly affect liver Zn in MT^{+/+} and MT^{-/-} mice (Table 2). All mice fed the Zn400 diet and then fasted had significantly higher Zn levels compared with their counterparts fed the Zn10 diets. This appeared to result more from reduction in the liver Zn levels after the Zn10 diet. Metallothionein concentrations in the liver of MT^{-/-} mice were less than 3 nmol Cd bound/g wet weight and were not affected by the content of Zn in the diet or fasting. Metallothionein concentrations were similar in fed MT^{+/+} mice irrespective of the dietary Zn content. Fasting resulted in much higher metallothionein concentrations than in their fed counterparts with the greatest differences (3-fold) in the group previously fed the Zn10 diet.

Plasma zinc

Dietary Zn did not influence plasma Zn in either fed group (Table 3) and was similar to those in MT^{+/+} mice fasted for 20 h. In contrast, fasted MT^{-/-} mice fed the Zn400 diet had significantly higher (40%) plasma Zn concentrations than their fasted MT^{+/+} counterparts.

Gut metallothionein

Metallothionein concentrations in the gut of MT^{-/-} mice were less than 2 nmol Cd bound/g wet weight and were neither affected by the content of Zn in the diet nor fasting. In MT^{+/+} mice (Table 4), there was a trend from higher to lower metallothionein values from stomach to colon. The Zn400 diet elicited a metallothionein response in the small intestine, reaching significance in the duodenum. Fasting resulted in metallothionein concentrations one- to two-fold higher than those of Zn10 fed mice. In contrast to the fed study, dietary Zn content had no influence on metallothionein. In a further experiment

Table 2. Liver zinc concentration (nmol/g wet weight) in MT^{+/+} (normal) and MT^{-/-} (metallothionein-null) mice fed diets of different zinc content. MT^{+/+} and MT^{-/-} mice (n=8/group) were fed diets containing 10, 150 or 400 mg zinc/kg diet for 7 d before either being sacrificed or fasted for a further 20 h.

Dietary zinc (mg/kg)	Zinc				Metallothionein	
	Fed		Fasted		Fed	Fasted
	MT ^{+/+}	MT ^{-/-}	MT ^{+/+}	MT ^{-/-}	MT ^{+/+}	MT ^{+/+}
10	386	422	343	372	8.1	33.6 ^b
150	440	467	418	394	7.7	27.1 ^a
400	457	431	430 ^a	463 ^a	9.4	21.6 ^{ab}

Zinc: LSD = 80.4 Metallothionein: LSD = 7.4

Shown are the means (n=4) with the least significant differences (LSD) for liver zinc and metallothionein. When the difference between two means is greater than the appropriate LSD the results are statistically significant at p<0.05. All MT^{-/-} mice had no metallothionein concentrations. ^a Statistically significant from mice fed the 10 mg zinc/kg diet. ^b Statistically significant from the fed mice.

Table 3. Plasma zinc ($\mu\text{mol/L}$) of $\text{MT}^{+/+}$ (normal) and $\text{MT}^{-/-}$ (metallothionein-null) mice fed diets of increasing zinc content. $\text{MT}^{+/+}$ and $\text{MT}^{-/-}$ mice ($n=8/\text{group}$) were fed diets containing 10, 150 or 400 mg zinc/kg diet for 7 d before either being sacrificed or fasted for a further 20 h.

Zinc (mg/kg)	$\text{MT}^{+/+}$		$\text{MT}^{-/-}$	
	Fed	Fasted	Fed	Fasted
10	9.0	9.0	9.2	9.7
150	9.2	9.0	9.0	10.2
400	8.7	8.6	10.1	12.0 ^a
				LSD = 1.7

Shown are the means ($n=4$) with the least significant difference (LSD). When the difference between two means is greater than the LSD the results are statistically significant at $p < 0.05$. ^a Statistically significant from all other groups.

Table 4. Gut metallothionein (nmol/g wet weight) of MT^{+/+} (normal) mice fed diets of different zinc content. MT^{+/+} mice (n=8) were fed diets containing 10, 150 or 400 mg zinc/kg diet for 7 d before either being sacrificed or fasted for a further 20 h.

Tissue	Fed			Fasted		
	10	150	400	10	150	400
Stomach	12.9	14.8	15.3	25.4 ^{ab}	24.2 ^{ab}	19.3 ^b
Duodenum	9.2	7.1	16.2 ^a	21.8 ^{ab}	27.1 ^{ab}	18.8
Jejunum	7.3	7.5	11.7	17.6 ^{ab}	21.1 ^{ab}	16.9 ^{ab}
Jejunum/Ileum	6.4	7.1	9.8	16.8 ^{ab}	24.1 ^{ab}	16.2 ^{ab}
Ileum	6.7	6.2	8.0	15.3 ^{ab}	15.8 ^{ab}	13.0 ^{ab}
Caecum	5.3	4.8	5.7	10.3 ^{ab}	9.0	6.7
Proximal Colon	5.3	6.3	7.2	10.6 ^{ab}	12.1 ^{ab}	9.3
Distal Colon	4.6	4.7	6.7	10.9 ^{ab}	9.3	8.5

within mice set: LSD = 4.3, between mice sets: LSD = 4.8

Shown are the means (n=4) with the least significant difference (LSD). There are two values for the LSD between treatment means, according to whether the treatment means concerned correspond to the same set of mice (down columns) or between sets of mice (across columns). When the difference between two means is greater than the appropriate LSD the results are statistically significant at p<0.05. ^a Statistically significant from fed 10 and 150 mg zinc/kg groups. ^b Statistically significant from the all fed groups of the same tissue. ^c Statistically significant from fed group of the same tissue and dietary zinc counterpart.

with MT^{+/+} mice, metallothionein concentrations were detected in the mucosal scraping of the small intestine with 4.5 ± 0.7 and 12.0 ± 1.2 nmol Cd bound/g wet weight after 7 d feeding of Zn10 or Zn400 diets, respectively.

Metallothionein response to glucagon

Metallothionein accumulation averaged over the small intestine was 50% greater than controls in glucagon-treated mice (11.0 ± 1.5 for glucagon vs 7.2 ± 0.5 nmol Cd bound/g wet intestine for controls). This increase was significant over the entire intestine (Table 5). Liver metallothionein was 73% greater with glucagon (31.3 ± 4.2 nmol Cd bound/g wet liver) compared with saline-injected controls (18.1 ± 1.2 nmol Cd bound/g wet liver) and the latter were markedly higher than those obtained in the livers of mice (7.7 ± 0.9 nmol Cd bound/g wet liver) that had not undergone the stress of injection (Fig. 1).

DISCUSSION

The concentrations of metallothionein in both the liver and gastrointestinal tract of mammals have been well reported (Richards and Cousins 1976; 1976a; Menard et al. 1981; Olafson 1983). Many agents including, heavy metals, hormones, cytokines and a variety of other stimuli have been shown to induce metallothionein synthesis in the liver, however, the control of metallothionein synthesis in the intestine is poorly understood. The purpose of this chapter was to assess the effect of feeding various levels of dietary Zn on intestinal and hepatic Zn, in addition, determine the influence of fasting on the expression of these levels in normal and metallothionein-null mice.

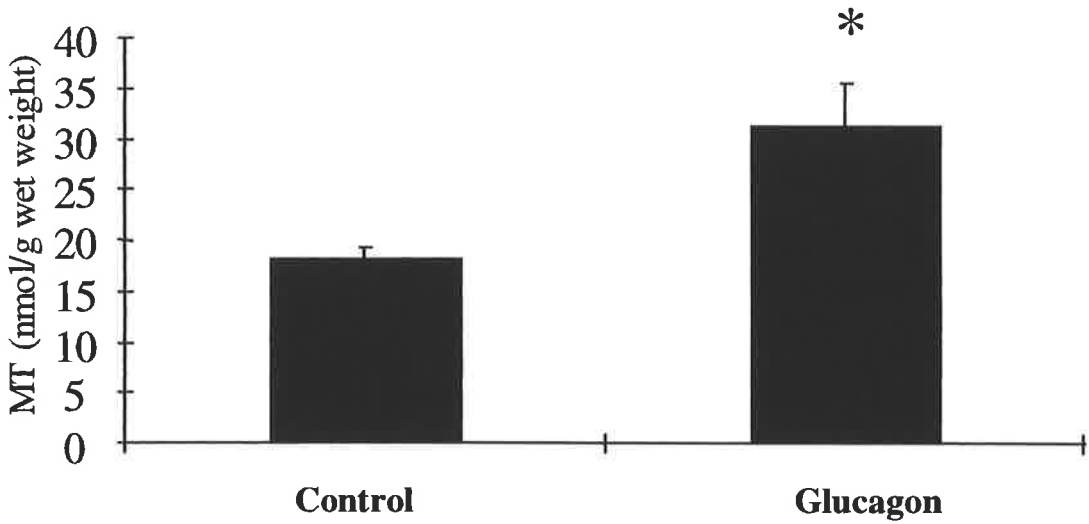
Table 5. Gut metallothionein (nmol Cd bound/g wet weight) of control and glucagon treated mice.

Tissue	Control	Glucagon
Stomach	12.8	17.1*
Duodenum/Jejunum	9.9	14.7*
Jejunum	6.4	9.9*
Jejunum/Ileum	6.2	9.4*
Caecum	5.7	9.8*
Colon	6.9	10.9*

within mice set: LSD = 2.4, between mice sets: LSD = 3.2

Shown are the means (n=6) with the least significant difference (LSD). There are two values for the LSD between treatment means, according to whether the treatment means concerned correspond to the same set of mice (down columns) or between sets of mice (across columns). When the difference between two means is greater than the appropriate LSD the results are significantly different at $p < 0.05$. * Statistically significant compared to control tissues.

Figure 1. Hepatic metallothionein concentrations of mice treated with glucagon and control. Results are expressed as nmol Cd bound/g wet weight and data are expressed as mean \pm SEM (n=6). * Significantly (p<0.05) different compared to control.



Dietary Zn induction of intestinal metallothionein has been demonstrated in rodents made Zn deficient and then subsequently replenished with Zn (Cousins, 1985; Olfason, 1983; Richards and Cousins, 1976a; Menard et al., 1981). Olafson, (1983) showed a 25% increase in intestinal metallothionein in Zn-deficient mice two weeks after supplementation with 300 mg Zn/kg. Four-fold inductions have been achieved in rats (Menard et al., 1981), such studies however, compare the increase in metallothionein following supplemental Zn with the trace levels of metallothionein caused by Zn depletion. There have been few reports of intestinal metallothionein induction at normal or excess Zn levels. In experiments with rats fed diets containing 60 or 350 mg Zn/kg, intestinal metallothionein was found to be increased by 44% after 3 weeks on the high Zn-containing diet (Reeves and Rossow, 1996).

Results from this chapter indicate that dietary Zn has no effect on metallothionein accumulation in the stomach, caecum, colon or the small intestine except the duodenum of mice when fed diets containing 10-400 mg Zn/kg for 7 d, whereas Zn levels increased only in the distal small intestine, caecum and colon. However, studies in the rat (Chapter 3) demonstrated that dietary Zn induced gut metallothionein in all regions of the gastrointestinal tract when fed dietary Zn level of 400 mg Zn/kg or above this level. This was consistent with Menard and co-workers (1981), who found that in rats, intestinal metallothionein synthesis was stimulated 3-fold above control levels, 6 h after feeding Zn-containing diets of up to 125 mg Zn/kg and this coincided with a 35% increase in mucosal Zn content. The findings indicated that gut metallothionein is induced only at very high dietary Zn intake, independent of the mucosal Zn concentration. Gut metallothionein was

more responsive to dietary Zn in rats (Chapter 3) than in mice, in which levels remained unchanged in most gut regions after feeding 400 mg Zn/kg.

Metallothionein-null mice have similar total gut Zn concentrations compared with control mice after feeding marginal and adequate Zn-containing diets. However, feeding a Zn excess diet to the metallothionein-null mice showed a marked decrease in gut Zn, primarily in the distal small intestine and the colon compared with controls identically fed, suggesting that metallothionein-null mice were incapable of sequestering excess Zn in the gut compared with normal mice. It can also be concluded from the present study that intestinal metallothionein does not fluctuate greatly in response to the luminal Zn contents within the range normally encountered in the diet (10-150 mg Zn/kg), nor were there major metallothionein-genotype-associated differences in total gut Zn concentrations between these diets.

A Zn400 diet, however, stimulated much higher increments in gut Zn concentrations of normal mice in comparison to metallothionein-null mice, especially in the ileum, caecum, and distal colon, indicating that metallothionein contributes to Zn sequestration within the gut. These results suggest that metallothionein may be linked to the high membrane-bound Zn in the normal compared to the metallothionein-deficient mice and that metallothionein is important for some export or secretion mechanism of Zn. This was supported by the finding of Hoadley et al., (1988) who demonstrated that intestinal metallothionein was linked to a vascular to luminal efflux of Zn in fasted mice.

Elevated Zn concentrations in the caecum and colon could be expected because of progressive dehydration of the contents and much reduced flow of luminal contents. The action of metallothionein appears to be governed more by its dynamic state than by static concentrations, presumably due to increase metallothionein turnover and transfer of Zn to other mucosal protein ligands. The situation in the gut contrasts with that in the liver, where incorporation into metallothionein is responsible for virtually all of the hepatic Zn uptake in the rat (Coyle et al., 1995).

An adaptation of normal mice to low dietary Zn intakes is also indicated by each of the eight segments of the gastrointestinal tract since normal mice fed the Zn10 diet contain less Zn than its metallothionein-null counterpart. Over the entire gut, the mean reduction of tissue Zn was 21%. However, in the small intestine, the difference was 27%, with a peak in the jejunum/ileum segment of 45%. The Zn10 diet, which is barely sufficient to maintain normal mice in positive Zn balance, was adequate for metallothionein-null mice, as shown by very similar liver and plasma Zn concentrations in both genotypes. The difference between genotypes in the caecal and colonic Zn averages only 11% (not significant). Overall, there are measurable differences between normal and metallothionein-null mice in their gastrointestinal responses to dietary Zn concentrations within the range of 10-400 mg Zn/kg.

In normal mice fed the Zn10 and Zn150 diets, the metallothionein concentrations in the stomach were approximately double those found in the small intestine and three times those in the caecum and colon. These high metallothionein levels are not described by Olfason (1983), who reported that metallothionein in the stomach and small intestine of

female Swiss White mice were of similar concentrations, indicating that these metallothionein levels may be strain-specific. Nonetheless, high levels of metallothionein could play a role in transferring Zn to essential Zn proteins, or protect the stomach wall from reactive oxygen species.

The mean plasma Zn concentrations in normal mice to the six test conditions covered a very narrow span of values, with only 0.57 μM separating the lowest from the highest group. The corresponding span for metallothionein-null mice was 3.0 μM , with a tendency for higher fasting values, particularly following the 400 mg Zn/kg diet, where the plasma Zn was 40% higher than its normal counterpart. The higher plasma Zn in this situation may represent, in metallothionein-null mice, a more rapid mobilisation of Zn from the liver and other tissues whereas, in normal animals, induction of metallothionein followed by its gradual degradation would release Zn back into the plasma compartment at a more controlled and lower rate. Consistent with the findings in rats (Chapter 3), liver Zn and metallothionein concentrations in mice were unchanged by dietary Zn. Plasma Zn levels increased in parallel to dietary Zn reflecting increased Zn absorption.

When mice were subjected to a 20 h fast, metallothionein was increased by 100-200% in the gut and 400% in the liver. The increase in metallothionein was similar throughout the entire gut, a finding indicating that the metallothionein response to fasting may be a general adaptation in tissues of endodermal origin to sequester Zn. Mice previously fed Zn10 and Zn150 diets before fasting had a higher gut metallothionein response than those fed the Zn400 diet. This was more pronounced in the liver, where fasting induced a quadrupling of metallothionein following the Zn10 diet, but only a doubling after the

Zn400 diet. These observations may suggest that the metallothionein response to fasting is greater in Zn-depleted mice, leading to a greater Zn sequestration and presumably lower Zn loss from the body when superimposed on marginal Zn nutrition, an adaptive response. The other interpretation is that prior feeding of the Zn400 diet resulted in a blunting of fasting-induced metallothionein synthesis.

The fasting response in terms of metallothionein seen in the liver was markedly higher than that in the gut, suggesting the liver is more sensitive to fasting than the gut. The induction of metallothionein after fasting prompts speculation this may act via a hormonal response, particularly an increased glucagon level. It is well documented that short-term starvation is associated with a fall in blood glucose, which switches the glycolytic pathway to glucose production. This change is under hormonal control of the glucagon : insulin ratio, catecholamines, vasopressin, angiotensin II and glucocorticoids. These hormones provoke via cAMP-Ca²⁺/Calmodulin-dependent activation/inactivation and by direct enzyme induction a rapid breakdown of liver glycogen, a degradation of liver, plasma and muscle proteins and an increase in lipolysis, similarly fasting is accompanied by an increase in the same hormones.

Several hormones have been suggested to be the primary inducer of metallothionein during fasting with the mostly likely being glucagon. This hypothesis was verified by treating normal mice with three injections of glucagon and determining the level of induction of metallothionein concentrations in the liver and intestine. In the present chapter, 9 h after injecting normal C57Bl/6 mice with glucagon, there was on average, a 50% increase in metallothionein accumulation along the gut and a 73% increase in the

liver. The metallothionein response to glucagon was not unlike that due to fasting and, in common with this perturbation, was similar in each segment of the gut. Interestingly, saline-injected controls had liver metallothionein levels more than double those of the non-injected controls, but unchanged intestinal metallothionein. This agrees with the findings of others showing that, unlike the gut, the liver is sensitive to stress hormones (Cousins, 1985; Coyle et al., 1993; Etzel and Cousins 1981). In studies on intestinal metallothionein gene expression in rats, only Zn was found to be an effector, not dexamethasone and interleukin -1 α (Hempes et al., 1991). Experiments in rats and in freshly isolated (Coyle et al., 1993) and cultured (Schroeder and Cousins, 1990) rat hepatocytes have demonstrated additive and independent effects of hepatic metallothionein by glucagon, epinephrine, and dexamethasone. As glucagon may affect metallothionein transcription, via cAMP, as well as promote translation of pre-existing metallothionein mRNA in the liver (Cousins, 1985), one or both of these processes may account for the rise in intestinal metallothionein following food deprivation.

In conclusion, our studies have shown that for the first time feeding dietary Zn showed differential distribution of gut Zn and metallothionein along the gastrointestinal tract. Increased gut Zn and metallothionein was demonstrated with corresponding dietary Zn, with normal mice having markedly higher Zn values than those unable to express metallothionein. On the other hand, dietary Zn did not affect liver Zn and metallothionein corresponding with constant levels of plasma Zn in control compared with metallothionein-null mice. Indicating that gut metallothionein responds more to Zn diet and may be important in regulating Zn homeostasis compared to liver metallothionein, in this dietary setting. In addition, this chapter has demonstrated that fasting markedly

induced metallothionein in the liver compared to the gut, the mechanism of action may in part be due to elevated levels of glucagon.

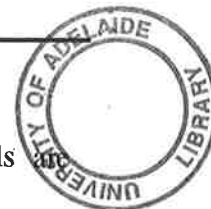
CHAPTER 5

Characterisation of Gut Zinc and Metallothionein in Rats Treated with Methotrexate

INTRODUCTION

The source of zinc (Zn) available for protection and repair of the mucosal wall is unknown. Several pools of Zn may be involved, including; (a) Zn attached to, but not internalised by the mucosal cell, (b) Zn that is either in the process of being absorbed or secreted through the mucosal cell, (c) excess Zn attached to low affinity ligands within the enterocyte, or (d) Zn mobilised from other parts of the body. Zn has been shown to have potential antioxidant roles in defined chemical systems (Bray and Bettger, 1990). Cousins (1985), Chen et al. (1977) and Blalock et al. (1988) demonstrated that very high (pharmacological) levels of dietary Zn can potentially exert an antioxidant function by significantly increasing (8-fold or greater) the size of the Zn-metallothionein pool in the liver and intestine.

The Zn binding ligand, metallothionein, with a cysteine-rich structure, has also been shown to protect against oxidative stress and DNA damage (Ebadi et al., 1996; Kondo et al., 1995; Schwarz et al., 1995). Zn bound-metallothionein is considered to be a pool of exchangeable Zn, which can donate Zn to higher affinity ligands on other proteins and therefore may complement Zn in processes involved in protection and repair. It is not known how the levels of both metallothionein and Zn changes during the course of



intestinal damage and recovery, in addition, whether these endogenous levels sufficient to promote mucosal protection and repair.

Methotrexate is used widely as a chemotherapeutic agent in the treatment for malignancies. Intestinal damage resulting from methotrexate administration to rats is characterised by histological parameters such as, crypt loss, villus fusion and atrophy, gross capillary dilatation and mixed cellular infiltrate (Taminiau et al. 1980, Vanderhoof et al. 1990). Howarth et al., (1996) have established a model of methotrexate-induced intestinal damage in the rat, which will be used in the present chapter to describe changes in both metallothionein and Zn concentrations in the liver and gastrointestinal tract during the course of intestinal damage.

MATERIALS AND METHODS

Animals and diets

88 male Sprague Dawley rats weighing 140 g were housed in metabolic cages and given free access water and to the Zn10 purified casein-based diet (described in Chapter 2).

Methotrexate time course

Rats were maintained in metabolic cages for 2 d prior to commencing the experiment to allow acclimatization. The procedure for injecting methotrexate was based on the method of Howarth et al. (1996). Briefly, methotrexate (Lederle Laboratories, Baulkham Hills, NSW, Australia) was administered to rats by once daily subcutaneous injection [2.5 mg/kg, given as an injection of 0.5 g methotrexate/L sterile isotonic (9 g/L) saline] in the suprascapular region on three consecutive days. Groups of rats (n=8 per group) were

killed on 10 consecutive days (d 1- d 10). A control group of un-infected rats were killed on d 0 for comparison.

Tissue collection

Gut tissues and liver were collected as previously described (Chapter 2).

Zinc and metallothionein analysis

Zn and metallothionein concentrations were analysed as previously described (Chapter 3).

Assessment of intestinal severity score

For histological analysis, methacarn-fixed specimens were embedded in paraffin wax, sectioned transversely at 4µm, stained with haematoxylin and eosin and examined with a light microscope. The semi-quantitative histological assessment for intestinal damage was described by Howarth et al. (1996). Briefly, a total score of each region of the intestine was derived from the sum of scores for 11 histological criteria (Table 1). These criteria were villus fusion and stunting (atrophy), disruption of brush border and surface enterocytes, reduction in goblet cell number, reduction in numbers of mitotic figures, crypt loss/architectural disruption, disruption or distortion of crypt cells, crypt abscess formation, infiltration of polymorphonuclear cells and lymphocytes, dilatation of lymphatics and capillaries, together with the thickening and oedema of the submucosal and muscularis externa layers. Each histological variable was scored from 0 (normal) to 3 (maximal damage) to give a maximum possible score of 33 for each intestinal region.

Table 1. Histological parameters used to derive severity score for methotrexate-induced intestinal damage.

Intestinal wall component	Histological parameter
<i>Mucosa</i>	Villus fusion and stunting Enterocyte disruption Reduction in goblet cell numbers Reduction in mitotic figures Crypt disruption Crypt cell disruption Crypt abcess formation Villus:Crypt ratio Lymphocytic and Polymorphonucleocyte infiltration Capillary and lymphatic dilatation
<i>Submucosa</i>	Thickening /Oedema
<i>Muscularis externa</i>	Thickening

Cell proliferation assessment using BrdU immunohistochemistry

All rats were injected intraperitoneally with 50 mg/kg BrdU (Bromodeoxyuridine) (Sigma Chemicals Inc., Castle Hill, NSW, Australia) prior to sacrifice. Incorporation of the thymidine analogue, BrdU, into the DNA occurs during the S-phase of the cell cycle, providing a specific indication of epithelial cell proliferation (deFazio et al., 1987). The BrdU technique was conducted on a single segment of the jejunum/ileum, the region maximally affected by methotrexate treatment. The immunohistochemical staining technique for BrdU detection was performed on methacarn fixed, paraffin wax-embedded, 3 μ m sections of the jejunum/ileum. The tissue sections were incubated in 1% hydrogen peroxide for 30 min to quench any endogenous peroxidase activity and then incubated for 8 min in 1 M HCl to denature double-stranded DNA. Sections were then blocked with 10% normal rabbit serum diluted in TBS for 30 min, rinsed in TBS for 10 min and incubated with mouse anti-BrdU (Dako, Carpinteria, CA, USA) at a dilution of 1:50 (in 1% normal rabbit serum in TBS) for 90 min. Sections were then washed twice (in 0.01% Tween 20 in TBS) for 15 min and biotinylated rabbit anti-mouse IgG (Dako, Carpinteria, CA, USA) was applied at a dilution of 1:400 in TBS for 60 min. BrdU stained jejunum/ileum sections were viewed under a light microscope using a 20 \times objective. BrdU labelling indices for each sample of the jejunum/ileum were determined by counting all of the positively stained epithelial cells in 12-15 well-orientated full-length crypts then expressing the number as a percentage of the total number of epithelial cells in the 12-15 crypts.

Statistical analysis

For the semi-quantitative histological scoring of intestinal damage, data of each region were compared statistically using a non-parametric Mann-Whitney U test. For all other measurements, data are expressed as mean \pm sem and comparisons were analysed by one-way ANOVA with unpaired t-tests to identify between group differences where a significant F value ($p < 0.05$) was attained.

RESULTS

Time course of methotrexate effects

In the present study, methotrexate administration for three days reduced feed intake (Fig. 1) and subsequently induced weight loss (Fig. 2) without substantial mortality, and this was supported by previous work from Howarth et al., (1996). Furthermore, control rats gained an average of 9.5 g/d with a food intake of 18-20 g/d. The loss of body weight and appetite in the methotrexate-rat reached a nadir on d 5, after which rats resumed normal eating habits. The semi-quantitative histological assessment of the relative damage in the small intestine shown in Table 2, indicated that small bowel damage increased in severity up to d 5 with maximal damage occurring at d 6 in the proximal jejunum segment (Fig 3), followed by rapid recovery and returned to normal by d 7-8. Methotrexate treatment had no significant effect on the stomach, caecum and colon.

Time course of intestinal zinc and metallothionein after methotrexate

Three consecutive methotrexate injections (d 1, 2 and 3) did not change Zn levels throughout the 10 d time course in the duodenum, jejunum and jejunum/ileum segments, with average concentrations of 394, 372 and 415 nmol/g wet weight respectively,

Figure 1. Effects of subcutaneous methotrexate injection on food intake in 140 g male Sprague Dawley rats. Food intake was measured daily in rats injected subcutaneously with 2.5 mg/kg/day methotrexate on d 1, 2 and 3. * Significantly ($p < 0.05$) different from controls (d 0).

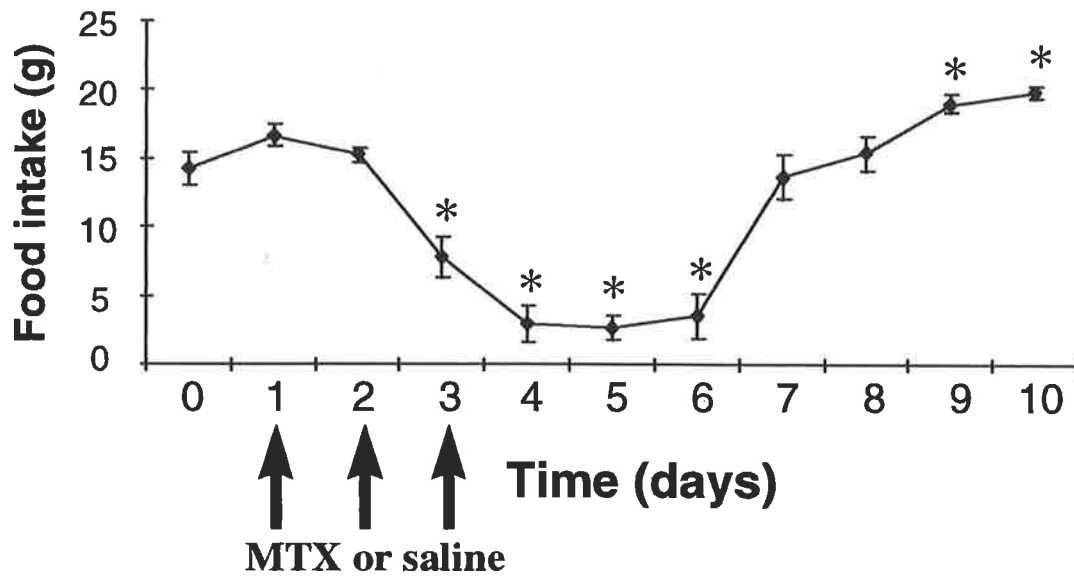


Figure 2. Effects of subcutaneous methotrexate injection on body weight in 140 g male Sprague Dawley rats. Body weight was measured daily in rats injected subcutaneously with 2.5 mg/kg/day methotrexate on d 1, 2 and 3. * Significantly ($p < 0.05$) different compared to controls (d 0).

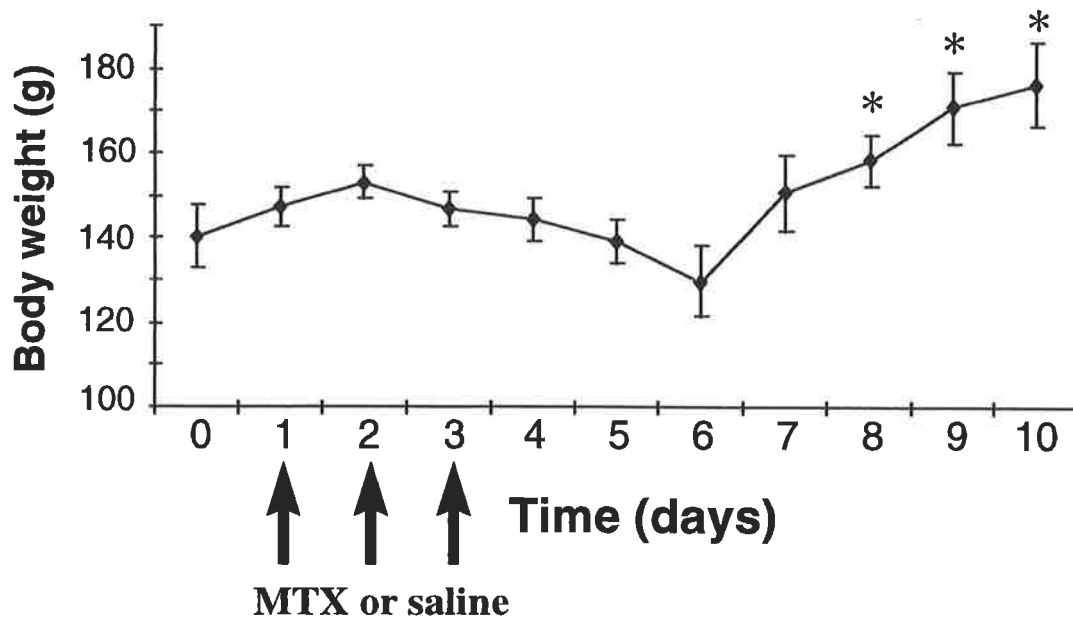
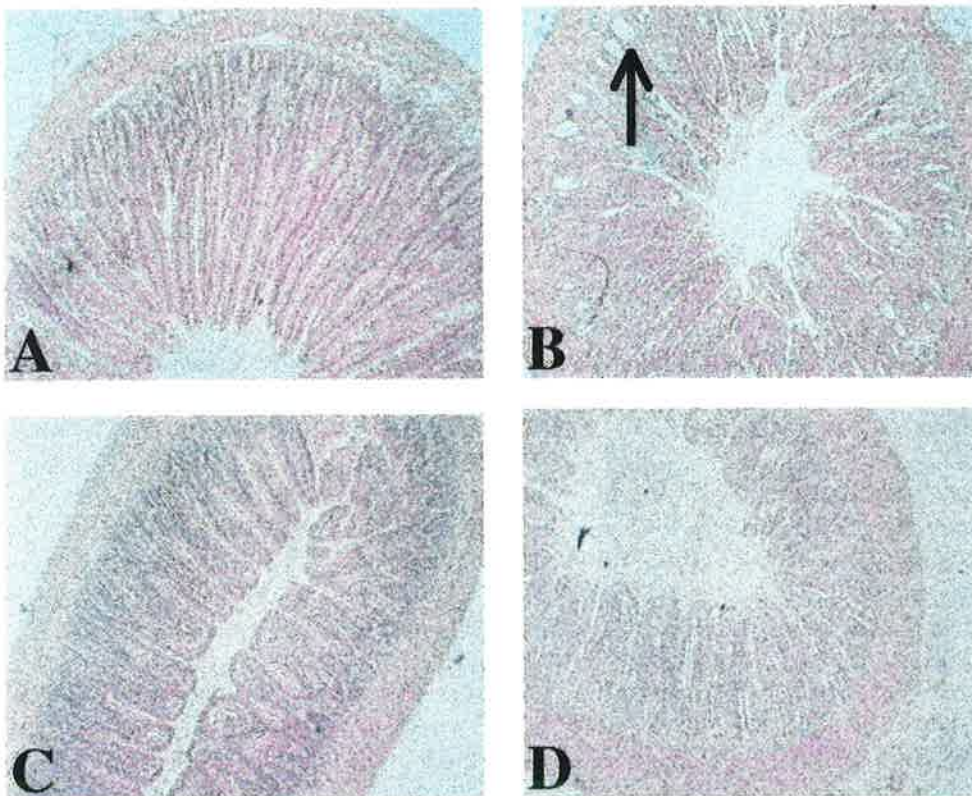


Table 2. Semi-quantitative histological assessment of methotrexate-induced intestinal damage at different regions of the small intestine in rats over a 10 d time course of methotrexate (2.5 mg/kg/day) treatment.

Time course of severity score in the small intestine after methotrexate treatment ¹				
	Duodenum	Jejunum	Jejunum/Ileum	Ileum
d 0	1 (0-4)	2 (0-4)	1 (0-7)	1 (0-7)
d 1	2 (0-9)	4 (1-5)*	2 (0-14)	2 (0-8)
d 2	4 (0-18)	6 (2-7)*	4 (0-13)	1 (0-8)
d 3	11 (3-21)*	16 (13-23)*	7 (3-15)*	2 (0-12)
d 4	14 (3-21)*	20 (18-24)*	17 (4-23)*	12 (0-22)
d 5	18 (8-21)*	22 (18-27)*	14 (8-16)*	10 (1-22)*
d 6	12 (2-13)*	14 (6-24)*	11 (1-17)*	11 (1-15)*
d 7	3 (0-8)	6 (0-7)	2 (0-4)	4 (0-10)
d 8	2 (0-7)	3 (0-6)	0 (0-4)	3 (0-8)
d 9	2 (0-6)	3 (0-5)	1 (0-4)	1 (0-8)
d 10	1 (0-5)	4 (1-4)	0 (0-3)	0 (0-9)

¹Values are the sum of scores for 11 independent histological criteria as detailed in Materials and Methods. The severity of each histological parameter was scored from 0 to 3. Values are expressed as medians, with the ranges in parentheses. * Significantly ($p < 0.05$) different compared to d 0 of each tissue region.

Figure 3. Effects of subcutaneous methotrexate injection on histological architecture of the jejunum and ileum of male Sprague Dawley rats (hematoxylin and eosin stain; $\times 100$). Methotrexate (2.5 mg/kg, s.c.) was given on d 1, 2 and 3. Histological parameters were assessed from d 0 to d 10, d 0 represents before MTX treatment, d 5 represents the period of maximal intestinal damage and from d 7 onwards represents natural recovery. **A:** Normal jejunum (non-methotrexate-treated rat). **B:** Jejunum from rat at d 5 after the initial methotrexate injection. Note obvious villous atrophy, crypt dilatation and loss of intact crypts (arrow). **C:** Normal ileum (non-treated-treated rat). **D:** Ileum from rat at d 5 after the initial methotrexate injection. Note comparatively mild histological changes in the ileum compared to the jejunum on the same day.



compared with control values. However, the ileal Zn was significantly ($p < 0.05$) elevated by 62% on d 4, 24 h after the final methotrexate injection, with overall concentrations over the time course of 571 nmol/g wet weight, higher than the other proximal regions (Table 3). Zn levels in the stomach, caecum and distal colon were unaffected after methotrexate treatment.

Intestinal metallothionein in control rats was highest in the duodenum compared to the other distal gut segments. However, methotrexate administration did not change the metallothionein levels in the duodenum throughout the 10 d time course. Jejunum metallothionein was doubled 72 h after the last methotrexate injection and then returned to normal levels. Mid jejuno-ileal and ileal metallothionein was increased much earlier, 24 h after the final methotrexate injection, mid jejuno-ileal and ileal metallothionein remained high until d 7 and d 10 respectively, with marked increases in the ileum (average of 134%) then the jejunum/ileum (92%) occurring at 72 h after the final methotrexate injection (Table 4). Stomach metallothionein concentrations remained unchanged throughout the methotrexate time course. Caecal metallothionein levels were elevated from d 2 and were high until the end of the time course, with an average increase of 78%. Colonic metallothionein was only elevated during the days associated with histological damage, with average increases of 88% and 78% in the proximal and distal colon, respectively.

Time course of liver zinc, metallothionein and plasma zinc after methotrexate

The time course of liver Zn and metallothionein responses following methotrexate treatment were closely correlated with intestinal damage. Hepatic Zn levels were significantly increased ($p < 0.05$) on d 3 and reached a maximum on d 6, corresponding to

Table 3. Zinc concentrations (nmol/g wet weight) of rats treated with methotrexate (2.5 mg/kg) on d 1, 2 and 3 to induce intestinal damage. Gut zinc was assessed in representative regions along the gastrointestinal tract of rats throughout a 10 d time course.

	Tissues							
	Stomach	Duodenum	Jejunum	Jejunum/Ileum	Ileum	Caecum	P. Colon	D. Colon
d 0	373 ± 28	431 ± 51	369 ± 15	405 ± 30	568 ± 48	406 ± 34	488 ± 14	459 ± 39
d 1	354 ± 12	385 ± 35	436 ± 51	446 ± 28	500 ± 27	288 ± 42	383 ± 34	401 ± 27
d 2	339 ± 25	436 ± 75	433 ± 49	385 ± 29	600 ± 51	378 ± 60	490 ± 77	411 ± 40
d 3	293 ± 61	348 ± 48	421 ± 76	403 ± 61	630 ± 98	351 ± 67	365 ± 55	371 ± 63
d 4	377 ± 45	436 ± 40	338 ± 64	561 ± 82	918 ± 129*	322 ± 65	369 ± 69	385 ± 74
d 5	334 ± 35	356 ± 33	307 ± 38	437 ± 63	587 ± 133	349 ± 72	341 ± 44	359 ± 33
d 6	320 ± 34	352 ± 35	369 ± 23	406 ± 70	431 ± 25	242 ± 26	282 ± 37	327 ± 39
d 7	394 ± 23	383 ± 17	352 ± 13	343 ± 20	486 ± 59	343 ± 39	365 ± 33	377 ± 21
d 8	312 ± 25	354 ± 18	355 ± 11	433 ± 92	452 ± 32	424 ± 28	441 ± 19	420 ± 11
d 9	351 ± 12	412 ± 22	406 ± 34	356 ± 22	515 ± 40	371 ± 18	432 ± 22	406 ± 21
d 10	392 ± 39	424 ± 6	360 ± 14	386 ± 17	605 ± 28	400 ± 22	450 ± 22	436 ± 30

Values are means ± sem (n=8). * Significantly (p<0.05) different from d 0 in the same region.

Table 4. Metallothionein concentrations (nmol Cd bound/g wet weight) of rats treated with methotrexate (2.5 mg/kg) on d 1, 2 and 3 to induce intestinal damage. Gut metallothionein was assessed in representative regions along the gastrointestinal tract of rats throughout a 10 d time course.

	Tissues							
	Stomach	Duodenum	Jejunum	Jejunum/Ileum	Ileum	Caecum	P. Colon	D. Colon
d0	8.0±1.0	11.0±1.1 ^a	7.6±1.0	6.0±1.4	5.9±0.6	2.5±0.2	3.8±0.5	3.6±0.6
d1	7.9±1.0	9.5±1.7	7.3±1.6	8.3±2.3	6.8±1.3	2.8±0.5	4.1±0.9	3.3±0.5
d2	7.8±0.9	11.0±1.7	9.2±1.8	9.7±1.3*	7.2±1.4	4.6±0.5*	6.6±0.7	4.6±0.8
d3	8.9±0.7	7.8±0.7	7.3±0.4	7.5±0.9	7.4±0.7	4.4±0.6*	6.8±1.4*	5.8±0.9*
d4	8.6±1.1	10.3±1.5	9.8±1.0	9.9±0.6*	14.3±2.2*	5.6±0.8*	9.6±1.4*	7.4±0.9*
d5	9.5±1.3	18.1±4.6	9.5±1.1	10.6±2.4	8.2±1.9	4.5±0.6*	7.1±1.2*	6.0±0.9*
d6	9.9±2.8	14.1±1.4	15.7±2.0*	17.7±3.5*	23.5±5.0*	4.7±0.7*	5.6±0.7*	4.8±0.4*
d7	6.9±0.8	9.9±0.3	8.8±0.6	10.6±1.3*	11.9±1.2*	4.4±0.3*	4.7±0.2	4.5±0.3
d8	6.9±0.8	9.3±0.8	8.8±0.8	9.8±0.8*	10.9±0.6*	5.0±0.3*	4.5±0.2	4.7±0.5
d9	4.8±0.6	10.4±1.8	9.0±1.7	8.0±0.8	8.4±0.7*	3.5±0.2*	4.5±0.5	3.5±0.6
d10	6.1±0.2	11.3±2.5	7.5±0.5	7.8±0.2	8.9±0.6*	3.3±0.3	4.7±0.5	3.6±0.4

Values are means ± sem (n=8). * Significantly (p<0.05) different from d 0 in the same region. ^a Significantly (p<0.05) different from other small intestinal regions on the same day.

the period of onset of intestinal damage (d 3), maximal damage (d 5) and recovery phase, d 6-7. During d 3-6, liver Zn (Fig. 4) was increased on average by 40% and liver metallothionein (Fig. 5) by 332%. The plasma Zn levels during the time course of methotrexate treatment (Fig. 6) were significantly ($p<0.05$) decreased (18%) by d 3 and progressively fell to a minimum by d 8 (51%) compared with controls. By d 10 plasma Zn levels had gradually increased but were still 27% lower than control levels.

BrdU labelling indices after methotrexate

Proliferative activity was assessed by BrdU labelling (Table 5). On the day of the final methotrexate injection, d 3, the BrdU labelling significantly decreased by 48% compared to d 0. 48 hr later when intestinal damage was maximal, d 5, there was an increased BrdU labelling of 46% and this was maintained during the early repair phase. As the intestine recovered (d 8-10) the BrdU labelling index decreased (Table 5).

DISCUSSION

Methotrexate is used as a component of numerous chemotherapeutic treatment regimens for malignancies affecting adults and children. It exerts its chemotherapeutic effects by inhibition of the enzyme dihydrofolate reductase, with subsequent disturbance of folate metabolism (Jolivet et al., 1983). In the present study, we administered methotrexate to rats to provide a model of small intestinal damage. Consistent with other reports (Howarth et al., 1996; Vanderhoof et al., 1990; Taminiou et al., 1980) of methotrexate toxicity in rats, we found that methotrexate damage was most severe in the proximal small bowel, 48 h after the final methotrexate injection was given with marked crypt and villus ablation. It is not known whether endogenous levels of Zn and metallothionein are

Figure 4. Effects of subcutaneous methotrexate injection on hepatic zinc in 140 g male Sprague Dawley rats. Hepatic zinc was measured daily in rats injected subcutaneously with 2.5 mg/kg/day methotrexate on d 1, 2 and 3. * Significantly ($p < 0.05$) different compared to controls (d 0).

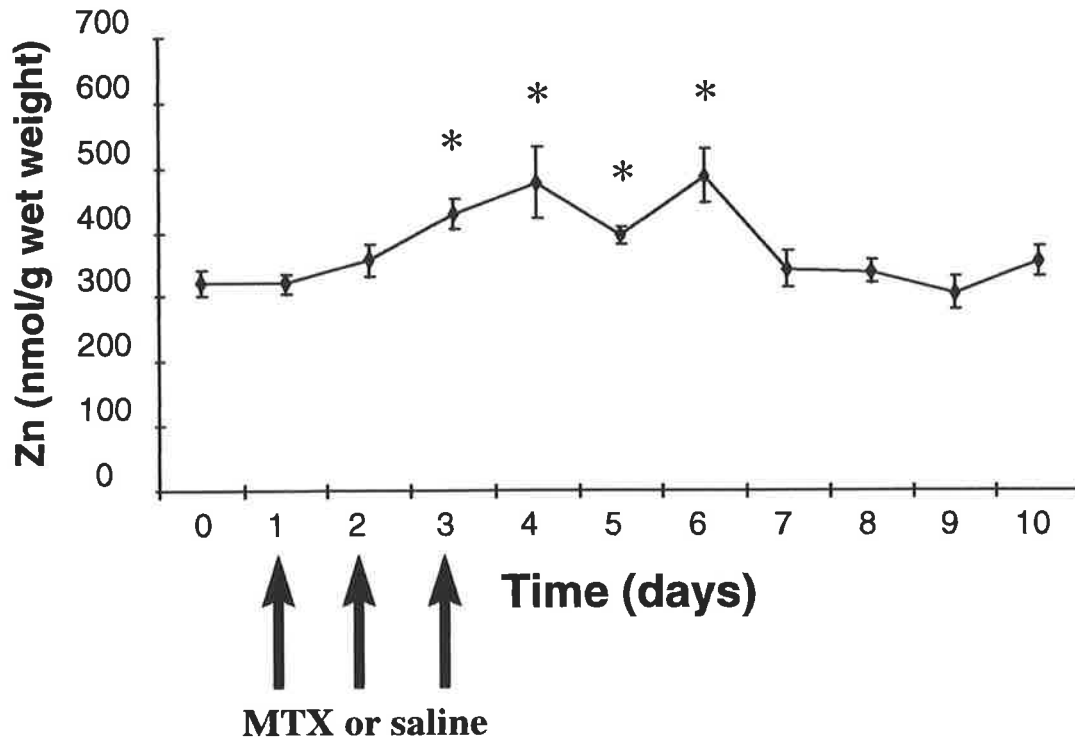


Figure 5. Effects of subcutaneous methotrexate injection on hepatic metallothionein in 140 g male Sprague Dawley rats. Hepatic metallothionein was measured daily in rats injected subcutaneously with 2.5 mg/kg/day methotrexate on d 1, 2 and 3. * Significantly ($p < 0.05$) different compared to controls (d 0).

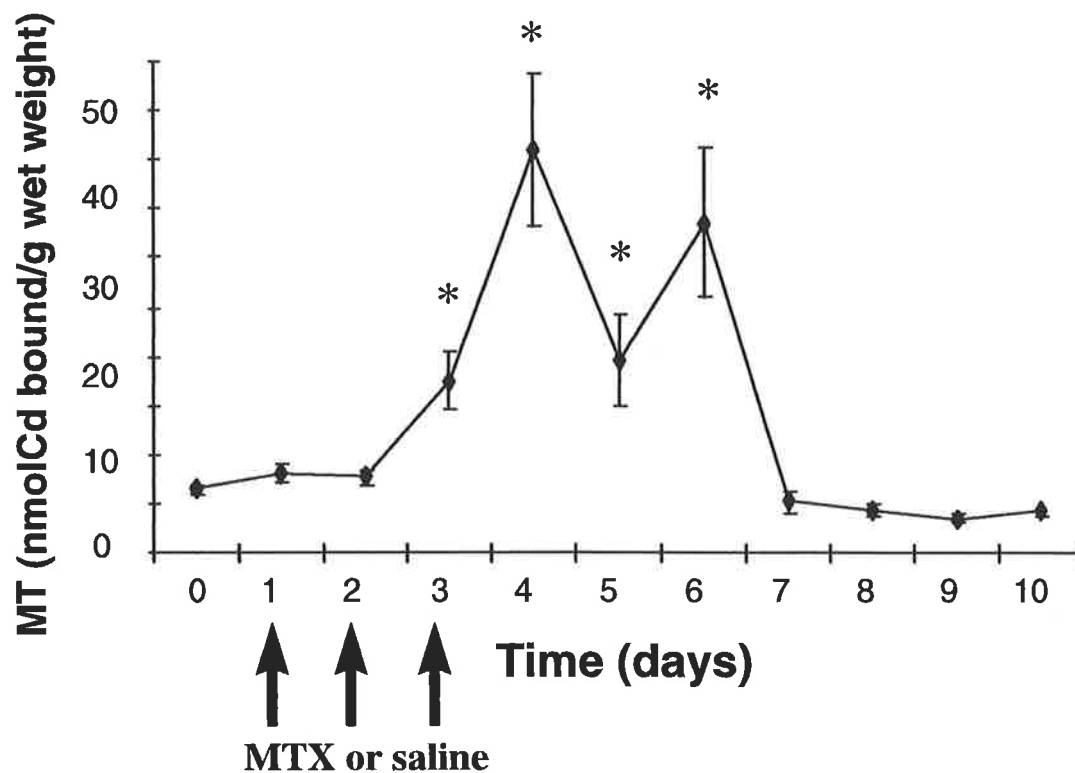


Figure 6. Effects of subcutaneous methotrexate injection on plasma zinc concentration in 140 g male Sprague Dawley rats. Plasma zinc levels were measured daily in rats injected subcutaneously with 2.5 mg/kg/day methotrexate on d 1, 2 and 3. * Significantly ($p < 0.05$) different compared to controls (d 0).

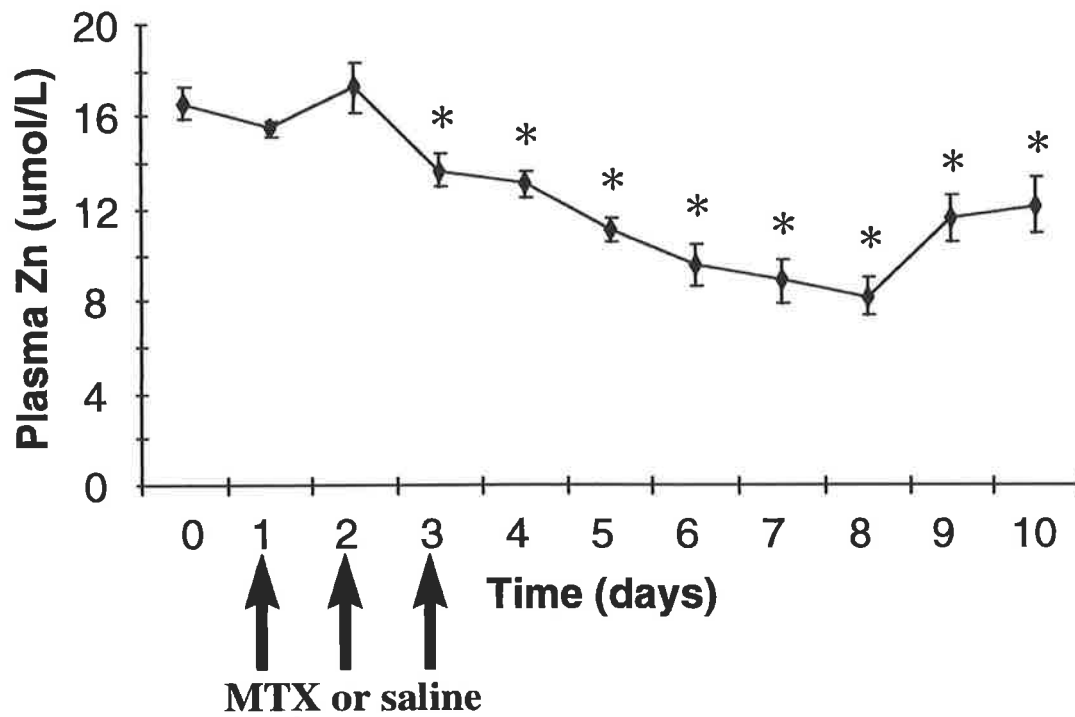


Table 5. Percentage of BrdU labelled cells per crypt in the jejunum/ileum region of the small intestine in rats before (d 0), during (d 1-3) and after (d 4-10) treatment with methotrexate (2.5 mg/kg).

% of BrdU labelling indices per crypt after methotrexate	
d 0	39 ± 1
d 1	37 ± 1
d 2	40 ± 3
d 3	20 ± 3*
d 4	44 ± 6
d 5	57 ± 5*
d 6	57 ± 1*
d 7	40 ± 1
d 8	31 ± 2*
d 9	32 ± 1*
d 10	35 ± 2

Data are expressed as mean ± sem of at least n=8 crypts. * Significantly (p<0.05) different from d 0.

sufficient to aid in the protection and/or recovery of chemotherapy-induced mucosal damage and this has not been described. Thus, in the present chapter, changes in endogenous concentrations of gut Zn and metallothionein after methotrexate-induced intestinal damage have been characterised.

Gut Zn concentrations throughout the 10 d methotrexate time course were remarkably stable even in the most severely damaged region. However, the ileum showed a significant ($p < 0.05$) increase in Zn levels only 24 h after the final methotrexate injection compared to control levels. Zn concentrations in the ileum were generally higher than the other small intestinal regions throughout the methotrexate time course. It was previously shown that the ileal region in rats fed diets containing low, normal, or high Zn content contained 20% greater mucosal Zn than the other gut regions (Chapter 3). This suggests that the ileum may be protected from methotrexate-induced damage compared to the jejunum, which has the lowest basal Zn concentration and the duodenum, which has a moderate Zn concentration. Since Zn is required for genetic expression of cells and proliferation in situation of regrowth from damage, it can be argued that Zn supplementation in rats treated with methotrexate may have had a protective effect on the duodenum and jejunum. Indeed Zn supplementation from 10 mg Zn/kg to 1000 mg Zn/kg diet to normal rats showed a marked increase in Zn levels in most regions of the gastrointestinal tract (Chapter 3).

Metallothionein concentrations were increased mainly in the jejunum and ileum with marked increases occurring in the ileum, this was also noted in the large intestine. This finding is consistent with the distribution of Zn within the small intestine, suggesting that

both Zn and metallothionein are important factors in protection of the epithelial mucosa from damage, as demonstrated in the ileum. High levels of metallothionein have been shown to protect against free radical damage in the mucosa and we have shown that feeding excess Zn to rats for one week increased intestinal metallothionein (Chapter 3). Hence, Zn supplementation may be a potential therapeutic agent for chemotherapy-induced intestinal mucositis.

Methotrexate administration to rats has resulted in a decrease in plasma Zn levels, which coincide with an increase in liver metallothionein. The decreased plasma Zn concentration from d 3 onwards is indicative of re-distribution of body Zn to tissues where metallothionein is induced. This is reflected in the increased liver Zn and hepatic metallothionein concentrations. This flux of Zn would also occur at the site of damage in the small intestine and is consistent with the premise that this pool of Zn could be used for tissue repair. Plasma Zn concentration has been shown to decrease after the onset of physiological stress such as tissue injury and inflammation. Such a decrease is believed to be mediated by hormones such as, interleukin-1 and -6 (Philcox et al., 1994; Coyle et al., 1993) and other cytokines. Interleukin -1 increases the synthesis of metallothionein, which binds plasma Zn, in the liver (Watanabe et al., 1995), and also possibly in other organs and injured tissues, resulting in increased tissue Zn and decreased plasma Zn concentrations.

In this study, this relationship between plasma Zn and hepatic metallothionein was demonstrated during phases of intestinal damage induced by methotrexate. Closer

examination of the interaction between Zn uptake and hepatic metallothionein induction during the damage phase is required to better define this relationship.

Methotrexate treatment caused significant anorexia and moderate weight loss, which was closely associated with histological damage to the gut. Anorexia and weight loss are markers of neuroendocrine disturbance as well as the presence of humoral cytokines (TNF- α) and IL-1 (Beutler and Cerami, 1989; Kern and Norton, 1988). TNF- α (cachectin) is thought to be the key mediator of anorexia and weight loss in inflammation (Beutler and Cerami, 1989).

In conclusion, the methotrexate-induced model of intestinal mucositis provides an ideal animal model with which to investigate the effects of exogenous Zn administration and/or agents on intestinal protection and repair. These advantages include that there are damage and natural repair phases and confined mainly to the small bowel as well as induced anorexia. In addition, the model is very reproducible especially in rodents characterised by diarrhoea, villus atrophy, and impaired digestive and absorptive function. Methotrexate injury is exacerbated by malnutrition but alleviated by administration of various growth factors and nutrients, suggesting that diet plays an important role in this model of injury. Thus, the roles of Zn in enhancing repair and promoting growth can be investigated in order to design more effective nutritional and adjunctive therapies that involve the administration of this cation for chemotherapy-induced mucositis in humans. The observations in this chapter suggest that Zn could either play a direct role or via metallothionein induction in cellular defense and repair mechanisms.

CHAPTER 6

The Effects of Zinc and Whey Growth Factor Extract (WGFE) Supplementation on Methotrexate-Induced Small Intestinal Damage and Intestinal Permeability in the Rat

INTRODUCTION

Intestinal permeability has been validated as a useful proxy indicator of mucosal damage in children with diarrhoea (Ford et al., 1985; Weaver et al., 1985), Crohn's disease and coeliac disease (Pearson et al., 1982). Intestinal diseases such as inflammatory bowel disease and toxigenic diarrhoea are associated with intestinal dysfunction that is linked to enhanced permeability indicating that function of the intestinal barrier is altered (Rodriguez et al., 1996). It is well established that chemotherapy alters mucosal morphology and gut barrier function. Keefe et al., (1997) and Sundstrom et al., (1998) have demonstrated that chemotherapy used for the treatment of various cancers is associated with increased intestinal sugar permeability. Zinc (Zn) supplementation has been shown to reduce intestinal permeability in children with diarrhoea living in developing countries (Roy et al., 1992; Alam et al., 1994; Sazawal et al., 1995; Bhutta et al., 1999). However, little is known about the influence of Zn supplementation on the modulation of intestinal permeability in gastrointestinal disorders, in particular chemotherapy-induced intestinal mucositis.

There is now evidence to suggest that growth factors can modify the severity of gut mucositis. Recent studies suggest a protective effect of exogenously administered

epidermal growth factor (EGF) in prevention of acute changes associated with experimental radiation enteritis (McKenna et al., 1994). In addition, administration of transforming growth factor- β (TGF- β) has been shown to reduce the severity of oral mucositis following 5-fluorouracil treatment in hamsters (Sonis et al., 1992). Growth factors, such as the fibroblast growth factors (FGF) and insulin-like growth factors (IGF), which have been shown to affect gut growth and repair (Howarth et al., 1998) may also influence the severity of gut mucositis.

Milk is a rich, natural source of growth factors that may enhance growth and repair of the gut in newborns (Cox and Burk, 1991; Read et al., 1984; Zumkeller, 1992). Human milk is particularly rich in EGF, whereas bovine milk contains high concentrations of IGF (Read et al., 1984; Zumkeller, 1992). Milk growth factors have now been extracted and enriched by cation-exchange chromatography from bovine cheese whey (whey growth factor extract; WGFE) obtained as a by-product of cheese making (Francis et al., 1995; Rogers et al., 1995). Howarth and co-workers (1996) demonstrated that oral administration of this growth factor extract improved chemotherapy-induced mucositis in rats.

The role of growth factors in intestinal growth and function, and the interaction between growth factors and specific micronutrients (such as Zn) on the improvement of growth, adaptation, repair and intestinal permeability of the damaged mucosa is unclear. Thus, this chapter investigates the efficacy of Zn, WGFE and a combination of Zn and WGFE (Zn+WGFE) supplementation on methotrexate-induced gut damage in the rat. It is

hypothesized that the varying concentrations of Zn in different parts of the gut may provide varying levels of susceptibility to methotrexate.

MATERIALS AND METHODS

Whey growth factor extract (WGFE)

The whey growth factor extract (WGFE) was kindly provided by G. S. Howarth of the Cooperative Research Centre for Tissue Growth and Repair and The Child Health Research Institute, SA, Australia and was prepared as described by Francis et al., (1995). Briefly, fresh cheddar whey was pasteurised (72°C for 15 sec) prior to microfiltration through a 0.2 µm pore membrane to remove fat. Purification of the growth factor was achieved by Sepharose Fast Flow-S cation exchange chromatography, taking advantage of the basic isoelectric point of many growth factors, compared with the major whey proteins, which is predominantly acidic. The growth factor activity was eluted from the cation-exchange column with 400 mmol/L NaCl and 10 mmol/L NaOH prior to diafiltration against deionised water followed by concentration. The desalted concentrates were then filtered through a 1 µm glass filter, freeze-dried and stored at 4°C. The final WGFE represented an approximately 100-fold concentration of the growth factor content of cheese whey, as determined by cell growth stimulation in cultured Balb/3T3 cells, L6 myoblasts and human skin fibroblast (Belford et al., 1995; Francis et al., 1995; Rogers et al., 1995). The concentrations of several known growth factors were measured in WGFE and are shown in Table 1. The WGFE contained 99.2 g protein/100 g WGFE, with lactoperoxidase and immunoglobulins as the most abundant proteins (Francis et al., 1995).

Table 1. Growth factor content and proportions in Whey Growth Factor Extract (WGFE).

Component	Content	% total WGFE
IGF-I	22.7 ng	0.00227
IGF-II	23.9 ng	0.00239
IGFBP's	200 ng (approx.)	0.02
acidic FGF	192 pg	0.00000192
basic FGF	662 pg	0.00000662
TGF- β	10 ng	0.001
PDGF	Detected	not done
EGF	not detected	not applicable

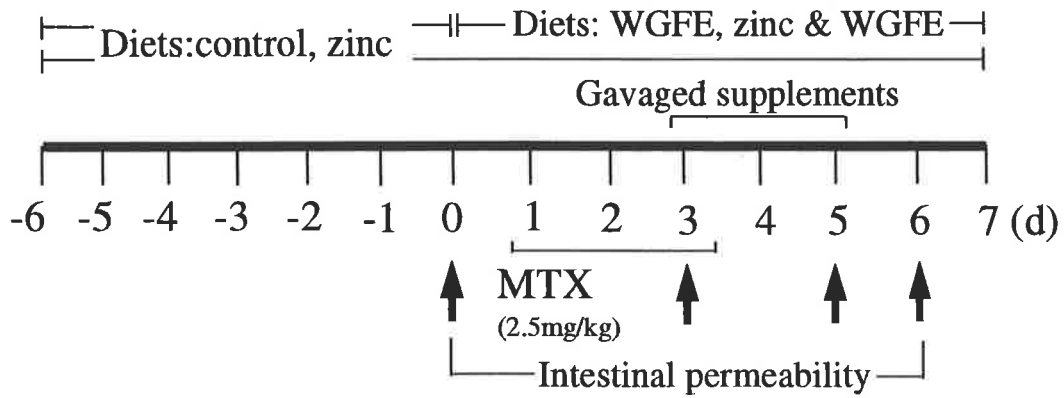
The growth factor content of WGFE was determined by radioimmunoassay for IGF-I and radio receptor assay for IGF-II (Owens et al., 1994). Indirect measurements of IGF binding protein content were based on interference in the assays for IGF-I and -II. Acidic FGF and basic FGF were detected by radio receptor assay using confluent monolayers of BHK-21 cells (Rogers et al., 1995). TGF- β content was estimated by a bioassay of growth inhibition in milk lung epithelial cells (Absher et al., 1991).

Zinc and whey growth factor extract supplementation and methotrexate-treated rats

Rats (n=64) were fed either a 10 mg Zn/kg (n=32) purified casein-based diet or supplemented with 1000 mg Zn/kg (n=32) for 7 d and then each group was further divided into two equal-sized subgroups. Sixteen rats were maintained on the Zn10 diet [control; 10 mg Zn/kg] and the other 16 rats were supplemented with WGFE (31.2 g/kg diet); [WGFE]. Similarly, 16 rats of the Zn-supplemented group were maintained on the same diet [Zn], the other 16 were allocated to a combination diet containing Zn and WGFE (1000 mg Zn/kg and 31.2 g WGFE/kg [Zn+WGFE], respectively, figure 1).

Animals were then injected subcutaneously with 2.5 mg/kg of methotrexate on three consecutive days (d 1, 2 and 3). Rats were maintained on their respective diets until d 5 and d 7 after the initial methotrexate injection, when they were sacrificed (see figure 1). These days were chosen because d 5 represented maximal damage and d 7 represented the onset of recovery (derived from Chapter 5). This allowed determination of whether gut damage was lessened (d 5) or whether there was an increased rate of recovery (d 7). From the preliminary methotrexate time course study (Chapter 5), body weight, food and water intakes were reduced between d 3-5. To compensate for this reduced food intake, rats were gavaged a 3 ml solution containing the average amount of Zn, WGFE or Zn+WGFE consumed per day. The average food intake of these animals in the first week was approximately 18 g/d. Orogastric gavage was performed without anaesthesia by passing a polyethylene tube (1.2 mm o.d., 0.8 mm i.d.) through the mouth into the stomach.

Figure 1. The experimental time frame used to investigate the role of zinc and whey growth factor extract (WGFE) supplementation on gut permeability and small intestinal damage in methotrexate-treated rats.



Tissue collection

At the end of each time point (d 5 and 7) gut tissues and liver were collected as previously described (Chapter 2).

Zinc and metallothionein analysis and severity scores

Zn and metallothionein concentrations were analysed as previously described (Chapter 3) and severity score assessment were described in Chapter 5.

Intestinal permeability using ⁵¹Cr-EDTA

Intestinal permeability was assessed by oral administration of ⁵¹Cr-EDTA (Amrad Biotech, Victoria, Australia) as described by Davies and co-workers (1994). Unanaesthetised control animals and rats supplemented with either Zn, WGFE or Zn+WGFE were gavaged 0.5 ml of 10 µCi/ml ⁵¹Cr-EDTA by passing a polyethylene tube (1.2 mm o.d., 0.8 mm i.d.) through the mouth into the stomach. 24 h urine samples were collected after administration of the radiolabel probe on d 0 (after 7 d of feeding either a control or Zn supplemented diet) and on d 3, 5 and 6 after the initial MTX injection. Urine volume was measured and 25 µl of Thiomersal (20 mg/ml; Merck Pty. Ltd., Victoria, Australia) was added to inhibit bacterial growth. The radioactivity in urine was counted using an LKB Wallac Multigamma 1261 gamma counter (Turku, Finland). Two standards of a 10 µCi/ml ⁵¹Cr-EDTA solution were also counted with each set of urine samples. Relative permeability was determined by calculating the activity present in each urine sample as a percentage of the administered dose after correcting for background radioactivity.

Statistical analysis

Data were analysed as previously described in Chapter 5.

RESULTS

Severity scores

On the basis of a preliminary time course study of methotrexate, d 5 represented maximal intestinal damage and d 7 an appropriate recovery time for assessment of effects of intervention after damage. These time points were selected as the critical points to assess the potential effect of pre-supplementation of Zn, WGFE and Zn+WGFE on methotrexate-induced gut damage. Semi-quantitative assessment of the histological severity of gut damage from methotrexate treatment on d 5, in all regions of the small intestine gave scores that were similar between control, Zn, WGFE and Zn+WGFE demonstrating no beneficial effect of treatment (Table 2). Similarly on d 7, the severity score was the same in the control and after WGFE in all segments of the gut (Table 3). However, damage was reduced in the ileum with Zn and Zn+WGFE treatments and the latter significantly ($p<0.05$) decreased the severity of damage in all regions of the small intestine compared to feeding the control diet, reducing the semi-quantitative scores by approximately 50% in all regions (Table 3).

Gut zinc

On d 5 Zn levels were mainly unchanged along the gut with the exception of the ileum where levels of Zn were 2-fold higher than the other regions (Table 4). WGFE treatment did not affect Zn levels on d 5. However, Zn and Zn+WGFE treatment had markedly increased tissue Zn concentration in all segments of the gut on d 5. Animals fed Zn or

Table 2. Semi-quantitative histological assessment of methotrexate-induced intestinal damage at different regions of the small intestine on d 5 after the first methotrexate injection in rats.

	Small intestinal regions			
	Duodenum	Jejunum	Jejunum/Ileum	Ileum
Diets				
Control	21 (19-22)	21 (19-23)	18 (16-22)	15 (10-23)
Zn	24 (18-16)	22 (18-24)	21 (13-26)	17 (8-21)
WGFE	23 (19-27)	19 (14-26)	18 (11-25)	18 (8-21)
Zn+WGFE	20 (11-25)	17 (14-23)	17 (9-23)	16 (5-17)

Values are the sum of scores for 11 independent histological criteria as detailed in Materials and Methods (Chapter 5). The severity of each histological parameter was scored from 0 to 3. Values are medians, with the ranges in parentheses. WGFE = whey-derived growth factor extract.

Table 3. Semi-quantitative histological assessment of methotrexate-induced intestinal damage at different regions of the small intestine on d 7 after the first methotrexate injection in rats.

	Small intestinal regions			
	Duodenum	Jejunum	Jejunum/Ileum	Ileum
Diets				
Control	15 (10-22)	19 (9-22)	13 (10-22)	12 (8-21)
Zn	14 (12-22)	18 (12-21)	12 (7-21)	10 (6-16)*
WGFE	15 (12-18)	17 (11-25)	13 (10-20)	11 (6-17)
Zn+WGFE	7 (3-9)*	8 (5-12)*	9 (3-14)*	6 (2-11)*

Values are the sum of scores for 11 independent histological criteria as detailed in Materials and Methods (Chapter 5). The severity of each histological parameter was scored from 0 to 3. Values are expressed as medians, with the ranges in parentheses. * Significantly ($p < 0.05$) different compared to the control diet. WGFE = whey-derived growth factor extract.

Table 4. Zinc (nmol/g wet weight) concentrations in segments of the gut wall of methotrexate-induced damage on d 5 after the first methotrexate injection in rats with prior feeding of various diets for 7 d and continued throughout the methotrexate treatment.

	Diets			
	Control	Zn	WGFE	Zn+WGFE
Tissues				
Stomach	500 ± 47	2424 ± 624*	618 ± 67	1209 ± 228*
Duodenum	677 ± 80	4350 ± 1449*	816 ± 104	1693 ± 203*
Jejunum	558 ± 58	3012 ± 816*	618 ± 64	1501 ± 233*
Jejunum/Ileum	835 ± 81	2793 ± 277*	975 ± 61	2534 ± 240*
Ileum	1231 ± 115 ^a	4296 ± 219*	1945 ± 146	3526 ± 235*
Caecum	586 ± 58	5492 ± 958*	771 ± 103	4233 ± 719*
Proximal colon	619 ± 55	5160 ± 1283*	605 ± 66	2727 ± 621*
Distal colon	689 ± 58	3802 ± 717*	642 ± 119	2372 ± 272*

Data are expressed as mean ± sem (n=8). * Significantly (p<0.05) different compared to the control diet. ^a Significantly (p<0.05) different compared to other regions within the same diet.

Zn+WGFE, had gut Zn concentrations on average 6- and 4-fold the controls, respectively (Table 4). On d 7, gut Zn concentrations were still raised after treatments with Zn and Zn+WGFE however, the Zn levels across all treatments were significantly ($p<0.05$) decreased in most regions of the gut compared to d 5 (Table 5).

Gut metallothionein

Methotrexate-treated rats fed the control diet had similar metallothionein concentrations along the gut on d 5, except in the ileum, where metallothionein levels were 1- to 3-fold greater than other regions of the gut (Table 6). Metallothionein concentrations were increased in most gut regions in rats fed Zn, WGFE, or Zn+WGFE. The average increase of gut metallothionein with Zn, WGFE, or Zn+WGFE was 23-, 2-, and 24- fold greater than the control diet, respectively (Table 6). The increase in metallothionein was more marked in the large intestine being 3-fold greater than levels in the small intestine, particularly in the Zn and Zn+WGFE groups.

Gut metallothionein concentrations on d 7, were similar to those on d 5 in the controls but lower than those of d 5 with Zn, WGFE and Zn+WGFE in most regions (Table 7).

Hepatic zinc and metallothionein

No difference was detected in the hepatic Zn or metallothionein concentrations between d 5 and d 7 for given treatment groups (Table 8). Rats treated with Zn or Zn+WGFE had markedly higher liver Zn and metallothionein concentrations than control or WGFE fed rats on d 5 (2.1- and 3.5-fold, respectively) and d 7 (13.5- and 6.6-fold, respectively). While liver Zn was increased on d 5 with WGFE compared to the controls, liver

Table 5. Zinc (nmol/g wet weight) concentrations in segments of the gut wall of methotrexate-induced damage on d 7 after the first methotrexate injection in rats with prior feeding of various diets for 7 d and continued throughout the methotrexate treatment.

	Diets			
	Control	Zn	WGFE	Zn+WGFE
Tissues				
Stomach	454 ± 79	2805 ± 789*	547 ± 52	1140 ± 214
Duodenum	368 ± 63 ^a	1865 ± 277*	385 ± 26 ^a	1013 ± 110* ^a
Jejunum	356 ± 77 ^a	804 ± 81* ^a	426 ± 75 ^a	664 ± 48* ^a
Jejunum/Ileum	339 ± 65 ^a	846 ± 61* ^a	486 ± 69 ^a	879 ± 52* ^a
Ileum	384 ± 67 ^a	1281 ± 125* ^a	557 ± 54 ^a	2202 ± 384* ^a
Caecum	332 ± 34 ^a	1437 ± 318* ^a	389 ± 26 ^a	1169 ± 112* ^a
Proximal colon	346 ± 51 ^a	2197 ± 549* ^a	436 ± 41 ^a	2221 ± 243*
Distal colon	490 ± 99 ^a	1545 ± 218* ^a	563 ± 71	1750 ± 271*

Data are expressed as mean ± sem (n=8). * Significantly (p<0.05) different compared to the control diet. ^a Significantly (p<0.05) different compared to d 5 of the same tissue region.

Table 6. Gut metallothionein (nmol Cd bound/g wet weight) concentrations in segments of the gut wall following methotrexate-induced damage on d 5 after the first methotrexate injection in rats with prior feeding of various diets for 7 d and continued throughout the period of methotrexate treatment.

	Diets			
	Control	Zn	WGFE	Zn+WGFE
Tissues				
Stomach	4.6 ± 0.9	39.6 ± 10.2*	9.5 ± 1.1*	30.6 ± 7.5*
Duodenum	6.0 ± 1.3	99.4 ± 20.7*	17.8 ± 3.2*	107 ± 16*
Jejunum	7.5 ± 1.8	62.6 ± 14.0*	10.5 ± 1.1	81.5 ± 2.2*
Jejunum/Ileum	7.2 ± 2.2	113 ± 26*	17.7 ± 3.6*	114 ± 26*
Ileum	12.4 ± 3.8 ^a	103 ± 30*	17.3 ± 1.6	113 ± 24*
Caecum	3.0 ± 0.6	130 ± 25*	17.8 ± 5.0*	134 ± 26*
Proximal colon	4.2 ± 0.8	136 ± 25*	21.9 ± 5.4*	137 ± 24*
Distal colon	3.2 ± 0.5	136 ± 24*	18.3 ± 4.9*	139 ± 25*

Data are expressed as mean ± sem (n=8). * Significantly (p<0.05) different compared to the control diet. ^a Significantly (p<0.05) different compared to other regions within the same diet.

Table 7. Gut metallothionein (nmol Cd bound/g wet weight) concentrations in segments of the gut wall following methotrexate-induced damage on d 7 after the first methotrexate injection in rats with prior feeding of various diets for 7 d and continued throughout the period of methotrexate treatment.

	Diets			
	Control	Zn	WGFE	Zn+WGFE
Tissues				
Stomach	5.0 ± 1.4	65.3 ± 18*	6.6 ± 1.8	47.0 ± 15.4*
Duodenum	8.9 ± 2.7	94.7 ± 24*	19.3 ± 2.4 ^a	96.1 ± 28*
Jejunum	11.0 ± 3.6	28.3 ± 8.5 ^a	10.1 ± 2.9	29.1 ± 9.2 ^a
Jejunum/Ileum	10.6 ± 3.3	30.6 ± 9.9 ^a	13.3 ± 3.9	43.5 ± 27.1 ^a
Ileum	13.6 ± 4.0	50.8 ± 16* ^a	24.8 ± 8.2	77.7 ± 27*
Caecum	3.6 ± 0.9	49.5 ± 17* ^a	4.6 ± 1.3 ^a	87.7 ± 30 ^a
Proximal colon	3.1 ± 0.9	103 ± 28*	6.0 ± 2.4 ^a	122 ± 31*
Distal colon	3.5 ± 0.7	54.8 ± 18* ^a	4.6 ± 1.5 ^a	98.5 ± 28*

Data are expressed as mean ± sem (n=8). * Significantly (p<0.05) different compared to the control diet. ^a Significantly (p<0.05) different compared to d 5 of the same tissue region.

Table 8. Liver zinc (nmol/g wet weight) and metallothionein (nmol Cd bound/g wet weight) of methotrexate-induced intestinal damage on d 5 and 7 after the first methotrexate injection in rats with prior feeding of various diets for 7 d and continued throughout the period of methotrexate treatment.

	Liver zinc		Liver metallothionein	
	Day 5	Day 7	Day 5	Day 7
Diets				
Control	490 ± 38	521 ± 43	9.2 ± 2.2	17 ± 4.2
Zn	1531 ± 260*	2335 ± 302*	131 ± 23*	130 ± 26*
WGFE	586 ± 24*	523 ± 41	26 ± 6.8*	23 ± 7.0*
Zn+WGFE	1425 ± 159*	1501 ± 302*	131 ± 23*	127 ± 28*

Data are expressed as mean ± sem (n=8). * Significantly (p<0.05) different compared to the control diet on the same day.

metallothionein was significantly higher than the controls on both days. Methotrexate-treated rats fed either the control, Zn, WGFE or Zn+WGFE had higher plasma Zn concentration on d 5 compared to d 7 (Table 9). Zn and Zn+WGFE groups had significantly higher plasma Zn concentrations on both d 5 and d 7 (2-fold) compared to the control diet (Table 9).

Intestinal permeability measured by ⁵¹Cr-EDTA

Intestinal permeability was assessed by measuring 24 hr urinary excretion of labelled ⁵¹Cr-EDTA in methotrexate-treated rats fed either a control, Zn, WGFE or Zn+WGFE diet (Table 10). Intestinal permeability was measured on d 0 (before methotrexate treatment), 3, 5 and 6 after the initial methotrexate injection. On d 0, there was no change in intestinal permeability in control or Zn fed rats. However, after the three-methotrexate injections (d 3) rats fed either a Zn or Zn+WGFE diet had significantly reduced intestinal permeability by 63% and 65% compared to control rats, respectively. Furthermore, feeding a WGFE diet also reduced (by 40%) the excretion of ⁵¹Cr-EDTA, although this was less marked than the reduction with Zn or Zn+WGFE. By d 5 much of the intestinal epithelium was excoriated, as demonstrated by the marked increase in permeability compared to d 3 and there was no significant difference between treatments. By d 6, intestinal permeability was still increased in the controls but was approximately 50% less permeable in the Zn and Zn+WGFE group compared with these controls. For the WGFE group intestinal permeability had almost returned to d 0 levels and was 80% lower when compared to controls on the same day (d 6).

Table 9. Plasma zinc ($\mu\text{mol/L}$) of methotrexate-induced intestinal damage on d 5 and 7 after the first methotrexate injection in rats with prior feeding of various diets for 7 d and continued throughout the methotrexate treatment.

Diets	Plasma zinc	
	D 5	D 7
Control	17.8 ± 1.1	12.9 ± 0.6^a
Zn	$61.2 \pm 5.6^*$	$39.3 \pm 6.1^{*a}$
WGFE	17.9 ± 1.6	14.0 ± 0.8^a
Zn+WGFE	$60.7 \pm 6.1^*$	$38.5 \pm 3.1^{*a}$

Data are expressed as mean \pm sem (n=8). * Significantly ($p < 0.05$) different compared to the control diet on the same day. ^a Significantly ($p < 0.05$) different compared to d 5.

Table 10. Percentage of 24 hr urinary excretion of labelled ^{51}Cr -EDTA prior to methotrexate-induced intestinal damage and on d 3, 5 and 6 after the first methotrexate injection in rats with prior feeding of various diets for 7 d and continued throughout the methotrexate treatment.

Diets	% of urinary excretion of labelled ^{51}Cr -EDTA after 24 hr			
	D 0	D 3	D 5	D 6
Control	3.3 ± 0.4	5.2 ± 0.9 ^a	16.4 ± 1.8 ^a	19.6 ± 2.9 ^a
Zn	3.0 ± 0.6	1.9 ± 0.3*	29.4 ± 4.7 ^a	8.5 ± 3.2*
WGFE	-	3.1 ± 0.5*	20.3 ± 4.9 ^b	3.9 ± 1.3*
Zn+WGFE	-	1.8 ± 0.2*	33.0 ± 12.8 ^b	10.5 ± 1.6 ^{*b}

Data are expressed as mean ± sem (n=8). * Significantly (p<0.05) different from control diet on the same day. ^a Significantly (p<0.05) different from day 0. ^b Significantly (p<0.05) different from d 3.

Crypt height and villus length

BrdU labelling (previously described in Chapter 5) was performed on the jejunum/ileum segment on d 5 and 7 after the initial methotrexate injection to determine the proliferative effect of Zn, WGFE or Zn+WGFE supplementation in the diet. The proliferative activity was unchanged irrespective of diet on either d 5 or d 7. The length of 12-15 full-length crypts was measured in the jejunum/ileum region of each rat on d 7 (Table 11). The crypt height was not significantly different between treatments. The villus length however was increased with Zn+WGFE and there was a trend for longer villi with Zn treatment although this did not reach significance.

DISCUSSION

Inert probes provide an indirect technique for monitoring intestinal mucosal changes and the impact of micronutrient and therapeutic adjuncts on intestinal morphology and function. In the present study the effects of dietary Zn, WGFE or Zn+WGFE on intestinal permeability were investigated in rats with gastrointestinal damage induced by methotrexate. It was demonstrated that all treatments diminished urinary excretion of ⁵¹Cr-EDTA in the early phase (d 3) of methotrexate-induced intestinal damage compared to controls. However, the most marked effect on intestinal permeability (63-65% reduction) was in rats fed Zn or Zn+WGFE compared to rats fed WGFE. These findings suggest that Zn plays a more dominant role than WGFE in reducing intestinal permeability, and this premise is supported by others who have shown Zn supplementation increases enterocyte size, epithelial growth and improves abnormalities of the intercellular tight junctions (Roy et al., 1992). An adequate Zn supply is also required for the regeneration of gut epithelium and restoration of its function and for the activation of

Table 11. Crypt height (mm) and villus length (mm) in the jejunum/ileum region of the small intestine of methotrexate-induced damage on d 7 after the first methotrexate injection in rats with prior feeding of various diets for 7 d and continued throughout the methotrexate treatment.

Diets	Crypt height	Villus length
Control	0.20 ± 0.005	0.34 ± 0.015
Zn	0.21 ± 0.011	0.39 ± 0.034
WGFE	0.20 ± 0.006	0.33 ± 0.015
Zn+WGFE	0.22 ± 0.006	0.41 ± 0.011*

Data are expressed as mean ± sem (n=8) of crypt height and villus length measured in 12-15 well-orientated, full-length crypts in the jejunum/ileum region from each animal. * Significantly (p<0.05) different compared to the methotrexate-treated rats receiving a control diet.

many of the brush border enzymes (Sazawal et al., 1995).

During the period of maximal damage (d 5) however, supplementation with Zn, WGFE or Zn+WGFE did not alter intestinal permeability compared to unsupplemented rats. This might be expected since a significant epithelial loss was observed at this stage and presumably most of the ⁵¹Cr-EDTA would have permeated through the damaged mucosa. Normally the epithelial cells lining the villi have a higher number of intercellular tight junctional strands and are more restrictive to permeability compared to epithelial cells lining the crypts (Hollander, 1992). However, the finding that most of the villi were denuded on d 5 makes it difficult to make any distinction regarding the site of the altered permeability. Interestingly, rats supplemented with Zn tended to have higher intestinal permeability than those without, indicating that the Zn chloride salt may have been acting as an irritant thus exacerbating the damage due to methotrexate.

During the recovery phase (d 6), intestinal permeability was reduced with all treatments compared to controls, the greatest reduction (80%) with WGFE followed by Zn (57%) then Zn+WGFE (46%). This suggests that WGFE was far more effective in enhancing recovery after methotrexate-induced gut damage than feeding Zn alone, furthermore Zn tended to retard the recovery when given in combination with WGFE, which could be due to the irritant properties of the Zn salt as mentioned above. Alternatively, Zn could inhibit the action of one or more growth factors or their receptors, as WGFE contains a number of growth factors that have been isolated and there are potentially more that have not been identified, which may be important in recovery from methotrexate. Feeding Zn+WGFE significantly increased the villus height during the recovery phase (d 7), suggesting that

Zn and WGFE have multifactorial effects on the intestinal epithelium after methotrexate administration.

Mucosal Zn and metallothionein concentrations were increased after Zn supplementation in all small intestinal segments on d 5 and d 7 but were lower on d 7 than d 5. Rats with a damaged intestine (d 5) bound 2- to 4-fold more Zn than controls without gut damage when fed diets containing Zn or Zn+WGFE. It might be expected that the increased Zn would reduce the degree of damage to the small intestine. However, this was not the case, possibly because of the severity of damage in the current methotrexate model. On the other hand during recovery (d 7) gut Zn concentrations were 3-fold higher in rats fed Zn or Zn+WGFE compared to rats fed the control or WGFE diets. Thus during the recovery period, it is possible that Zn facilitated repair and re-epithelialization of the intestinal mucosa.

The marked increase in gut metallothionein in rats fed Zn or Zn+WGFE diets suggests that Zn is the predominant inducer of gut metallothionein rather than inflammation following or during injury caused by methotrexate administration. WGFE can also induce gut metallothionein but to a lesser extent than Zn or Zn+WGFE. Although, little is known about the mechanism by which WGFE can induce gut metallothionein it is known that other growth factors primarily induce metallothionein via stress mediators (Moffat and Denizau, 1997). Possible candidates include EGF and TGF- α (found in WGFE; Table 1) since these can induce metallothionein expression in cultured rat hepatocytes (Moffat et al., 1995). In liver, the transcription factor AP-1 appears to play a role in the mechanism of metallothionein gene expression induced by EGF, because the AP-1

transcription factor consists of either Jun homodimer or Fos/Jun heterodimeric complexes (Angel and Karin, 1991) and AP-1 has been identified as a factor responsible for the basal level expression of the human metallothionein-IIa gene (Lee et al., 1987). Furthermore, EGF is known to induce both c-jun and c-fos (Kruijer et al., 1986), thus activating AP-1 and subsequently metallothionein. Possibly WGFE, containing EGF and an array of other growth factors (Table 1) may act in a similar manner in the gut as EGF in the liver to induce intestinal metallothionein.

In the previous chapter it was demonstrated that the ileum had the least damage induced by methotrexate and also had the highest basal Zn levels compared to other gut regions. This may indicate that high levels of Zn in the ileum may have offered protection prior to and during methotrexate damage. Based on the semi-quantitative histological score, Zn supplementation did not reduce the severity of damage in any region examined on d 5 and 7 compared to controls, despite raised levels of gut Zn and metallothionein. The severity of damage in the ileum was however reduced with Zn supplementation on d 7. One explanation may be that methotrexate-induced damage was too severe and that Zn supplementation was not sufficient to prevent or reverse the damage to the epithelium. In addition, the time that Zn is given may be crucial to the repair process. Watanabe et al., (1995) demonstrated that Zn is crucial in the healing of gastric ulcers, especially in the early stages of ulcer development. Zn may increase the rate of healing by increasing cell proliferation in regenerating mucosa or alternatively by exerting antioxidant activity by protecting sulfhydryl groups and stabilising cell membranes (Dalton et al., 1996).

Gut integrity and recovery from methotrexate damage was not improved by feeding WGFE and this agrees with the findings of Howarth et al., (1996). They showed that administration of WGFE had no significant effect on the overall semi-quantitative damage score 5 d after treating rats with methotrexate. However, in that study WGFE improved specific histologic parameters including villus surface indices in the jejunum and ileum by 52% and 56%, respectively and the jejunal crypt area index by 64%. Howarth and co-worker postulated that WGFE may have caused lengthening of residual intact crypts, or increased the regeneration of new crypts. Presumably one or more of the growth factors in WGFE (Table 1) contributed to its protective role. These growth factors include IGF, PDGF, and FGF and all have been implicated in the wound healing process. More specifically, IGF-I has been shown to enhance epithelial proliferation in rat intestine (Read et al., 1992; Steeb et al., 1994). In the present study, the concentration of growth factors in the WGFE diet may have been too low to have a mucosal protection effect. Hence, studies are warranted with a higher proportion of WGFE in the diet that may ameliorate the effect of methotrexate-induced intestinal damage.

Zn+WGFE did not reduce the severity of damage in the small intestine on d 5 but during recovery (d 7) it did decrease in severity score in all regions by 50%. Huang et al., (1999) demonstrated that high concentrations of extracellular calcium can interact with Zn and specific growth factors to stimulate DNA synthesis in fibroblast cultures. In addition, Ninh et al., (1996) showed that Zn supplementation was associated with increased plasma IGF-I concentrations in malnourished Vietnamese children and suggested that the growth stimulating effect of Zn might be mediated through changes in circulating IGF-I. It is

possible that Zn may activate the individual growth factors in WGFE or upregulate their cell membrane receptors.

In chapter 5, it was shown that plasma Zn declined rapidly after methotrexate treatment, however, in the current study feeding Zn alone or Zn+WGFE markedly increased plasma Zn levels during maximal damage and during the recovery period. The decreased plasma Zn concentrations during infection or inflammatory states can be accounted for by an accelerated flux of Zn from plasma to liver due to the induction of metallothionein (Klasing, 1984). The increased liver Zn may be required for metalloenzymes that may have a specific role in host defense functions.

In conclusion, Zn improved the recovery from methotrexate-induced damage only in the ileum which was the region least affected by methotrexate. A significant improvement in recovery from damage was however found in all regions of the intestine when animals were fed Zn+WGFE. Zn was more effective than WGFE in maintaining mucosal integrity in the early phase of damage. However, WGFE was more effective than Zn in improving mucosal integrity during the recovery phase.

Rats fed diets with WGFE showed signs of improved epithelial integrity but poorer recovery from damage by d 7 while those fed Zn+WGFE had poorer permeability on d 6 but had recovery from damage by d 7. These findings indicate that measurement of intestinal permeability on d 6 does not predict the speed of recovery. Further experiments are warranted using a Zn salt with less irritant properties than Zn chloride used here. This would reduce the severity of the methotrexate damage and possibly give better

permeability indices and a more rapid return of the normal intestinal mucosa. Nonetheless, the experiments provide evidence that orally administered Zn used in combination with WGFE might be useful in the treatment of intestinal mucositis induced by high dose chemotherapy or radiotherapy. Further studies are warranted to investigate the additive or synergistic mechanism(s) of Zn and WGFE on the reparative responses of the intestinal epithelium.

CHAPTER 7

Conclusions, Clinical Application and Future Directions

Conclusions

The gastrointestinal mucosa undergoes continual renewal and is in constant need of nutrients to maintain normal growth. The epithelium is sensitive to physiological disturbances such as stress and noxious agents that can cause the development of lesions and ulcerations. Anti-cancer drugs such as methotrexate can cause gut mucositis. Unfortunately, there are few effective treatments for this condition. Zn and growth factors are essential for maintaining a healthy gut and it is argued here that their interaction may protect and aid in the repair of damaged mucosa following the administration of methotrexate.

It was shown in Chapter 5 that administration of methotrexate to rats provides a model of small intestinal damage in which the efficacy of Zn and WGFE supplementation could be assessed. In agreement with findings of others (Taminiau et al., 1980; Vanderhoof et al., 1990; Howarth et al., 1996), it was demonstrated that methotrexate-induced damage was most severe in the proximal small bowel of rats, as evidenced by marked crypt and villus ablation. It is unclear why methotrexate selectively damages the proximal small intestine, however an explanation could be that the activity of dihydrofolate reductase (DHFR) is poorly expressed in this region and more susceptible to inhibition by methotrexate. Other possibilities include increased uptake of the drug, or more prolonged retention due to defective breakdown and clearance of methotrexate or increased binding of methotrexate to DHFR (Banerjee et al., 1995) in the jejunum compared to other gut regions. Goldie et

al., (1981) have identified that there are two distinct forms of DHFR with different heat stability, isoelectric points and molecular weights in methotrexate-resistant L5178Y lymphoma cells and these variants have varying responses to methotrexate. This indicates that there may be a difference in DHFR isoforms along the gut with varying resistance to methotrexate.

Maintenance of normal intestinal barrier integrity is essential for health, and previous studies have shown that Zn has beneficial effects on epithelial barrier function *in vitro* (Hennig et al., 1993), and *in vivo* reducing intestinal permeability in malnourished children with diarrhoea (Roy et al., 1992;). Integrity of the mucosal barrier function of the gut can be estimated by the intestinal permeability to molecules of large molecular weight using probes such as ⁵¹Cr-EDTA. The results of intestinal permeability of ⁵¹Cr-EDTA in normal rats shown in Chapter 6 were consistent with those of Bjarnason et al. (1985). Nakamaru et al., (1998) demonstrated that methotrexate administration disrupts the paracellular barrier function of the small intestinal epithelium, resulting in increased permeation of ⁵¹Cr-EDTA and this is in agreement with the results shown in Chapter 6. In addition, it was demonstrated that Zn, WGFE or Zn+WGFE supplementation to rats with methotrexate-induced intestinal mucositis reduced ⁵¹Cr-EDTA permeability during the early phase of damage. During the recovery phase intestinal integrity was improved by all treatments, with a marked reduction in permeability in rats supplemented with WGFE alone compared to those with Zn (Chapter 6). These findings indicate that there may be a synergistic effect between Zn and WGFE during the early phase of damage resulting in improvement in gut integrity. However, this was not noted during the recovery phase, in which WGFE was more effective than Zn and Zn+WGFE.

IGF-I and PDGF and an EGF-like molecule betacellulin are found in WGFE, a growth factor extract derived from bovine whey (Table 1; Chapter 6). General nutritional status, that is, specific nutrients (eg Zn, glutamine), and certain growth factors (eg insulin-like growth factors; IGF) have important interactions relevant for intestinal growth and function (review; Ziegler et al., 1999). MacDonald et al., (1998) found that Zn is essential for IGF-I stimulation of cell division. Zn in combination with IGF-I, EGF, or PDGF resulted in a significant increase in thymidine uptake while the absence of Zn resulted in the inhibition of IGF-I stimulation of thymidine uptake (MacDonald et al., 1998). Mediation of cell division by growth factors requires binding of the ligand to its receptor, which then activates intracellular signalling pathways (review; Pouyssegur et al., 1988). The type I IGF-I receptor possesses an intrinsic tyrosine kinase, which is thought to initiate a cascade of phosphorylations. It has been suggested that Zn deficiency may result in the failure to activate the phosphorylation cascade within cells in response to IGF-I (reviewed; De Meyts, et al., 1994). The addition of physiological concentrations of Zn to the enterocytes increased the mitogen-activated protein kinase activity and induced phosphorylation of cellular proteins (De Meyts et al., 1994). The above studies indicate that Zn availability affects membrane signalling systems and intracellular second messengers that coordinate cell proliferation in response to IGF-I. Thus, WGFE may mediate cell division via IGF-I intracellular signalling pathways and Zn may be vital to this process. In this regard, Roth and Kirchgessner (1994) showed that force-feeding a Zn-depleted diet to rats for 14 d resulted in a 28% decrease in serum IGF-I compared to rats fed a Zn-adequate diet even when the food intake was similar.

The absence of an interaction between Zn and WGFE in improving mucosal integrity during the recovery phase (Chapter 6) suggests that WGFE alone may enhance barrier function by regulating the tight junctions of enterocytes. There is evidence that some of the growth factors found in WGFE regulate intercellular tight junctions (Walsh et al., 2000). In particular, epidermal growth factor (EGF), a 6.4 kDa protein, has been shown to influence the apical actin cytoskeleton and tight junction proteins of A431 human epidermal carcinoma cells (Van Itallie et al., 1995). More recently, it was postulated that EGF activates zona occludin (ZO)-1 in A431 cells to improve the tight junctions between these cells (Van Itallie et al., 1995). Zona occludins are a group of proteins, (ZO-1, ZO-2 and ZO-3) that have been implicated in tight junction assembly (review; Tsukamoto and Nigam, 1999).

Transforming growth factor (TGF)- β 1 is also present in WGFE and is a 25 kD disulfide-linked homodimer produced by platelets, lymphocytes, macrophages and endothelial cells. TGF- β 1 has been shown to enhance the barrier function of T84 intestinal epithelial monolayer and to promote intestinal resistance (Planchon et al., 1999; Beltinger et al., 1999). TGF- β 1 not only upregulates colonic epithelial barrier function and inhibits the barrier reducing effects of cytokines such as IFN γ , IL-4 and IL-10 (Planchon et al., 1999). Studies in immortalised rat prostatic basal epithelial cells have shown that TGF- β 1 induces cellular differentiation and facilitates intracellular bridging by increasing the expression of ZO-1 (Danielpour et al., 1999).

The improvement of gut integrity after Zn and WGFE supplementation in methotrexate-treated rats may also be due to restoration of the glycocalyx. The glycocalyx serves as a

crucial barrier to noxious intestinal agents and is also the site of digestion of proteins and carbohydrates (Ito, 1969). Many chemotherapeutic agents produce morphologic and functional changes in gastrointestinal mucosa which can result in the thinning of the glycocalyx and a reduction in the number of microvilli (Taminiau et al., 1980). It is possible that in the absence of the glycocalyx barrier, the enterocyte is exposed to the noxious agents resulting in greater mucosal damage. Kelly and Hunter (1990) showed that EGF stimulated mucous production by increasing the synthesis of glycoproteins which are the main constituent of the mucous defence barrier. In addition, Katayama et al., (2000) showed that daily administration of polaprezinc (10 mg/kg) for 7 d increased the thickening of the mucous membrane after acetic acid-induced stomatitis in hamsters. Therefore, increasing delivery of Zn and WGFE may decrease the susceptibility to methotrexate damage by restoration of the mucus layer covering the brush border, and subsequently mucosal barrier and gut functions (DeWitt and Kudsk, 1999).

During the phase of maximal intestinal damage, Zn, WGFE and Zn+WGFE supplementation did not reduce intestinal permeability. This result was not surprising since much of the epithelium had been denuded at this time-point. Indeed, a trend of increased permeability on d 5 with Zn supplementation suggested that Zn chloride, a known gut irritant may have exacerbated the damage caused by methotrexate. In addition, methotrexate administration may have enhanced the production of the inflammatory cytokines, tumour necrosis factor-alpha, interleukin-1 and interleukin-6 and these have been associated with poorer intestinal mucosal integrity in trinitrobenzene sulfonic acid-induced colitis (Tateishi et al., 1997). Zn deficiency has been shown to increase inflammatory cytokines while Zn supplementation has been found to block the

release of cytokines in endothelial cells (Hennig et al., 1999). Indeed, it was demonstrated that Zn improved intestinal permeability during the early phase of gut damage and also in the recovery phase (Chapter 6). Thus Zn supplementation may provide a pharmacologic block against the release of inflammatory cytokines induced by methotrexate in the gut, thereby improving intestinal integrity.

In Chapter 3 it was shown that metallothionein concentrations are lowest in the proximal small intestine and greatest in the ileum. In addition, rats fed low- and normal-Zn diets showed an increase in metallothionein levels (40%) in the ileum compared to other gut regions. Although a number of functions have been proposed for metallothionein (reviewed in Chapter 1), the major biological roles may be in storage of Zn during rapid growth and development as well as sequestering free radicals (Cai et al., 1999). It is most probable that the latter function plays a dominant role in protection of the intestinal mucosa during methotrexate treatment. Gressier et al., (1994) demonstrated that methotrexate increases the amount of hydrogen peroxide released by polymorphonuclear neutrophils *in vitro* in a dose-dependent manner with a maximum increase of 43.7% for 500 μ M of methotrexate. In this regard, it was shown that following methotrexate administration (Chapter 5), small intestinal metallothionein concentrations increased markedly in the ileum and least in the proximal small bowel. This suggests that high levels of metallothionein in the ileum may protect against methotrexate-induced damage whereas the lower concentrations found in the proximal small bowel may increase the susceptibility to damage.

MT can donate Zn to Zn-requiring proteins for biochemical processes such as cell division and differentiation (Maret and Vallee 1998; Maret, 2000). The transfer of Zn from metallothionein is thought to be dependent on the intracellular redox state with Zn released from metallothionein when glutathione is oxidised (GSSG) and bound when in the reduced (GSH) form (Maret, 2000, see Fig. 1). Zn released by this mechanism is free for binding to other proteins as described in Chapter 1. Berendji and co-workers (1997) have demonstrated that nitric oxide (NO) can also release Zn^{2+} from Zn-metallothionein complexes within the cytoplasm or the nucleus of cells. NO is a reactive oxygen species that can cause damage and also aid in defense mechanisms against foreign antigens. NO is induced in acute or chronic disease states and is involved in defending the body against pathogens. The generation of NO by NO-synthase (NOS) depends upon the availability of L-arginine (Fig. 2). NO is thought to cause Zn release from metallothionein via electrophilic attack on the thiolate anion groups (reviewed; Aravindakumar et al., 1999).

In chapter 3, the regional distribution of Zn levels along the gastrointestinal tract in response to dietary Zn was described. It was shown that Zn concentrations increased in all gut regions and there was a concomitant increase in metallothionein levels in rats fed diets containing more than 100 mg Zn/kg. Zn bound to metallothionein represents a small exchangeable Zn pool. It was calculated approximately 1-3% and 2% of the total in the small intestine and liver, respectively was bound to metallothionein, irrespective of dietary Zn intake (Chapter 3). Buhler and Kagi (1974) reported that Zn bound to metallothionein represents 5-10% of the total Zn in uninduced human hepatocytes.

Figure 1. The oxidative pentose pathway provides NADPH essential for antioxidant functions (review; Porter et al., 2000). NADPH is formed at the steps catalysed by glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. NADPH is necessary to convert oxidised glutathione (GSSG) to glutathione (GSH) thus maintaining the intracellular redox state. GSH also causes the retention of Zn by metallothionein (MT; Zn-MT) whereas Zn is released in the presence of GSSG. The mechanism of the release of Zn^{2+} ions from MT is believed to involve oxidation of the cysteine sulphur ligand (S_{red} to S_{ox}) with GSSG. Zn released from MT may be used for biochemical reactions or further stimulate MT synthesis by binding to cytoplasmic transcription factors.

OXIDATIVE PENTOSE PATHWAY

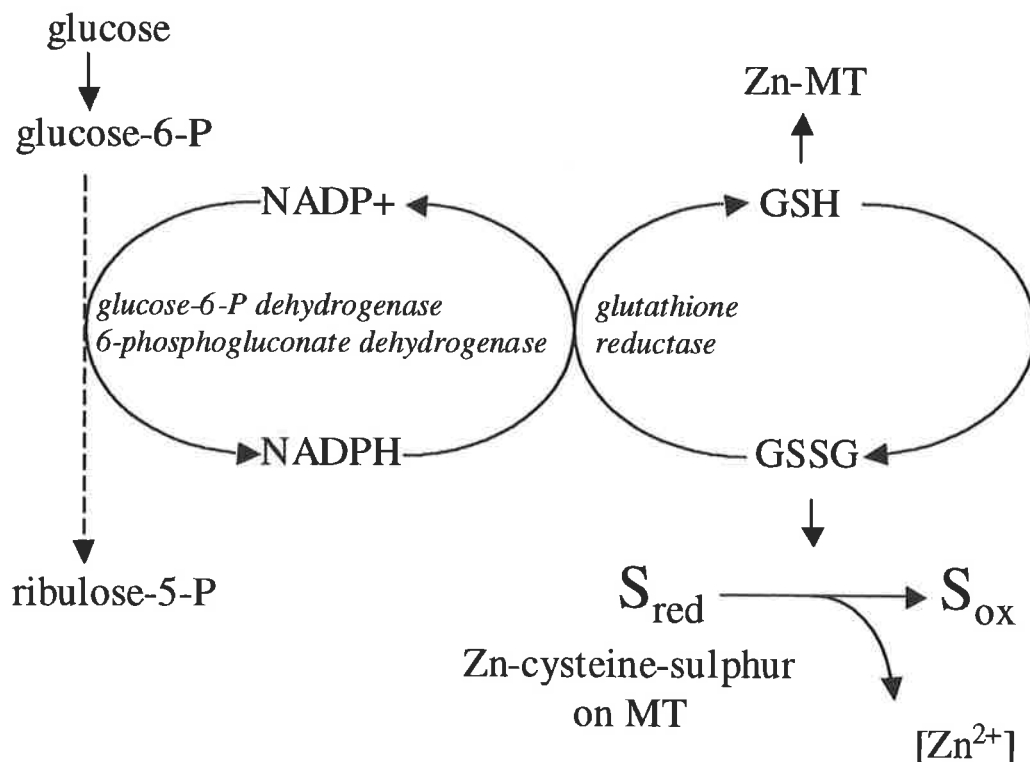
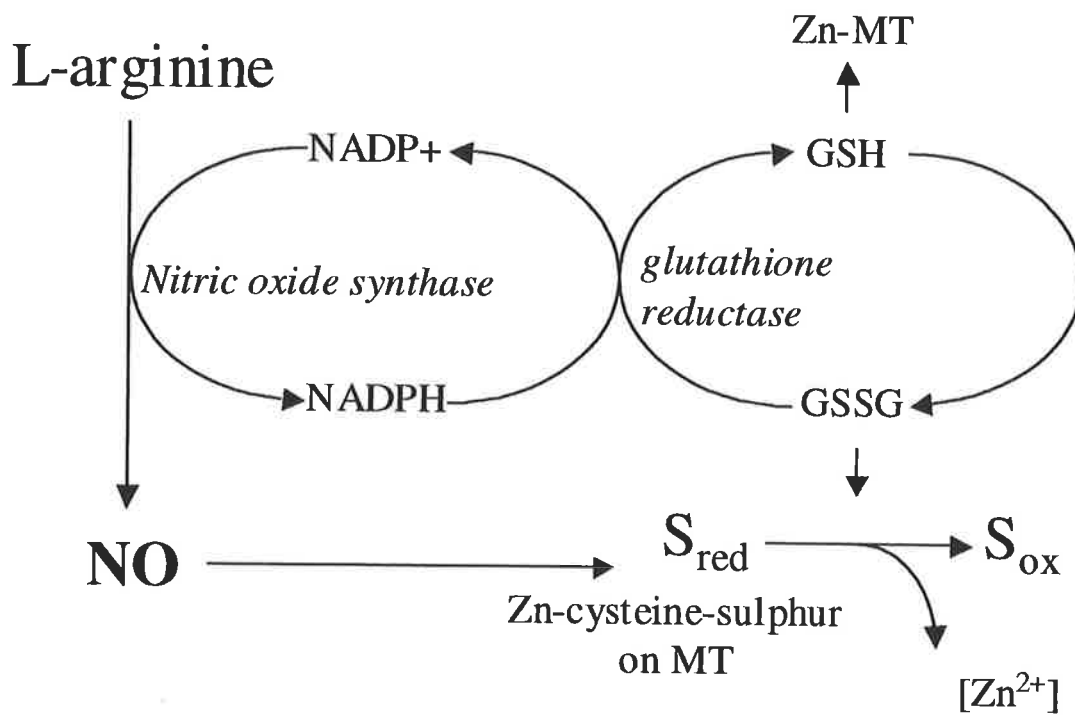


Figure 2. NO synthase catalyses a reaction by which nitric oxide and NADPH are produced from L-arginine and NADP⁺. NADPH reduces oxidised glutathione (GSSG) to glutathione (GSH). The change in redox state causes Zn release from the Zn-metallothionein complex via the mechanism explained in Figure 1. In addition, NO disrupts the metal-thiolate bonds of MT thus mediating Zn release.



It was demonstrated that the greatest proportion of Zn in the intestinal wall was present in the mucosal scrapings, with 94% membrane-bound and 6% cytosolic, irrespective of dietary Zn (Chapter 3). As the biochemical processes required for cell proliferation or cell death take place at the sub-cellular level it is likely that the cytosolic Zn either bound to metallothionein or “free” is responsible for mucosal protection and repair. The intracellular free Zn concentration was estimated by Zalewski et al., (1993) in Jurkat lymphoid cells as detected by Zinquin to be in the order of $2 \text{ nmol}/10^7$ cells, which would correspond to an intracellular concentration of 10^{-5} to 10^{-4} M. This level has been argued to be too high by Brand and Kleinke (1996) since Zinquin not only binds free Zn but also a component of protein-bound Zn relative to binding affinities (Coyle et al., 1994). In contrast, Simon et al., (1991) estimated intracellular free Zn to be of 10^{-11} M, thus the measurement of free intracellular Zn awaits more sensitive methods.

It was demonstrated in Chapter 2 that Zn and metallothionein were co-localised in the basal cells of the intestinal crypt, mainly in stem and Paneth cells. The Zn requirement of these cells is high, since stem cells require Zn for replication and differentiation. Paneth cells are morphologically well characterised and are found only in the small intestine and are localised at the base of the crypts, but their precise function has not been elucidated. However, these cells secrete anti-microbial polypeptides into the gut lumen, including alpha-defensins, termed cryptdins (Ouellette et al., 2000). Fernandes et al., (1997) found that cysteine-rich intestinal protein (CRIP) that contains a double zinc finger motif, was localised in high abundance in the cytoplasmic granules of Paneth cells. In addition, Sawada et al., (1993) identified and purified a 90 kD Zn-binding protein from rat Paneth cells however, the structure and function of this protein remains to be elucidated. These

Zn-containing proteins may thus explain the intense Zinquin fluorescence seen in the ileum.

Mullins and Fuentealba (1998) found high concentrations of metallothionein in Paneth cells by immunohistochemical staining of the duodenum of copper-loaded rats. In Chapter 2, the Paneth cells of rats fed normal diets were also observed to have high staining for metallothionein in particular in the jejunum and ileum. These observations indicate that Paneth cells may be important in Zn and copper homeostasis within the small intestine and in the functioning of these cells.

The role of metallothionein in regulating Zn metabolism was examined using metallothionein-knockout mice in chapter 4. Zn concentrations were higher in the distal gut with increasing Zn intakes in normal compared to metallothionein-knockout mice. This was consistent with the study of Rofe and co-workers (1999) who showed metallothionein-null mice excreted more endogenous Zn in the gut via increased pancreatic and mucosal secretions. Thus normal mice might be expected to have higher mucosal Zn levels than metallothionein-knockout mice. Furthermore, Coyle et al (1999) demonstrated that normal mice had greater Zn absorption from an egg-white diet compared to metallothionein-null mice. In a subsequent study, Zn-depleted mice were found to absorb more Zn from an intragastric solution by a metallothionein-facilitated process (Coyle, et al., 2000), indicating that gut metallothionein confers an absorptive advantage. Kelly et al., (1996) showed that metallothionein-I and -II can protect against both Zn deficiency and Zn toxicity. In addition, Dalton et al., (1996) found that transgenic mice that over-express metallothionein have a significantly larger metallothionein-bound

Zn pool compared to normal mice which could be used during periods of low Zn intake. Thus, gut metallothionein accumulates when animals are exposed to excess Zn and may be used as a source of Zn when intake is low.

The mechanism of induction of metallothionein in the small intestine is poorly understood although, its mediators in the liver have been well described (Cousins, 1985). Since gut metallothionein levels are induced by fasting (Chapters 3 and 4) when pancreatic secretions switch from anabolic (insulin) to catabolic (glucagon) hormones, it was hypothesised that glucagon would be a likely mediator. Coyle et al., (1993) demonstrated that in freshly isolated hepatocytes glucagon increased metallothionein synthesis by 28-35% after 5 hr. Cousins and Coppen (1987) and Hidalgo and co-workers (1987) showed that the induction of metallothionein in liver cells is co-ordinately regulated by glucagon through changes in cAMP levels and glucocorticoids. In Chapter 4, it was demonstrated that glucagon administration stimulated gut as well as liver metallothionein. In addition, the degree of metallothionein induction of glucagon administration was similar to that following fasting in both rats and mice shown in Chapters 3 and 4, respectively. The induction of metallothionein by glucagon in the gut presumably occurs by a cAMP mediated mechanism similar to that found in the liver (Kramer et al., 1993). Cousins and Coppen, (1987) showed that metallothionein mRNA levels were increased by glucocorticoids and cAMP in rats and isolated liver cells.

Clinical application

Measurements of intestinal permeability have been used as an indicator of intestinal damage in children with diarrhoea (Ford et al., 1985; Weaver et al., 1985) and Crohn's

and coeliac disease (Pearson et al., 1982). Intestinal mucosal injury resulting from chemotherapy can be detected by early increases in intestinal permeability that precedes histological and biochemical changes. Orally administered Zn and WGFE resulted in a reduction in intestinal permeability after methotrexate-induced damage (Chapter 6), indicating that these treatments may have potential benefits for patients suffering from chemotherapy-induced intestinal mucositis. This therapeutic regimen may also be useful in children debilitated with diarrhoea from enteric pathogens. In addition, Zn+WGFE may have benefits in malnutrition, intestinal diseases such as inflammatory bowel disease, gastrointestinal allergy or coeliac disease, and in patients with poor gut health.

Future Directions

The protective role of metallothionein in chemotherapy-induced intestinal mucositis using metallothionein-null mice was investigated, however, the mice were found to be resistant to methotrexate with no damage to the gut at doses (50-100 mg/kg); much higher than that used in rats. The resistance of these mice to methotrexate could be explained by high gut folate levels and this needs to be examined as it could be reduced by lowering dietary folate intake.

Several aspects of the methotrexate-induced intestinal mucositis model in the rat could be modified. Firstly, the severity of small bowel damage by methotrexate could be reduced by either administering a lower dose of methotrexate or reducing the number of doses given. Secondly, other Zn salts (ZnSO₄, Zn acetate) which have less irritant properties than ZnCl₂ need to be investigated, as well as using different concentrations of Zn. Other

animal models (eg. colitis or gastritis models) with less severe mucositis could be used to investigate the potential beneficial effects of Zn supplementation.

Further studies are needed to establish the mechanism by which Zn and/or WGFE decrease intestinal permeability after methotrexate administration. ⁵¹Cr-EDTA permeability is a useful marker to assess gut integrity, however for more precise assessment, the absorption of two sugars of different molecular sizes (e.g. lactulose/rhamnose) would provide a more sensitive index of small intestinal damage. The dual sugar permeability test has been used in screening small intestinal disease and in assessing the response of treatment (Uil et al., 1997; Haase et al., 2000). A further advantage of the dual sugar test is the elimination of error due to non-mucosal factors, since variables such as rate of gastric emptying, intestinal transit and completeness of urinary collection would affect both sugars equally (Sorensen et al., 1993).

The research in this thesis opens the door to a large number of important future directions. In particular, in unravelling normal mammalian Zn physiology, comparison of the data derived from this research with those of similar methodologies after intravenous administration of Zn would be of great importance. In addition, the investigation of pancreatic acinar cells that have been postulated to have a key role in Zn homeostasis (Rofe et al., 1999; De Lisle et al., 1996) would be important.

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PUBLICATIONS ARISING FROM THIS THESIS

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