FACTORS AFFECTING ZINC AND COPPER METABOLISM DURING DEVELOPMENT IN THE PIGLET MODEL

By

ZHENG WANG, MD, MSc.

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AUTHOR: Zheng Wang, MD (Shanghai University of Traditional Chinese Medicine and Pharmacology, Shanghai, China)
MSc (Shanghai Second Medical University, Shanghai, China)

SUPERVISOR: Dr. S.A. Atkinson, Ph.D

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ABSTRACT

This research investigated absorption, distribution and utilization of Zn and Cu in developing piglets as a model for human premature infants, specifically focusing on the impact of diet (amount and ratios of minerals), drugs (dexamethasone) and stage of development on mucosal cell membrane Zn and Cu transport and body Zn and Cu storage. In 32 and 52 h old piglets, velocity of Zn transport across intestinal brush border membranes (BBM) was higher than in 10 and 20 d old piglets but was occurred via a nonsaturable mechanism. Cu transport across BBM was greater in the 20 d group than in 10 d, 32 h and 52 h groups and occurred mainly via a saturable mechanism in 32 h, 10 d and 20 d groups. Plasma Zn and Cu were significantly lower but tissue Zn, Cu and metallothionein (MT) contents were higher in the younger groups than the 10 and 20 d groups. Competition for transport between elements may also be important in infants since Cu transport across BBM was suppressed by Zn but enhanced by Fe at the ratios of Zn:Cu and Fe:Cu which are similar to those in premature infant formulas. Further studies indicated that Fe mainly increases Cu binding to BBM. High dietary Zn intake did

not alter Zn transport across BBM but induced Zn accumulation in the intestinal mucosa and liver in piglets. These results are valuable in determining appropriate upper limits for Zn, Cu and Fe contents in infant formula. Exogenous dexamethasone (DEX) when used therapeutically to induce lung maturation in premature infants may compromise Zn and Cu status. Zn influx across BBM was significantly greater but Zn efflux was significantly lower in DEX treated compared to control piglets. The effect of DEX on Zn influx was abolished by dietary Zn supplement. Intestinal Cu uptake was also enhanced by DEX treatment. However, DEX-induced mucosal uptake of Zn and Cu did not appear to result in increased net Zn and Cu absorption since intestinal MT was induced by DEX. This study provides evidence that DEX treatment alters Zn and Cu metabolism in early life. Zn and Cu status should be carefully monitored in premature infants receiving long-term DEX therapy.

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LIST OF ABBREVIATIONS

AAS	Atomic absorption spectrom
BBM	intestinal brush border membrane
BBMV	intestinal brush border membrane vesicles
BLM	intestinal basolateral membrane
BPD	bronchopulmonary dysplasia
CRIP	cysteine rich intestinal protein
Cu	copper
d	day
DDI	distilled deionized water
DEX	dexamethasone
Fe	iron
FPLC	fast protein liquid chromatography
GI	gastrointestinal tract
GRE	glucocorticoid responsive element
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
h	hour
LBW	low birth wight
min	minute
MT	metallothionein
PG	prostaglandin
TRE	trace element responsive element
Vmax	maximum uptake rate
Zn	zinc

Chapter 1

GENERAL INTRODUCTION

1.1 Rationale, Hypothesis and objectives

Zn and Cu are essential for growth and development of neonates. They are co-factors of numerous enzymes in all areas of metabolism. In term newborns Zn and Cu contents per kilogram body weight are significantly greater than in adults (Shaw 1979, Shaw 1980). These large stores of Zn and Cu may serve as fetal reserves for the high requirement of rapid growth and development after birth. Studies examining body Zn and Cu contents of the human fetus at different developmental stages suggest that most Zn and Cu in newborns accumulate during the third trimester of gestation (Widdowson 1961). Prematurely born infants do not experience this important period of nutrient accretion in utero. Consequently, their body Zn and Cu stores were found to be much lower than term infants at birth (Sutton 1985). At full term a healthy infant contains 12 mg Cu, while a premature infant may contain 10 mg Cu or less depending on how premature he is at birth (Widdowson 1961). Since body stores are low at birth, the requirements for Zn and Cu are much higher in preterm infants than in term infants if preterm infants are to achieve accretion of metals at the rate of fetal accretion in late

pregnancy (Shaw 1979; Shaw 1980).

Prematurely born infants may not have a high capacity for Zn and Cu absorption in order to fulfil the requirement for growth and to compensate for the low body stores. Indeed, negative Zn and Cu balances have been documented in premature infants at least until a post-conceptional age of 37 to 40 weeks (Haschke et al, 1985, Atkinson et al, 1990). The immaturity of their gastrointestinal tract is considered to be the major reason for the malabsorption. Other possible reasons for negative Zn and Cu balances include inappropriate amounts and ratios of Zn and Cu in premature formula as well as interactions of dietary Zn and Cu with other nutrients or medications. Therefore, recommendations for Zn and Cu requirements of human premature infants must consider not only the predicted accretion of Zn and Cu over the third trimester of fetal growth but also the factors mentioned above. This research project is focused on the mechanisms by which these factors affect Zn and Cu absorption in developing infants. The hypotheses of the investigations were as follows:

a) High permeability of intestinal BBM and low levels of intestinal Zn and Cu binding/transport proteins resulting from gut immaturity are the major causes of Zn and Cu malabsorption in premature infants.
b) Exogenous glucocorticoid hormone used to treat severe lung disease in premature infants will alter normal growth due

to intestinal absorption and overall metabolism of Zn and Cu.

c) High dietary Zn inhibits intestinal Zn and Cu absorption by down-regulation and/or up-regulation of intestinal Zn and Cu transport/binding proteins, such as MT.

d) Dietary Zn supplementation reverses the effects of glucocorticoids on Zn metabolism and normalizes growth and development.

The overall goals of this study were to investigate absorption, distribution and utilization of Zn and Cu in early-weaned piglets as a developing animal model for premature infants. Specifically, I focused on the impact of stage of development, diet (amounts and ratios of minerals) and drugs (glucocorticoids) on cell membrane Zn and Cu transport and overall Zn and Cu metabolism.

1.2 Review of literature

1.2.1 Zn and Cu metabolism in early development

The mechanisms which control and regulate absorption, transport and excretion of Zn and Cu may be immature at birth. To protect themselves from Zn and Cu deficiency, term newborns have high body Zn and Cu stores (Widdowson 1961). Preterm newborns are more vulnerable to Zn and Cu deficiency than term newborns because their body Zn and Cu stores are lower as compared to term newborns (Shaw 1979, Shaw 1980, Sutton 1985).

1.2.1.1 Zn

At birth, plasma Zn concentration in human term and preterm infants is similar to that of adults. But there is a progressive decline in preterm infants during the first 10 weeks of life with a nadir (9-10 $\pm$2.6 μmol/L) at about 6 - 12 weeks of age (Gibson and DeWolfe, 1981). Some preterm infants develop Zn deficiency during this period when the demands of growth have used up body reserves (Krebs and Hambidge 1986). At the 6th week postnatally, 45% of premature infants had plasma Zn concentrations below 7 μmol/L (Thorp et al, 1981). Malabsorption of Zn has been suggested to be the principal reason for Zn deficiency in human premature infants (Ziegler et al, 1989). Dauncey (1977) reported that preterm infants receiving breast milk were in negative Zn balance on the tenth day of life, and the negative balance may persist for more than 60 days in some cases. The possible mechanism for negative Zn balance in these infants could be high endogenous loss of Zn (Lonnerdal 1989). However, it is unknown whether the high endogenous Zn loss is the result of high excretion of Zn from liver, from pancreas or from intestinal mucosa.

The distribution and metabolism of Zn in neonates are also different from those in adults. Infants have a higher storage of Zn in liver, bone and muscle (Widdowson 1961). Hepatic Zn contributes about 25% of the total body Zn in neonates compared with 6% calculated for adults (Widdowson

1961). Most Zn in liver is in the form of Zn-MT complex, which is suggested by the observation that hepatic Zn and MT concentrations correlate significantly (Zlotkin and Cherian, 1988). Hepatic Zn-MT may function as a fetal reserve for Zn. In human infants this reserve declines rapidly to reach a plateau at about 4 months postnatally (Zlotkin and Cherian, 1988). Preterm infants have lower hepatic Zn reserves which are depleted more quickly than term infants (Shaw 1979). An alternative explanation for hepatic Zn accumulation in the fetus and newborns is that the mechanisms for Zn excretion from the liver are immature (Cousins 1985). Two observations are against this hypothesis: a) Endogenous loss of Zn is high in premature infants (Lonnerdal 1989). b) Deprivation of dietary Zn for dams during pregnancy significantly reduced Zn content in their newborn pups, consequently these newborns had an earlier depletion of Zn-MT in liver compared to non-deprived animals (Keen et al, 1989). This observation suggests that Zn stored in the liver can be re-used to meet body requirements, and is not just excreted in bile. Since rats are not a good model for infants, further studies are required to examine Zn storage and excretion in early life in a more appropriate animal model of human infants.

1.2.1.2 Cu

Plasma Cu and ceruloplasmin concentrations in human neonates at birth are only one-third those of adults; they are

also lower in preterm than in term neonates and are related to post-conceptional age (Sutton 1985). Plasma concentrations of Cu and ceruloplasmin rise rapidly in term infants during the first 10 - 12 weeks of life and then gradually increase during infancy (Hillman 1981). The low plasma Cu in the neonatal period is probably caused by the immaturity of the liver to synthesize ceruloplasmin. This immaturity is suggested by the observation that neither the amount of Cu intake nor the timing of Cu supplementation has any influence on plasma or serum Cu concentration in term or preterm neonates (Hillman et al, 1981). Another possible factor related to low circulating Cu during early life could be a tendency to Cu accumulation in intestinal mucosa. This accumulation increases quickly in the first 2 days of life, then falls gradually to adult levels at the end of the three week suckling period in rats (Mason 1981). It was found that the accumulated Cu is bound to MT in intestinal mucosa (Linder 1989). A relatively high concentration of Cu in the milk of dams at this period and immaturity of the mechanism for transporting Cu across the BLM of the mucosal cell and to other parts of the body could be responsible for intestinal Cu accumulation (Linder, 1989).

Because most Cu in neonates is obtained from maternal circulation in the third trimester of gestation, prematurely born infants are born with lower Cu stores compared to infants born at term. During early neonatal life, Cu absorption may be

limited since a negative Cu balance has been demonstrated in human premature infants during the first month of life (Dauncey, 1977). Most reported cases of human Cu deficiency have occurred in premature infants (Danks 1988). The immaturity of the GI tract could cause malabsorption and/or high endogenous loss of Cu in these infants. These possible mechanisms should be studied in an appropriate animal model of premature infants.

In contrast to lower plasma Cu concentration, Cu content per kilogram body weight is greater in fetus and neonates than in adults. The distribution of Cu in the fetus and neonate also differs from that in adults. The hepatic Cu content in newborns is much higher than that of adults in almost all animal species (Widdowson, 1961). Subsequently, liver Cu concentration falls quickly in infancy and continues to fall in childhood. Compared to term infants, preterm infants have a more rapid depletion of hepatic Cu reserves (Linder, 1989). The Cu in the liver of neonates is mainly bound to MT in the lysosomal fraction of hepatic cells. In contrast to Zn, there is no quantitative correlation between hepatic MT and Cu (Zlotkin and Cherian, 1988). As for Zn, the immaturity of biliary Cu excretion mechanisms was also proposed to be the reason for hepatic Cu accumulation (Bremner and Beattie 1990). However, in addition to liver, the concentrations of Cu in muscle, skin, adrenal and thyroid are

all significantly higher in the fetus and newborn as compared to those in adults. Because the milk of most animal species is not rich in Cu, Cu accumulation in the fetus is likely intended to meet the high requirement of rapid growth after birth (Linder, 1989).

1.2.2 Regulation of Zn and Cu metabolism

Although the mechanisms for Zn and Cu metabolism have not been well defined, there is some evidence indicating that the digestive system plays a central role in the maintenance of body Zn and Cu homeostasis. It regulates absorption and excretion of Zn and Cu according to body requirements. Because of this homeostatic regulation, deficiencies of Zn and Cu rarely occur in adults.

1.2.2.1 Zn and Cu absorption

The mechanisms of Zn and Cu absorption especially during development are not completely understood. Three steps which are generally considered to take place in Zn and Cu absorption are depicted in Figure 1 (Cousins 1985). A detailed discussion of the mechanisms and regulation follows.

1.2.2.1.1 Cell uptake from intestinal lumen

Dietary Zn and Cu are mostly bound by protein and low molecular weight chelates, eg, amino acids and citrate. The proportions of free Zn and Cu in total dietary Zn and Cu are very small (Solomons and Cousins 1984). The ligands present free Zn and Cu to metal binding molecules on intestinal BBM

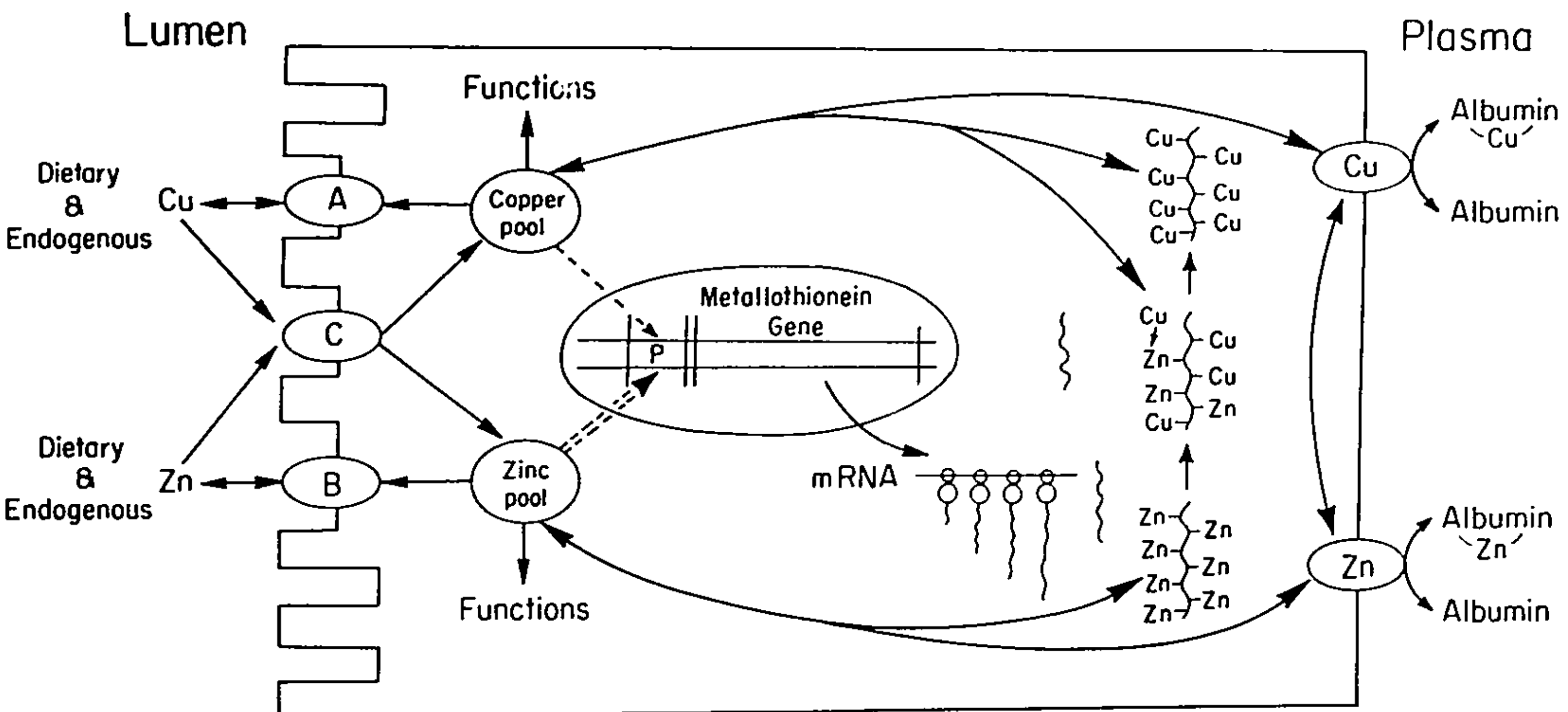


Figure 1, Mechanisms of Zn and Cu transport across an intestinal epithelial cell (Cousins 1985)

after passing the mucus coat of the intestine. Although some data suggest that Zn and Cu are transported across BBM of the small intestine as a form of metal-ligand complex, the kinetics of Zn and Cu uptake by intestines do not support the possibility of Zn- or Cu-ligand co-transport (Menard and Cousins 1983; Fischer and L'Abbe 1985).

The mechanism of Zn uptake by BBM is documented to be the combination of passive and carrier-mediated diffusion (Menard and Cousins, 1983). At low luminal Zn concentrations, the carrier-mediated mechanism is dominant, whereas passive diffusion dominates at high luminal Zn concentrations. The major sites for Zn absorption are in the duodenum and proximal jejunum (Menard and Cousins, 1983).

The mechanism by which Cu transports across the intestinal BBM is uncertain. Because low dietary Zn intake enhanced both Zn and Cu uptake by intestinal mucosa, a shared transporter for Zn and Cu on BBM was suggested (Schwarz and Kirchyessner, 1974). However, Cu deficient animals did not have up-regulated Zn absorption (Danks 1988). Many questions remain to be studied in this area. Cu absorption occurs mainly in the stomach and duodenum in humans. This conclusion comes from the observations that ^{64}Cu appears in plasma rapidly after oral administration (Van Bareveld and Van den Hamer, 1984).

1.2.2.1.2 Intracellular compartmentalization

In the cytoplasm of intestinal enterocytes, Zn and Cu are bound by low molecular weight chelates and binding proteins and traverse the cell to the BLM (Camakaris 1987). If dietary Zn and Cu intake or body Zn and Cu stores are high, Zn and Cu may induce the expression of MT gene by interacting with the trace element responsive elements which are located in the 5′ flanking region of the MT gene that controls the expression of the gene. MT in intestinal mucosa was proposed by Cousins (1985) to have three functions in maintaining body Zn and Cu homeostasis:

a) As a Zn and Cu buffer in the small intestine, MT limits Zn and Cu absorption when dietary Zn and Cu intake or body Zn and Cu stores are elevated.

b) When the synthesis of MT is induced in response to a stimulus other than Zn and Cu loading, ie, fasting, an increased portion of dietary Zn or Cu taken up by the intestine would go to the MT-associated mucosal Zn and Cu compartment, resulting ultimately in increased Zn and Cu absorption, albeit at a lower rate.

c) When intestinal MT is induced in response to elevated serum Zn, intestinal MT could work as a sink, facilitating the transfer of Zn from vascular spaces to mucosal cells. Net absorption of Zn and Cu would then be reduced because of the increased vascular to mucosal Zn and Cu flux. This

store of Zn and Cu would be lost finally when the mucosa cell is sloughed off from the intestine.

In addition to MT, a cysteine rich intestinal protein (CRIP) in intestinal mucosa has been cloned and sequenced recently. As for MT, the expression of CRIP gene is induced by high dietary Zn intake or DEX treatment (Hempe and Cousins, 1991). CRIP was considered to function as a ferry for Zn in intestinal enterocytes (O'Dell 1992).

1.2.2.1.3 Zn and Cu transport across BLM

As in the BBM, the mechanism of Zn transport across BLM can be separated into saturable and nonsaturable components, while the carrier mediated process is dominant (Oestreicher and Cousins, 1989). The kinetics of Zn transport across BLM did not appear to be markedly influenced by dietary Zn status. Similarly, dietary Zn did not induce specific BLM proteins. An energy requirement for BLM translocation of Zn has been suggested (Oestreicher and Cousins, 1989). The mechanism of Cu transport across the BLM is unknown.

1.2.2.2 Zn and Cu metabolism following absorption

1.2.2.2.1 Transfer from intestine to liver via portal circulation

Following release from the BLM of the intestinal mucosa, Zn and Cu are bound either with albumin or amino acids in the portal circulation. It was demonstrated that Zn and Cu do not compete with each other for binding with albumin

because they have different binding sites on albumin (Disilvestro and Cousins 1983).

1.2.2.2.2 Plasma Zn and Cu

Plasma Zn concentration is about 10-23 μmol/L in human adults. About 66% of plasma Zn is bound with albumin (Perkins 1964). Therefore, plasma albumin concentration can affect plasma Zn levels. The rest of Zn in plasma is bound to α2-macroglobulin and low molecular weight ligands, usually histidine and cysteine. Amino acid bound Zn could act as a Zn donor for a high affinity Zn transport system on cell membranes or could act as an intermediate between protein bound Zn and cell uptake mechanisms (Cousins 1988). α2-macroglobulin has a high affinity for Zn. The role of this protein in Zn metabolism is not clear (Disilvestro and Cousins 1983).

Plasma Cu concentration is about 15 - 32 μmol/L in human adults. Unlike in the portal circulation, Cu released from hepatocytes is principally bound with ceruloplasmin (60%-70%). The rest of plasma Cu is bound largely to albumin (15%-20%) and to a lesser degree to amino acids (10%), especially to histidine, threonine and glutamine (Bremner 1980). There is no interchange in the blood stream between ceruloplasmin Cu and other forms of Cu. The Cu bound with ceruloplasmin and amino acids was demonstrated to be available for non-hepatic tissues for synthesis of cuproenzymes while the Cu bound with

albumin is mainly transported to the hepatocytes (Camakaris 1987). The mechanism by which Cu in the form of albumin and amino acid complexes is transported across cell membranes is not clear. Ceruloplasmin is more efficient than albumin and amino acids in donating Cu to apoenzymes. Cu bound with amino acid and albumin and free ionic Cu compose the labile plasma pool which may increase with dietary intake while Cu bound with ceruloplasmin is less sensitively influenced by dietary Cu level during early development (Linder 1989).

Recently, another Cu binding protein in plasma, transcuprein, has been purified and sequenced. It consists of two subunits (about 10 and 200 KDa). The dissociation constant of rat transcuprein for Cu is very close to that of human albumin. Because there is a rapid equilibration between transcuprein and albumin Cu pools, Weiss and Linder (1985) suggest that transcuprein is an intermediate in the 'flow' of Cu from albumin pools to hepatocytes. The function of transcuprein in Cu metabolism needs further study.

1.2.2.2.3 Zn and Cu storage

Zn is primarily an intracellular ion. 95% of the total body Zn is in the cell. Tissue Zn content is from 0.15 to 1.5 μmol/g wet weight. Liver, the major Zn storage site, stores, releases and excretes Zn according to body requirement and dietary intake. It has been found that there is a good correlation between tissue Zn and MT concentrations, which

suggests that MT-Zn is the major form of Zn storage (Zlotkin and Cherian 1988).

Liver and spleen are major Cu storage organs. The regulation of Cu storage is principally via the induction of MT. The factors which induce MT synthesis such as high Zn intake, hormones, stress, inflammation and fasting could affect Cu distribution and storage (Linder 1989).

1.2.2.2.4 Zn and Cu excretion

Zn excretion is mainly by pancreatic, biliary and mucosal secretion or by desquamated mucosal cells (Cousins 1985). Transepithelial Zn transfer from the vascular system into the intestinal lumen may occur through mucosal cells or intercellular junctions (Pekas 1966). Pancreatic secretions could contain factors that influence Zn absorption. However, Jackson et al (1981) found that intra-luminal endogenous ligands do not regulate Zn absorption because exchanging duodenal washings of Zn deficient and control rats did not induce any change in Zn absorption. Biliary Zn was associated with low molecular weight moieties, while Zn in pancreatic juice completely binds to proteins (Pekas 1971). Unlike Cu excretion, biliary Zn excretion is small and less important than pancreatic Zn excretion. Serosal to mucosal Zn flux through intestinal enterocytes has been demonstrated by several experiments: restriction of bile and pancreatic secretions did not prevent the secretion of endogenous Zn via

feces (Jackson et al, 1981); ^{65}Zn injected intravenously appeared rapidly in intestinal mucosa (Smith et al 1978); an active transport mechanism was shown in the serosal to mucosal direction (Cousins 1985) and Davis and Nightingale (1975) estimated that <1% of ^{65}Zn taken up by intestinal mucosa cells returned to the intestinal lumen. Compared to Zn absorption, Zn excretion is a more important regulatory mechanism for body Zn homeostasis in the condition of high dietary Zn intake (Davis and Nightingale, 1975).

Body Cu is excreted principally via the gastrointestinal tract with <3% of the intake appearing in the urine. Bile is the major excretion route of body Cu. Only 10-15% of biliary Cu is reabsorbed (Gollan 1975). The biliary content of Cu responds rapidly to changes in the plasma concentrations of non-ceruloplasmin Cu. Pancreatic secretion is another route of Cu excretion. Cu secretion by pancreatic fluid has been estimated to be about 500 μg per day (Gollan 1975).

Human gastric mucosa secretes approximately 1 mg Cu/day. Compared to the average daily intake of 1.2-1.7 mg Cu/day, this secretion is high. However, unlike Cu in bile and pancreatic fluid, gastric juice does not inhibit Cu reabsorption. About 40-60% of gastrically secreted Cu is reabsorbed. A large part of secreted Cu is bound to substances

of low molecular weight (Kressner, 1984). Because body Cu status has less effect on Cu absorption than on Cu excretion, Cu excretion is more important than Cu absorption in maintaining Cu homeostasis (Bremner 1980).

1.2.3. Factors affecting Zn and Cu absorption

The absorption of Zn and Cu is affected by many endogenous and exogenous factors in early life. The capacity as well as the mechanisms for Zn or Cu absorption change with the maturity of the gut in infants. The amount and ratio of Zn and Cu in the diet may affect their absorption. Some other dietary factors, e.g. phytate, reduce the bioavailability of Zn and Cu. Zn is absorbed more efficiently from breast milk than it is from cow's milk or formula. The latter effect can be eradicated by removing phytate from the formula (Lonnerdal et al, 1988).

1.2.3.1 Stage of development

There are different ontogenic processes of Zn and Cu absorption between species. The rate of absorption was found to be higher in neonatal rats than in adult rats (Ghishan and Sobo 1983; Mann et al, 1979)). However, studies in human infants suggested that they have lower capacity for the absorption of Zn and Cu than adults (Haschke et al 1985; Dauncey 1977). The GI tract of rats is less mature than that of humans at birth, but rat pups grow and develop much more quickly than human infants. Pinocytosis was considered to be

the major mechanism for Cu absorption in infant rodents by Linder et al (1989). The mechanisms for Zn and Cu absorption in human infants have not been defined. The different mechanisms for the absorption of these elements between neonatal rodents and humans may explain their different absorptive capacities for Zn and Cu.

1.2.3.1.1 Zn

Ghishan and Sobo (1983) studied Zn absorption in rat pups at various developmental stages by in situ intestinal perfusion. They found that significantly more radioactive Zn was absorbed into blood and liver in suckling than in adolescent rats. The net absorption rate, on the basis of dry weight of the segments, was 2 - 8 fold greater in segments of the suckling compared to corresponding segments of the adolescent rats. The transport kinetics indicate the saturable process gradually replaced the non-saturable process during intestinal maturation, and there was a progressive rise in mean Km values for intestinal Zn transport with age.

Although suckling rats were shown to have a higher rate of Zn absorption, sub-optimal Zn status and negative Zn balance have been found to occur much more frequently in human infants and children than in adults as discussed in Section 1.2.1 (Shaw 1980; Atkinson et al, 1993; Lonnerdal 1989).

1.2.3.1.2 Cu

Mann et al (1979) compared the net absorption of Cu

using ^{64}Cu tracer between suckling (10-12 d) and adult mice. They found the suckling mice absorbed much more ^{64}Cu than adults, consequently the muscle and blood of the sucklings retained proportionately more ^{64}Cu tracer than those of adults. Similarly, Mistilis and Mearrick (1969) found that intestinal uptake of ^{64}Cu from aqueous solution, plasma or biliary origin was more efficient (over 75%) in suckling rat pups than adult rats. After weaning, the uptake of biliary Cu falls from 75% to 8% and that of plasma Cu from 96% to 20%. This decline is related to the gut closure because it may be induced by glucocorticoid administration (Mistilis and Mearrick 1969).

In contrast to the results from mice, there is evidence that Cu deficiency may occur in preterm infants with low birth weight but without evidence of malnutrition. These patients had hypocupremia, anemia, neurologic symptoms and bone changes. Their positive response to Cu supplement has been demonstrated (Halliday 1985). Malabsorption and low body store of Cu was suggested to be the major reasons for Cu deficiency in these premature infants as discussed in Section 1.2.1 (Al-Rashid and Spanglet, 1971).

The above observations suggest rat or mouse pups may not be good models for human infants in studying Zn and Cu absorption. For this reason, I investigated the ontogeny of Zn and Cu absorption mechanisms in piglets that represent an

animal model which is closer to the human.

1.2.3.2 Diet

1.2.3.2.1 Amount of Zn and Cu in diet

Because of the relatively high incidence of Zn and Cu deficiency observed in human premature infants, some investigators suggest giving them dietary Zn and Cu supplements regularly to prevent deficiency (Shaw 1979; Shaw 1980). However, the effects of dietary Zn and Cu supplements on Zn and Cu absorption have not been defined in early life. High dietary Zn intake was found to reduce the absorptive capacities of the intestine for Zn, Cu and Fe in adult animals (Cousins 1985). The rate of Zn uptake by the intestinal BBMV was significantly lower in adult rats fed with high Zn diet than in the control (Hunt 1981). In contrast, dietary Zn depletion enhanced Zn uptake by the intestinal BBMV in rats (Menard and Cousins 1983). The induction of a membrane protein of ca 45,000 daltons when restricting dietary Zn was shown by analysis of proteins of BBM using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in rats (Cousins 1985). Because of the correlation between membrane protein induction and increased Zn uptake by BBM, the association of this protein to membrane Zn transport was suggested. Neonates are more vulnerable to the excess or deficiency in dietary Zn or Cu contents because of the immaturity of the intestinal regulating mechanism.

Similar to the effect of high dietary Zn intake on Zn absorption, excess dietary Cu reduced Cu absorption in animals (McNaughton et al, 1974). Negative Cu balance has been observed in human premature infants receiving high dietary Cu intake (Casey and Hambidge, 1985). However, premature infants fed with a formula providing 300 μg Cu/100 kcal for 1 month did not have negative Cu balance (Tyrala 1986). Further studies are required to determine whether dietary Cu intake suppresses Cu absorption and how much dietary Cu will suppress Cu absorption in infants. These studies are necessary for setting an appropriate upper limit for Cu content in infant formula.

1.2.3.2.2 Mineral-mineral interactions

Because of the similarity in physicochemical properties of some elements, there are various interactions affecting their metabolism (Hill and Matrone 1970). These interactions may be either positive or negative. Positive interaction means the dependence of one or several other elements for the normal metabolic efficacy of the element in question, the best example of this is the interaction of Cu and Fe (Frieden 1983). Negative interaction means the normal metabolism of some elements is inhibited by the relative excess of other elements; this interaction is exemplified by Zn and Cu (O'Dell 1989). The interactions between elements are also characterized as direct (competitive) and indirect

(noncompetitive). If two or more elements compete for the same binding site on a ligand, the interaction between them is direct, all other interactions are indirect.

The sites of interaction for absorption may occur:

a) in the lumen of the small intestine where two or more elements form an insoluble complex such as Cu and Ca, Zn and Ca.

b) at the brush border membrane where they compete for the binding site on specific transport proteins.

c) in the cytosol of intestinal enterocytes where they compete for intracellular transport or storage proteins.

d) at the BLM where they compete for binding to transport proteins (Davis 1974).

1.2.3.2.2.1 Zn and Cu interactions

The negative interaction between Zn and Cu is probably the most significant in mineral-mineral interactions (Cousins 1985). Zn and Cu may interact during absorption, compartmentalization and excretion. The molecular mechanism of the interaction between Zn and Cu lies in their similar electron configurations and ionic radii. Because of this similarity, Zn may be displaced from its complexes by Cu and vice versa (O'Dell 1989).

Dietary Zn^{2+} and Cu^{2+} may compete for the metal-binding sites on intestinal metallo-proteins. For example, high

dietary Zn may induce MT in intestinal mucosa but MT has higher affinity for Cu than for Zn. Therefore Cu is retained in the intestinal mucosa and the net absorption of Cu is reduced (Cousins 1985). Whether Zn and Cu may compete for their transport proteins located on intestinal BBM or BLM has not been determined.

Because the interaction for absorption becomes particularly distinct when one element is in excess compared to the other, the ratio of absorbable Zn and Cu is more important than the absolute amount of Zn and Cu in the diet. For example, high dietary Cu intensified clinical Zn deficiency symptoms in rats fed Zn deficient diet (Suttle and Mills, 1966); excess dietary Zn in combination with adequate dietary Cu lowered tissue and plasma Cu concentrations in rats (Magee and Matrone 1960).

Since most of the studies on Zn and Cu interaction at the level of absorption were conducted in adult rodents, the mechanism of Zn and Cu interaction in early life is not defined. This information is particularly important for determining the appropriate ratios of these two elements in infant formula. Because of the difficulty in examining the mechanism of Zn and Cu interaction in infants, I studied the interaction in a developing piglet model and an in-vitro system. In this model the interaction between Zn and Cu, at a ratio similar to that in infant formula, for transport across

the BBM of intestinal enterocytes was defined.

1.2.3.2.2.2 Cu and Fe interactions

Fe-fortified infant formula is effective in preventing Fe deficiency in early life (Lonnerdal 1989). However, high levels of dietary Fe may interfere with the absorption of other trace elements, such as Cu. Because of the function of Cu in Fe utilization, the reduced Cu absorption may impair Fe nutrition in infants (Frieden 1983).

The importance of Cu in Fe utilization was first suggested by Hart et al (1928). They found that Fe deficiency anaemia in rats could be cured only if, along with Fe, Cu was also supplied. Cu deficiency may impair Fe absorption, intermediary metabolism and erythropoiesis (Frieden 1983). Ceruloplasmin, the major Cu transport protein, acts as a ferroxidase in the oxidation of Fe^2 as it is released from hepatocytes and converted to Fe^3-transferrin. This Fe mobilization is critical for body Fe utilization (Frieden 1983).

The direct effect of excess dietary Fe on Cu absorption is controversial. Haschke et al (1987) found that preterm infants fed $FeSO_4$ fortified preterm formula had negative Cu balance. However, Keen et al (1984) did not detect any alterations in Cu absorption in infants fed a formula fortified with Fe as Fe-lactoferrin. The different Fe

supplements used in these two studies may explain different results. If $FeSO_4$ does suppress Cu absorption, what is the effect of other Fe compounds? Fe-lactoferrin may be a good Fe supplement, but it is relatively more expensive than simple Fe compounds. Since infants often receive oral Fe supplements, determining an appropriate amount and form with less interactive effects for the absorption of other trace elements is desired.

1.2.4. The effects of glucocorticoids on development and Zn, Cu metabolism in neonates

Exogenous glucocorticoids are commonly used to treat chronic lung disease caused by lung immaturity and long-term mechanical ventilation in premature infants (Yeh et al 1990). Although exogenous glucocorticoids have been demonstrated to induce the maturity of lungs and several other organs in neonates (Smith and Post 1989), they may also alter the metabolism of many nutrients, such as protein and carbohydrate (Balazs and Cotterrell 1972). Some effects of glucocorticoids on Zn and Cu metabolism have also been reported, such as the induction of hepatic MT. However, most of these results were obtained in adult animals. The influence of glucocorticoids on nutrient metabolism is more complicated in infants because glucocorticoids may induce the maturity of several important enzymes which participate in nutrient metabolism (Yuwiler and

Celler 1973; Celano et al, 1977).

1.2.4.1 Glucocorticoids and development

Glucocorticoids have been documented to play an important role in fetal development and maturation. During pregnancy the overall production and metabolism of cortisol is characterized by increased transcortin concentration in the maternal compartment, which results in a 3-4 fold higher plasma level than in non-pregnant women. The fetal adrenal gland starts to produce cortisol in the first trimester of pregnancy, utilizing either placental progesterone or 3ß-hydroxy-5-ene C_{21} precursors of fetal origin. Most of the cortisol produced circulates as cortisone and cortisol becomes predominant only during the last trimester. The fetus also receives a contribution from the maternal compartment. Using isotopic infusion studies, it was calculated that at term the maternal contribution to fetal cortisol is 25-50%, while the fetal contribution to maternal cortisol is negligible (Fisher 1992).

At present, it is well established that cortisol induces lung maturation and controls the synthesis of pulmonary surfactant in the fetus (Smith and Post, 1989). Glucocorticoids have also been demonstrated to induce the maturation of liver, pancreas and intestine in fetal and neonatal animals (Balistreri 1992; Puccio 1988; Peitsch 1984).

In the liver, many maturational events may be controlled by glucocorticoids. They may induce structural differentiation of the liver, such as involution of hepatic hemopoietic cells, development of bile canaliculi and fluidization of microsomal membranes (Kaufman 1992). Furthermore, some enzyme activities, such as hepatic glutathione S-transferases and selected P450 related activities, are also evoked by glucocorticoids in infant animals (Kaufman 1992). In newborn animals, glucocorticoid treatment induced the hypertrophy and hyperplasia of the pancreas and the increase in intestinal amylase and hydrolase activities (Puccio 1988). Corticosteroids enhance gastrointestinal mucosal growth and the responsiveness of the parietal cell to secretory stimuli. These effects could result from promoting gene transcription and protein synthesis in the mucosal cell by glucocorticoids (Peitsch 1984). In addition, exogenous glucocorticoids were demonstrated to increase the fluidity of cell membranes and decrease the permeability of intestinal mucosa, thereby inducing earlier gut closure in infant animals, particularly in rats (Daniels 1973).

Although glucocorticoids selectively induce the maturation of some organs, the overall body DNA, RNA and protein synthesis is decreased in glucocorticoid treated infant animals. Balazs and Cotterrell (1972) found that daily treatment of infant rats with steroids from 1-5 days of

postnatal life resulted in a significant decrease in the incorporation of labelled thymidine into brain cells which indicates impaired cell replication. Howard (1968) reported that injection of corticosterone reduced the body weight by 10% and brain weight by 21% in infant rats. Dexamethasone therapy significantly retarded normal growth of human premature infants (Yeh et al, 1990; Kazzi et al, 1990; Brownlee et al, 1993).

1.2.4.2 Glucocorticoids and Zn and Cu metabolism

In adult animals, glucocorticoids have been documented to induce the synthesis of tissue MT and thereby redistributing body Zn and Cu (Bremner and Beattie 1990). A glucocorticoid responsive element has been identified in the 5′ upstream region of the MT gene. The binding of glucocorticoid-receptor complex to this element effectively induces the expression of the gene (Hager and Palmiter, 1981). Glucocorticoids also stimulate Zn uptake by Hela cells, hepatic cells in culture and intestinal enterocytes. It was suggested that the elevated Zn uptake by these cells is the result of intracellular MT induction (Cousins 1988). However, glucocorticoids up-regulated Zn uptake by intestinal enterocytes but did not induce intestinal MT (Hempe et al, 1991). A direct effect of glucocorticoids on membrane Zn transport protein(s) or membrane phospholipid composition could be the mechanism responsible for the elevated Zn uptake

observed in these studies. Bonewitz et al (1983) found that DEX increased intestinal BBM Zn transport by 70% and decreased BLM Zn transport by 40%, further supporting the direct regulation of membrane protein by DEX. The physiological significance of the body Zn redistribution induced by glucocorticoids may be related to the host defence in the conditions of stress and infection.

The effect of glucocorticoids on Zn and Cu metabolism in fetal and newborn animals have not been investigated. However, the impact of glucocorticoids on Zn and Cu absorption is implicated by their effect on intestinal maturation.

1.2.5 Biochemical indices of body Zn and Cu status

At present, no sensitive and specific index of Zn status exists clinically. Diagnosis of Zn deficiency in humans must be confirmed by a positive response to Zn supplementation (Gibson 1989). Serum or plasma Zn concentration is a most frequently used index of body Zn status. However, this index is subject to the effects of many other factors, such as infection, stress, plasma albumin concentration etc (Gibson 1989; Markowitz et al, 1985), most of these factors induce the redistribution of Zn from plasma to liver. Urinary Zn measurement may provide information about the status of Zn excretion. All other biochemical indices of Zn status have not been well established, for example, erythrocyte Zn content is not always lower during experimental Zn deficiency; Zn levels

in neutrophils, platelets, saliva and sweat are difficult to measure, and more work is needed to confirm the validity of these measurements (Hambidge et al, 1988). Recently erythrocyte MT concentration was suggested by Cousins et al (1990) to be a good index of body Zn status in humans, but further research is required to validate its application. Compared with erythrocyte MT, liver MT concentration is a much better index of Zn status because liver is the major Zn storage organ in humans. The measurement of hepatic Zn and MT and plasma Zn could provide accurate information about body Zn status. In addition, the capacity of the intestine to absorb Zn is also a good indicator of body Zn status because Zn absorption is regulated according to body requirements.

Hypocupremia is an early and consistent manifestation of experimental Cu deficiency (Suttle and Angus, 1976). However, as in the case of Zn, plasma binding proteins, infection, stress, diurnal variation and exogenous hormones may change Cu concentration in blood circulation (Solomons 1979). Because these factors redistribute Cu from plasma to liver, the measurement of liver Cu in addition to circulating Cu may overcome the effects of these factors in animal models. The measurement of erythrocyte Cu, hair Cu, urinary Cu in Cu deficiency all have limitations which preclude their use as a single, valid index of body Cu status (Solomons 1979). The activities and quantities of Cu metalloenzymes have been

assessed in the condition of Cu deficiency. Apoceruloplasmin quantification by sensitive immunoassays showed some potential as an index of Cu nutriture and erythrocyte Cu,Zn-superoxide dismutase content was found to fall rapidly in Cu deficiency in swine. L'Abbe and Friel (1992) found that erythrocyte Cu,Zn-superoxide dismutase activity is a more appropriate indicator of Cu status in the very low birth weight infants than plasma Cu. Other Cu metalloenzymes, such as serum amine oxidase and leucocyte cytochrome C oxidase decline in patients with Menke's disease (O'Dell 1990). The combination of plasma Cu and Cu metalloenzyme indices may improve the assessment of Cu nutrition in adults. However, the activities and contents of Cu metalloenzymes experience maturational change during the first several months of life which preclude them as useful indices of Cu status in infants or young animals.

MT is a major Cu storage protein which is induced by excess body Cu status (Bremner and Beattie, 1990). Although there is no evidence for using tissue MT content as a single index of Cu status, the measurement of hepatic MT in combination with hepatic and plasma Cu may help us to understand Cu status more completely.

We used plasma and hepatic Zn, Cu and MT concentrations in piglets as indices to determine body Zn and Cu status, including their distribution and storage.

1.2.6 Advantages of using piglets as experimental model

Due to the similarity in digestive, respiratory, renal and cardiovascular systems between human and pigs, neonatal piglets are a good model for studying nutrient metabolism in human infants (Cooper, 1975). Although humans have a gestation period more than twice that of pigs, the degree of maturity at birth are comparable between human and pigs. A normal sized newborn piglet weighs about 1.4 kg, which is closer to the human newborn (2.4-3.8 kg) than the dog (0.4 kg) and the rat (0.005 kg). The chemical compositions of fat-free body tissue (water, protein and ash) at birth are similar for man and pigs. The nutrient requirement of piglets resembles that for human infants in more ways than any other non-primate species. Pig's milk has more than 6 times the protein content of human milk (7.1 vs 1.2) while fat and water contents are similar. Zn and Cu concentrations in pig milk are 76 μmol/L and 11.4 μmol/L respectively, which are closer to those in human milk (Zn=45 μmol/L, Cu=3.46 μmol/L) than those of other species. On the other hand, the piglet has a more rapid growth rate, higher body temperature, high metabolic rate and lesser fat reserve than human infants (Glauser 1966).

Zn was shown to be an important nutrient for pigs. Young pigs fed Zn deficient diets had severe dermatitis, diarrhea, vomiting, anorexia, loss in weight, thymic atrophy, suppressed T cell functions and finally death (Tucker and

Salmon, 1955). Cu deficiency in young growing pigs is manifest by microcytic hypochromic anaemia, low ceruloplasmin levels, reduced erythrocyte life span, crooked legs with bone disorders, poor appetite, retarded growth, cardiac hypertrophy, aortic rupture and ataxia (Hill et al, 1983). These symptoms are very similar to those of severe Zn and Cu deficiency observed in human infants. The fetal pig stores a large amount of Cu in the liver which is released into the blood circulation after birth. This release results in the seven fold increase in plasma Cu concentration within two weeks after birth. When a sow was fed high Zn diet during gestation, there was little fetal storage of Cu (Hill et al, 1983). The competition between Zn and Cu for absorption has also been shown in humans and high Zn intake has been used successfully to treat Wilson's disease (Hoogenraad et al, 1987). Because of the similarities in development as well as in Zn and Cu metabolism, the piglet is an appropriate model to study Zn and Cu metabolism in neonates and infants.

Chapter 2

METHOD DEVELOPMENT FOR ASSESSING Zn AND Cu ABSORPTION

2.1 Introduction

There are various biochemical and physiological approaches to determine Zn and Cu absorption in humans and animal models. These methods can be generally classified as *in-vivo, in-vitro* and *in-situ* experiments. Compared to the *in-vivo* methods, the other two approaches are more direct and may assess the function of each step of Zn and Cu absorption separately. In this chapter I will discuss how I developed and validated the *in-vitro* and *in-situ* experimental methods to study intestinal uptake and transfer of Zn and Cu in a neonatal piglet model. The applications of these methods to study Zn and Cu absorption in relation to ontogeny and drug and dietary interventions will be presented and discussed in following chapters.

2.2 Method development and validation

2.2.1 Methods for determining BBMV Zn uptake

2.2.1.1 Preparation of mucosa samples

Male Yorkshire piglets aged 3-4 d were obtained from Arkell Research Farm (Guelph, Ontario, Canada). The animals

were weaned to a pig formula (Appendix I) and euthanized at 20 d of age by injecting an overdose of sodium pentobarbital, i.p. One hundred cm of the proximal, medial and distal small intestine were excised at necropsy and immediately immersed in ice-cold 0.9% saline. The intestine was slit lengthwise and then rinsed with ice-cold saline. Epithelial mucosa was scraped from the muscle tissue with a glass slide and stored at -70° C for further preparation. Scraping was performed on ice to minimize enzymatic activity.

2.2.1.2 Preparation of BBMV

Intestinal BBMV were obtained by homogenizing the mucosa and employing a differential centrifugation method (Davidson and Lonnerdal 1988)(Appendix II). Since sucrase is located at the outside surface of intestinal BBM only, the enrichment in sucrase activity is a indicator of the purity of BBMV preparation. Several researchers suggest that it is an reliable marker for BBMV purity and the extent of variation between BBMV preparations (Davidson and lonnerdal 1988; Menard and Cousins 1983). They found that a BBMV preparation with a 10-15 fold increase in sucrase activity over that of the original homogenate is appropriate for trace element transport study. In this study sucrase activity was assayed regularly as the liberation of glucose on incubation with sucrose (Dahlqvist 1968)(Appendix VII) in BBMV preparation and the

original homogenate. The measurement of sucrase activity before and after lysing vesicles with hypotonic solution was conducted to asses vesicle resealing and orientation. The BBMV preparation was used in the ^{65}Zn transport study the same day to avoid vesicle damage caused by freezing and thawing.

2.2.1.3 Zn uptake at different Zn concentrations

A modification of the technique of Menard and Cousins (1983) was used to perform transport experiments. Approximately 100 μg of vesicular protein, and an incubation buffer containing 300 mM D-mannitol, 10 mM Tris-HEPES at pH 6.7 with a total volume of 50 μL, were added to a test tube containing 50 μL of the same buffer but containing increasing $ZnCl_2$ concentrations (0.1 to 0.5 mM) and 37 kBq $^{65}ZnCl_2$ (DuPont Chemical Co, Wilmington, DE). After a 1 min incubation at 37° C, uptake was terminated with 1 mL of ice-cold stop solution (300 mM D-Mannitol, 10 mM Tris-HEPES, pH 6.7). The BBMV were collected on 0.22 μm filters (Millipore, Groton, CT) using vacuum. The tubes were rinsed twice more with stop solution to ensure quantitative collection of the vesicles. The filters were assayed for radioactivity by a gamma counter (Beckman Gamma 5500, Irvine, CA). Samples were prepared in triplicate with a blank (no BBMV) included to correct for non-specific binding of ^{65}Zn to filters. Uptake rates (V) were expressed as nmol Zn^{2+} per mg total vesicular protein per min (Appendix

III).

Total protein was measured by the dye-binding procedure of Bradford (Bradford 1976) using crystalline bovine serum albumin as a standard. The mean between batch precision for vesicle protein determination was 4.3% ± 0.7% (CV).

2.2.1.4 Determining kinetic parameters for Zn uptake by BBMV

Maximum uptake rate (V_{max}) and the half saturation constant (K_m) were determined using the method described by Eadie (1942) and Hofstee (1952)(Appendix V). The Eadie-Hofstee plot is considered the best technique for the analysis of uptake data (Zivin and Waud, 1982). Our Eadie-Hofstee plot regression curves for individual studies had correlation coefficients in the range 0.70 to 0.99.

The Zn uptake data were further analyzed by fitting to an equation describing the combination of carrier mediated and passive diffusion (Herd S.M. et al, 1987):

$$v = \frac{V_{max}*S}{K_m + S} + P*S \qquad (1)$$

by non-linear regression, using the computer program Fig P 6.0 (Biosoft, Cambridge, UK).

V_{max} is the maximum velocity for saturable Zn uptake, K_m is the apparent Michaelis-Menten constant and P is the first order rate constant for non-saturable Zn uptake. S is

the concentration of Zn in medium.

2.2.1.5 Time course of BBMV Zn uptake

Because the flux of Zn across BBMV is bi-directional, only the measurement of initial uptake (when the amount of Zn in BBMV is negligible) represents the net uptake. To determine the appropriate incubation time to measure the initial uptake, the time course of Zn uptake was first studied. The uptake study was performed at increasing incubation times (0.25 to 20 min) with constant Zn^{2+} concentration (0.2 mmol/L).

2.2.1.6 Zn uptake at different BBMV concentrations

The uptake study was also performed using BBMV concentrations as the changing variable. Zn^{2+} concentration (0.3 mmol/L) and incubation time (1 min) were held constant while BBMV concentrations were increased from 17 to 280 $\mu g \cdot 50\ \mu L^{-1}$.

2.2.1.7 The effect of temperature on BBMV Zn uptake

A Zn uptake experiment was also performed at 4°C instead of 37 °C to estimate the contribution of binding and passive diffusive components in the Zn uptake measured by this method.

2.2.1.8 Zn binding to BBMV during uptake

The amount of ^{65}Zn binding to BBMV was further determined by using hypotonic buffer to terminate Zn uptake. Because membrane vesicles were broken in the hypotonic

condition, the Zn^{2+} uptake measured in this condition showed the amount of Zn^{2+} binding to BBMV.

2.2.2 Method for determining Zn efflux across BBMV

BBMV were first loaded with ^{65}Zn via incubation with 0.3 mmol/L $ZnCl_2$ and 2 μCi ^{65}Zn in the incubation buffer at 37°C for 60 min. After examining the amount of Zn loaded in BBMV (the amount of ^{65}Zn loaded in BBMV after a 60-min incubation was estimated to be 4.64±0.48 μmol/mg protein), the incubation solution was diluted 19-fold with transport buffer (10 mmol/L Tris-HEPES, 300 mmol/L D-mannitol, pH 6.7) containing 10 mmol/L EGTA. At different time intervals after the dilution, the percentage of ^{65}Zn remaining in BBMV was determined by rapid filtration and counting of filters in a gamma counter (Beckman Gamma 5500).

Incubation was also performed at 4°C instead of 37°C for 60 min to determine whether BBMV Zn accumulation occurred via active uptake or passive diffusion and binding to the membrane.

2.2.3 Methods for measuring BBMV Cu uptake

2.2.3.1 Preparation of $^{64}CuCl_2$

Cu powder (1 mg, laboratory reagents, BDH Chemicals, Toronto, Canada) was irradiated at the Nuclear Reactor of McMaster University for 2.5 h to produce ^{64}Cu with a specific activity about 3.7 x 10^7 Bq/mg. ^{64}Cu powder was first dissolved

in a few drops of concentrated ultrapure HNO_3 (J.T.Baker Inc., Phillipsburg, NJ, USA). This solution was evaporated to dryness under a heat lamp. Three drops of HCl (certified A.C.S., Fisher Scientific, Nepean, Ont, Canada) were added to convert $Cu(NO_3)_2$ to $^{64}Cu_2Cl_2$. The excess acid was evaporated and $^{64}CuCl_2$ was redissolved in incubation buffer (300 mmol/L D-mannitol and 10 mmol/L Tris-HEPES, pH 6.7) containing 0.2 mmol/L $CuCl_2$ for the time curve and 1 mmol/L $CuCl_2$ for the kinetic measurement of Cu transport.

2.2.3.2 Cu uptake at different Cu concentrations

A modification of our technique for Zn transport was used to study Cu uptake by BBMV (Appendix IV). Vesicular protein (ca 40 μg) was incubated with incubation buffer (300 mmol/L D-mannitol, 10 mmol/L Tris-HEPES, pH 6.7) containing 2 μCi ^{64}Cu and increasing concentrations of $CuCl_2$ (total volume 200 μl) in a 96-well vinyl assay plate (Costar, Cambridge, MA) at 25 °C for 1 min. Cu uptake by BBMV was terminated by rapid filtration of the incubation solution on 0.45 μm filters of 96 filter well plates which were set on the Millipore MultiScreen™ Filtration System (Millipore, Groton, CT) under vacuum. Filters were rinsed twice more with the incubation buffer containing 5 mmol/L EDTA and then punched off the plate and measured for radioactivity in a gamma counter (Beckman Gamma 5500, Irvine, CA). Samples were prepared in triplicate

with a blank (no BBMV) included to correct for non-specific binding of ^{64}Cu to filters. Radioactivity measurements were corrected for decay by a decay curve (Appendix IV).

2.2.3.3 Time course of Cu uptake by BBMV

$^{64}CuCl_2$ (2 μCi) was added into 0.75 mL incubation buffer containing 0.2 mmol/L $CuCl_2$. After adding three 20 μl aliquots of this isotope solution to filters to determine non-specific binding, about 700 μg BBMV protein was added into the isotope solution incubated at 37 °C. At different time intervals (0-30 min) triplicate 20 μl samples were filtered to terminate Cu uptake by BBMV.

2.2.3.4 Cu binding to BBMV during uptake

$^{64}CuCl_2$ (1.85 kBq) was added into 0.3 mL incubation buffer containing 0.2 mmol/L $CuCl_2$ and 200 - 800 mmol/L D-mannitol incubated at 37 °C. After determining non-specific binding of ^{64}Cu to filters, about 300 μg BBMV protein was added. Uptake was terminated at 5 min. Results were plotted as nmol Cu uptake per mg protein per 5 min *vs* 1/osmolarity. Binding of Cu to BBMV was then determined by extrapolating the plotted line to the ordinate.

2.2.4 Method for in-situ intestinal perfusion

2.2.4.1 Determining Zn absorption by in-situ intestinal perfusion

Piglets were fasted overnight before the intestinal

perfusion experiment. They were anaesthetized by isoflurane (Aerrane[R], Anaquest, Mississauga, Ontario) inhalation. After opening the abdominal cavity with a middle incision, a 10 cm segment of proximal jejunum was isolated, cannulated and flushed with 60 mL of air. The cannulated segment was then returned to the abdominal cavity. Body temperature of the piglets was maintained at 36-37°C by a heating pad and infrared lamp during the perfusion. The cannulated intestinal segment was perfused with 30 mL perfusate in-situ at a rate of 0.5 mL/min for 30 minutes in a recirculating manner with the aid of an infusion pump (IVAC 530, IVAC Corporation, San Diego, CA)(the composition of the perfusate: D-mannitol 260 mmol/L, D-glucose 20 mmol/L, Tris-HEPES 10 mmol/L, $CaCl_2$ 2 mmol/L, $ZnCl_2$ 0.2 mmol/L, Poly R478 6 mg, $^{45}Ca^{*}$ 550 kBq/30 mL, ^{65}Zn 740 kBq/30 mL, pH 6.7, osmolarity 315 mosmo/L. [$^{*45}Ca$ was used by another graduate student to study Ca absorption in the same piglets]. Perfusate samples of 200 µL were collected at time '0' and every 5 min thereafter for 30 min. A 0.5 mL blood sample was drawn from the portal vein at time '0' of the perfusion and every 8 min thereafter for 60 min. After taking each blood sample, 0.5 mL of 0.9% saline was infused into the portal circulation to compensate for blood loss. At the end of blood sampling, the intestinal segment was flushed with 60 mL air and then excised.

After the perfusion, Poly R-478 concentration in the perfusate samples was measured directly by a spectrometer at 515 nm (Stahl et al 1991). Zn concentration in the perfusate samples was determined directly by atomic absorption spectrometry (Perkin Elmer, model 703) to calculate net Zn absorption. Dry weight of the ligated intestinal segment was determined after drying the segment at 100°C for 48 hr. The dry intestinal segment was crumbled with a glass pestle. The total liver was removed, rinsed with ice-cold 0.9% saline and homogenized. ^{65}Zn radioactivity in the perfusate, blood, liver and perfused intestinal segment was measured in a gamma counter (Beckman Gamma 5500, Irvine, CA). The volume of liquid or solid samples was kept same between piglets when they were measured by the gamma counter.

2.2.4.2 Calculation of net Zn absorption

Disappearance of elemental Zn from the lumen was taken to indicate net Zn absorption during intestinal perfusion. The calculations are shown below.

Net Zn absorption:

μmols/30 min= $V_i[Zn_i - Zn_f \cdot \text{Poly } R_i/\text{Poly } R_f]$; (2)

V: volume perfused in mL/30 min;

[Zn]: total concentration of Zn measured by AAS as μmole/mL;

Poly R: Poly R478 concentration in μg/mL;

i or f: initial or final values in the perfusate.

2.3 Results

2.3.1 Zn uptake by BBMV

Periodic sucrase assays before and after the BBMV preparation produced 10 $\pm$ 2.5 fold increases in specific activity. There was no significant change in sucrase activity before and after lysing the vesicles.

Fig 2 shows the saturation curve of BBMV Zn uptake. The results of curve fitting indicates that Zn uptake by BBMV in piglets is via a combined mechanism of saturable and non-saturable transport.

Fig 3 shows the time course of BBMV Zn uptake when extravesicular Zn concentration was 0.2 mmol/L. The appropriate incubation time for measuring the initial uptake was estimated by this curve to be 1 min.

Fig 4 presents Zn uptake at various BBMV concentrations. This curve indicates that the range of BBMV concentrations which produce consistent study results is from 30 to 200 μg BBMV protein.

Zn^{2+} uptake was significantly lower at 4°C compared to 37°C (Fig 5).

Fig 6 shows the effect of changing osmolarity of the termination solution on Zn^{2+} uptake by BBMV. Compared to the Zn^{2+} uptake using isotonic termination solution, the Zn^{2+} uptake was significantly lower and was not saturable when

using hypotonic termination solution.

No significant differences were detected in the kinetic parameters of Zn transport among the proximal, medial, and distal sections of the small intestine. Duodenal transport was not measured because an insufficient amount of mucosa was available from this segment.

2.3.2 Zn efflux across BBMV

After a 0.5 min efflux, there was 7.2% of loaded ^{65}Zn remained in BBMV and decreased to 3.6% after 5 min efflux (Fig 28, Chapter 5, curve for the control group). Compared with the amount of ^{65}Zn accumulated at 4°C, there was 21.5% more ^{65}Zn loaded in BBMV after a 60 min incubation at 37°C ($p<0.05$).

2.3.3 Cu uptake by BBMV

Figure 7 shows the change in Cu uptake as extravascular Cu concentration increased from 0 to 0.6 mmol/L. The hyperbolic curve strongly suggests saturation kinetics. Uptake data was fitted to equation (1). The calculated parameters from the equation were: V_{max} 8.33 nmol Cu/min/mg protein, K_m 39 μmol/L and P value of 3.79 μmol/L. These parameters indicate that transport mechanism for Cu is a combination of facilitated and passive diffusion.

At an extravesicular Cu concentration of 0.2 mmol/L, Cu uptake increased quickly with time. A steady-state of intravesicular Cu level that represents the balance between

influx and efflux was reached by 2 min (Fig 8) and this steady-state was maintained for 30 min. At higher extravesicular Cu concentrations (1 and 2 mmol/L), steady-state of Cu uptake and efflux still started at about 2 min and was maintained for 30 min, but the amount of Cu associated with BBMV at this state were greater than that at 0.2 mmol/L Cu. The uptake increased proportionally with the Cu concentration in incubation medium.

Fig 9 demonstrates the Cu uptake time course at two different temperature: 4°C and 37 °C. The lower temperature did not change the time course for Cu uptake but significantly reduced the uptake at steady-state. The temperature dependence of Cu uptake suggests a Cu carrier protein could be involved in Cu transport.

As extravesicular osmolarity increased, Cu uptake decreased because of the reduced intravesicular space. If extravesicular osmolarity increased infinitely the intravesicular space would be negligible. Under such a condition the amount of Cu associated with BBMV represents the extent of binding. We determined the extent of Cu binding by extrapolating the plot of osmolarity versus Cu uptake to negligible intravesicular space (the intercept at y axis). The results are shown in Fig 10, which suggest there is about 3 nmol Cu/mg protein bound to BBMV at equilibrium under

conditions of 0.2 mmol/L extravesicular Cu.

2.3.4 Zn absorption determined by in situ intestinal perfusion

About 50% ^{65}Zn radioactivity in the perfusate disappeared during the first 5 min perfusion. Then the disappearance curve reached saturation. This steady-state that represents the balance of ^{65}Zn influx and efflux across intestinal BBM was maintained until the end of 30 min perfusion (Fig 35, Chapter 6, curve for the cotrol group). As the ^{65}Zn radioactivity in the perfusate gradually decreased, the ^{65}Zn radioactivity in portal blood increased and reached the highest point at the end of 30 min perfusion. After this point, the radioactivity in portal blood gradually declined but did not go back to the baseline at the end of experiment (Fig 37, Chapter 6, curve for the control group). The calculated net Zn absorption during 30 min perfusion is 108$\pm$39 μg/g dry wt of intestine as mean$\pm$SD from 6 normal 20 d old male piglets (Fig 36, Chapter 6, data for the control group).

2.4 Discussion

Zn uptake by intestinal epithelial cells is the first step in Zn absorption. This step controls and regulates the amount of dietary Zn entering the epithelial cells. Compared to other steps in Zn and Cu absorption, this step is more easily affected by various dietary and pharmacological factors

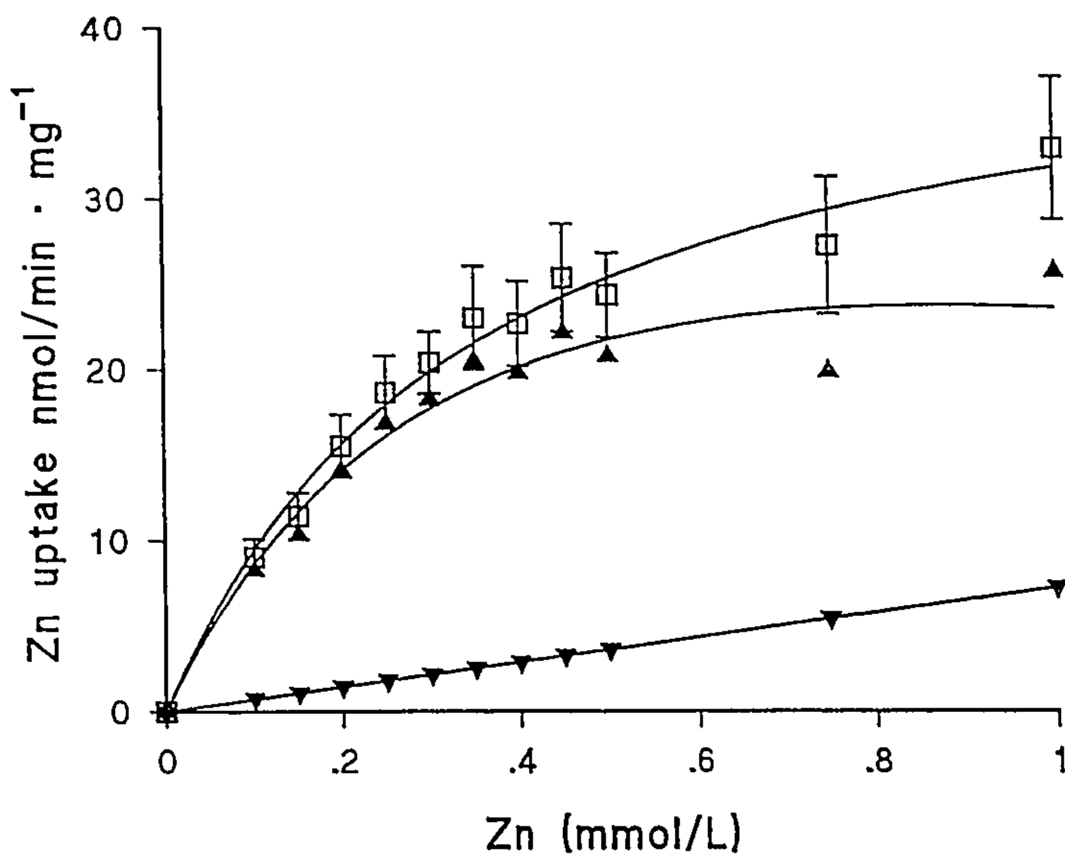


Figure 2, Initial rates of Zn uptake by BBMV with extravesicular Zn concentrations ranging from 0 mM to 1 mM. BBMV were obtained from 6 normal 20 d old male piglets fed an adequate amount of Zn. Data represent mean ± SEM. (■) The curve obtained by fitting the data to equation (1) represents the sum of a saturable and a linear components. The proposed saturable (▲) and linear (▼) components of Zn uptake are calculated according to the constant (P) obtained from the curve fitting.

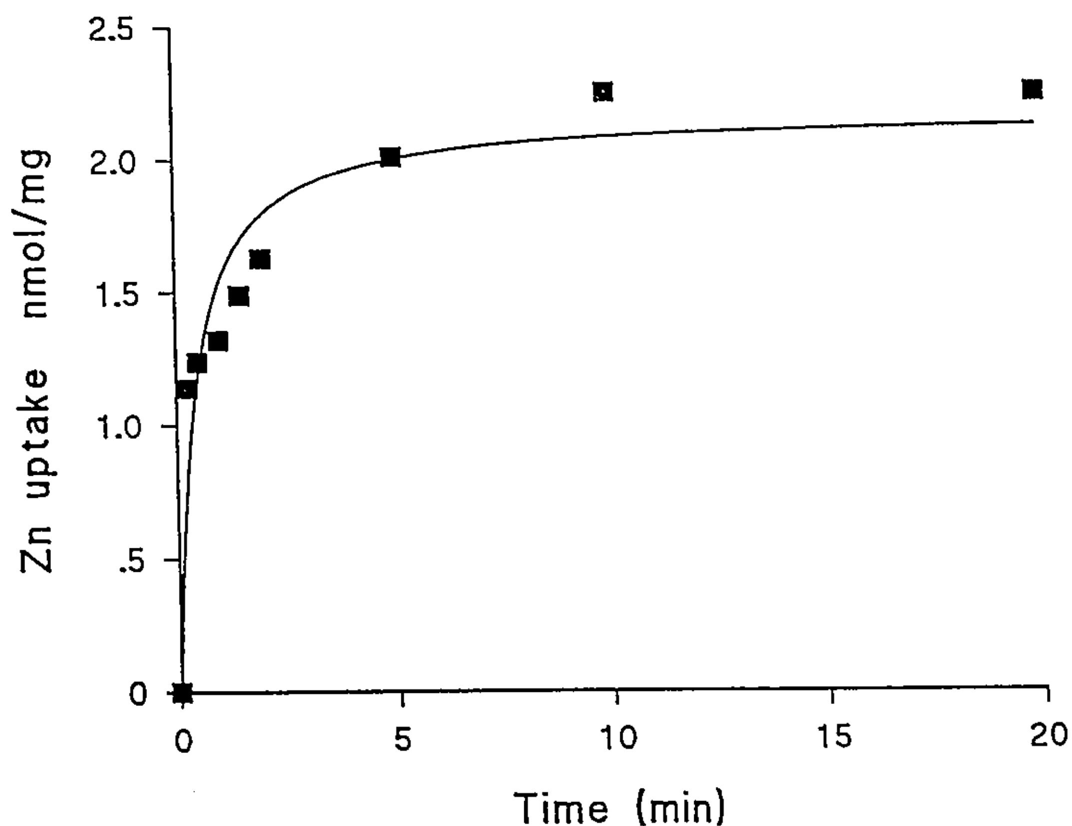


Figure 3, Rate of Zn uptake by BBMV expressed as a function of time ranging from 0.25 to 20 min. Zn concentration of the medium was kept constant at 0.2 mmol/L. The BBMV were recovered from a normal 20 d piglet not involved in this study. The curve was obtained by fitting data with equation (1).

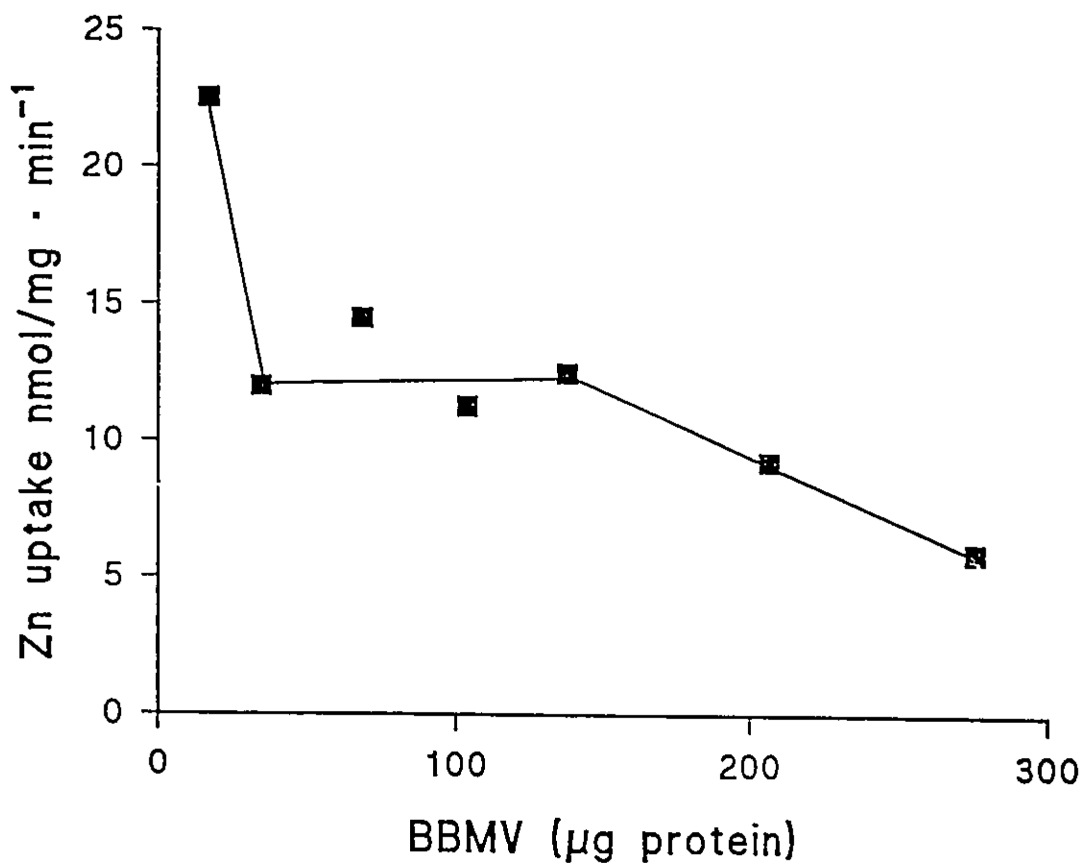


Figure 4, Rate of Zn uptake expressed as a function of BBMV concentrations from 17 to 280 μg membrane protein·50 μl^{-1}. Zn concentration of the medium was kept constant at 0.2 mmol/L. The incubation time was 1 min. The BBMV were recovered from a normal 20 d piglet.

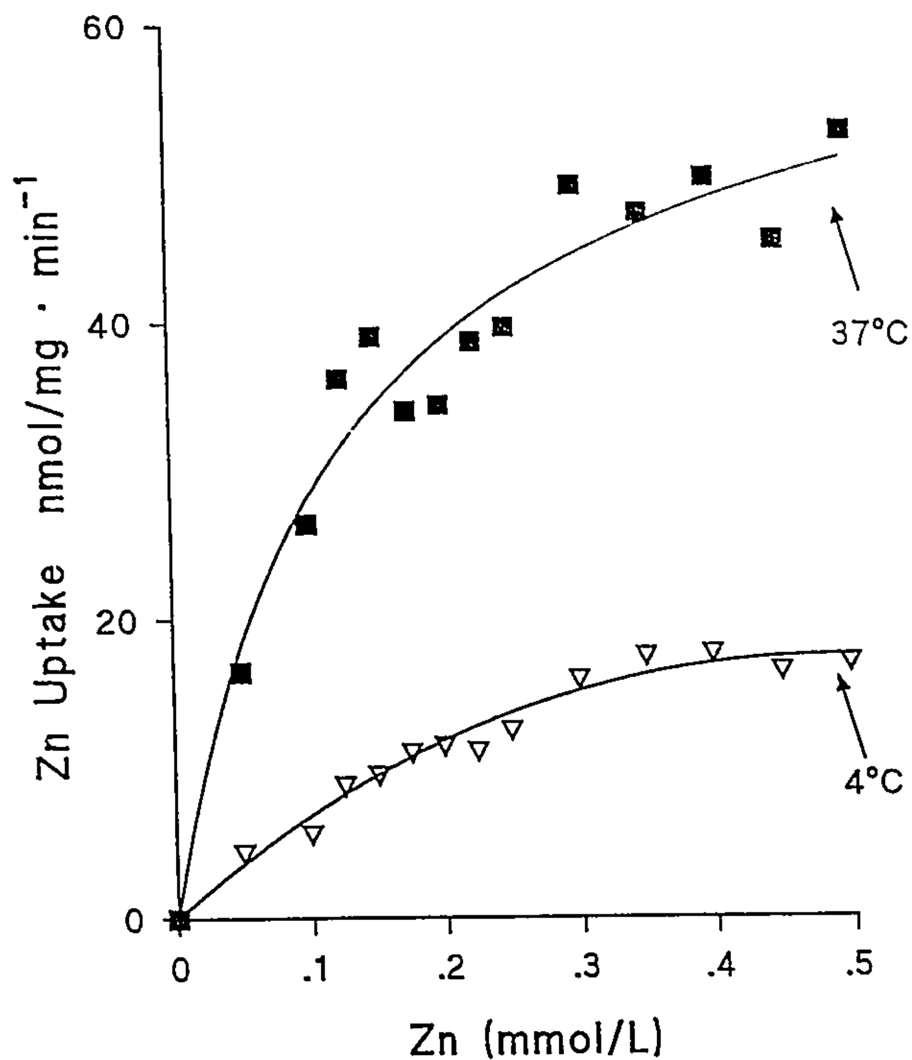


Figure 5, The effect of temperature on Zn uptake by BBMV. BBMV were incubated with Zn^{2+} at different concentrations at either 37°C or 4°C for 1 min. The BBMV were recovered from a normal 20 d piglet.

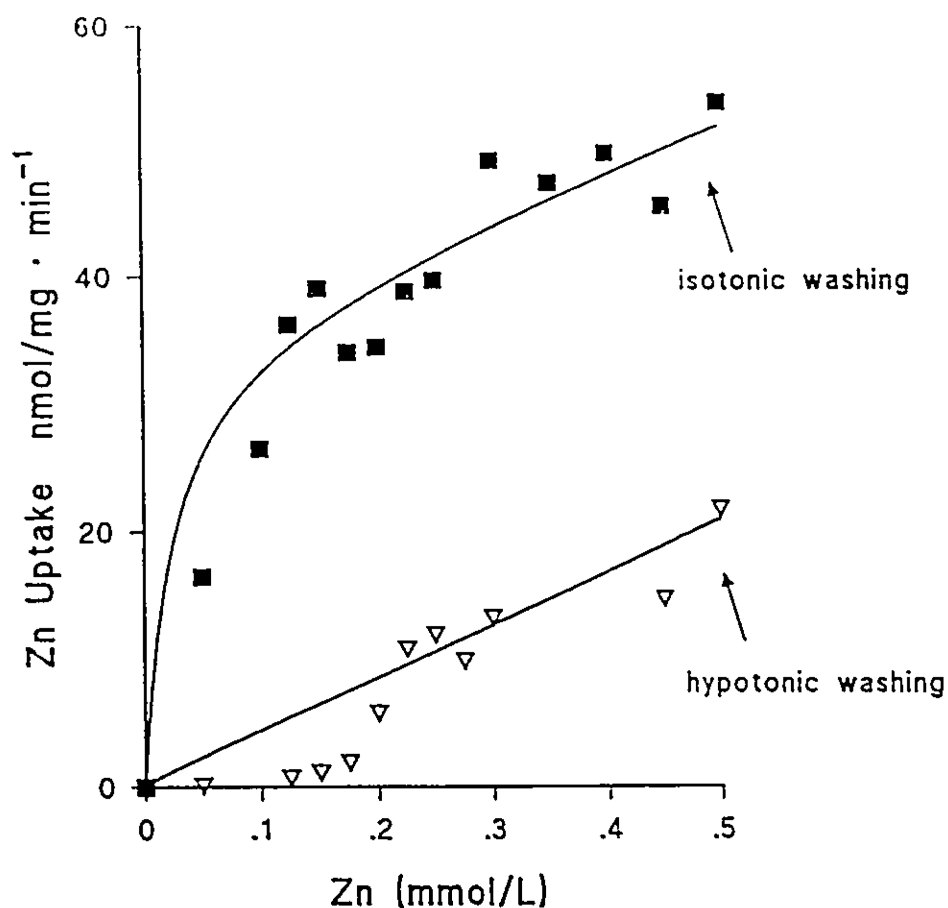


Figure 6, The effect of the osmolarity of the termination solution on Zn uptake. A BBMV preparation was divided into two equal parts. Both were incubated with Zn^{2+} at different concentrations at 37 °C for 1 min. After the incubation, 1 ml ice cold isotonic (10 mmol/L Tris-HEPES, 300 mmol/L D-manitol, pH 6.7) or hypotonic (0.1 mmol/L Tris-HEPES, pH6.7) buffer was added to terminate Zn uptake. The membrane vesicles were quickly separated from free Zn^{2+} via rapid filtration. The filters were then washed twice with either isotonic or hypotonic buffer. The curves were obtained by fitting data with equation (1) (isotonic washing) or an equation for first order kinetics (hypotonic washing).

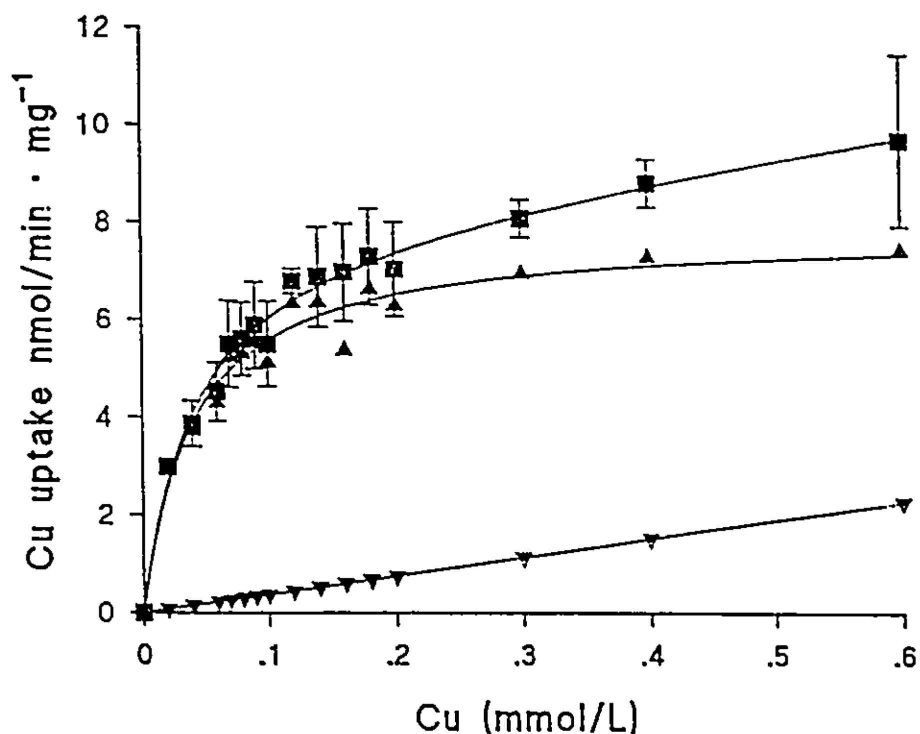


Figure 7, Initial rates of Cu^{2+} uptake (1 min) with extravesicular Cu concentrations ranging from 0.05 mmol/L to 0.6 mmol/L. BBMV were prepared from the intestine of 6 normal 20 day old piglets fed an adequate amount of Cu. Data represent the mean ± SEM. (■) The curve obtained by fitting the data to equation (1) represents the sum of the saturable and a linear components. The proposed saturable (▲) and linear (▼) components of copper uptake are calculated according to the constant (P) obtained from the curve fitting.

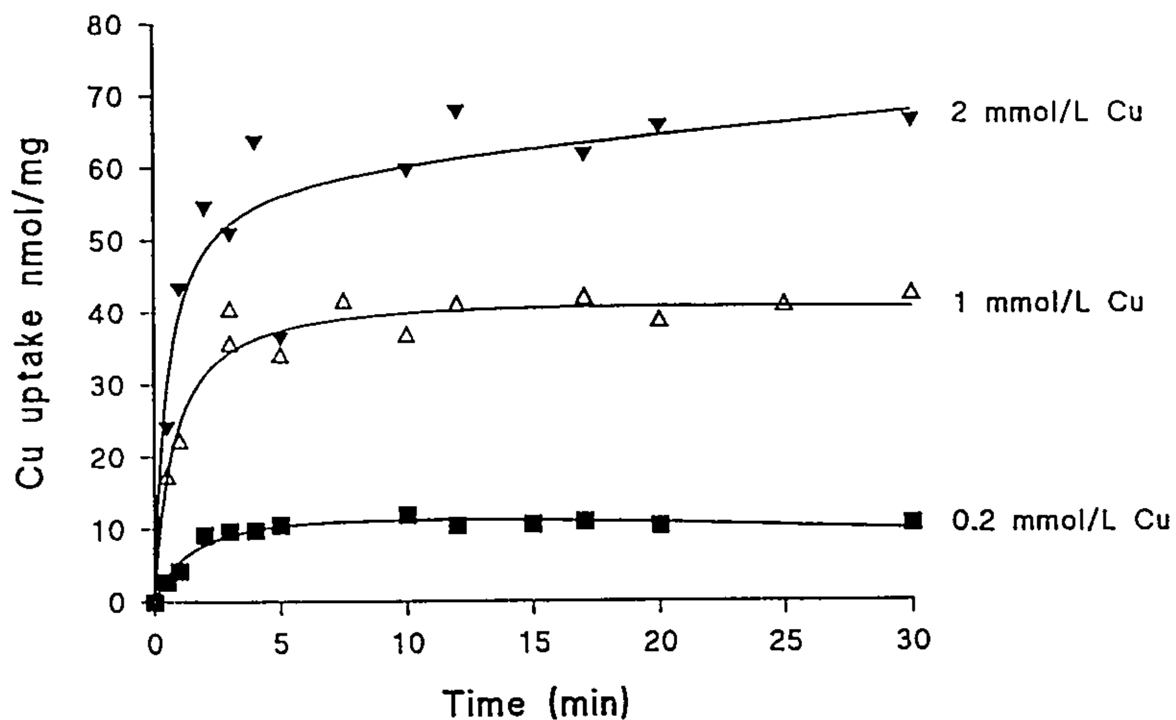


Figure 8, The time course of Cu^{2+} uptake by BBMV at several extravesicular Cu concentrations. The BBMV were recovered from a normal piglet fed an adequate amount of Zn and Cu. The curves were obtained by fitting the data to equation(1).

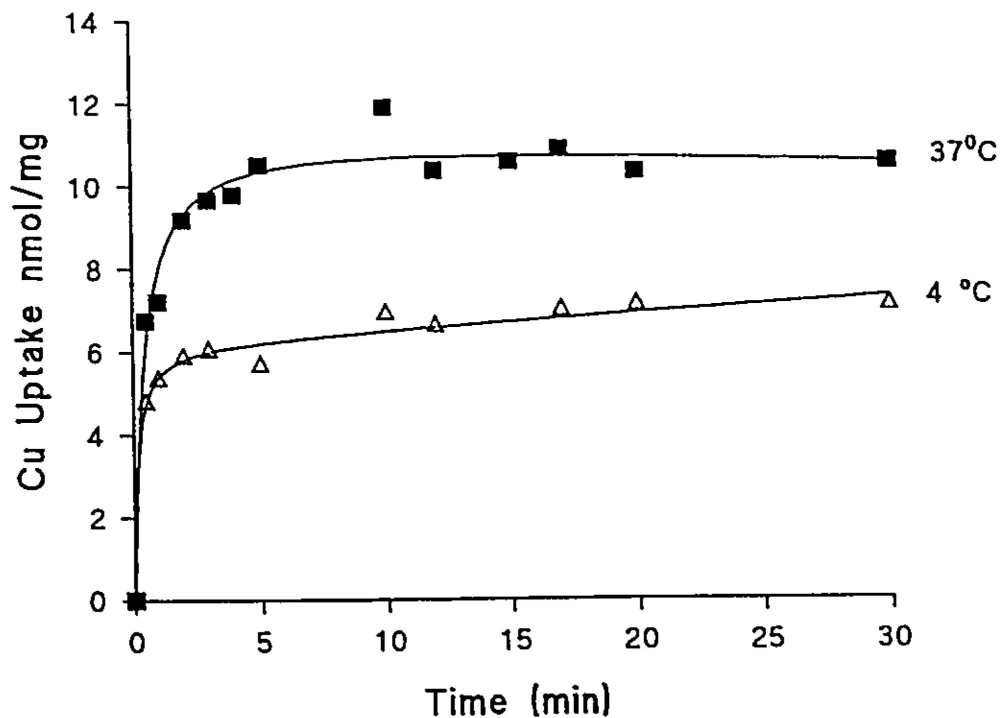


Figure 9, Effect of temperature on Cu^{2+} uptake. A BBMV suspension was divided into two equal parts and each was equilibrated at 37 °C or 4 °C for 10 min before adding ^{64}Cu tracer. The BBMV were recovered from a normal piglet fed an adequate amount of Zn and Cu.

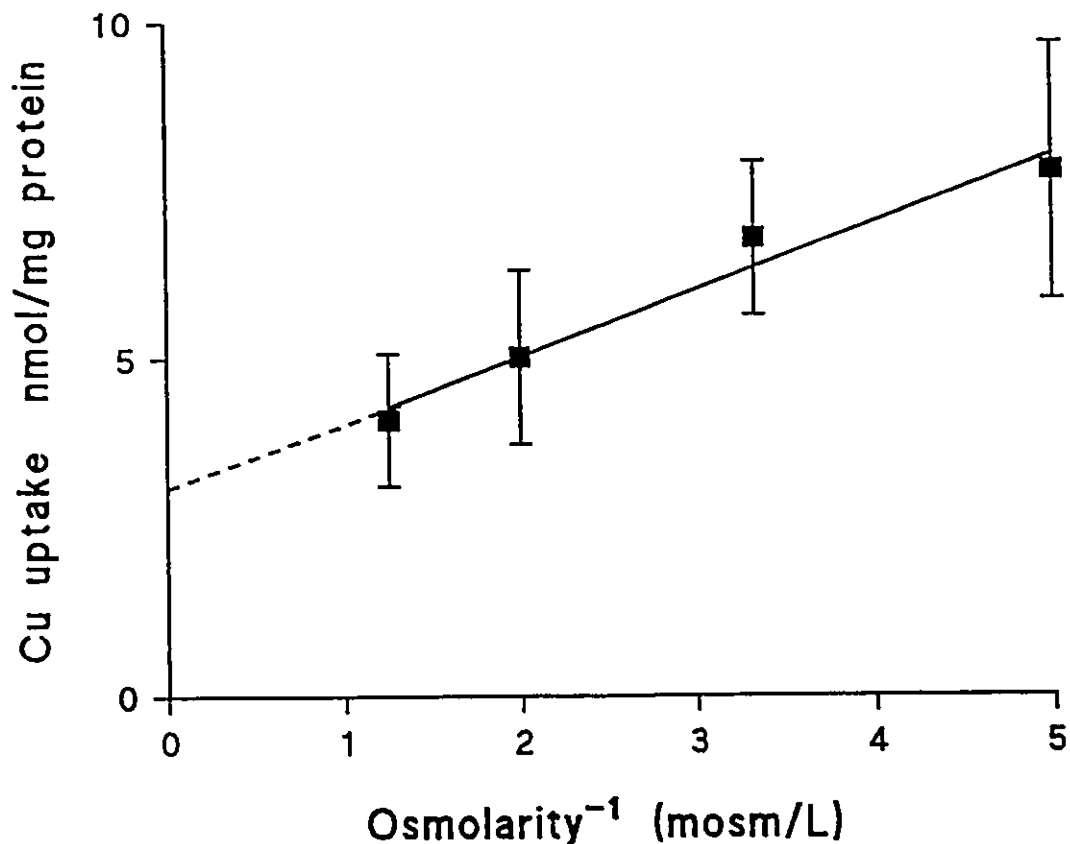


Figure 10, Cu binding to vesicles during uptake in medium containing 0.2 mmol/L Cu. BBMV were incubated with the above medium containing increasing concentrations of D-Mannitol for 5 min. Uptake rates were plotted as function of 1/osmolarity. The intercept of the Y axis is the proposed amount of Cu bound to the vesicle. The results represent the mean ± SEM of 6 experiments with BBMV preparations from 6 normal 20 d old male piglets fed an adequate amount of Cu.

(Cousins 1985). Several research methodologies have been developed to study intestinal Zn uptake in animals. The intestinal perfusion technique measures Zn uptake at the whole organ level. To study Zn uptake at the cell level one may use the isolated and cultured intestinal enterocytes (Raffaniello and Wapnir, 1989). However, because of the polarity of intestinal epithelial cells and the directional movement of Zn across the cell, a separation of the uptake mechanisms on BBM and BLM of epithelial cells is desirable.

Since Miller and Crane (1962) first prepared BBMV of intestinal epithelial cell by a differential centrifugation method, prepared intestinal BBMV have been widely applied in nutritional, pharmacological and biochemical studies (Murer and Hildmann 1984). The BBMV Zn uptake method was first established by Menard and Cousins in adult rats (1983). The BBMV Cu uptake method was first applied by Fischer and L'Abbe to study the response of Cu uptake to high Zn or low Cu feeding (1985).

The major difficulty in making intestinal BBMV is the contamination from other parts of cell membranes. The purity of our BBMV preparation as indicated by 10 $\pm$2.5 fold increase in sucrase activity is close to the reported value in a Zn transport study (Menard and Cousins 1983). Another difficulty in intestinal BBMV preparation is the detrimental effects of various digestive enzymes on prepared BBMV. To suppress the

activities of these enzymes, all preparation procedures were strictly conducted at 4°C in this study.

Our finding that Zn uptake by BBMV was by a combination of facilitated transport and passive diffusion in piglets agrees with the results of Menard and Cousins in adult rats (1983). The lower uptake at 4°C suggested the involvement of membrane transport protein(s) in BBMV Zn uptake. We found that ^{65}Zn binding to BBMV during uptake was small compared to the amount of ^{65}Zn taken up by BBMV (Fig 6).

The flux of Zn across the BBM of intestinal epithelial cell is bi-directional. Zn efflux across BBM has been suggested to be a physiological process for the excretion of excess Zn in intestinal mucosa and blood circulation (Cousins 1985). The mechanism by which Zn is released from BBMV is generally considered to be passive diffusion. Because of this passive property, the rate of Zn efflux could be an indication of membrane permeability to Zn (Bikle et al, 1983). Since the permeability of BBM is usually higher in infants than in adults and high membrane permeability could result in high endogenous loss of Zn and Cu (Neu et al, 1986, Lonnerdal 1989), the determination of BBM permeability is an important aspect in evaluating Zn and Cu absorption in early life.

The high efflux rate observed in this study (Fig 28, Chapter 5, Zn efflux curve for the control group) resulted

from the use of high concentration of EGTA in the incubation medium which created a large outward Zn concentration gradient across BBMV. Since this outward Zn concentration gradient was created by chemical means, the rate of efflux determined here does not represent a physiological state.

Current knowledge is limited about the mechanism(s) by which Cu is transported into the intestinal mucosa. Cu transport across the intestinal BBM was reported to occur principally via passive diffusion (Fischer and L'Abbe, 1985). The control and regulation of Cu absorption could occur in the cytosol and BLM of intestinal enterocytes by mechanisms that are not well defined. In contrast, in hepatocytes, fibroblasts, erythrocytes and lymphocytes, Cu uptake occurs by a carrier-mediated mechanism in humans and rats (Linder, 1989). Furthermore, Zn and Fe, elements with similar physicochemical properties, are documented to be transported across the BBM by specific carriers (Flanagan, 1989, Menard and Cousins, 1983).

Our results suggest that a carrier-mediated process plays a major role in intestinal Cu uptake over a range of Cu concentrations from 0.2 mmol/L to 2 mmol/L (Fig 8). This range of Cu concentration should be similar to or higher than the normal Cu concentrations in the intestinal lumen of human infants, thus, Cu uptake by the small intestinal mucosa is mainly via a carrier-mediated mechanism under conditions of

normal dietary Cu intake. Menard and Cousins (1983) found that Zn uptake by intestinal BBM was principally via a carrier-mediated mechanism in the presence of 0.2 mmol/L Zn in the incubation medium, while passive diffusion played a major role at extravesicular Zn concentration of 1 mmol/L. Unlike Zn transport, the transport of Cu is saturable even in the presence of 2 mmol/L Cu (Fig 8). The difference between Zn and Cu transport suggests that the transport systems for the two elements in intestinal BBMV are regulated separately.

Some difficulties involved in measuring Cu transport kinetics are the short half-life of ^{64}Cu tracer (12.7 h) and the high non-specific binding of ^{64}Cu to polyvinylidene difluoride filters. The first problem was solved by making the radioactive ^{64}Cu on site at our institution's nuclear reactor and by correction of ^{64}Cu decay when calculating uptake rates (Appendix IV, 4c). To decrease non-specific binding of ^{64}Cu, some researchers have converted Cu to Cu-histidine$_2$ (Schmitt et al, 1983) or Cu-nitrilotriacetate (Allerton and Linder, 1985). We used $^{64}CuCl_2$ as a tracer to avoid the possible effect of histidine and nitriloacetate on Cu absorption (Darwish et al, 1984, Keen et al, 1980). Non-specific binding of Cu to filters was minimized by completely converting ^{64}Cu to $^{64}CuCl_2$ and washing the filters with buffer containing 5 mM EDTA.

In the study for Cu uptake, we used a new rapid

filtration system, the Millipore Multiscreen™ Filtration System. The 96 filter plate very effectively improved the efficiency of each experiment and allowed us to use samples of 20-50 μg membrane protein to measure the rate of transport as compared to 100 μg membrane protein used in Zn uptake study (Wang et al, 1993). Fig 10 demonstrates that the rate of uptake we measured is not just the amount of Cu bound to BBMV. The amount of Cu binding to vesicles found in this study is very similar (3.2 vs 3 nmol/mg) to those of Fischer and L'Abbe (1985) who used 300 μg vesicular protein to measure Cu uptake.

We used two methods measuring the binding of Zn or Cu tracer to membrane surface. The hypotonic washing method was suggested by the reviewer when we submitted a paper to "Pediatric Research". We chosen the method modifying intravesicular space to estimate Cu binding because the hypotonic washing method could wash out some tracers bound to the membrane.

Although the measurements of Zn and Cu influx and efflux across prepared intestinal BBMV provided important information about Zn and Cu transporters or channels on BBMV and the permeability of BBMV to these elements under various physiological and pharmacological conditions, only the first step of Zn and Cu absorption is assessed by these measurements. To evaluate the function of overall Zn and Cu

absorption, one must use other methods.

A relatively simple method to determine the rate of Zn and Cu absorption is measuring the amount of Zn or Cu in incubation media taken up by an isolated, everted gut segments (Csaky 1984). However, this technique is completely in vitro; the gut segment lacks a blood supply as well as nerve and hormonal regulation. Compared to the everted gut sac technique, in situ intestinal perfusion is conducted in a more physiological condition. The gut is kept in the abdominal cavity and with blood supply during the experiment. The primary perfusion methodology involves injecting radioisotope tracers of Zn or Cu and a volume marker which indicates the movement of water into a two end ligated intestinal segment in situ and measuring the disappearance of isotope tracers from the perfusate after a certain time interval (Johnston 1932). The shortcoming of this method is that tracer and volume marker stay in the intestinal segment much longer than the normal transit time of luminal dietary contents. To overcome this shortcoming, an open ended single pass perfusion system and a closed recirculation perfusion system was introduced (Csaky 1984). The former mimics the single passage of food through the intestinal segment and therefore is more physiological. However, this method requires a larger amount of isotope tracer than the latter method and the rate of disappearance of isotope tracer

from the perfusate can not be determined. The recirculation method allows less radioactivity exposure to the researcher and the whole time course of isotope tracer disappearance can be monitored. Furthermore, the slope of the disappearance curve can be used to indicate the rate of absorption. The drawback of this method is that the concentration of isotope tracer in the perfusate gradually decreases as the solution recirculates.

All perfusion methods measure the disappearance of isotope tracer or elemental Zn or Cu from the perfusate, which only indicates the flux of isotope tracer from lumen to intestinal mucosa. Net absorption means the amount of dietary Zn or Cu entering the portal blood circulation. To determine the net absorption of Zn, a dual intestinal lumen and its blood vessel perfusion method was established by Smith et al (1978). In this method both intestinal segment and its blood vessels were perfused. The amount of isotope tracer from luminal perfusate which appears in the perfusate of the blood vessel during a certain time period is the rate of net Zn absorption. Because the composition of buffer cannot mimic the whole blood completely, some investigators used whole blood from a donor animal to perfuse the blood vessel (Windmueller and Spaeth 1984). An alternative method is to take blood samples from the portal vein during intestinal perfusion (Smith et al, 1978). Although this method cannot

give the value of net absorption, the appearance curve of isotope tracer in portal blood and the area under this curve provide information about the rate of net absorption. Portal blood sampling methods are especially applicable for comparing the absorption rate between different groups.

We chose the recirculating intestinal perfusion plus portal blood sampling method to measure net Zn absorption because we wanted to compare the whole Zn absorption process between different treatment groups and it was not necessary to know their exact values.

One limitation of perfusion methods mentioned above is that they can not distinguish the flux of Zn and Cu via transcellular and paracellular routes. Furthermore, with the expansion of the isolated intestinal segment during perfusion the movement of Zn or Cu via paracellular routes could be increased.

The advantage of using elemental Zn instead of ^{65}Zn to calculate Zn absorption is that ^{65}Zn transport could be affected by the size of the mucosal Zn pool (isotope dilution effect). However, although many investigators used the same equation as used here to calculate "net Zn absorption", elemental Zn measurement actually indicates the amount of elemental Zn taken up by the intestinal segment during intestinal perfusion, not net absorption.

Chapter 3

THE ONTOGENY OF INTESTINAL ABSORPTION OF Zn AND Cu AND BODY Zn AND Cu DISTRIBUTION IN THE PIGLET MODEL

3.1 Introduction

There has been little research into the absorptive ability of the small intestine for Zn and Cu at various stages of development. As a result, it is uncertain whether current recommendations of dietary Zn and Cu for human infants are meeting their needs. The availability of dietary Zn and Cu in the LBW infant is of particular concern because these infants are born with extremely low stores of Zn and Cu (Sutton 1985). Neonatal stores of Zn and Cu can rapidly be depleted due to the increased demand for growth and development combined with an insufficient dietary intake of Zn and Cu (Krebs and Hambidge, 1986).

The GI tract plays a central role in the maintenance of whole body Zn and Cu homeostasis (Cousins 1985). It absorbs and excretes Zn and Cu according to dietary Zn and Cu and body Zn and Cu stores. The negative balances of Zn and Cu observed in human premature infants fed with mother's breast milk suggests that their GI tract is immature so that it cannot absorb adequate amounts of Zn and Cu to fulfil body

requirements (Dauncey 1977, Atkinson 1990). In contrast, studies in rodents reveals that suckling rats and mice have a higher rate of net Zn and Cu absorption than adolescent animals. Whether this is a result of developmental differences in carrier-mediated or diffusional processes was not defined (Ghishan and Sobo, 1983; Mann et al, 1979). The discrepancy between the observations in rodents and humans suggests that rodents may not be the most appropriate model in which to study the ontogeny of Zn and Cu absorption in human infants.

The association between the mechanisms for Zn and Cu uptake by the intestinal BBM has not been determined. Zn and Cu may share receptor(s) in the BBM because low dietary Zn intake was found to enhance Cu absorption (Schwarz and Kirchyessner, 1974). We have demonstrated that high dietary Zn suppresses Cu uptake by the intestinal BBMV independent of the induction of MT in cytoplasm (Wang et al, 1993a).

The objective of this study was to understand the mechanism by which Zn and Cu are absorbed across intestinal BBM in early life and whether the kinetics of Zn and Cu transport are altered as a consequence of development. The change in whole body Zn and Cu compartmentalization during development and its correlation to tissue MT were also studied. Piglets were used as a model for the growing preterm infants because of the anatomical and physiological similarities between their digestive tract (Pekas 1991; Cooper

1975).

3.2 *Methods*

3.2.1 *Animal and diets*

The piglets used in this study are all male Yorkshire strain. After birth, piglets were fed by the sow for 1 to 4 d at the Arkell Research Farm (Guelph, Ont). The piglets for study at 32 and 52 h of age were sacrificed within 1 h of being removed from the sow. The piglets studied at 10 or 20 d of age were brought to the McMaster University Central Animal Facility when they were 3-4 d old. They were weaned onto a specially designed formula (18 ppm Zn, 0.75 ppm Cu) (Appendix 1). On the first, second, and third d after arrival the piglets were fed half strength, 3/4 strength and full strength formula. Once weaned onto the full strength formula they remained on it for the duration of the study period. The total amount of formula fed was based on the fasting weight of piglets in the morning (400 ml/kg/d). Feedings were three times daily. The piglets received a quarter portion of formula at 9:00 am and 3:00 pm and a final half portion at 9:00 pm. They were housed in stainless steel metabolic cages under infra-red heat lamps to maintain an ambient temperature of 30°C.

3.2.2 *Necropsy and tissue collection*

The piglets were sacrificed at a postnatal age of 32

h, 52 h, 10 d and 20 d by an intraperitonal injection of pentobarbital. Blood samples were collected by heart-puncture. The liver was removed, washed with ice-cold 0.9% saline and stored at -70°C until analysis. The first 100 cm of the proximal jejunum was excised, slit lengthwise and flushed with ice-cold 0.9% saline. Epithelial mucosa was scraped from the muscle using a glass slide at 4°C. The mucosa sample was stored at -70°C before measuring Zn and Cu uptake kinetics.

3.2.3 Preparation of intestinal BBMV

The method of BBMV preparation has been described in Chapter 2. In brief, BBMV was obtained by $MgCl_2$ precipitation and differential centrifugation. The extent of BBMV purity was monitored by measuring the increase in sucrase (EC 121.1.1.36) activity (Dahlqvist 1968). The BBMV preparations were prepared fresh for each transport study to ensure optimal physiological activity.

3.2.4 Zn and Cu uptake experiments

To measure the uptake of Zn and Cu across isolated BBMV, a rapid filtration technique was used. ^{65}Zn was purchased from DuPont Chemical Co (Wilmington, DE)(3.7 x 10^7 Bq/mg Zn) and ^{64}Cu was irradiated at the Nuclear Reactor at McMaster University (3.7 x 10^7 Bq/mg Cu). Approximately 40 μg of vesicular protein was incubated with increasing concentrations of ^{65}Zn or ^{64}Cu and elemental Zn or Cu ($ZnCl_2$ or $CuCl_2$) in the

incubation medium (300 mmol D-mannitol, 10 mmol Tris-Hepes, pH 6.7) as a total volume of 200 μl in a 96 well vinyl assay plate (Costar, Cambridge, MA) at 25°C for 1 min. Uptake was terminated by rapid filtration of 100 μl incubation solution on 0.22 μm filters which were set on the Millipore multiscreen™ filtration system (Millipore, Groton, CT) under vacuum. The filters were then rinsed twice with 100 μl of the incubation medium containing 5 mM EDTA, punched from the multiscreen plate, and measured for radioactivity using a gamma counter (Beckman Gamma 5500, Irvine, CA). Samples were prepared in triplicate with a blank which did not contain BBMV to correct for the nonspecific binding of ^{65}Zn or ^{64}Cu to the filters.

3.2.5 Analysis of kinetic data

Measurements of the initial rates of uptake at various substrate concentrations were fitted to the equation (1) which represents both facilitated and passive diffusion

$$V = \frac{V_{max} \times S}{K_m + S} + P \times S$$

by non-linear regression or the equation for first order kinetics (passive diffusion)

$$V = P \times S$$

by linear regression using the computer program Fig P 6.0 (Biosoft, Cambridge, UK).

3.2.6 Tissue Zn and Cu content

Plasma Zn and Cu were measured using an atomic absorption spectrometer (model 703, Perkin-Elmer, Norwalk, CT). Certified standard reference plasma (A67 and A69 for Zn and E9302 and E9309 for Cu; Interlaboratory Comparison Program, Centre de Toxicologie du Quebec, Sainte-Foy, Quebec, Canada) was used as a control throughout assay procedures. The Zn concentrations of the standard reference samples were determined to be 19.0 and 17.5 μM compared with known values of 18.35 and 17.5 μM, respectively. The Cu concentrations of the standard reference samples were 15.22 and 21.42 μM compared to the target value 15.1 and 21.6 μM, respectively. Liver and intestinal mucosa were quantitatively assayed for Zn and Cu by atomic absorption spectrometry after ashing at 500°C for 72 h and dissolved in 10 ml 1.25 M nitric acid. Certified standard reference nonfat milk powder (National Bureau of Standards, Washington, DC, no.1549) was analyzed as control. Zn concentration for this reference material was 47.2 ± 1.4 μg/g dry weight (C.V.=2.9%) compared with the known value of 46.1 ± 2.2 μg/g dry weight. Cu concentration for this reference material was 1.42±0.2 μg/g dry weight compared to the known value of 1.11 ± 0.1 μg/g dry weight(C.V.=4.49%).

3.2.7 Tissue MT concentration

MT levels in liver and intestinal mucosa were

determined by a cadmium saturation assay using ^{109}Cd (Appendix VI).

3.2.8 Statistical analysis

Multiple comparisons of group means were performed using Tukey's method on the different age groups after performing a one-way analysis of variance (ANOVA) between the groups using Minitab Statistics software (Minitab Inc., State College, PA). Results were considered statistically significant if $p<0.05$. Data were presented as mean$\pm$SD unless otherwise noted.

3.3 Results

3.3.1 Tissue Zn, Cu and MT

The compartmentalization of tissue Zn and Cu varied by stage of development. The 32 and 52 h old piglets had significantly lower plasma Zn ($P<0.001$)(Fig 11), plasma Cu ($P<0.001$) (Fig 12) and intestinal mucosa Zn ($P<0.001$)(Fig 13) than the 20 d old piglets. Liver Zn ($P<0.05$)(Fig 14) and intestinal mucosal Cu ($P<0.05$) (Fig 15) were significantly higher in younger animals. Liver Cu was similar at all age (Fig 16). The 32 h and 52 h old piglets had a significantly higher concentration of liver MT while the 20 d old piglets had a significantly higher concentration of intestinal MT (Fig 17).

3.3.2 The kinetics of Zn, Cu transport

The kinetic studies of ^{65}Zn and ^{64}Cu uptake by isolated BBMV demonstrate that the transport mechanism of Zn and Cu is also altered by stage of development (Fig 18, 19). For Cu, carrier mediated transport appeared to dominate at all stages studied. In contrast, uptake of Zn occurred via passive diffusion in the first 52 h of life whereas carrier mediated transport was established around 10 d.

3.4. Discussion

In this study, the mechanisms of Zn and Cu transport across isolated intestinal BBMV were shown to change during intestinal maturation. Zn uptake occurred via a nonsaturable mechanism in the 32 h and 52 h old piglets, however, a saturable mechanism replaced the nonsaturable process by 10 d postnatal age. The transport rate of Zn was higher in 32 h and 52 h old piglets than in 10 d old piglets. These results are consistent with the concept that the BBM of newborn animals is immature and therefore has a higher permeability than that of mature animals. This higher permeability allows for an increased transport of components by passive diffusion. However, the ontogeny of Zn and Cu was not similar. Cu uptake by BBMV occurred predominantly via a saturable process from 32 h to 20 d postnatal age. It appears that Cu transport experienced the alterations from high to low then to high

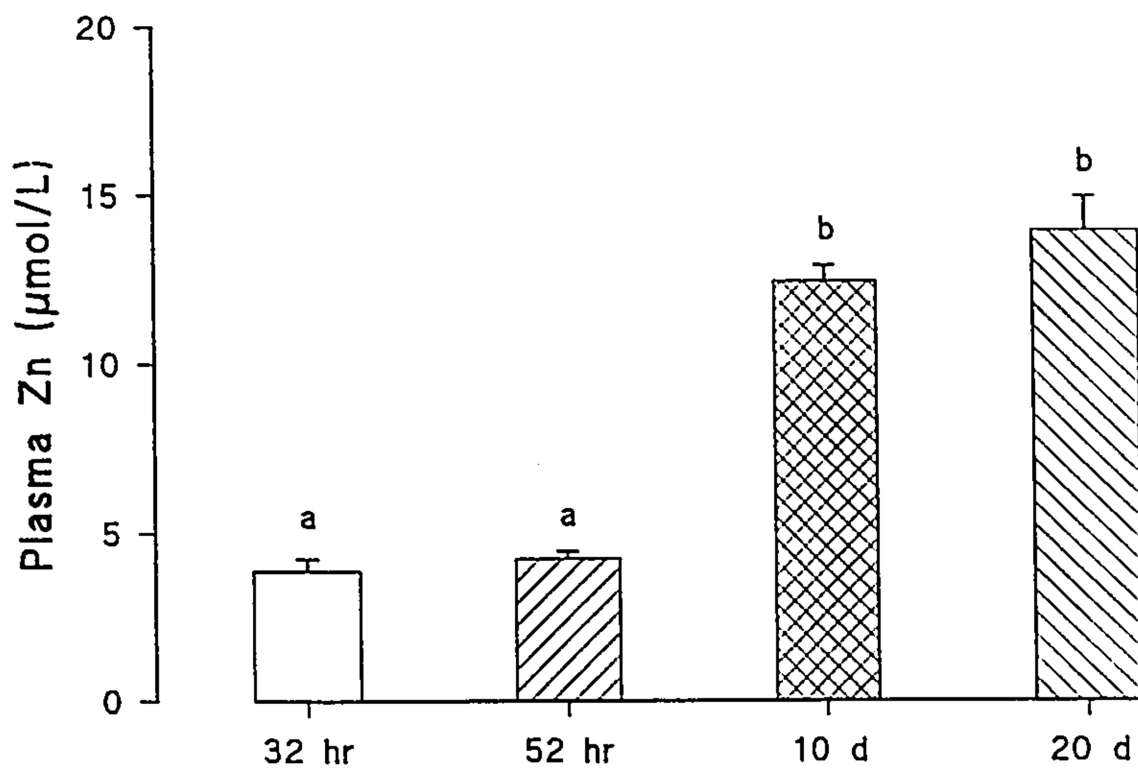


Figure 11, Comparison of plasma Zn concentrations between the piglets at 32 h (N=4), 52 h (N=3), 10 d (N=6) and 20 d (N=6) postnatal age. Bar represents mean ± SEM, a vs b, $p<0.001$.

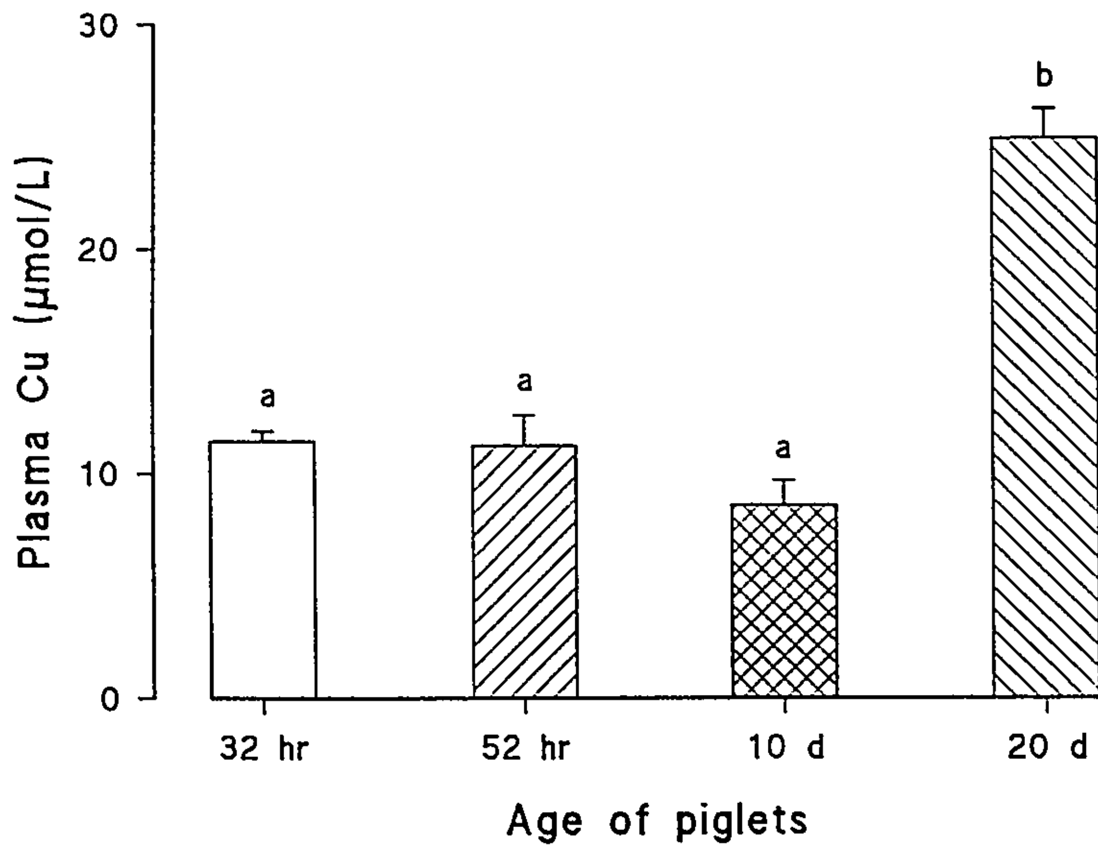


Figure 12, Comparison of plasma Cu concentrations between the piglets at 32 h (N=4), 52 h (N=3), 10 d (N=6) and 20 d (N=6) postnatal age. Bar represents mean ± SEM, a vs b, $p<0.001$.

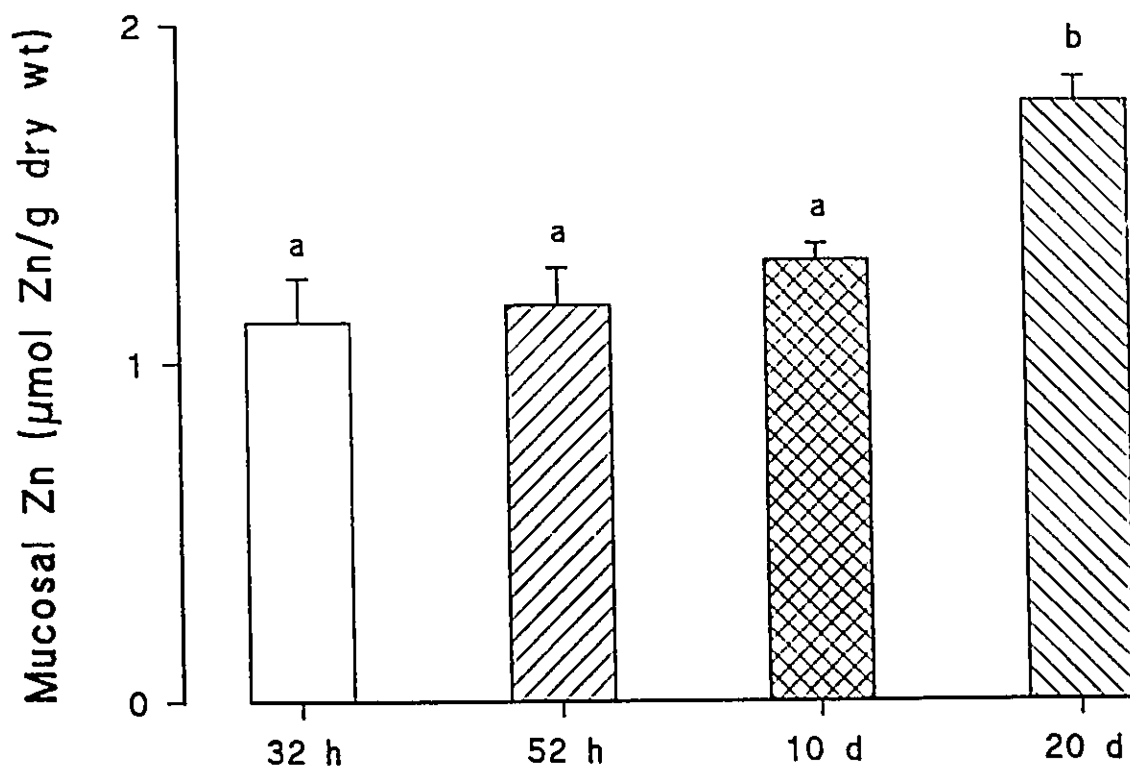


Figure 13, Comparison of intestinal mucosa Zn contents between 32 h (N=4), 52 h (N=5), 10 d (N=6) and 20 d (N=7) old piglets. Bar represents mean ± SEM, a vs b, $p<0.001$.

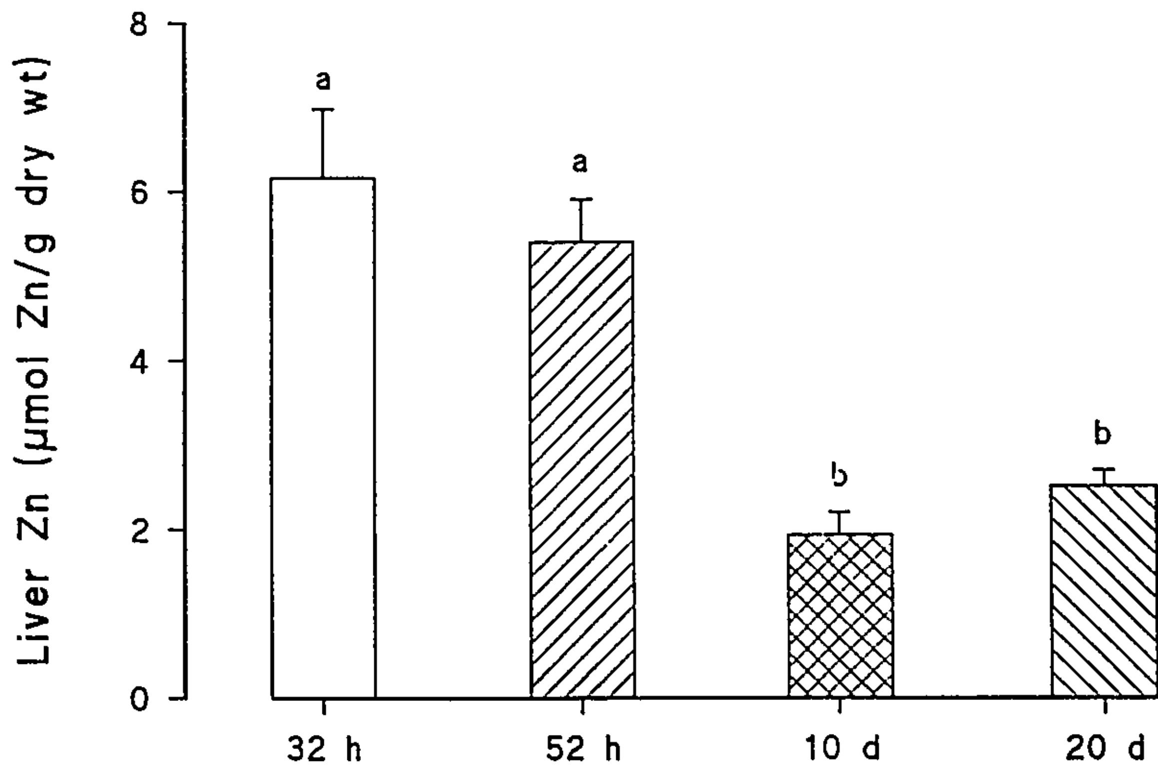


Figure 14, Liver Zn contents of 32 h (N=4), 52 h (N=4), 10 d (N=6) and 20 d (N=7) old piglets. Bar represents mean ± SEM, a vs b, $p<0.001$.

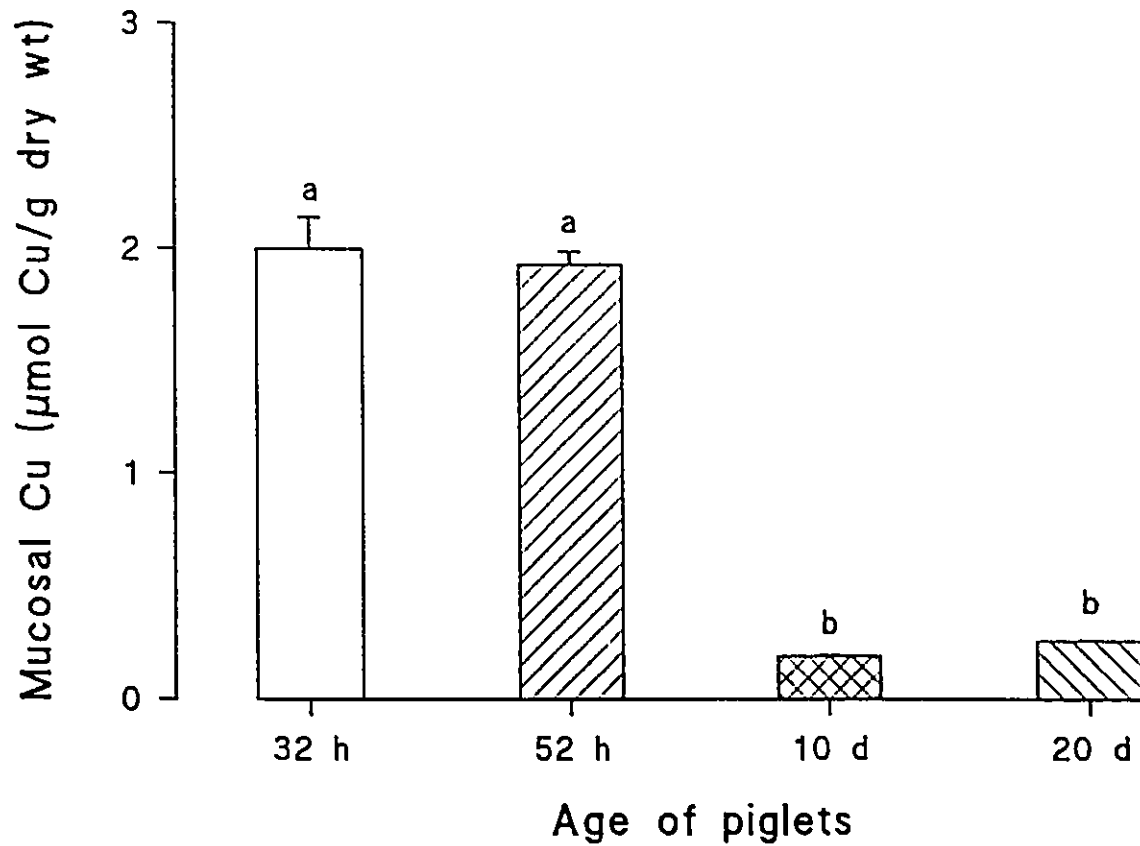


Figure 15, Comparison of intestinal mucosa Cu contents between 32 h (N=4), 52 h (N=3), 10 d (N=6) and 20 d (N=7) old piglets. Bar represents mean ± SEM, a vs b, $p<0.001$.

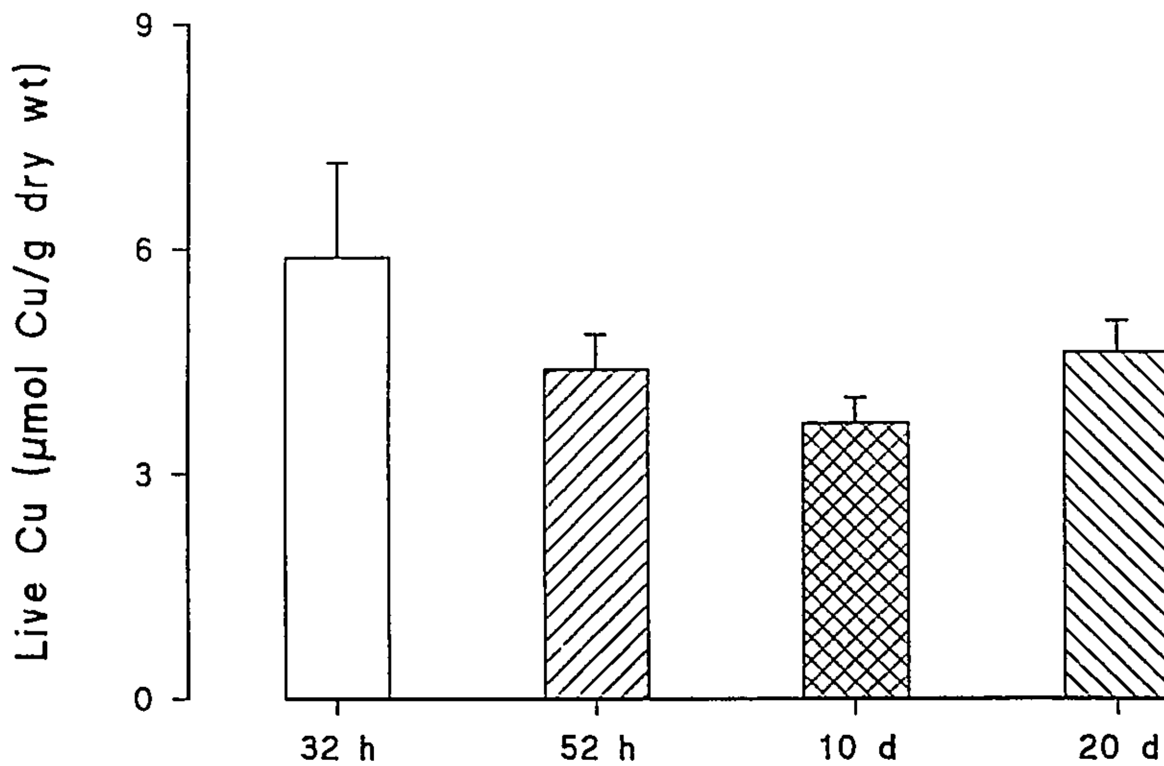


Figure 16, Liver Cu contents of 32 h (N=4), 52 h (N=3), 10 d (N=5) and 20 d (N=4) old piglets. Bar represents mean ± SEM.

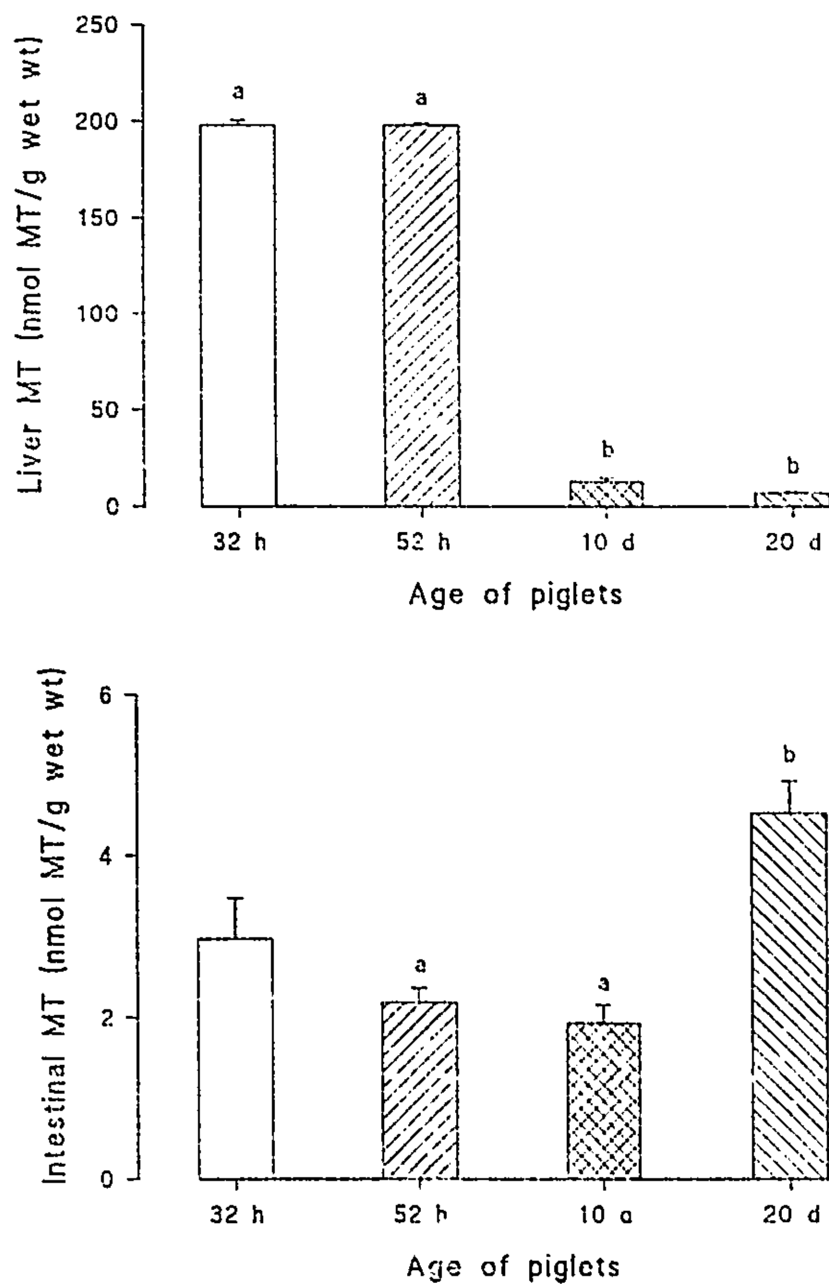


Figure 17, Liver and intestinal mucosa MT contents of 32 h (N=4), 52 h (N=3), 10 d (N=5) and 20 d (N=6) old piglets. Bar represents mean ± SEM, a vs b, $p<0.001$.

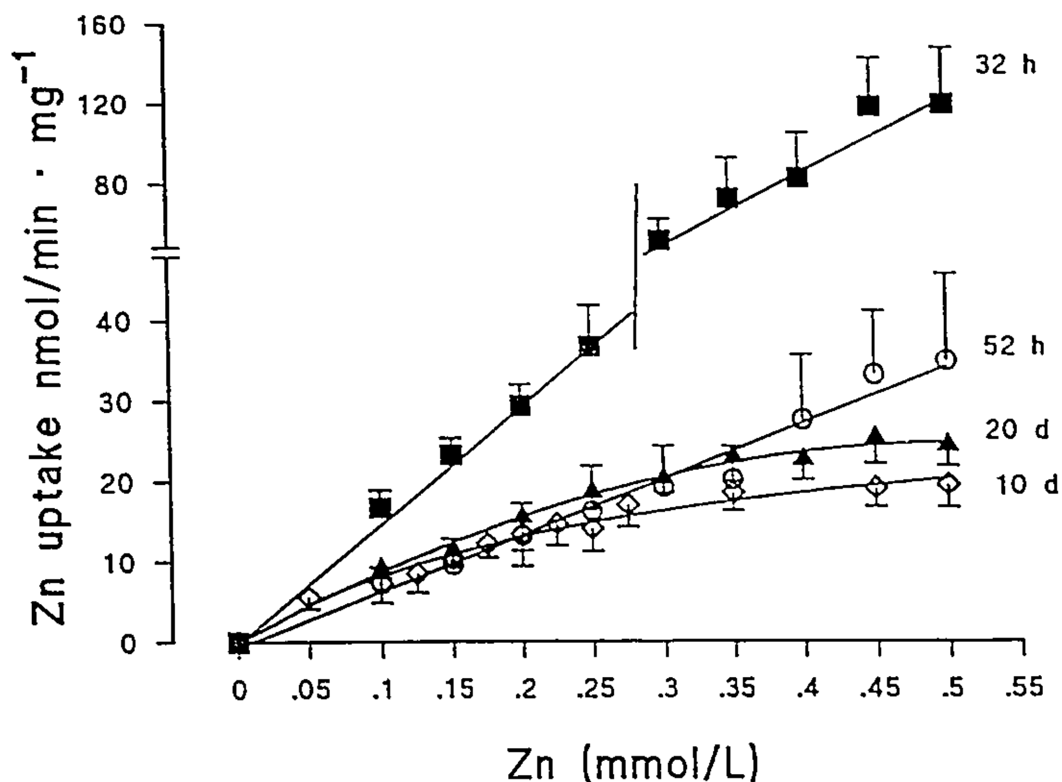


Figure 18, Ontogeny of intestinal Zn uptake mechanism. The BBMV were obtained from normal male piglets at 32 h (N=4), 52 h (N=5), 10 d (N=7), 20 d (N=6) postnatal age. Data represent mean ± SEM. The curves were obtained by fitting the data with a equation for first order kinetics (32 h and 52 h) or equation (1)(10 d and 20 d).

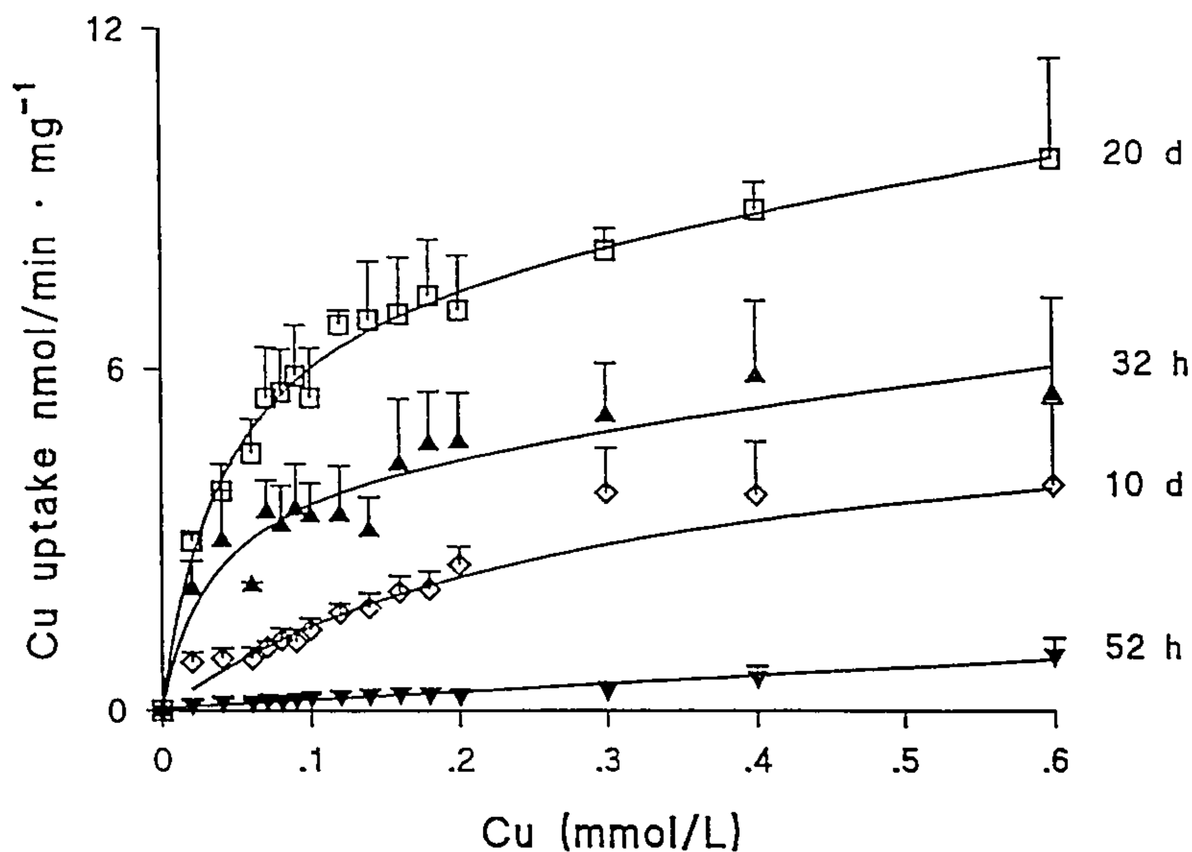


Figure 19, Ontogeny of intestinal Cu uptake mechanism. The BBMV were obtained from normal male piglets at 32 h (N=4), 52 h (N=7), 10 d (N=7), 20 d (N=6) postnatal age. Data represent mean ± SEM. The curves were obtained by fitting the data with equation (1).

again in piglets during the first 20 d of life. The difference in the ontogeny of Zn and Cu transport suggests that Zn and Cu are taken up by different transport systems.

The mechanisms of Zn and Cu uptake by intestinal BBM are not completely understood. Studies by our group (Wang et al, 1993b) and others (Menard and Cousins, 1983) suggest that the uptake of Zn occurs via a combination of carrier mediated and passive diffusion. In adult animals and animals in later infancy (10 d), a carrier mediated mechanism appears to have a major role in Zn uptake by brush border membranes. In the presence of high dietary Zn, passive diffusion is responsible for a part of Zn transport. The rate of passive diffusion is proportional to the Zn concentration in the intestinal lumen. In contrast, Cu uptake occurs mainly via passive diffusion (Fischer and L'Abbe 1985). A few studies have compared Zn or Cu absorption between suckling and adolescent rats or mice. Suckling mice were shown to have a higher net Cu absorption than adult mice by an *in-vivo* study using ^{64}Cu tracer (Mann et al, 1979). Studies (Ghishan and Sobo, 1983) which used an *in-situ* intestinal perfusion technique in rats at different ages demonstrated that suckling rats had a higher lumen to mucosa Zn flux than adolescent rats. Furthermore, the lumen to mucosa Zn flux was saturable in proximal small intestine in both suckling and adolescent rats. It is difficult to compare the

findings of these studies with our results since the *in-vivo* or *in-situ* technique used in these studies measured both paracellular and transcellular Zn transport. As well, rats and mice are likely not the most appropriate models for studying Zn and Cu absorption in human neonates (Glauser 1966).

There were also differences between Zn and Cu compartmentalization during the first 20 days of life. The 32 h and 52 h old piglets had significantly lower plasma Zn, Cu and intestinal mucosa Zn concentrations but higher liver Zn and intestinal mucosal Cu content than 20 d old piglets. The Zn in liver in 32 h and 52 h old piglets could be bound to MT because there was a good correlation between hepatic Zn and MT concentrations in these age groups. Cu accumulated in the intestinal mucosa in 32 h and 52 h old piglets could bind with other proteins or ligands because intestinal MT content was not proportionally high in these age groups.

Although the piglets at 32h and 52 h of age had higher Zn influx across BBMV than those at 10 d or 20 d age, the net absorption of Zn could be lower in the younger groups because of the higher efflux of Zn across the BBM which was due to higher membrane permeability to Zn. This explanation is supported by the significantly lower plasma and intestinal mucosa Zn concentrations observed in 32 h and 52 h piglets compared to those of 20 d piglets. The Cu accumulation in intestinal mucosa in 32 h and 52 h groups may result in

reduced Cu absorption. The higher BBM Cu transport but relatively lower rate of BLM Cu transport could be the reason of intestinal Cu accumulation. Ghishan (1989) reported that Ca uptake by basolateral membrane is significantly lower in suckling than in adolescent rats. It is also possible that there is a greater concentration of Cu binding proteins other than MT in intestinal mucosa which caused intestinal Cu accumulation in 32 h and 52 h groups. The significantly lower plasma Cu found in 32 h and 52 h piglets could be due to poor absorption, high organ storage or high excretion.

In conclusion, our findings suggest that there were different ontogenic processes between intestinal Zn and Cu transport across BBM in piglets. The carrier mediated mechanism for Cu transport matured earlier than that for Zn transport. The immaturity of carrier mediated transport and high permeability of intestinal BBM to Zn could be related to lower plasma Zn and intestinal mucosal Zn in newborn piglets. While intestinal Cu accumulation could be a reason for the significantly lower plasma Cu in these piglets.

This study provides some insight into the biological mechanisms which explain why Zn and Cu deficiency is observed in some premature infants. Since the mechanism that controls Cu absorption matures earlier than the mechanism for Zn absorption, it is logical that the incidence of Cu deficiency is lower clinically than the incidence of Zn deficiency. High

permeability of intestinal BBM as a result of immaturity is related to the high fecal loss of Zn found in human premature infants. It is clear that the stage of development is an important factor affecting Zn and Cu metabolism. Therefore when establishing dietary recommendations for Zn and Cu for human infants at different developmental stages, the mechanism of Zn and Cu metabolism at the correspondent stage should be considered.

Chapter 4

The interactive effects of dietary Zn and Fe on Cu uptake by Intestinal BBMV in the Developing Piglet

4.1 Introduction

Cu is essential for normal growth and development of human infants. Because breast milk of most species is not rich in Cu, fetus stores Cu in the liver and other organs during the third trimester of gestation to meet the high requirement of Cu for rapid growth after birth. Prematurely born infants do not experience this important period of intrauterine Cu accumulation thus their body Cu store is much lower than term infants at birth. Most reported cases of Cu deficiency occurred in this population. An appropriate dietary Cu intake is therefore critical for these infants to maintain a normal Cu balance.

One of the important dietary sources of Cu for infants is infant formula. Because the Zn and Fe contents of formula are usually higher than Cu and the ratios of Zn:Cu or Fe:Cu are extremely high when formula is fortified with Zn or Fe, the interactive effects of these elements on Cu bioavailability should be carefully considered when

determining the upper limits for Zn and Fe contents in infant formula.

Current knowledge is limited with respect to the possible mineral-mineral interactions in infant diets that may impact on Cu absorption in the developing infant. Since Cu shares similar physicochemical properties with Zn and Fe, these elements have the potential to compete for transport across membranes. An antagonistic effect of Zn on whole body Cu absorption has been demonstrated in humans and animals (Patterson et al, 1985). The interaction between Zn and Cu is not likely at the site of transport in the brush border membrane since Zn has specific carriers (Menard and Cousins, 1983). Rather the mechanism to explain the competition of Zn and Cu for absorption is thought to be the 'bystander phenomenon'. In the presence of high dietary Zn, induction of intestinal MT occurs which acts as a mucosal trap for Zn and Cu (Cousins,1985). However, this is an unlikely mechanism in young (piglets) as compared to adult animals since we demonstrated (Wang et al, 1993a) that MT was not induced in the intestinal mucosa in piglets fed a high Zn formula (1000 ppm/kg dry weight). Perhaps in early life the regulation of MT synthesis is immature and not responsive to the same signals as in adult animals (Wang et al, 1993a). An alternative mechanism such as competition between Zn and Cu for a carrier in the brush border and/or basolateral membranes, is therefore

postulated. Although dietary Fe was suggested to suppress Cu absorption and retention in some in vivo studies (Sherman and Tissue, 1981; Haschke et al, 1986), other studies demonstrated no effect of Fe on Cu absorption (Keen 1984, Bremner 1987).

The objective of this study was to determine the effects of dietary Zn and Fe on intestinal Cu uptake in the developing animal.

4.2 Material and methods

4.2.1 Animals and diets

Eleven 10-12 day old male Yorkshire piglets were purchased from the Arkell Research Farm (Guelph, Ontario, Canada). Care of the piglets in this study conformed with the "Guide for Care and Use of Experimental Animals" (Canadian National Research Council, 1984). Piglets were weaned onto a specially designed liquid formula (Appendix 1). After 3 days of adaptation to the diets piglets were randomly allocated to normal (18 mg/kg Zn) or high Zn (1000 mg/kg Zn) formula groups. Following the 5 day treatment, the piglets were euthanized and samples of plasma, liver and proximal jejunum were collected. The mucosa was scraped from the intestine at 4°C and stored at -70°C for future preparation of membrane vesicles for in-vitro metal uptake experiments.

4.2.2 Preparation of intestinal BBMV

BBMV were obtained by $MgCl_2$ precipitation and

differential centrifugation (Davidson and Lonnerdal, 1988). The preparation was conducted on the same day as the ^{64}Cu transport study in order to avoid potential vesicle damage caused by freezing and thawing.

4.2.3 Determination of Cu uptake kinetics

Cu uptake by BBMV was measured by rapid filtration method using ^{64}Cu tracer ($^{64}CuCl_2$ was produced by irradiation of elemental Cu at the Nuclear Reactor at McMaster University). Samples were prepared in triplicate with a blank (no BBMV) included to correct for non-specific binding of ^{64}Cu to filters. Radioactivity measurements were corrected for decay by a decay curve established in our laboratory (Appendix IV).

To determine Cu uptake kinetics, BBMV were incubated with increased concentrations of Cu for 1 min. Measurements of the initial rates of uptake (v) at various Cu concentrations were fitted to the equation (1) describing a combination of facilitated and passive diffusion (Herd et al, 1987).

The time course of Cu uptake by BBMV was established by the method described in Chapter 2.

4.2.4 Effect of Zn and Fe on the time course of Cu uptake

$ZnCl_2$ or $FeCl_3$ solution (10 mM) was added to the incubation buffer containing 0.2 mM $CuCl_2$ to a final concentration of Zn or Fe of either 1 or 2 mM. Binding of Cu to BBMV in the presence of 1 mM Fe was determined by

increasing extravesicular osmolarity using the method described in Chapter 2.

4.2.5 Tissue Cu content

Cu levels in plasma, liver and intestinal mucosa were assayed by atomic absorption spectrometry (model 703, Perkin-Elmer, Norwalk, CT). Certified standard reference plasma was used as a control throughout assay procedures. Liver and intestinal mucosa were quantitatively assayed for Cu by atomic absorption spectrophotometry after ashing at 500°C for 72 h and dissolved in 10 ml 1.25 M nitric acid. Certified standard reference nonfat milk powder (National Bureau of Standards, Washington, DC, no.1549) was analyzed as control. Urine Cu was measured directly by atomic absorption spectrometry.

4.2.6 Tissue MT concentration

MT levels were determined in liver and intestinal mucosa of all pigs by a cadmium (Cd) binding assay using ^{109}Cd (Appendix VI).

4.2.7 Statistical analysis

Results are reported as mean±SEM and differences between groups were tested by the unpaired Student's t-test using Minitab Statistic Program (Minitab Inc., State College, PA). A 'p' value of less than 0.05 was considered to indicate a statistically significant difference between two mean values.

4.3 Results

4.3.1 Effect of Zn and Fe on Cu uptake

Addition of 5-10 fold molar excess of Zn or Fe did not change the time for approaching steady state of Cu uptake across BBMV. However, 1 and 2 mM Zn decreased the rate of Cu uptake at the steady state. There was no difference in the effect of suppression of Cu uptake between 1 and 2 mM Zn (Fig 20). Fe significantly enhanced Cu uptake by BBMV ($p<0.01$). Furthermore, 2 mM Fe notably increased the non-saturable component of Cu uptake (Fig 21). The results from increasing extravesicular osmolarity in the presence of 1 mM Fe as $FeCl_2$ (Fig 22) suggest that Fe mainly increases binding of Cu to BBMV. However, the different slopes between the two lines in Fig 22 indicate that Cu uptake was also increased when Fe was added to the medium.

4.3.2 Effects of 5 d high Zn feeding on Cu uptake by BBMV

Five day high zinc feeding resulted in significantly lower Jmax and Km of BBMV Cu uptake in 20 day old piglets ($p<0.01$ and $p<0.05$, respectively, Fig 23 a, 24). Another striking characteristic of BBMV Cu uptake in piglets fed high Zn was a dramatic increase in Cu uptake as extravesicular Cu concentration increased from 0.16-0.8 mM (Fig 23 b). This part of the curve was fitted by a straight line with a slope of 0.899. First order kinetics suggest that this increase in Cu

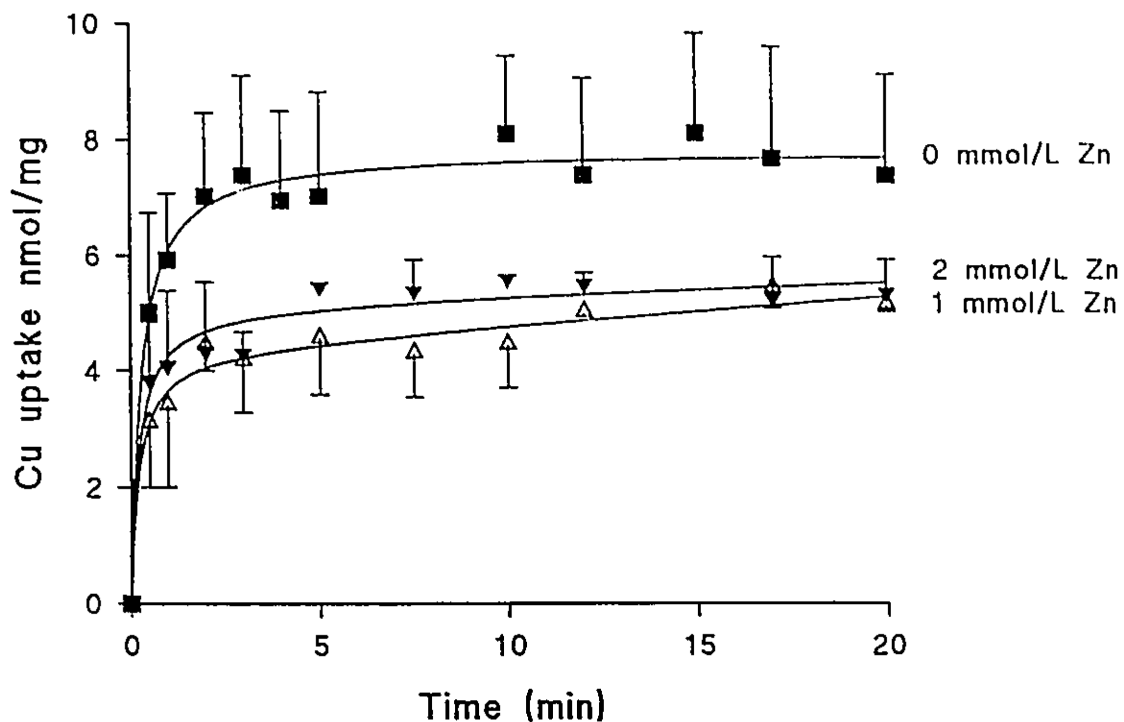


Figure 20, The effect of Zn on the time course of BBMV Cu^{2+} uptake. The vesicles were prepared from the proximal small intestine of normal 20 day old piglets and the uptake of 0.2 mmol/L Cu was conducted at 37°C under gradient condition (medium > vesicle). The results represent the means of a minimum of three preparations ± SEM.

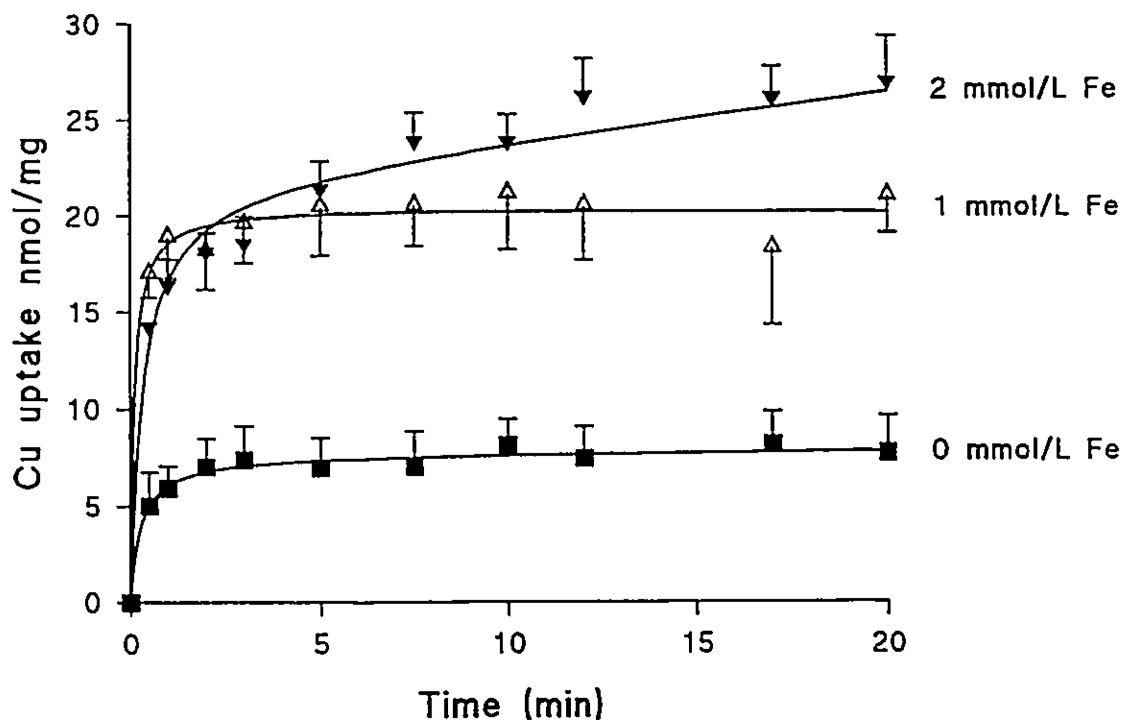


Figure 21, The effect of Fe on the time course of BBMV Cu^{2+} uptake. The vesicles were prepared from the proximal small intestine of 3 normal 20 day old piglets and the uptake of 0.2 mmol/L Cu was conducted at 37°C under gradient conditions (medium > vesicle) with 0, 1 or 2 mmol/L Fe in the medium. The results represent the means of a minimum of three preparations ± SEM.

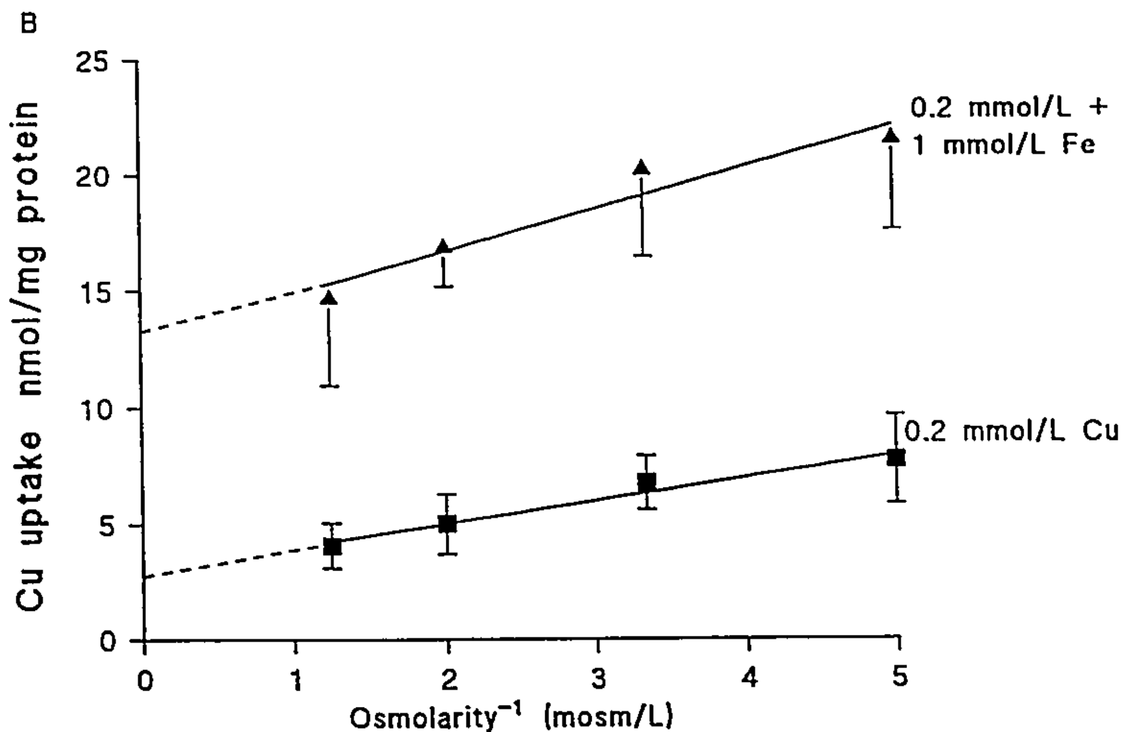
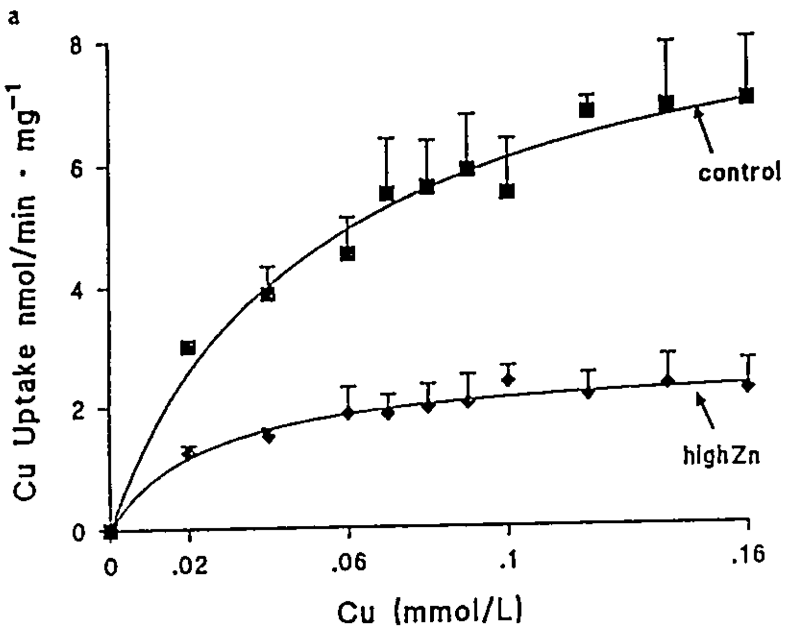
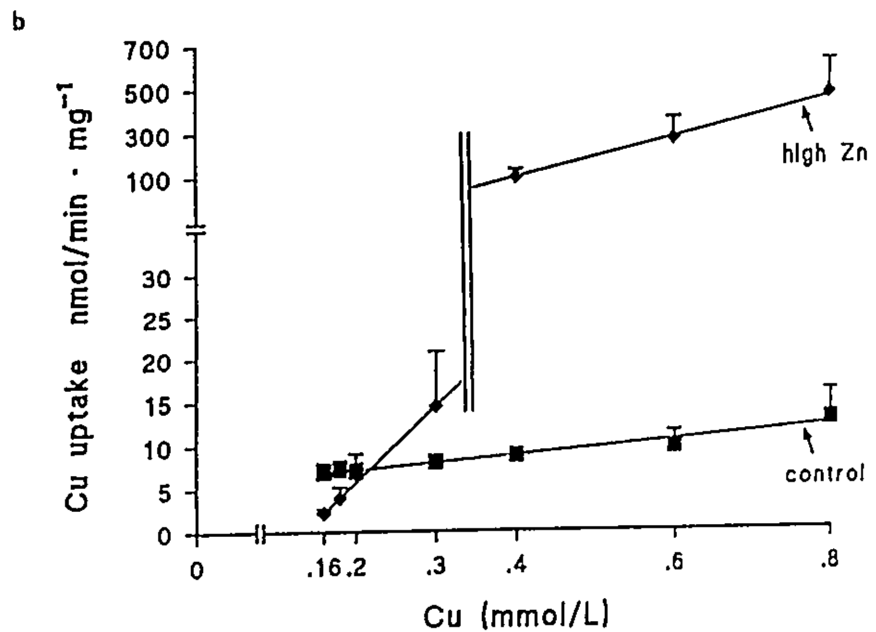


Figure 22, Cu binding to vesicles during uptake in medium containing 0.2 mmol/L Cu or 0.2 mmol/L Cu plus 1 mmol/L Fe. BBMV were incubated with the above two media containing increasing concentrations of D-Mannitol for 5 min. Uptake rates were plotted as function of 1/osmolarity. The intercept of the Y axis is the proposed amount of Cu bound to the vesicle. The results represent the mean $\pm$ SEM of 6 experiments with BBMV preparations from 6 normal 20 day old piglets.

Figure 23, Initial rates of Cu^{2+} uptake (1 min) by BBMV with extravesicular Cu concentrations ranging from 0 mmol/L to 0.16 mmol/L (a) and 0.16 mmol/L to 0.8 mmol/L (b). Data represent the mean ± SEM for vesicles from 5 control piglets and 5 piglets fed a high zinc diet (1000 mg/kg for 5 days).


a
Cu Uptake nmol/min · mg^{-1}
0
2
4
6
8
0
.02
.06
.1
.16
Cu (mmol/L)
control
highZn


b
Cu uptake nmol/min · mg^{-1}
0
5
10
15
20
25
30
100
300
500
700
0
.16.2
.3
.4
.6
.8
Cu (mmol/L)
high Zn
control

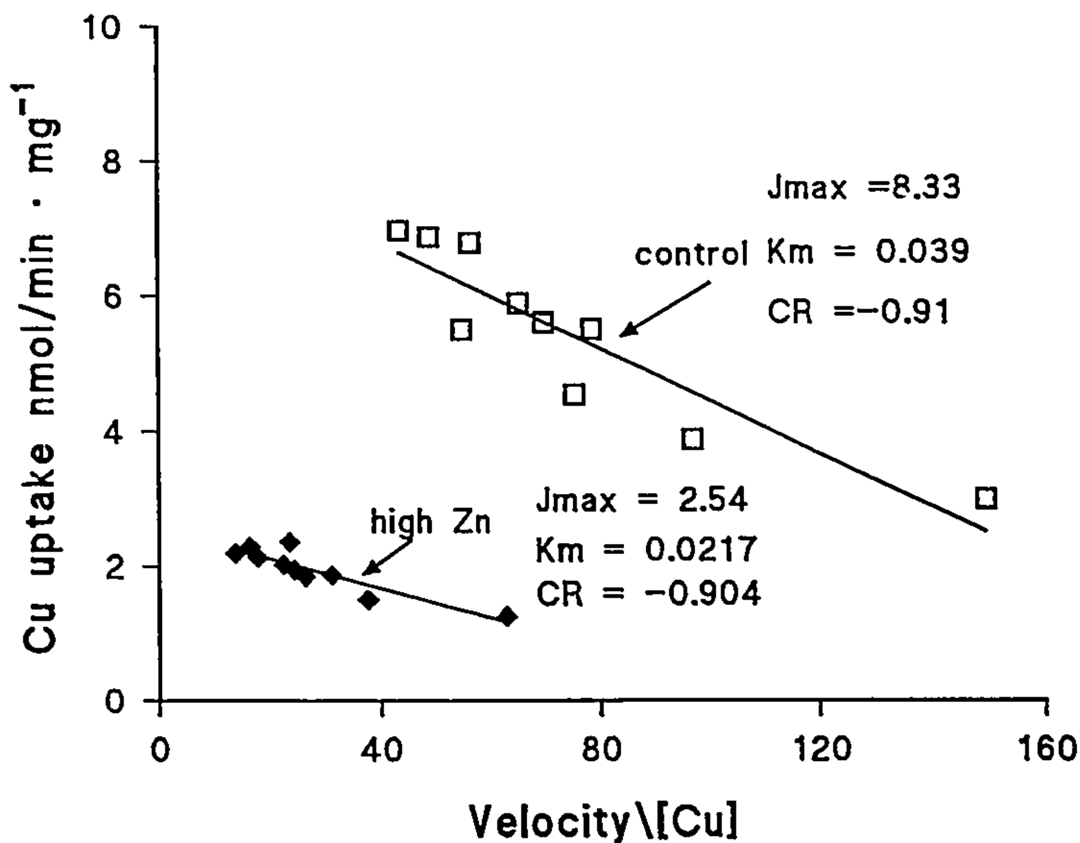


Figure 24, Eadie - Hofstee plot of the kinetics of Cu^{2+} uptake by BBMV from the data described in Fig 23 a. Jmax and Km for each group were determined from linear regression and are expressed as nmol Cu/mg protein.min^{-1} and mM, respectively.

uptake is via passive diffusion.

4.3.3 Tissue Cu and MT contents in pigs fed high Zn formula

Although hepatic Cu contents tended to be lower in high Zn fed pigs than in controls, there was no statistically significant difference detected between two groups (Table 1). High Zn fed animals had 10 times higher liver MT concentrations than control piglets (Table 2).

4.4 Discussion

In the present study we found that: a) Fe enhances Cu uptake by BBMV in-vitro. b) Zn suppress Cu uptake by BBMV in-vivo or in-vitro in developing piglets.

Our observation of reduced intestinal Cu transport in association with high Zn feeding is in contrast to previous research. Fischer and L'Abbe (1985) found that high Zn feeding (Zn:Cu=40:1) increased Cu uptake by intestinal BBMV in rats. We found that adding Zn into incubation media at a concentration 5 to 10 times higher than the Cu concentration decreased the velocity of Cu uptake over the time course of 20 min. In high Zn fed (Zn:Cu=500:1) piglets, Cu uptake was significantly reduced when extravesicular Cu concentration was below 0.16 mM. The difference in dietary Zn and Cu ratio, animal species and developmental stage of animals between our study and that of Fischer and L'Abbe (1985) may be responsible for the varying results. It is interesting to note there was not a very high passive component in the saturation curve of

TABLE 1, PLASMA AND TISSUE Cu CONCENTRATIONS OF PIGLETS FED WITH HIGH DIETARY Zn

	High Zn	**Control**
Plasma Cu (nmol/L)	25.05 ± 2	24.86 ± 1.4
Intestine Cu (μg/g dry wt)	19.2 ± 2.6	18.67 ± 3
Liver Cu (μg/g)	243.1 ± 50	317.5 ± 58

Data represent mean ± SEM.

TABLE 2, LIVER AND INTESTINAL MUCOSA MT CONCENTRATIONS IN PIGLETS TREATED WITH DEX OR HIGH DIETARY Zn

	Control	DEX+	High Zn
Liver MT (nmol/g wet wt)	7.1 ± 0.9	7.1 ± 0.7	79.0 ± 12.8*
mucosa MT (nmol/g wet wt)	4.5 ± 0.5	3.7 ± 0.8	4.6 ± 1

* significantly higher than DEX and Control groups, $p<0.001$.

+ MT data from DEX treated piglets are for Chapter 5.

Data represent mean ± SEM.

Zn uptake by BBMV for the high Zn fed group (Chapter 5). The difference in the passive component between Zn and Cu uptake in the same piglet group could be caused by different capacities for the facilitated transport systems on BBM (Zn > Cu).

High dietary Zn may affect Cu absorption by two mechanisms (Cousins, 1985). The first is by inducing MT in the intestinal mucosa. Because MT has a higher affinity for Cu than for Zn, most Cu in intestinal enterocytes would be bound to MT thereby reducing Cu absorption (Cousins, 1985). However, animals fed high Zn in this study had intestinal MT concentrations similar to controls. The insignificant MT induction by high dietary Zn in infant animals may be due to immaturity in the regulation of MT synthesis in intestinal mucosa.

Zn may also compete with Cu for a shared transporter on the BBM or it may suppress the synthesis of BBM Cu transport protein(s). Competition between Zn and Cu for binding to specific sites on BBM was demonstrated by the rapid decrease in Cu uptake when Zn was added to the incubation medium. During 5 days of high Zn feeding, synthesis of the Cu carrier protein could be specifically suppressed as indicated by the low Vmax in BBMV Cu uptake in high Zn fed animals. The lower Km observed in the high Zn fed group may indicate a higher affinity of the Cu carrier protein to Cu in order to

compensate for the smaller quantity of Cu carrier in BBM. Cu and Zn were reported to bind to a common site on intestinal brush border membranes as suggested by the observation that reduced dietary Zn supply enhanced uptake and absorption of Cu as well as Zn (Schwarz and Kirchgessner, 1974). However, our observation that high Zn fed piglets had normal Zn transport kinetics (Chapter 5) but altered Cu transport kinetics across intestinal brush border membranes suggests there are separate Zn and Cu transport systems on these membranes, at least in young animals.

The effect of Fe on intestinal Cu absorption is uncertain. Haschke et al demonstrated that Fe fortified formula (Fe:Cu=14:1) significantly suppressed Cu absorption in human infants measured by the balance method (Haschke et al, 1986). However, no effect of Fe on Cu absorption has been found in animal models (Keen et al, 1984, Bremner et al, 1987). Ferrous sulphate is often used as an Fe supplement in infant formula and sulphur (S) has been suggested to form unabsorbable copper sulphide with Cu within the intestinal lumen (Mills, 1960). The inhibitory effect of Fe on Cu absorption found by Haschke et al may be caused by the supplement of S with Fe. Using lactoferrin Fe as an Fe supplement, Cohen et al(1985) reported that tissue Cu levels were not consistently altered by Fe supplementation in weanling mice. The present study is the first in vitro

experiment showing that Fe enhances Cu binding to intestinal brush border membranes. Since non-specific binding of ^{64}Cu to the filter was not increased in the presence of 5 or 10- fold Fe, it seems unlikely that $FeCl_2$ could decrease the solubility of $^{64}CuCl_2$ or interfere with the binding of ^{64}Cu to EDTA. One possible mechanism for Fe induced enhancement of Cu binding to BBM is that the binding of Fe^{2+} to BBM activates some binding sites for Cu. Physiological levels of Cu may facilitate intestinal Fe absorption (Lee et al, 1968). Cu deficiency caused anaemia and hepatic Fe accumulation (Cohen et al, 1985). These observations suggest there could be a positive interaction between Fe and Cu for absorption.

We conclude that Cu transport can be suppressed by high luminal Zn concentration and enhanced by high Fe. In proprietary formulas designed for premature infants the ratio of Zn:Cu and Fe:Cu range from 6:1 to 11.4:1 and 1.5:1 to 4.3:1, respectively. These encompass the ratios of elements used in the present study. Further investigations are warranted to assess the importance of trace element interactions on Cu absorption in rapidly growing premature infants.

Chapter 5

ALTERATIONS IN INTESTINAL UPTAKE AND COMPARTMENTALIZATION OF Zn IN RESPONSE TO SHORT-TERM DEXAMETHASONE THERAPY OR EXCESS DIETARY ZN IN PIGLETS

5.1 Introduction

In very premature infants at risk of severe lung disease, medical management often includes use of glucocorticoid therapy and high Zn diets from mineral-fortified premature formulas. DEX, a synthetic glucocorticoid, is documented to stimulate lung maturation thereby reducing the incidence and severity of severe lung disease in LBW infants (Cummings et al, 1989; Yeh et al, 1990; Kazzi et al, 1990). Prematurely born infants are particularly vulnerable to Zn deficiency because of limited Zn stores at birth (Shaw 1979) and malabsorption as a result of an immature GI tract (Hambidge and Casey 1981). Negative Zn balances have been documented in LBW infants fed breast milk or preterm formula containing five times the amount of Zn in breast milk (Atkinson and Shah, 1991). From animal studies, it is apparent that DEX, especially in combination with a relatively high Zn diet may have a significant impact on intestinal Zn transport

and hepatic storage (Bonewitz et al, 1983; Menard and Cousins, 1983; Cousins 1985).

Glucocorticoid hormones and exogenous (dietary) Zn have been implicated in the regulation of Zn homeostasis principally via induction of the major cytosolic Zn-binding protein MT (Cousins 1985). Tissue-specific induction of MT synthesis has been associated with reduced Zn absorption (Richards and Cousins, 1975), lowered plasma Zn and enhanced Zn accumulation in liver (Cousins and Leinart 1988). Other studies have shown increased Zn absorption across intestinal cells following glucocorticoid treatment (Flanagan et al 1983; Pattison and Cousins 1986). High levels of dietary Zn may induce gene expression of MT in intestinal mucosal and hepatic cells (Cousins 1985). The increased MT synthesis correlates with a reduction in overall Zn absorption (Cousins et al,1986). Little is known, however, about the effects of DEX or high dietary Zn during the newborn period.

In order to investigate the possible consequences of short-term exogenous DEX or high dietary Zn on Zn homeostasis during early development of LBW infants, we have established an early-weaned piglet model.

5.2 Methods

5.2.1 Animals

Ten to twelve day old male piglets were removed from the sow at the Arkell Research Farm (Guelph, ON) and brought

to the McMaster University Central Animal Facility. The care of the piglets in this study conformed with the "Guide to the Care and Use of Experimental Animals" (Canadian National Research Council 1984). They were housed in stainless steel metabolic cages under infra-red heat lamps to maintain an ambient temperature of 30° C. The piglets were weaned onto a specially designed formula which is based on the composition of sow's milk (Appendix 1). On the first, second, and third days after arrival, the piglets were fed half strength, 3/4 strength and full strength formula, respectively. Afterwards, they were fed full strength formula. Piglets were weighed in the morning on arrival and on days 1, 3, and 5 of the study.

After an adaptation period of three to four days, the early weaned piglets were randomly allocated to the control, high Zn, or DEX treated group. They were fed 400 (mL/kg)/d of formula (quarter rations at 0900 h and 0300 h and half rations at 2100 h) (18 mg/kg Zn) for the study period of five days. The high Zn group was fed formula containing zinc at a concentration of 1000 mg/kg dry wt of diet ($ZnSO_4$ added to the vitamin/mineral mix). The DEX treated group was injected (intra-muscularly) with 1.5 mg/kg (bid) of DEX (sodium phosphate salt, 4 mg/mL, Hexadrol, Organon Teknika, Toronto, ON) at 0900 h and 2100 h. The control and high Zn piglets received a volume equivalent placebo injection of saline.

Since DEX treated infants are known to become hyperglycemic, the blood glucose status of the piglets was assessed (Kramer and Hultzen, 1978). Blood glucose was measured each morning before dosing and feeding using Chemstrip BG (Boehringer-Mannheim, Laval, PQ) on a drop of blood from a pin prick of an ear vein. Daily urine volumes were also measured to monitor glucose losses.

5.2.2 Blood and tissue samples

The piglets were euthanized on the morning of the sixth day of the study by a euthanol injection. Blood samples were taken by a heart puncture and urine samples by a bladder puncture. Plasma was retrieved by using heparinized syringes to draw the blood and subsequent centrifugation at 2000 x g for 15 min. Final urine-glucose values were measured using a modified peroxidase-glucose oxidase assay from Dahlqvist (1968).

One hundred cm of the proximal intestine were excised at necropsy and immediately immersed in ice-cold 0.9% saline. The intestine was slit lengthwise and then flushed with saline. Epithelial mucosa was scraped from the muscle tissue with a glass slide and stored at -70° C. for further preparation. Scraping was performed on ice to minimize enzymatic activity. Samples of liver were also removed from the animals.

5.2.3 Preparation of BBMV

Intestinal BBMV were obtained by homogenizing the mucosa and employing a differential centrifugation method (Davidson and Lonnerdal, 1988). The extent of brush border purification was determined by measuring changes in the specific activity of sucrase (Dahlqvist 1968)). The BBMV solution was used in the ^{65}Zn transport study the same day to avoid vesicle damage caused by freezing and thawing.

5.2.4 Zn uptake experiments

BBMV Zn uptake was measured by a rapid filtration method using ^{65}Zn tracer. Samples were prepared in triplicate with a blank of nonspecific binding (no BBMV) included to correct for background radioactivity. Uptake rates (V) were expressed as nmol Zn^{2+} per mg total vesicular protein per minute. Total protein was measured by the dye-binding procedure of Bradford (Bradford 1976) using crystalline bovine serum albumin as a standard. Maximum uptake rate (V_{max}) and the half saturation constant (K_m) were determined using the method described by Eadie (1942) and Hofstee (1952).

5.2.5 Zn efflux across BBMV

To compare the permeability of BBMV between DEX-treated and control piglets, we measured the rate of Zn efflux across BBMV. BBMV was first loaded with ^{65}Zn via incubation with 0.3 mM $ZnCl_2$ and 74 KBq ^{65}Zn in the incubation buffer at

37°C for 60 min. After examining the amount of Zn loaded in BBMV, the incubation solution was diluted 19-fold with transport buffer containing 10 mM EGTA. At different time intervals after the dilution, the percentage of ^{65}Zn remaining in BBMV was determined by rapid filtration and counting of filters in a gamma counter (Beckman Gamma 5500).

5.2.6 Tissue Zn content

Zn levels in plasma were measured using an atomic absorption spectrophotometer (Perkin-Elmer) following a 5:1 dilution with 1.25 M nitric acid (Beatty 1978). Certified standard reference plasma was used as a control throughout assay procedures.

Liver and mucosal tissue samples were quantitatively assayed for Zn by atomic absorption spectrometry (Perkin-Elmer) (Beatty 1978). These tissue samples were prepared by weighing ca 0.5 g wet mucosa and ca 1.0 g wet liver into crucibles which were heated at 100° C for 24 h in a muffle furnace and then weighed dry. The samples were then heated at 500° C for 72 h and dissolved in 10 mL 1.25 M Ultrapure nitric acid. The liver solutions were diluted 5:1 with 1.25 M Ultrapure nitric acid. Certified standard reference non-fat milk powder was analyzed.

5.2.7 Tissue MT concentration

MT levels were determined in liver and intestinal

mucosa of all pigs. Tissue homogenates (20% wt/vol) were centrifuged at 10 000 x g for 20 min, at 4° C. The supernatant was heated for 2 min in a boiling water bath. After another 10 000 x g centrifugation for 2 min, 100 μL of the supernatant was subjected to MT measurement by a Cd binding assay using ^{109}Cd (Eaton 1982).

5.2.8 FPLC analysis of intestinal Zn, Cu binding proteins

To study the effects of DEX and high Zn feeding on Zn and Cu binding proteins in intestinal mucosa, the mucosal homogenate was analyzed by a fast protein liquid chromatography (FPLC) system. Mucosa samples were homogenized in 20 mM HEPES-PBS buffer, pH 7.4 (20% wt/vol) in a Janke & Kunkel homogenizer for two 20 second intervals at medium speed. The homogenate was centrifuged at 169 000 x g at 4° C for 30 min. A 500 μL aliquot of the supernatant was transferred to a Superose 12 column (10 x 300 mm) of an FPLC system (Pharmacia, Uppsala, Sweden, Model LCC-500). The eluting solution was 20 mM HEPES-PBS, pH 7.4 and flow rate was 60 mL/h. The eluting fractions were collected at 1 min intervals and analyzed by AAS for Zn and Cu concentrations.

5.2.9 Brush border enzyme activity

Approximately 0.1 g wet mucosal tissue was suspended in 1.0 mL buffer (100 mM D-mannitol, 1 mM Tris-Hepes, pH 7.5), homogenized for 1 min and then centrifuged at 3000 x g for 10

min. The supernatant was assayed for sucrase and lactase according to the method described by Dahlqvist (1968). Enzyme activity was expressed as nmol substrate hydrolysed/mg protein·min^{-1}.

5.2.10 Statistical methods

Multiple comparisons were performed using the Tukey's method on the different groups after performing a repeated measurements procedure between the groups (SAS Institute, Cary, NC) (SAS Institute, 1985). Kinetic data were analyzed using the covariance technique (ANOCOVA). All tests were carried out with at most a 5% level of confidence. Data were represented as mean ± SD unless otherwise noted.

2.3 Rasults

2.3.1 Piglet characteristics

The characteristics of the piglets used in the experiment are shown in Table 3. There were no differences in age, initial weight, or dietary intake among the three groups. Daily blood glucose levels as measured by Chemstrip BG were significantly higher for the DEX group as compared to the control group (t=4.09, p=0.026) (Figure 25). The groups did not differ in urine glucose and volumes which were monitored to help assess glycemia status. This is consistent with previous studies which found the glucose renal threshold for 3-5 kg piglets to be 8.0 to 9.5 mM (Link 1953). One pig in

TABLE 3, THE CHARACTERISTICS OF THE PIGLETS TREATED WITH DEX OR HIGH DIETARY Zn

	Control	DEX	High Zn
Age (d)	13.9 ± 0.4	14.0 ± 0.8	13.9 ± 0.7
Initial weight (kg)	3.62 ± 0.34	3.68 ± 0.29	3.44 ± 0.24
Formula consumed (ml/kg/d)	360 ± 35	385 ± 30	355 ± 75
weight gain (g/d)	85 ± 50	51 ± 42	88 ± 52
Urine volume (ml/kg/d)	115 ± 35	145 ± 45	130 ± 40

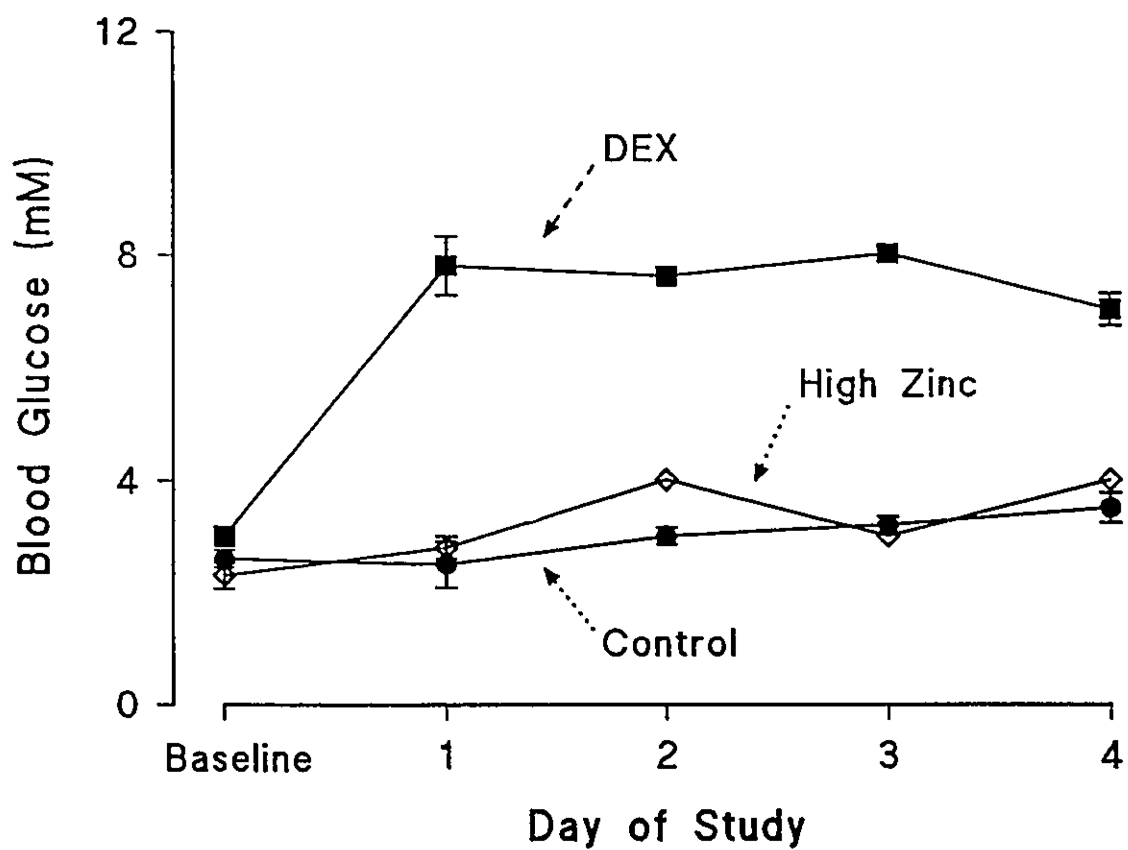


Figure 25, Daily blood glucose values measured at baseline and on each day of treatment. There are 7 piglets in each treatment group. Data represent mean ± SEM.

the DEX treated group was an outlier (>2 SD from the mean) for blood and urine glucose levels as well as for velocity of Zn transport. The high blood and urine glucose values suggest that this pig may have some disorder in glucose metabolism, probably diabetes. Therefore this pig was excluded from the data analysis.

5.3.2 The kinetics of Zn transport across intestinal BBM

Figure 26 shows saturation curves comparing the initial rate of Zn uptake by BBMV for the three groups. The values for the DEX treated group were higher than the control and high Zn diet group at each extravesicular Zn concentration. There was no difference in Zn uptake velocities between high Zn and control groups. The Eadie-Hofstee plots of the three groups are shown in Figure 27 where the data are presented as the means of each group. J_{max} and K_m values were calculated from linear regression analysis of these plots. The Y-intercept of the regression equation represents the J_{max} and the absolute value of the slope represents the K_m (Eadie 1942, Hofstee 1952). This figure shows that the DEX treated group not only had a 170% higher J_{max} but also an 80% higher K_m than the other two groups.

5.3.3 Zn efflux across BBMV

The amount of ^{65}Zn loaded in BBMV after a 60-min incubation was greater in DEX than control piglets(5.24±0.92

μmol/mg protein *versus* 4.64$\pm$0.48 μmol/mg protein) but the difference was not statistically significant. The Zn efflux rate was much higher in control piglets than in DEX piglets (Fig 28). After a 0.5 min efflux, there was 31.0% of ^{65}Zn left in BBMV of DEX-treated pigs, whereas there was only 7.2% of loaded ^{65}Zn in BBMV of the control animals ($p<0.001$). After a 5-min efflux, there was still 17.9% of ^{65}Zn left in BBMV of DEX treated animals compared with 3.6% in BBMV of control animals ($p<0.05$).

5.3.4 Tissue Zn and MT

Similar levels of plasma Zn were found among the three groups. Furthermore, there were no differences in liver and intestinal mucosal Zn content between the DEX treated and control groups. However, the high Zn diet group did have a significantly higher Zn content in both liver and intestinal mucosa than the other two groups (Fig 29).

Liver and intestinal MT concentrations are shown in Table 2 (in Chapter 4). High Zn fed piglets had 10 times higher liver MT concentration than those of the other two groups. There is no significant difference in MT concentration in intestinal mucosa among the treatment groups.

5.3.5 Zn, Cu binding proteins in intestinal mucosa

Figures 30 a-c show representative protein separations in mucosal samples from the three treatment groups following

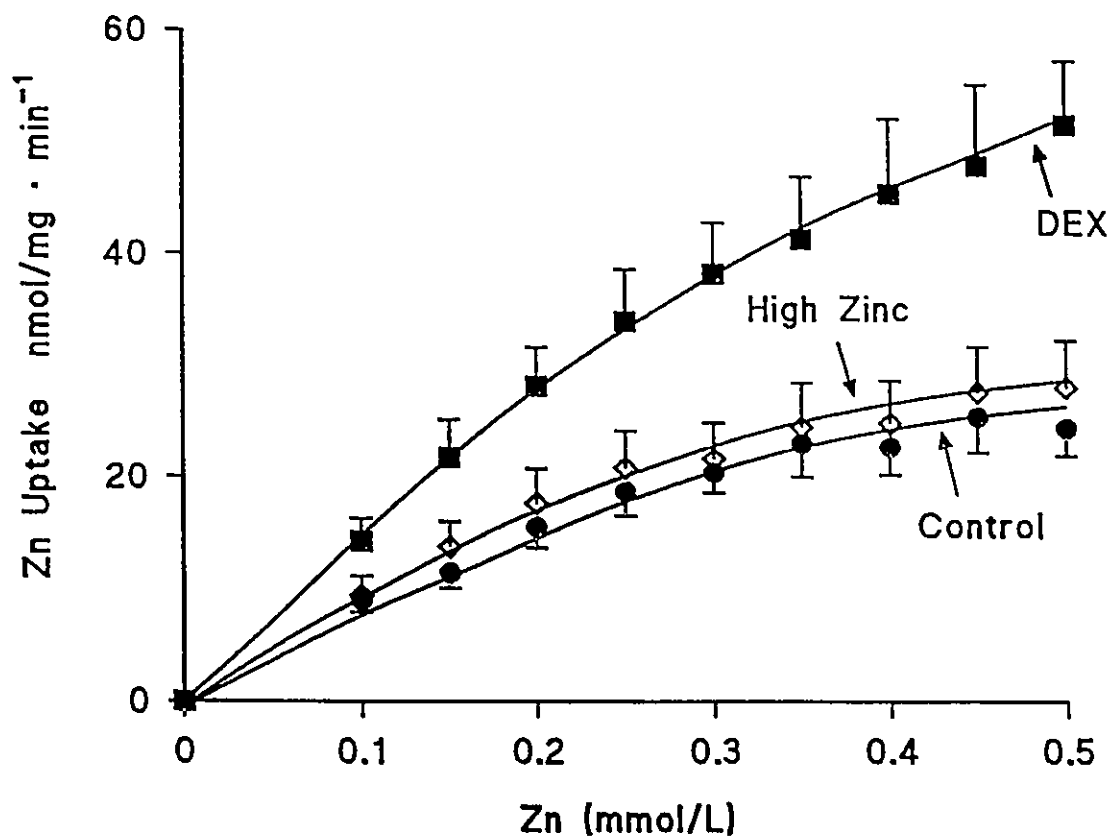


Figure 26, Initial rates of Zn^{2+} uptake (1 min) by BBMV with extravesicular Zn^{2+} concentrations ranging from 0 mmol/L to 0.50 mmol/L. Data represent the mean $\pm$ SEM for vesicles from 7 control piglets, 7 piglets fed a high Zn diet (1000 mg/kg), and 6 DEX-treated piglets (1.5 mg/kg, im, bid, and 4.0 mg/mL).

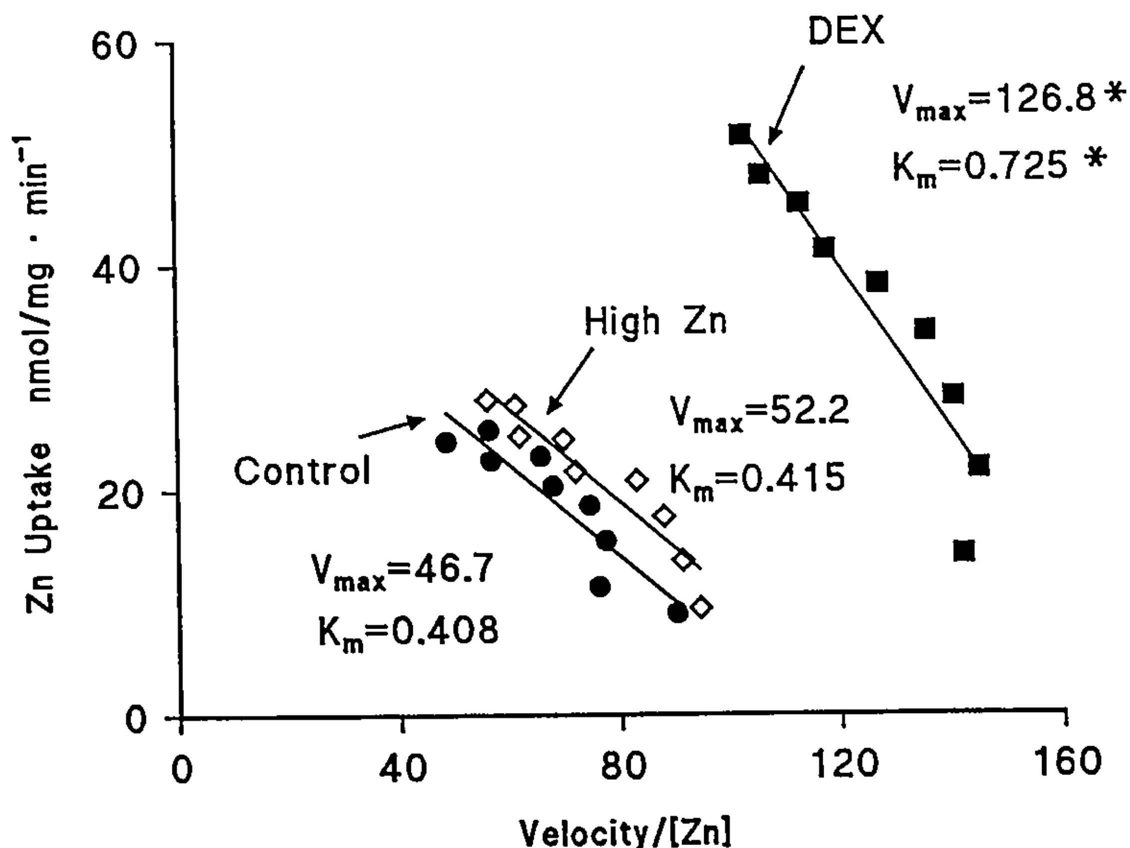


Figure 27, Eadie-Hofstee plot of the kinetics of Zn^{2+} uptake by BBMVs from the data described in **FIGURE 26.** Data are expressed as initial rates of uptake (1 min). J_{max} and K_m for each group were determined from linear regression equations and are expressed as nmol Zn/mg total protein·min^{-1} and mmol/L, respectively. * notates these values are significantly different ($p<0.05$).

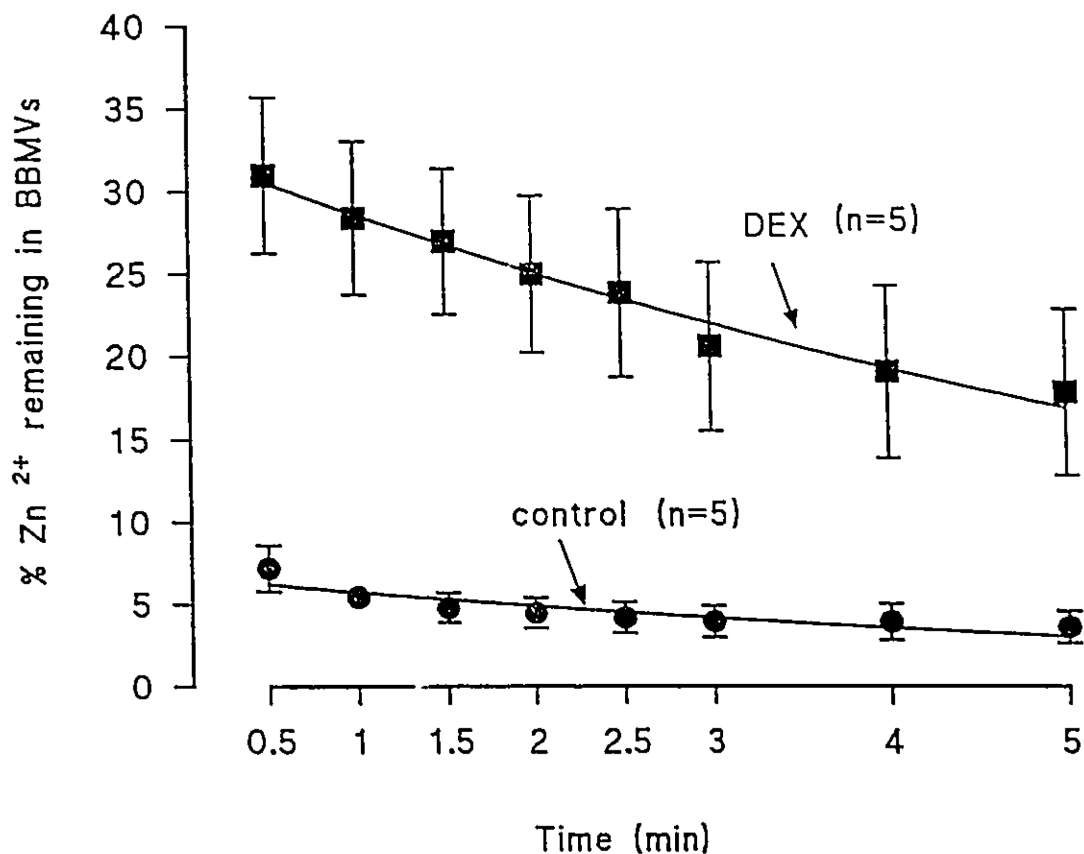


Figure 28, The effect of DEX treatment on Zn^{2+} efflux from BBMV preloaded with Zn^{2+}. Time 0 represents the amount of Zn^{2+} taken up by BBMV during preloading (*i.e.*100% Zn^{2+} remaining in BBMV). Each point is the mean ± SEM of five piglet samples.

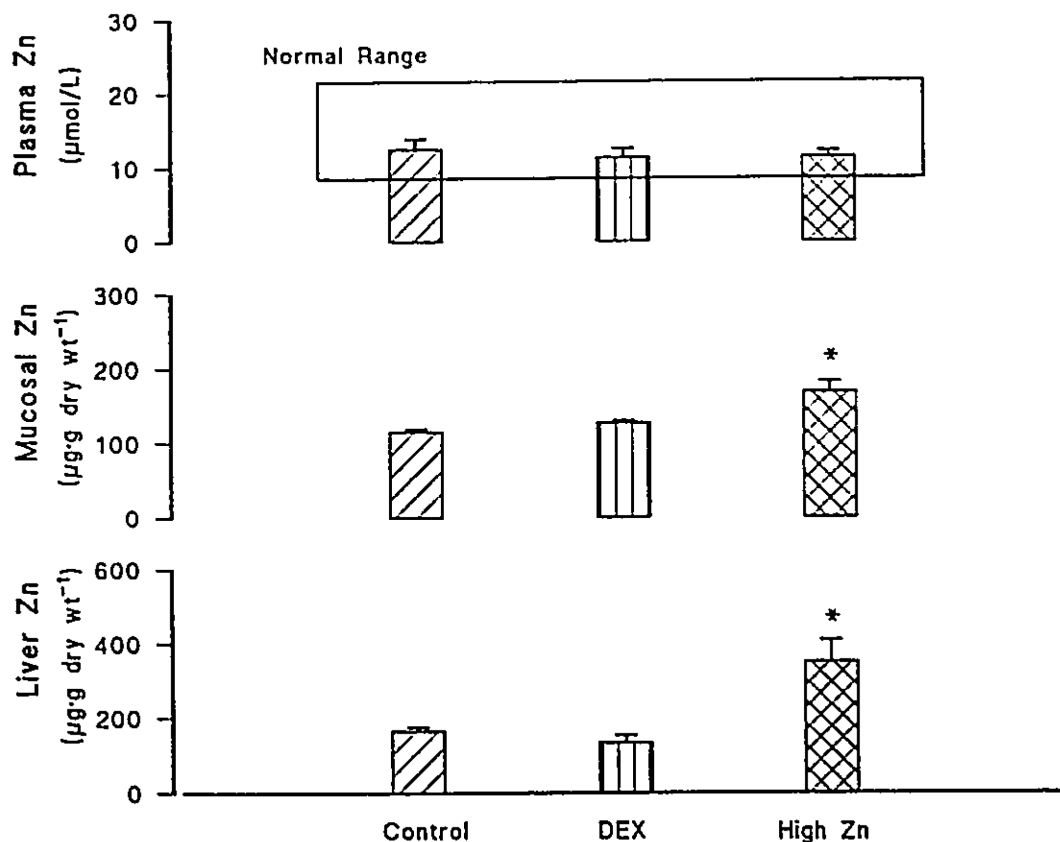
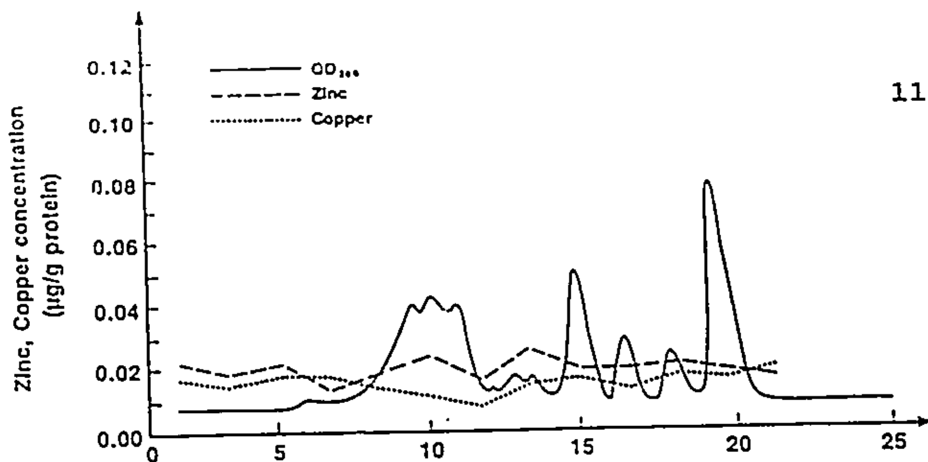


Figure 29, Comparisons of plasma, mucosal, and liver Zn values between treatment groups. Bars represent mean ± SEM for tissue from 7 control piglets, 6 DEX-treated piglets (3.0 mg/kg·d^{-1} and 4.0 mg/mL), and 7 piglets fed a high Zn diet (1000 mg/kg). The normal range for plasma Zn was determined in piglets up to 3 weeks old by Ullrey, et al (1967). * notates these groups were significantly different within tissues ($p<0.05$).

Figure 30, FPLC gel filtration chromatography of mucosal homogenate (20% wt/vol) on Superose 12 in 20 mM HEPES-PBS buffer at pH 7.4. Flow rate was 60 mL/h and a 500 μL sample was injected. Figure 5a: control; Figure 5b: DEX; Figure 5c: high Zn.



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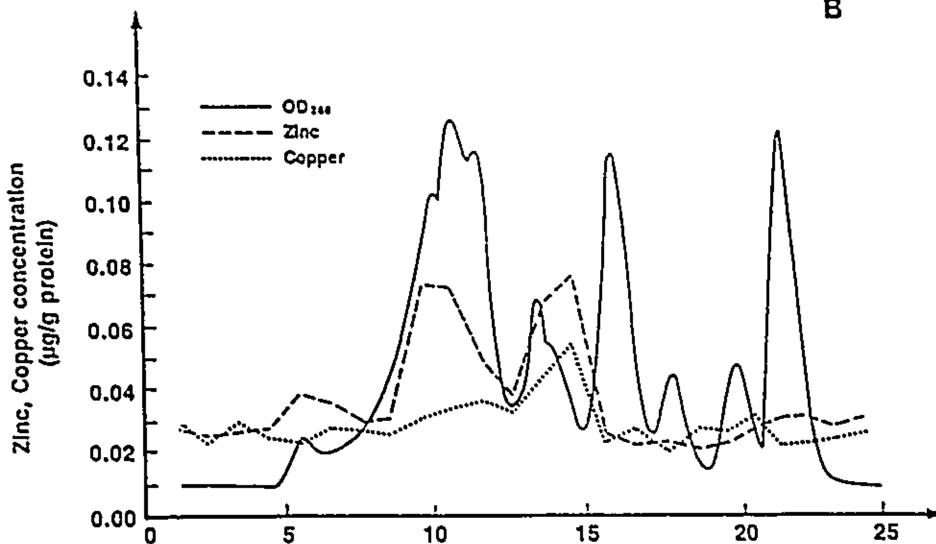

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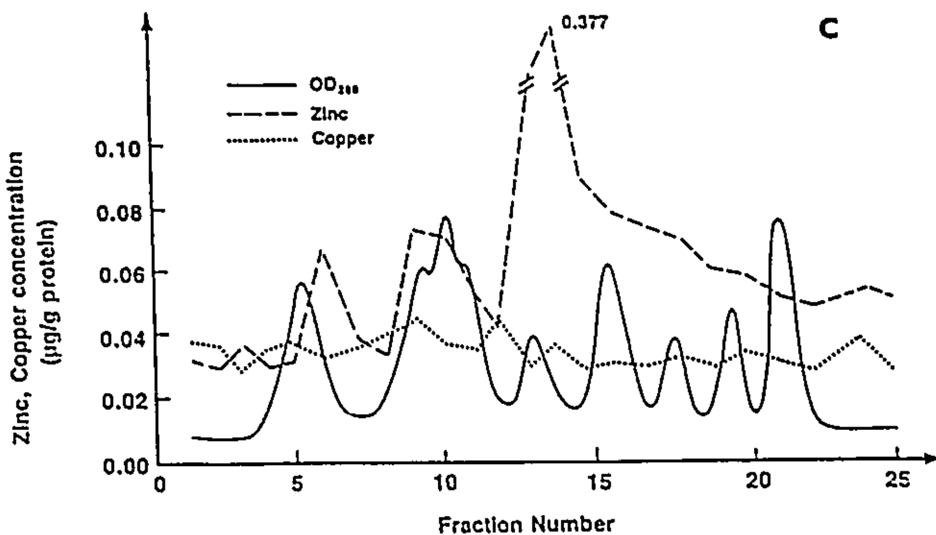

separation by the FPLC system. Protein, Zn and Cu concentrations are shown on the same graph. Comparing the three FPLC graphs, the pattern of protein peaks are similar with the exception of peak height. The peaks are notably higher in the DEX treated piglet sample than those of the other two treatment groups. This is consistent with the report that DEX can induce several enzymes and increase RNA to DNA ratio in intestinal mucosa (Neu et al, 1986). In Figure 30a, there are two small Zn peaks but no definite Cu peak. The height of both Zn peaks was more than doubled in DEX-treated piglets (Figure 30b) compared to that in Figure 30a. A Cu peak was also found in the same position of the second Zn peak. In Figure 5c, the height of the second Zn peak is more than ten times of that seen in Figure 5a. Since MT was not induced in intestinal mucosa of all piglets, the change in the height of these Zn and Cu peaks suggested that several Zn and Cu binding proteins other than MT were induced by DEX and high Zn feeding. The functions of these proteins and their physio-chemical and immunological characteristics need to be studied further.

Table 4 presents the results of intestinal lactase and sucrase activity from homogenate of the mucosal scrapes. Although there were no statistically significant differences among the three groups, the DEX treated group tended to have lower lactase activity and higher sucrase activity than the

TABLE 4, INTESTINAL MUCOSA LACTASE AND SUCRASE ACTIVITY IN PIGLETS TREATED WITH DEX OR HIGH DIETARY Zn

	Control	DEX	High Zn
Sucrase (U)*	66.7 ± 34.7	100.3 ± 35.8	74.0 ± 22.4
Lactase (U)	228.2 ± 51.1	85.2 ± 28.6	122.6 ± 56.2

* U, nmol substrate hydrolyzed/mg total protein/min

other two groups ($p=0.18$ and $p=0.12$, respectively).

5.4 Discussion

In this study, exogenous DEX given to developing piglets resulted in an increase in Zn uptake by BBMV that is suggestive of induction of maturation of intestinal epithelial cells. Five days of DEX treatment produced a significantly higher J_{max} for Zn uptake. Compared to controls, this implies greater quantities of Zn carrier protein in the brush border membrane, possibly as a result of up-regulated protein synthesis or prolonged half-life of the protein in the membrane. The higher K_m observed with DEX treatment indicates that the affinity of the carrier protein for Zn was lower. It is possible that in small intestinal epithelial cells, DEX induced the synthesis of a Zn carrier protein with a low affinity for Zn. This induction may imply maturation of the intestinal epithelia although the ontogeny of this protein in the piglet has yet to be studied.

Another possible mechanism to explain the increased Zn transport is that DEX inhibits prostaglandin (PG) synthesis in small intestinal enterocytes. Such a mechanism has been proposed for the improvement of respiratory parameters by DEX treatment in BPD infants (Mammel et al, 1983). Song et al (1988) found that PGE_2 and $PGF_{2\alpha}$ caused increased intestinal Zn absorption. They suggested that PGE_2 chelates Zn at the

intestinal lumen and carries it across the mucosal cell of the small intestine. They found that PGE_2 and $PGE_{2\alpha}$ doubled the amount of Zn taken up by BBM when the PG was added to the serosal side of the jejunal mucosal cells. However, it should be noted that the concentration of prostaglandins used was considerably lower than that of Zn, making it unlikely that a quantitative effect via chelation of Zn occurred. However, a systemic effect by the prostaglandins would be a possibility. In contrast, Cousins et al (Cousins et al, 1978) and Cunnane (1982) reported that physiological doses of PGF_2 inhibited Zn absorption. Differences in prostaglandin dose and location of treatment could account for the conflicting results. More research is required to clarify the roles of prostaglandins in Zn absorption and their relation to physiological perturbations caused by DEX treatment.

The observed similar levels of plasma, intestinal mucosa, and liver Zn between DEX and control groups suggested no differences in net transfer of Zn across the intestine. Bonewitz et al (1983) found that DEX-treated adult rats had a 75% higher lumen to mucosa transport of Zn and a 45% lower mucosa to plasma transport; thus, part of the transported Zn was retained in the mucosa cells. In rats given a bolus or a daily injection of DEX for 7 d, overall Zn absorption was not increased by DEX, but an accumulation of Zn in small

intestinal mucosa was found (Hempe et al, 1991). The failure of our DEX-treated piglets to accumulate Zn in intestinal mucosa may be the result of immaturity of their intestinal mucosa compared with the chronologically older animals previously studied (Hempe et al, 1991). High permeability of the brush border membrane could have allowed the leakage of the Zn from mucosal cells back into the intestinal lumen in the piglets. However, based on the efflux experiment, the permeability of BBM to Zn in DEX-treated piglets was much lower than that in the control piglets. These results are consistent with previous observations of glucocorticoid-induced inhibition of Ca transport by a reduction in permeability of the intestinal mucosa of 14-d-old rats (Ghishan and McNeely, 1982). Furthermore, glucocorticoid treatment in rats of the same age can cause premature "closure" of the small intestine (Daniels et al, 1973). The higher influx and lower efflux of Zn across intestinal BBM plus lack of Zn accumulation in the intestinal mucosa suggests that DEX enhances Zn absorption by a carrier-mediated mechanism (Hoadley and Cousins, 1988). Possible explanations for the unchanged plasma and tissue Zn levels may be the moderate Zn content of the pig formula, the short duration of DEX treatment, and/or Zn accumulation in intestinal mucosa. These possible mechanisms need to be clarified in future studies.

In the present study, DEX did not induce MT synthesis in liver or intestine, which is partially different from the results of Hempe et al (1991); they found that intraperitoneal administration of DEX to rats for 7 days markedly increased liver MT synthesis over control animals but did not alter intestinal MT concentration. Zlotkin et al (1988) measured liver MT levels in human premature and term infants during the first year of life. They found that hepatic MT levels were highest in the youngest infants, falling with increasing postnatal age. Our data (unpublished results) also suggest that there is a marked decrease in liver MT concentration during the first 20 days of life in piglets. Compared with liver MT, the level of intestinal MT changed very little in the same period. Our data suggest that the physiological fall in liver MT is not altered by DEX therapy.

To determine if exogenous DEX caused induction of other proteins located in the brush border membrane, we measured lactase and sucrase activities. Current literature suggests that lactase activity is altered by DEX due to stimulated maturation of the small intestine (Majumdar and Rehfeld,1983; Majumdar and Nielson, 1985). During development in rodents, lactase activity was highest in the first week postnatally and by the fourth week it decreased to normal adult levels (Majumdar and Rehfels, 1983). Majumdar and Neilsen (1985) reported that the presence of exogenous

glucocorticoids in suckling rats and mice stimulated a decrease in lactase activity around three weeks postnatally. Although the differences between the control and DEX treated piglets were not significant, a trend indicating a reduced (28%) lactase activity in the DEX treated animals was observed (p=0.18). The ontogeny of sucrase activity during early maturation of the small intestine can also be induced by exogenous glucocorticoids (Majumdar and Nielson, 1985). Unlike lactase, sucrase levels are lowest during the early postnatal weeks and then rise to higher levels. We observed a 34% increase in sucrase activity in the DEX treated compared to the control animals (p=0.12). Since there was no sucrose present in the piglet diet, the suggested induction of sucrase may be attributed to the presence of glucocorticoids. The high Zn diet did not alter either lactase or sucrase activities compared to control animals.

As experienced clinically in DEX treated BPD infants (Yeh et al, 1990), DEX treated piglets had significantly higher blood glucose levels than the other two groups. The elevated blood glucose probably resulted from stimulation of gluconeogensis and inhibition of glucose utilization by DEX. Furthermore, the DEX treated group of piglets had a lower mean weight gain than the other two groups; however, these differences were not statistically significant perhaps because of type II error (small sample size). Growth delay has been

associated with DEX therapy in infants treated for BPD (Yeh et al, 1990) and in the newborn rat model (Venkataraman et al, 1991). Changes in protein, carbohydrate and mineral metabolism caused by DEX are possible reasons for the observed reduced growth. The mechanism by which DEX influences protein and carbohydrate metabolism is better understood than its effects on mineral metabolism. The effects of glucocorticoids on the absorption, distribution, and utilization of Zn and other trace elements require further exploration.

A five day high Zn intake did not change the kinetic parameters of Zn transport, which is in contrast to our hypothesis. The significantly higher liver and intestinal mucosal Zn and liver MT in this group compared to the control group suggest that more Zn was transported across the brush border membrane and entered the systemic circulation. Hunt et al (1981) reported that in rats, a high Zn diet increased liver and intestinal Zn content and decreased overall Zn absorption as measured by whole body counting of administered tracer Zn. In their study, a four day moderately high Zn feeding (141 mg/kg dry wt of diet) decreased overall Zn absorption significantly compared to the same period of feeding with lower dietary Zn (11.2 mg/kg dry wt of diet). These results suggest that the amount and duration of high Zn feeding in our experiment should have been more than sufficient to decrease Zn absorption. The similar Zn transport

kinetics we found between high Zn and control piglets may suggest that a high Zn diet could decrease Zn absorption by a mechanism other than altered brush border membrane transport. It is also possible that the mechanisms down-regulating Zn absorption in older animals are immature in the very young animal.

The absence of induction of intestinal MT by high Zn feeding may also be a special phenomenon for animals in early infancy. The unchanged rate of Zn transport across BBMV and intestinal MT concentration will allow more dietary Zn to be absorbed in the condition of high dietary Zn. However, the high Zn binding protein peak shown by FPLC analysis of intestinal mucosa of high Zn group suggest some Zn binding protein other than MT was induced by high dietary Zn which could inhibit large quantities of Zn across intestinal mucosa.

We conclude that DEX treatment can induce several enzymes and proteins in apical membranes of small intestinal enterocytes in early-weaned piglets. As a result, Zn transport across the brush border membrane increased and Zn efflux across the membrane was decreased. The similar kinetic parameters we found in the Zn transport study between high dietary Zn and control piglets may suggest that a high Zn intake decreases overall Zn absorption via a mechanism other than lowered Zn transport across the brush border membrane. Five days of high Zn intake in piglets increased Zn content in

liver and small intestinal mucosa, probably as a result of high passive diffusion and induced hepatic MT synthesis.

During late gestation, the physiological peak of maternal glucocorticoid levels in late gestation has been shown to induce the maturation of the lung and small intestine (Majumdar and Nielson, 1985). This induction is probably important for the postpartum development of digestive and respiratory functions. Preterm infants have immature pulmonary and intestinal epithelial cells because they do not experience the intrauterine maturation in the last trimester. To date, exogenous glucocorticoids have proven therapeutically efficacious in the induction of pulmonary maturation (Cummings et al, 1989). Our interest is in knowing the impact of therapeutic use of exogenous glucocorticoids in the LBW infant on induction of maturation of intestinal enterocytes that in turn may alter regulatory mechanisms for trace element transport.

Because of the wide range of biological effects of glucocorticoids, DEX treatment in infants with BPD can cause many side effects. Some of these effects are harmful, including pulmonary infection, hyperglycemia, hypertension, retarded growth and bone demineralization (Yeh et al, 1990; Taeusch 1975); but several others may be beneficial for LBW infants (Yeh et al, 1990; Kazzi et al, 1990). It is conceivable that DEX therapy in very premature infants may

induce maturation of the small intestine and possibly the synthesis of Zn carrier protein(s) on the brush border membrane. This could be advantageous with respect to delivery of Zn to meet the nutrients for rapid growth and to compensate for the immaturity of the gastrointestinal tract in LBW infants. The effects of DEX treatment on the absorption and utilization of Zn and other trace elements in LBW infants should be evaluated systematically.

Chapter 6

Long-term consequence of chronic dexamethasone therapy ±high dietary Zn on growth and Zn, Cu metabolism in the piglet model

6.1 Introduction

Long term DEX therapy as used to ameliorate severe lung disease of premature infants is common practice in neonatal intensive care units in North America (Brownlee et al, 1993; Kazzi et al, 1990; Yeh et al, 1990). This therapy is thought to induce lung maturation, improve lung inflammation and reduce the dependence on mechanical ventilation (Kazzi et al, 1990; Yeh et al, 1990). However, because of its glucocorticoid activity, DEX has the potential to interfere with a number of metabolic pathways in premature infants (Yeh et al, 1990). Glucocorticoid hormone has been documented to induce maturation of liver (Yuwiler and Celler, 1973), pancreas (Puccio 1988) and the gastrointestinal tract (Arsenault and Menard 1985). This hormone stimulates the differentiation of gastric mucosal cells (Peitsch 1984), promotes the maturation of some digestive enzymes on intestinal brush border membranes (Celano et al, 1977), and

enhances the fluidity but decreases the permeability of intestinal BBM (Neu et al, 1986). As a result, DEX was suggested to cause premature gut closure in infant animals (Daniels 1973).

In a previous study we found that a short-term (5 day) treatment with high dose DEX (3 mg/kg/d) enhanced Zn uptake across isolated intestinal BBM in young piglets. At the same time the rate of Zn efflux across intestinal BBM was reduced (Wang et al, 1993a). Whether the accumulation of Zn within mucosal cells would result in increased delivery of Zn to the blood or whether the Zn becomes trapped in the mucosal cell and is subsequently lost through intestinal sloughing remains to be investigated.

DEX was documented to increase biliary Cu excretion in adult rats (Cousins 1985). We found a short-term (5 day), high dose DEX treatment (3 mg/kg/d) significantly reduced liver Cu content in piglets (Wendy and Atkinson, unpublished results). However, the effect of long-term DEX treatment on Cu metabolism in early life has not been investigated.

The objective of the present study was to determine the effect on growth and Zn and Cu metabolism of long-term low dose DEX representative of the drug regimen is currently used for prematurely born infants in neonatal intensive care units. A high dietary Zn intake was included as an intervention in order to see if excess dietary Zn would ameliorate the effect

of DEX on growth and Zn metabolism.

6.2 Material and methods

6.2.1 Animal and diets

A total of 18 male Yorkshire piglets aged 3-4 d were obtained from Arkell Research Farm (Guelph, Ontario, Canada). Upon arrival, they were weaned onto a special pig formula based on one designed in our laboratory to meet the nutrient requirements of infant piglets (Appendix 1). The care of the piglets conformed with the "Guide to the Care and Use of Experimental Animals" (Canadian National Research Council 1984). The piglets were housed in stainless steel metabolic cages under infrared heat lamps used to maintain an ambient temperature of 30°C. They were fed with pig formula as 400 ml/kg d^{-1} and DDI as 120 mL/d. After 3 days adaptation to the diet, the piglets from each litter were randomized to one of three treatments: regular pig formula containing 45 ppm Zn and oral DEX (Hexadrol 4 mg DEX/ml, Organon Teknika, Toronto) at a dose of 0.5 mg/kg/d, bid; the same DEX treatment plus high dietary Zn intake (150 ppm); or regular formula feeding and oral placebo (sterile water). The investigators were blinded to the treatments. The DEX and placebo were given twice daily at 0900 and 1700 h by an oro-gastric feeding gavage tube (#8 Frech) which was rinsed with 5-10 ml of sterile distilled water. The treatment was maintained for 15 days. Measurements

included daily weight, milk and fluid intake and urine volume. Length was accurately measured at day 0, day 7-8 and day 16 of treatment while the piglets were anaesthetized. Blood samples were taken while piglets were anaesthetized by gas inhalation at day 1 and 8 of treatment from the internal jugular vein and at day 16 from the heart. Blood glucose level was checked every third day through lancing of the ear vein and Chemstrip analysis (Boehringer-Mannheim, Laval, PQ).

6.2.2 In-situ intestinal perfusion

The rate of Zn absorption was determined on the 16th day of treatment by an in-situ intestinal perfusion technique (Ghishan and Sobo 1983). The piglets were fasted overnight and anaesthetized by isoflurane (Aerrane[R], Anaquest, Missisauga, Ontario) inhalation. After opening the abdominal cavity with a middle incision, a 10 cm segment of proximal jejunum was isolated, cannulated and flushed with 60 ml of air. The cannulated segment was then returned to the abdominal cavity. Body temperature of the piglets was maintained at 36-37°C. The cannulated intestinal segment was perfused with perfusate described on p41 in-situ at a rate of 0.5 mL/min for 30 minutes in a recirculating manner with the aid of a infusion pump (IVAC 530, IVAC Corporation, San Diego, CA). Perfusate samples of 200 μL were collected at time '0' and every 5 min thereafter for 30 min. During this time a 0.5 mL blood sample

was drawn from the portal vein at time '0' and every 8 min thereafter for 60 min. After taking each blood sample, 0.5 mL of 0.9% saline was infused into the portal circulation to compensate for blood loss. At the end of blood sampling, the intestinal segment was flushed with 60 mL air and then excised.

After intestinal perfusion, Poly R-478 and elemental Zn concentrations in the perfusate samples were measured. The total liver was removed, rinsed with ice-cold 0.9% saline and homogenized. ^{65}Zn radioactivity in the perfusate, blood, liver and perfused intestinal segment was measured in a gamma counter (Beckman Gamma 5500, Irvine, CA).

6.2.3 Necropsy and tissue collection

At the end of the intestinal perfusion, a blood sample was taken by heart-puncture. The piglets were euthanized by an overdose of sodium pentobarbital. A 100 cm segment of proximal jejunum immediately adjacent to the perfused segment was excised, slit open lengthwise and flushed with ice-cold 0.9% saline. Epithelial mucosa was scraped from the intestinal muscle layer by a glass slide on a glass plate at 4°C. The mucosa sample was kept at -70°C for later measurements of Zn and MT concentrations. Kidneys and spleen were carefully excised, blotted on paper tower and then weighed.

6.2.4 Preparation of intestinal BBMV

BBMV were prepared by homogenizing the mucosa of the proximal jejunum and employing a $MgCl_2$ precipitation method. BBMV were prepared on the same day as the ^{64}Cu transport study in order to avoid potential vesicle damage caused by freezing and thawing.

6.2.5 Cu uptake experiments

^{64}Cu was produced by irradiating elemental Cu at the Nuclear Reactor at McMaster University. Cu uptake across BBMV was measured by the rapid filtration method with ^{64}Cu tracer as described in Chapter 2. Samples were prepared in triplicate with a blank (no BBMV) included to correct for non-specific binding of ^{64}Cu to filters. Radioactivity measurements were corrected for decay by a decay curve of ^{64}Cu. Uptake rates were expressed as nmol Cu^{2+} per mg total vesicular protein per min. Total protein was measured by the dye-binding procedure of Bradford (Bradford 1976) using crystalline BSA (Sigma Co., St. Louis, MO) as a standard. V_{max} and Km were determined using the method of Eadie and Hofstee (Eadie 1942; Hofstee 1952).

6.2.6 Tissue Zn and Cu contents

Plasma Zn and Cu were measured using an atomic absorption spectrometer (model 703, Perkin-Elmer, Norwalk, CT). Certified standard reference plasma (A67 and A69 for Zn and E 9302 and E 9309 for Cu; interlaboratory Comparison

Program, Centre de Toxicologie du Quebec, Sainte-Foy, Quebec, Canada) was used as a control throughout assay procedures. Liver and intestinal mucosa were quantitatively assayed for Zn and Cu by atomic absorption spectrometry after ashing at 500°C for 72 h and dissolved in 10 ml 1.25 M nitric acid. Certified standard reference nonfat milk powder (National Bureau of Standards, Washington, DC, no.1549) was analyzed as control. Urine Zn and Cu were measured directly by atomic absorption spectrometry.

6.2.7 Tissue MT concentrations

MT concentration was measured in liver and intestinal mucosa of all pigs. Tissue homogenate (20% wt/vol) was centrifuged at 10 000 x g for 20 min at 4°C. The supernatant was heated for 2 min in a boiling water bath. After another 10 000 x g centrifugation for 2 min, 100 μl of the supernatant was assayed for MT by a cadmium binding assay using ^{109}Cd (Eaton 1982).

6.2.8 Statistical analysis

A two-way (treatment and time) repeated measurements ANOVA or a one-way ANOVA (across treatment groups) was performed as appropriate for outcomes of interest using Minitab software Program (Minitab Inc., State College, CA). Comparisons of mean values between treatment groups or between time points were analyzed by a post-hoc multiple comparison

test, the Student-Newman-Keuls Test (Glantz 1992). Data are represented as mean ± SD unless otherwise noted.

6.3 Results

The characteristics of the piglets are shown in Table 5. There were no differences in age, initial weight, formula intake, and urine volume, serum Zn and Cu or blood glucose at day 16 of treatment. Compared to DEX and DEX+Zn groups, the control group had a significantly higher velocity of weight growth (Fig 31) (slope of weight gain: 0.33 vs 0.14 and 0.14 kg/d) respectively; and length growth (Fig 32)(slope of length gain: 1.31 vs 0.55 and 0.6 cm/week respectively). The kidneys were significantly larger at necropsy in DEX and DEX+Zn groups than in the control (Table 6). Fig 33 and 34 show plasma Zn and Cu levels at day 0, day 7-8 and day 16 of the treatment. The DEX group had a lower plasma Zn level at day 7-8 than at day 0 ($p=0.05$). Plasma Cu increased significantly ($p<0.05$) from days 7 to day 16 and was significantly higher in DEX and DEX±Zn groups than control piglets ($p<0.05$).

Fig 35 shows ^{65}Zn disappearance from the perfusate during in-situ intestinal perfusion. Compared to the control, the DEX group had significantly lower radioactivity of ^{65}Zn remaining in the perfusate at each time point, which indicates a higher rate of intestinal Zn uptake. However, the effect of DEX on intestinal Zn uptake was abolished by high dietary Zn

intake. Fig 36 shows the uptake of total elemental Zn by the intestine during in-situ intestinal perfusion. As for the change observed in ^{65}Zn tracer, elemental Zn uptake tended to be higher in DEX group than in the control ($p=0.12$). The appearance of ^{65}Zn radioactivity in portal blood during and following in-situ intestinal perfusion is presented in Fig 37. Compared to DEX group, the control group had significantly higher ($p<0.05$) ^{65}Zn radioactivity in portal blood at 16 min of perfusion. Fig 38 shows ^{65}Zn radioactivity in the perfused intestinal segment. DEX+Zn group had significantly higher ^{65}Zn accumulation than the control group. Accumulation of ^{65}Zn tended to be higher in the intestinal segment (Fig 38) but lower in the liver (Fig 39) in DEX compared to the control group but this did not reach statistical significance.

Table 7 presents the results of Zn and Cu content of the intestinal mucosa and liver of the three groups. Compared to the control, DEX and DEX+Zn groups had significantly ($p<0.05$) lower liver Zn content.

Liver and intestinal MT concentrations are shown in Table 8. DEX and DEX+Zn piglets had significantly ($p<0.05$) higher intestinal MT but significantly lower liver MT ($p<0.05$) as compared to the control group.

Saturation curves comparing the initial rate of Cu uptake by BBMV for the three groups are presented in Fig 40.

The values for the DEX and DEX+Zn group were higher than the control at each extravesicular Cu concentration. The Eadie-Hofstee plots of the three groups are shown in Fig 41, in which the data are presented as the means of each group. The J_{max} and Km values were calculated from linear regression analysis of these plots. DEX and DEX+Zn groups had 138-158% higher Vmax. There was no difference in the Km among three groups.

6.4 Discussion.

The present study served to further elucidate the alterations in absorption and utilization of Zn and Cu that occur with exogenous dexamethasone treatment when given at doses that parallel those prescribed to treat severe lung disease in tiny premature infants. Intestinal uptake of Zn across intestinal BBM was induced by the 15 day treatment with DEX as evidenced by a greater disappearance of ^{65}Zn tracer from the perfusate over 30 min (Fig 35). This observation supports our previous observations in isolated BBMV of piglets who received higher dose of DEX for a shorter period (Wang et al 1993a). Since the alterations of Zn content of intestinal mucosa, plasma and liver were not detected in that study, we initially hypothesized that the DEX-stimulated influx of Zn was re-released into the intestinal lumen because of the high permeability of intestinal BBMV in the young piglets. However,

TABLE 5, THE CHARACTERISTICS OF THE PIGLETS TREATED WITH DEX ± Zn

	Control (N=6)	**DEX** (N=6)	**DEX+Zn** (N=6)
Age (d)	22 ± 2	22 ± 2	22 ± 2
Initial weight (kg)	2.2 ± 0.5	2.0± 0.6	2.0 ± 0.7
Formula intake (ml/kg.d^{-1})	391 ± 7	390 ± 16	392 ± 8
Weight gain (g/kg)	80 ± 10*	45 ± 14	44 ± 9
Urine volume (ml/kg/d^{-1})	197 ± 15*	232 ± 24	220 ± 38
Fluid intake (ml/kg/d^{-1})	410 ± 7	434 ± 13*	417 ± 6
Urine Zn (μg/ml)	0.18 ± 0.1	0.13 ± 0.1	0.09 ± 0.06
Urine Cu (μg/ml)	0.06 ± 0.12	0.03 ± 0.03	0.05 ± 0.08
Blood glucose (day 16th)	3 ± 0.9	5.5 ± 3.6	7 ± 4

*$P < 0.05$.

Data represent mean ± SD.

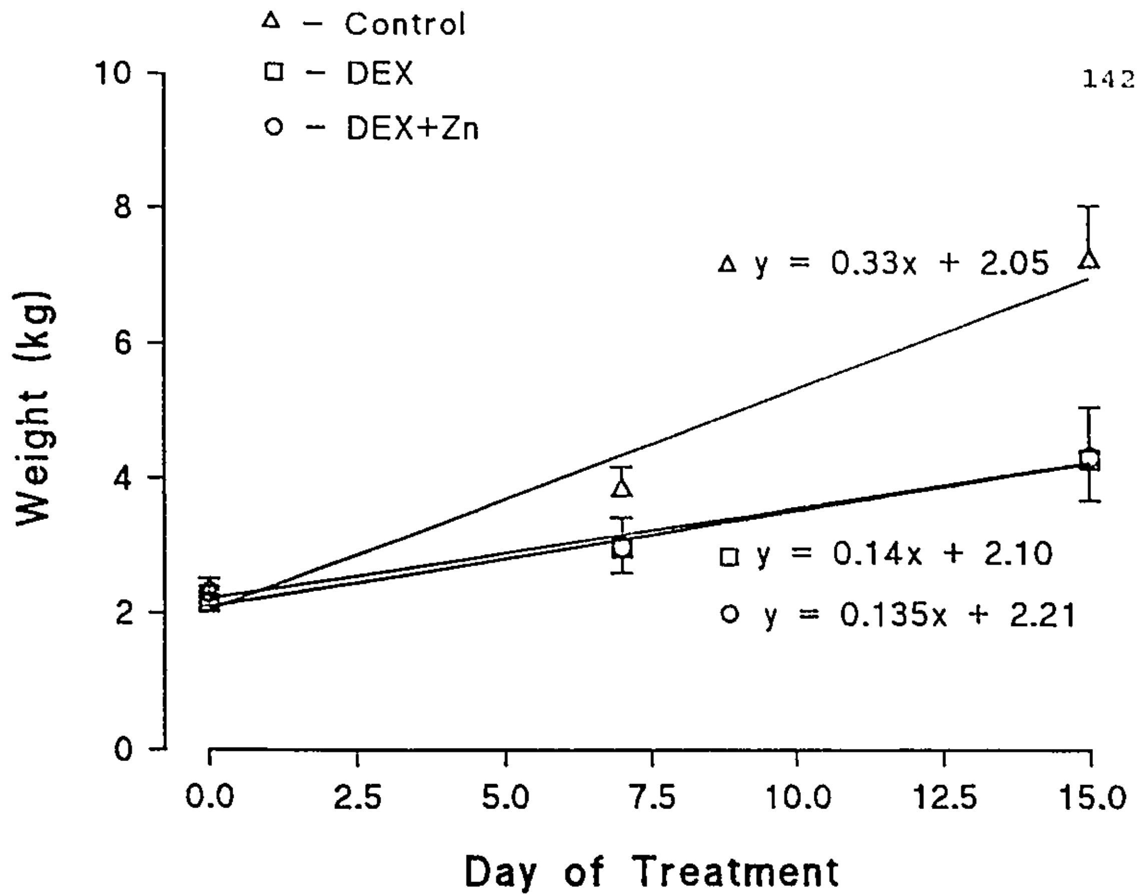

Figure 31, The rate of piglet weight gain during 15 day treatment. There are 6 piglets in each treatment group. Data represent mean $\pm$ SD. The lines were calculated by linear regression of data using computer program Fig P 6.0.

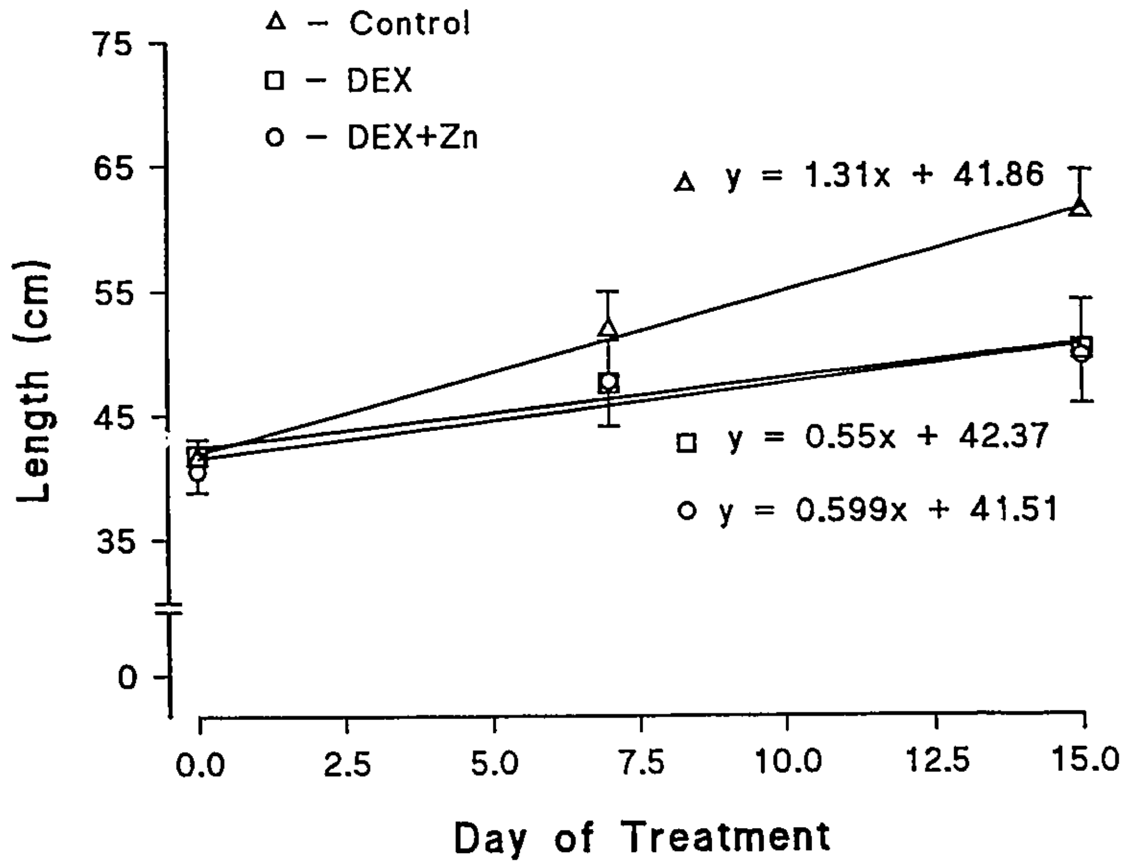

Figure 32, The rate of piglet length gain during 15 day treatment. There are 6 piglets in each treatment group. Data represent mean ± SD. The lines were calculated by linear regression of data using computer program Fig P 6.0.

TABLE 6, WEIGHT OF SPLEEN AND KIDNEY OF THE PIGLETS TREATED WITH DEX±Zn

	Control (N=4)	DEX (N=4)	DEX + Zn (N=4)
Spleen (g/kg)	2.0 ± 0.3	1.7 ± 0.5	1.4 ± 0.6
Kidney L(g/kg)	3.1 ± 0.6*	4.1 ± 0.6	4.2 ± 0.2
Kidney R(g/kg)	3.1 ± 0.6*	4.1 ± 0.4	3.8 ± 0.2

* significantly lower than DEX and DEX+Zn groups, $p < 0.05$.

Data represent mean ± SD.

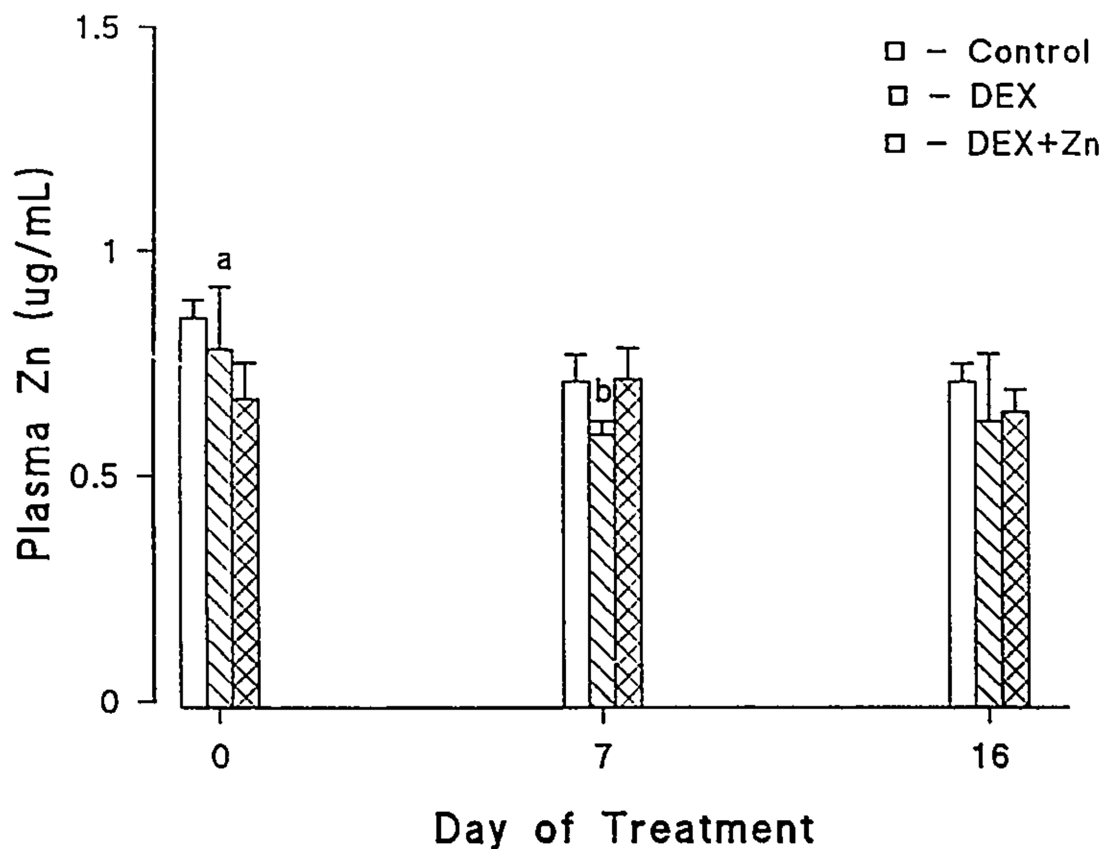

Figure 33, Comparison of plasma Zn values at d 0, d 7-8 and d 16 of treatment between the three groups. There are 6 piglets in each treatment group. Bar represents mean ± SEM. a versus b indicates that DEX group had significantly lower plasma Zn concentration at d 7-8 than that at d 0 ($p < 0.05$).

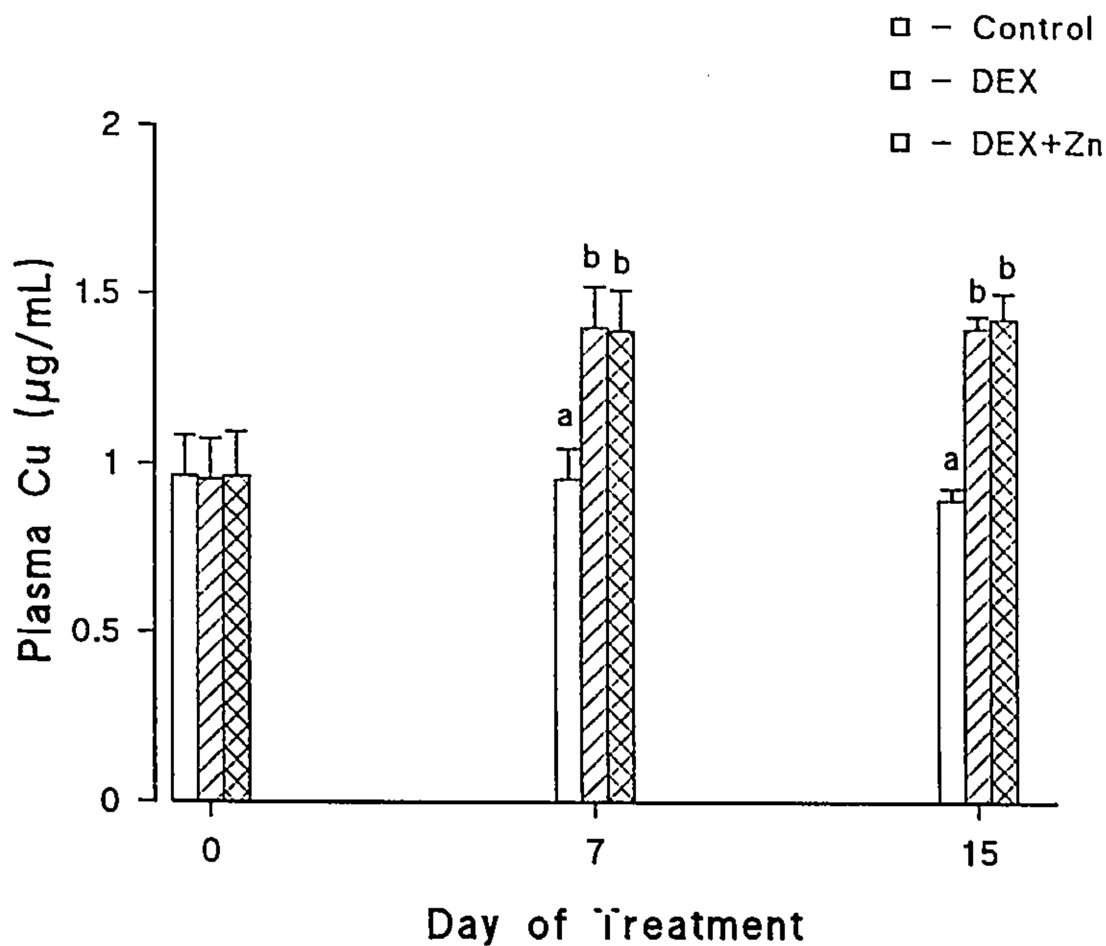

Figure 34, Comparison of plasma Cu values at d 0, d 7-8 and d 16 of treatment between the three groups. There are 6 piglets in each treatment group. Bars represent mean ± SEM. a versus b indicates that DEX and DEX±Zn groups had significantly higher plasma Cu concentrations at d 7-8 and d 16 than that at d 0 ($p < 0.01$).

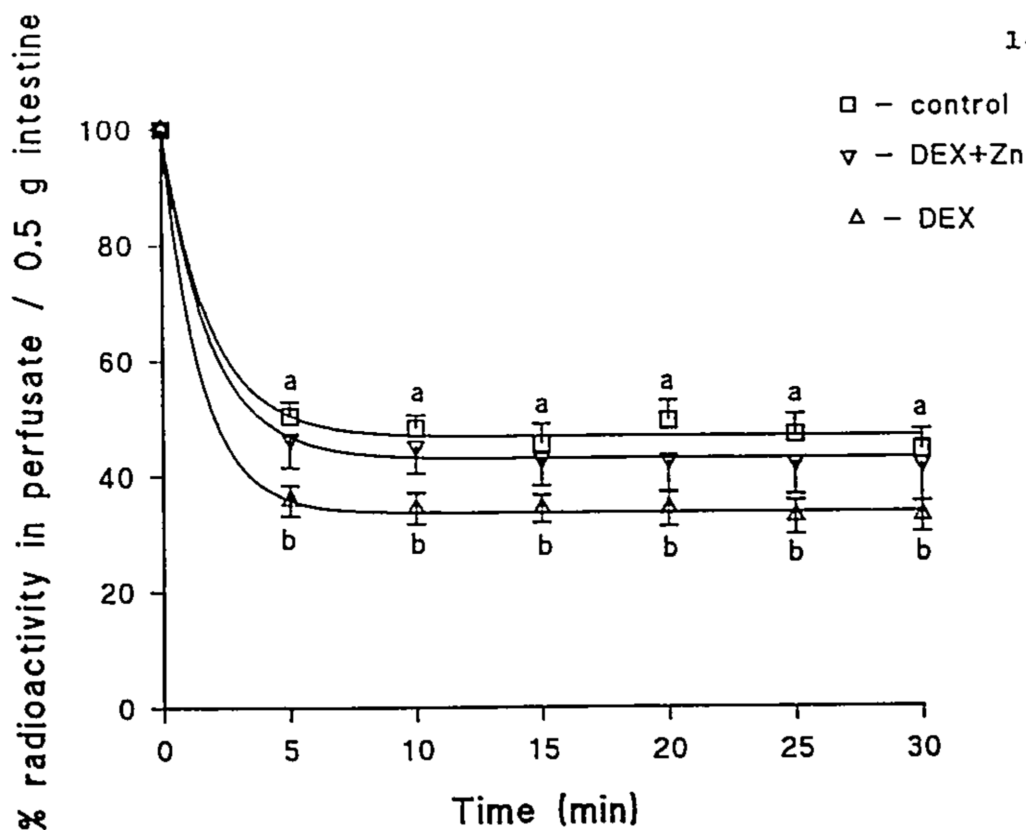

Figure 35, Time course of ^{65}Zn removal from luminal perfusion of proximal jejunum of 5 control piglets, 4 DEX treated piglets and 6 DEX treated piglets fed a high Zn diet. Initial Zn concentration in the perfusate was 0.2 mmol/L. Results are expressed as percentage of initial Zn remaining in the recirculating perfusate, normalized to 0.5 g dry tissue weight. Data represent mean ± SEM. The curves were calculated by nonlinear regression of data to a bi-exponential model using the computer program Fig P 6.0. Points notated with "a" are significantly ($p < 0.05$) higher than "b" by the Student-Newman-Keuls Test.

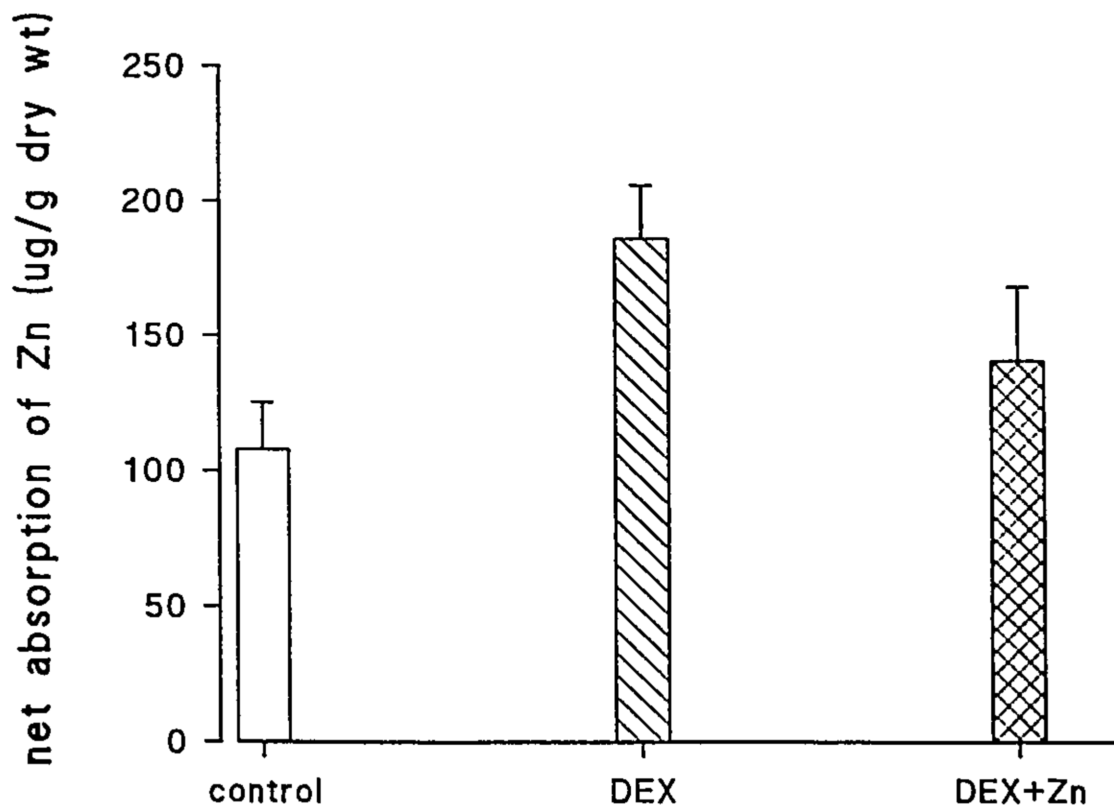

Figure 36, Net absorption of Zn calculated from the data of elemental Zn measurement in the perfusate before and after intestinal perfusion using equation (2). There are 5, 4 , 6 piglets in control, DEX and DEX±Zn groups, respectively. Bar represents mean ± SEM.

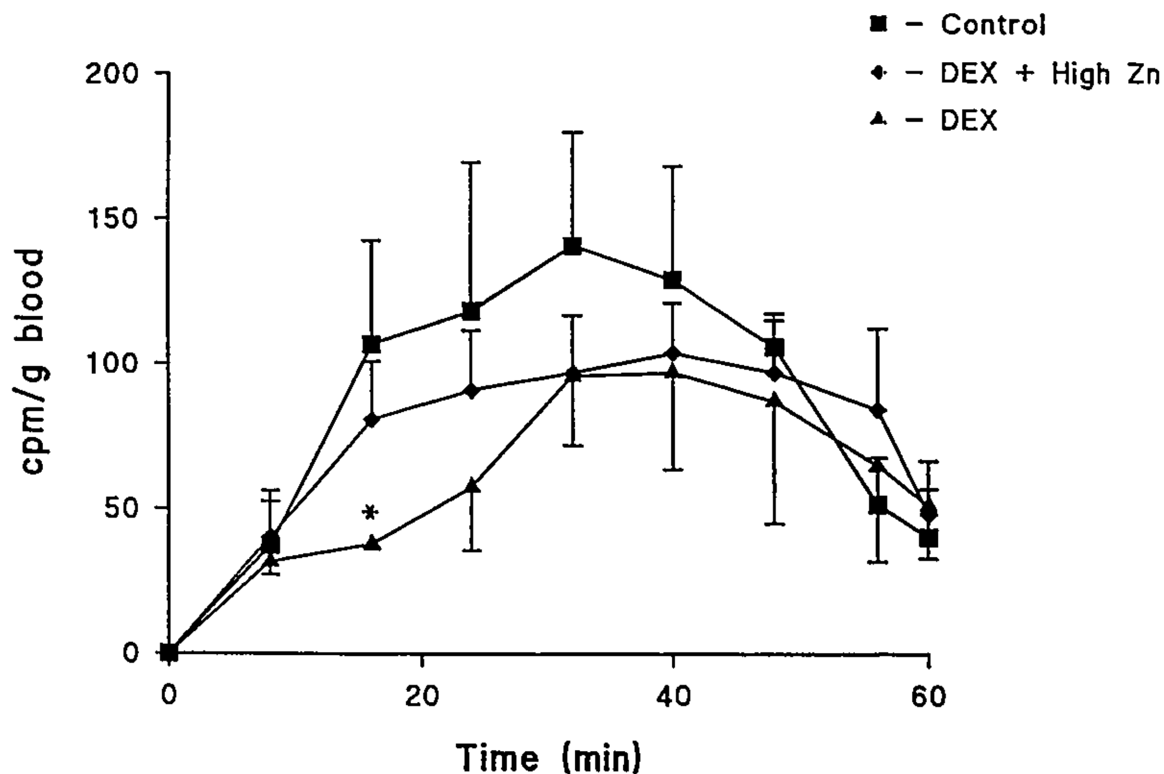

Figure 37, Transfer of ^{65}Zn from the lumen to portal circulation in perfused intestine from 5 control piglets, 4 DEX treated piglets and 6 DEX treated piglets fed a high Zn diet. The intestinal segment was perfused for 30 min. 0.5 ml blood was removed via the portal vein at time'0' of perfusion and every 8 min thereafter for 60 min. Data represent mean $\pm$ SEM. * notates this value is significantly lower than that of the control group ($p<0.05$).

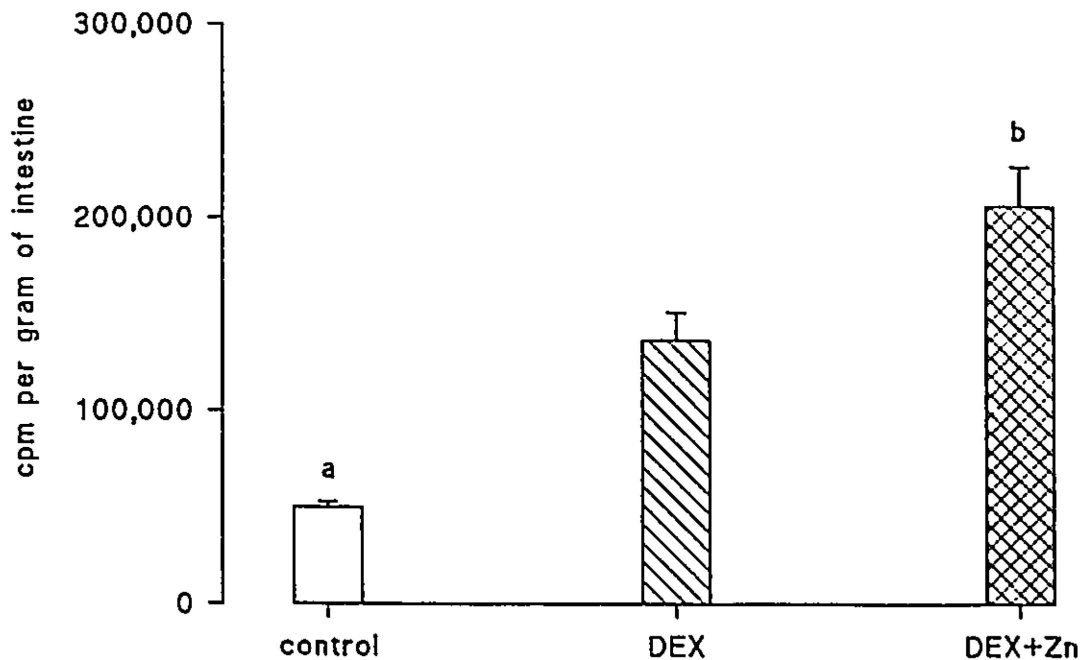

Figure 38, The ^{65}Zn radioactivity remaining in the intestinal mucosa after intestinal perfusion. There are 5, 4, 6 piglets in control, DEX and DEX±Zn groups, respectively. Bar represents mean ± SEM. a versus b indicates control is significantly lower ($p< 0.05$) than DEX±Zn.

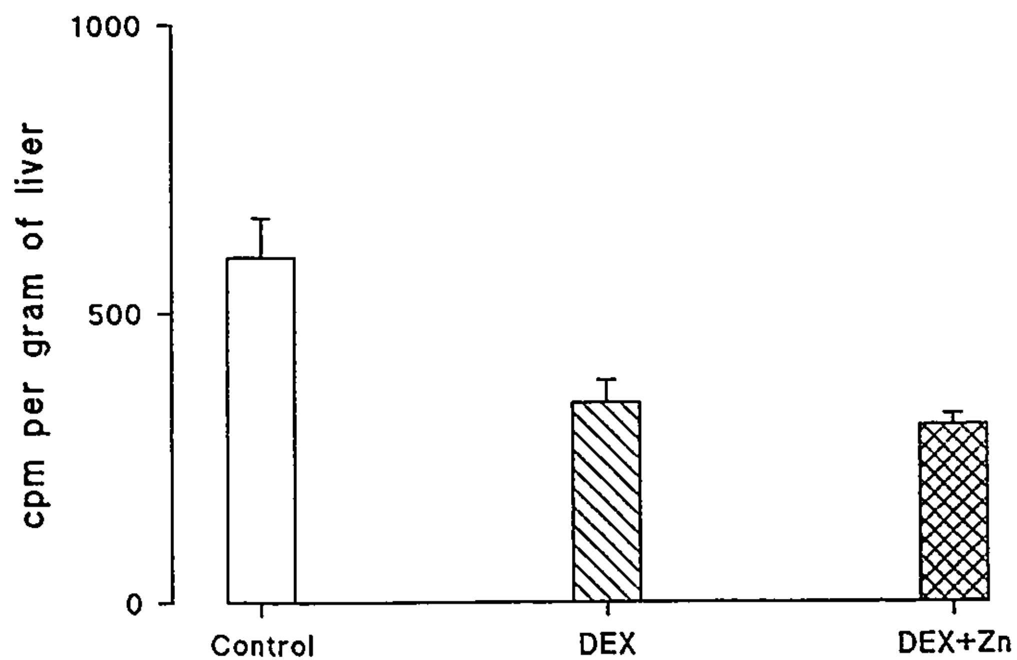

Figure 39, The ^{65}Zn radioactivity in liver after intestinal perfusion. There are 5, 4, 6 piglets in control, DEX and DEX±Zn groups, respectively. Bar represents mean ± SEM.

TABLE 7, TISSUE Zn AND Cu IN PIGLETS TREATED WITH DEX±Zn

	Control (N=6)	DEX (N=6)	DEX+Zn (N=6)
Mucosa Cu (μg/g wet wt)	3.6 ± 0.03	4.0 ± 0.2	4.4 ± 0.2
Liver Cu (μg/g wet wt)	11.7 ± 0.5	16.7 ± 0.6	13.7 ± 0.5
Mucosa Zn (μg/g wet wt)	15.3 ± 0.1	17.7 ± 0.3	16.4 ± 0.2
Liver Zn (μg/g wet wt)	58.7 ± 1.6*	35.7 ± 0.2	35.0 ± 1.1

* significantly higher than DEX and DEX+Zn groups, $p < 0.05$.

Data represent mean ± SD.

TABLE 8, LIVER AND INTESTINAL MUCOSA MT CONCENTRATIONS IN PIGLETS TREATED WITH DEX±Zn

	Control (N=5)	**DEX** (N=5)	**DEX + Zn** (N=5)
Mucosa MT (nmol/g wet)	4.6 ± 1.5*	11.7 ± 3.8	11.6 ± 2.8
Liver MT (nmol/g wet)	7.3 ± 2.8*	2.8 ± 1.5	3.5 ± 2.2

* significantly different from DEX and DEX+Zn groups, $p < 0.05$.

Data represent mean ± SD.

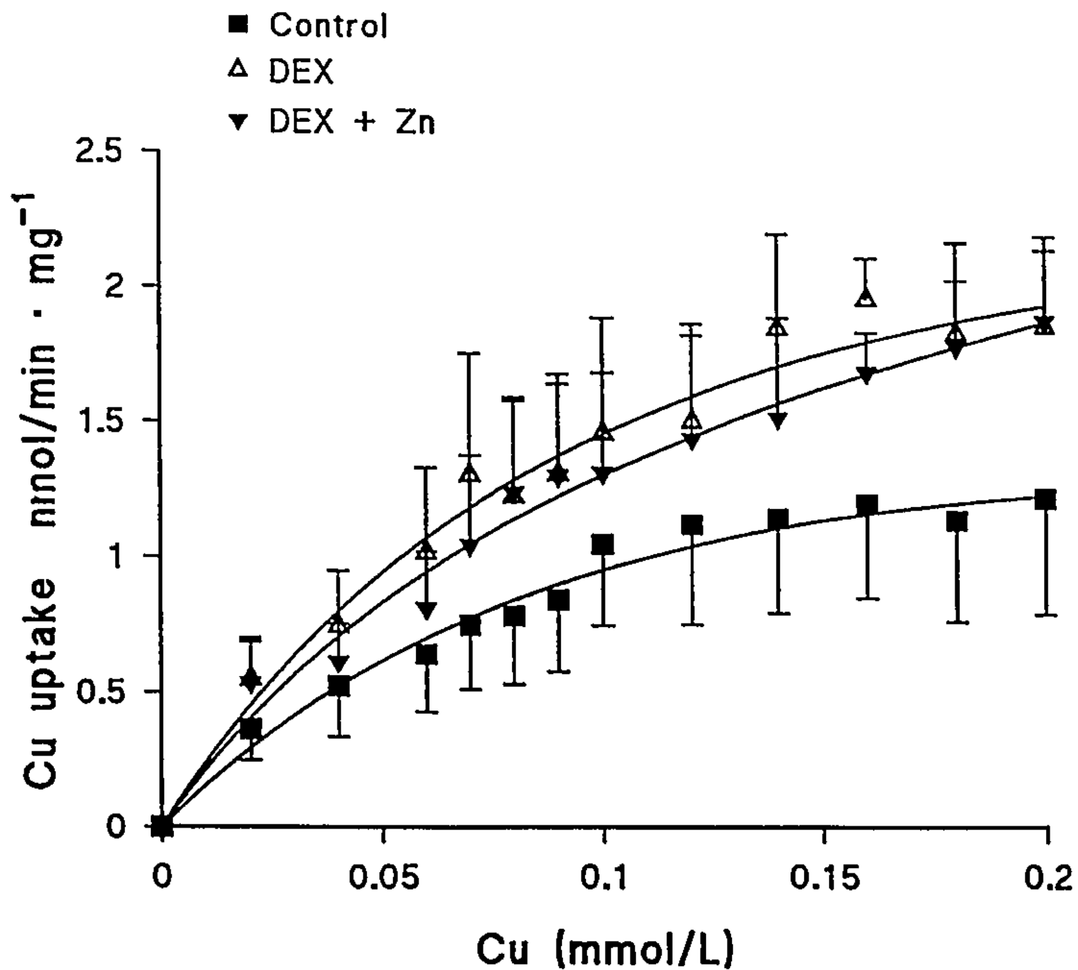

Figure 40, Initial rates of Cu^{2+} uptake (1 min) by BBMV with extravesicular Cu^{2+} concentrations ranging from 0 - 0.2 mmol/L. There are 6 piglets in each treatment group. Data represent mean ± SEM.

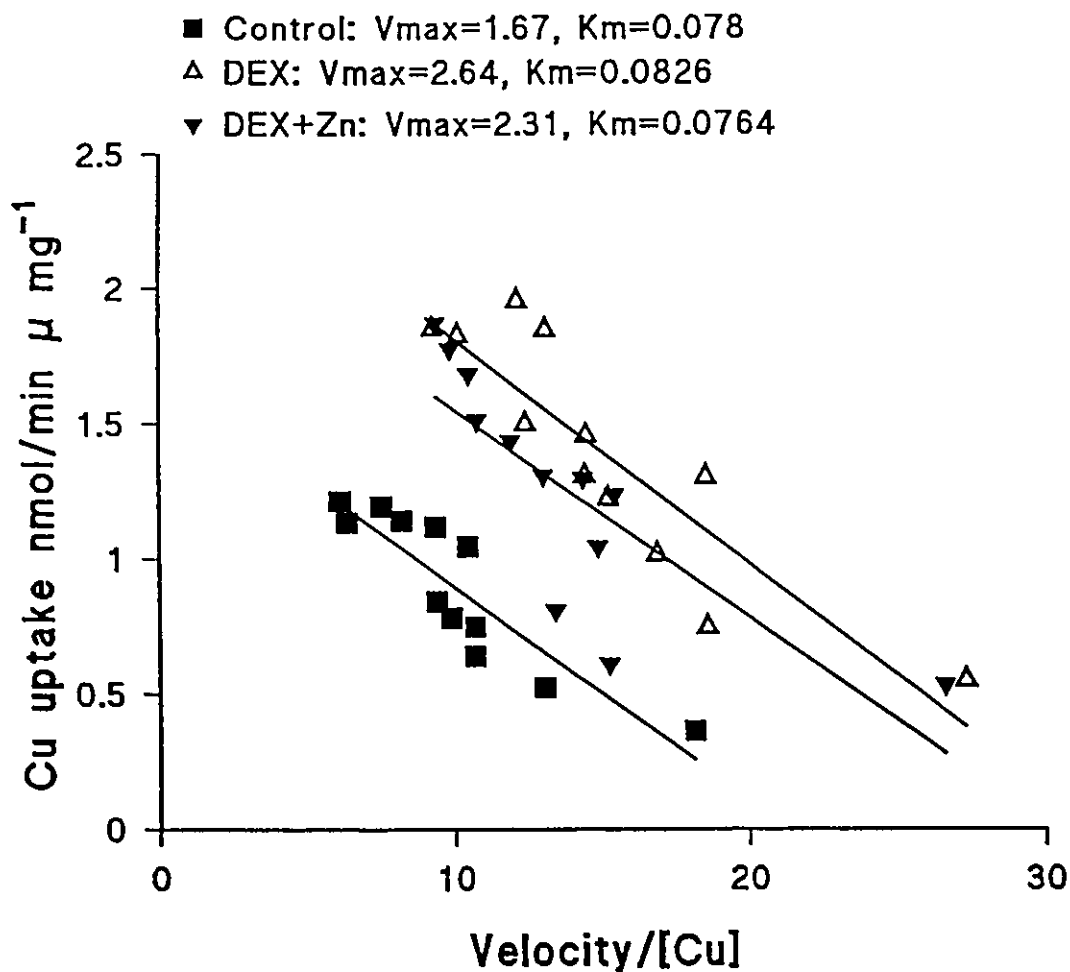

Figure 41, Eadie-Hofstee plot of the kinetics of Cu^{2+} uptake by BBMV from the data described in Fig 5a. Data are expressed as initial rates of uptake (1 min). J_{max} and Km for each group are determined from linear regression using computer program Fig p 6.0.

the rate of Zn efflux across BBMV in DEX piglets was found to be reduced compared to the control piglets (Wang et al,1993a), which suggested that Zn was either retained in the intestinal mucosa or was transported into the portal circulation after entering the mucosa.

In the present study a 3 time longer experiment albeit at only 1/6 the dose of DEX induced MT in intestinal mucosa. This support our previous speculation that Zn became trapped in intestinal mucosa since MT is known to be specific for Zn and Cu (Bremner and Beattie, 1990). We did find the trend of Zn tracer accumulation in the intestinal segment of the DEX treated pigs. In DEX+Zn group, the amount of Zn tracer retained by intestine was significantly greater than that of the control. Although the change in mucosal Zn contents was not significant in either DEX or DEX+Zn groups (may be due to lower sensitivity of the method applied to measure elemental Zn), the reduction in plasma Zn by just 7 days of DEX treatment supports the suggestion that Zn became trapped within the mucosal cells and was not transported into the circulation. Since MT bound with Cu could not be measured by the Cd saturation assay and we did not perfuse the intestinal segment with ^{64}Cu, it is impossible to estimate how much Cu was retained in intestinal mucosa. A interest observation is the difference in MT induction between this study and the short-

term, high dose study (Chapter 5). One explanation for the difference in MT induction between these two studies may be the difference in the route of administration of DEX - oral versus intramuscular. The DEX given orally may affect intestinal mucosa more directly.

While MT may be responsible for the accumulation of Zn within mucosal cells, it is likely that the up-regulation of transport at the BBM occurs via induction by DEX of specific membrane transport protein(s) for Zn and Cu or alterations in the phospholipid composition of the membrane (Neu et al, 1986).

Excess dietary Zn is known to impede intestinal Zn transport by down-regulating the synthesis of membrane Zn transport protein in adult animals (Cousins 1985). However, in young piglets (15 days old) we previously did not detect any change in Zn transport across isolated BBMV in response to high Zn feeding possibly because of the immaturity of this regulatory mechanism (Wang et al, 1993b). To determine if excess dietary Zn and DEX would have any interaction effects on Zn transport, we used older animals (22 d old) and long term DEX treatment plus high dietary Zn intake in this study. We observed that high dietary Zn when given with DEX abolished the DEX-induced Zn uptake. DEX and dietary Zn may regulate the same Zn transport system on intestinal BBM but with opposite

effects. The significantly higher ^{65}Zn accumulation in DEX+Zn group may be due to the induction of Zn binding protein(s) in addition to MT by high dietary Zn or down-regulated Zn transport across intestinal BLM.

In contrast to the intestine, liver uptake and storage of Zn appeared to be reduced with DEX treatment. MT and total elemental Zn content in the liver were significantly suppressed by DEX. High dietary Zn did not change the effect of DEX treatment. The difference between intestinal and hepatic synthesis of MT in response to DEX could be a result of the differing Zn status in the two organs. MT is not inducible with Zn deficiency (Aggett and Barclay 1991). The low Zn level in liver could impair the function of Zn fingers in the glucocorticoid receptor which is necessary for the binding of glucocorticoid-receptor complex to the GRE which is located in the 5′ upstream region of the MT gene (Chesters 1992). Thus, the down-regulation of hepatic MT expression could be an indirect effect of DEX treatment.

Similar to Zn, Cu uptake across the intestinal BBMV was induced by DEX, but the uptake was not altered by high dietary Zn intake. This observation suggests that Zn and Cu transport systems have the same response to DEX but different sensitivity to high dietary Zn intake. In a previous study we found that a high dietary Zn intake which was seven-fold

higher than the amount of dietary Zn used in this study, significantly suppressed Cu uptake by intestinal BBMV in 20 d old piglets (Wang et al, 1993b). Thus, dietary Zn may suppress Cu uptake but only under conditions of very high dietary Zn:Cu ratio in young animals.

The elevated plasma Cu observed at day 7-8 and day 15 of the treatment in DEX-treated piglets could be due to the induction of ceruloplasmin synthesis and/or release by DEX treatment. The release of tissue Cu storage as a result of protein catabolism in response to DEX treatment is another possible explanation for elevated plasma Cu. To our knowledge, there are no previous reports on the effect of DEX on tissue Cu release in animals or humans. Observations of elevated plasma amino acid (Williams and Jones, 1992) and blood urea nitrogen (Broenlee et al 1992) in human premature infants undergoing DEX therapy provide evidence for the protein catabolism effect of DEX.

In this study we investigated the effect of long term DEX treatment on Zn and Cu metabolism at the level of intestinal enterocytes and the major storage organs. Although DEX enhanced intestinal Zn uptake, the true absorption of Zn was not elevated and could be impaired by DEX because of the induction of MT, which could retain Zn in the cytosol of intestinal mucosa cells. DEX also enhanced Cu uptake by intestinal BBMV, but the net absorption of Cu was not measured

and can only be indirectly presumed from the higher intestinal MT concentration. The down regulation of Zn transport across BLM by DEX treatment could be another mechanism for reduced Zn absorption. Bonewitz et al (1983) found DEX increased BBM Zn transport by 75% but reduced BLM Zn transport by 45%. His finding supports our hypothesis.

The piglet chronically treated with DEX has proven to be an appropriate model for investigating the metabolic effects of this glucocorticoid in the developing infant. Both animals and infants develop side effects of reduced weight and length growth (Yeh et al, 1990), increased protein catabolism (Brownlee et al, 1993) and hyperglycaemia. No studies of trace element metabolism in DEX-treated infants are reported. Although glucocorticoid hormones induce the maturation of some organs, the overall DNA, RNA and protein synthesis was found to be impaired by glucocorticoid treatment in neonates (Balazs 1972). The alterations in protein, carbohydrates, fatty acids and mineral metabolism could be individually or in combination the pathophysiological basis for the impaired growth in DEX treated piglets.

If the present findings in piglets are extrapolated to human premature infants, then one could consider that Zn deficiency may be a contributing factor to the observed growth failure (Yeh et al, 1990). Unfortunately, supplementary dietary Zn did not reverse the Dex-induced growth failure nor

the reduced storage of Zn in liver.

Chapter 7

Overall conclusions and proposed future research

7.1 Conclusions

The present research has focused on two main areas:

1. Absorption of Zn and Cu at different developmental stages in early life.
2. The effects of dietary Zn and exogenous glucocorticoids on mucosal transport and storage of Zn and Cu during early development.

It was hypothesized that: a) the immaturity of metal ion transport mechanisms in the gastrointestinal tract during early life is an important mechanism which may be limiting to absorption of Zn and Cu and this may be a major cause of Zn and Cu deficiency observed in some premature infants; b) exogenous glucocorticoid used to treat severe lung disease in this population could alter intestinal absorption and storage of Zn and Cu which may contribute to reduced growth; c) supplementation with dietary Zn would improve Zn status and reverse the effects of DEX treatment on growth and Zn metabolism in developing animals.

Neonatal piglets were used as a model of human

premature infants in all studies. Efforts were first made to establish experimental methodologies to measure Zn and Cu absorption in vitro or in situ in infant piglets. The effects of dietary Zn supplement for Zn and Cu absorption were studied with different amounts and feeding duration. DEX was given intramuscularly or orally at different doses for short term (5 d) or long term (15 d), respectively. Results of these experiments indicated that piglets are an appropriate model for human premature infants. For example, 32 and 52 h old piglets had significantly lower plasma Zn and Cu than those of 20 d older piglets. Premature infants are known to have lower circulating Zn and Cu than term infants (Dauncey 1977). The observation that younger piglets had higher liver Zn and MT than the old ones is also consistent with the finding of Zlotkin and Cherian (1988) in human infants. The DEX treated piglets had impaired growth and elevated blood glucose which are frequent clinical findings in human premature infants undergoing DEX therapy.

Several research questions raised when we designed these studies have been answered using the piglet model:

1. What is the ontogeny of processes for Zn and Cu transport across intestinal BBM and compartmentalization of these elements in early life ?

The mechanism for Zn transport across the intestinal BBM was predominantly passive diffusion in piglets at 32 and

52 hr postnatal age. A carrier mediated transport was established by 10 d. The immaturity of the carrier mediated transport could be the cause to the lower net absorption of Zn in the younger groups because passive diffusion is bi-directional. The lower absorption and hepatic Zn accumulation may explain the significantly lower plasma and intestinal mucosa Zn observed in the youngest groups. The ontogeny of Cu transport was different from that of Zn transport. The velocity of Cu transport across BBM varied from high to low to high and carrier mediated transport predominated over the first 20 d of life. The significantly lower plasma Cu found in the youngest piglets could be caused by lower absorption, high organ storage or low transport protein (ceruloplasmin) in blood. The ontogeny data obtained from piglets provide some insight into the biological mechanisms which explain why Zn and Cu deficiency is observed in premature infants.

Previous knowledge about the ontogeny of process for Zn and Cu absorption was mostly obtained from studies in rodents. The current study provides information about mechanisms of mucosal transport of Zn and Cu in an animal model which is closer to human infants than the rodents. Results of the current study agree with the observation in human infants that newborns are vulnerable to Zn deficiency. Whether Zn deficiency in very young animals can be improved by dietary Zn supplement was investigated in the next study.

2. Does dietary Zn supplement alter Zn and Cu transport across intestinal BBM and improve body Zn status in neonatal animals?

Dietary Zn supplement (1000 mg Zn/kg dry diet) did not change the kinetics of Zn transport across the intestinal BBM in 20 d old piglets. The piglets fed with high dietary Zn had a significantly higher Zn content in both liver and intestinal mucosa but similar plasma Zn as compared to the litter-mates fed with regular formula. These observations suggest that net absorption of Zn was elevated by a dietary Zn supplement. However, most absorbed Zn was accumulated by the liver because hepatic MT was induced by about 10 times. Although dietary Zn supplements may improve Zn status in piglets, the significantly suppressed intestinal Cu uptake observed in this study indicates that long-term dietary Zn supplement may cause Cu deficiency.

Previous knowledge about the responses in Zn and Cu absorption to high dietary Zn intake was mainly from adult rats. The current study provided information about the role of dietary Zn in the regulation of Zn absorption in a neonatal piglet model. In contrast to the findings in adult rats, high Zn feeding did not suppress Zn transport across BBM and did not induce intestinal MT in piglets. The absence of these protective responses to high dietary Zn intake may increase the vulnerability to Zn toxicity in early life if animals or

infants are fed excessive dietary Zn.

3. Does treatment with exogenous glucocorticoid hormone improve Zn and Cu absorption by inducing intestinal maturation or impair Zn and Cu absorption because of its catabolic effect on Zn and Cu transport/binding proteins in early life?

Although DEX enhanced Zn uptake by intestinal BBM, the net absorption of Zn into portal blood may be reduced because of the induction of intestinal MT by DEX. MT appeared to trap Zn in the intestinal mucosa. The intestinal Zn accumulation could also result from down-regulation of Zn transport across BLM by DEX treatment. The significantly lower liver and plasma Zn found in long-term DEX treated piglets suggested that whole body Zn was becoming deplete. Cu uptake by the intestinal BBM was also up-regulated by DEX treatment. In contrast to plasma Zn, plasma Cu was significantly elevated by DEX treatment. The induction of hepatic synthesis of ceruloplasmin by DEX is a possible reason for the observed elevated plasma Cu. The retarded growth found in DEX treated piglets demonstrates that DEX altered the metabolism of multi-nutrients, not only Zn and Cu.

In clinical practice, DEX therapy is being used with success to ameliorate lung disease in small prematurely born infants. Although reduced growth has been observed in DEX-treated infants, the nutritional basis for this in relation to

alterations in nutrient metabolism in associate with exogenous DEX have been given little attention. Until now, the evidence for DEX-induced changes in Zn metabolism have primarily been studied in adult rats. The current studies are the first to assess the effects on Zn and Cu metabolism of exogenous DEX when given at therapeutic doses of relevance to the clinical management of premature infants. The results of the studies in piglets provide the basis to pursue the immediate and long-term effects of DEX therapy on Cu and Zn status of developing infants and the impact of this on growth.

4. Will a dietary Zn supplement reverse the effects of DEX on growth and Zn absorption in neonates?

Dietary Zn supplements to piglets treated with DEX abolished the DEX induced enhancement of intestinal Zn uptake and normalized their plasma Zn. But dietary Zn did not reverse the growth failure nor the reduced storage of liver Zn observed in the piglets treated with DEX. Our conclusions from these observations are as follows.

a) On the intestinal BBM, dietary Zn and DEX may regulate the same Zn transport system on intestinal BBM but with opposite effects.
b) In contrast, in the cytosol of intestinal enterocytes, dietary Zn and DEX have additive effects on the induction of Zn binding protein(s) in the cytosol of intestinal enterocytes. Because of this induction, most Zn was

retained within the cytosol of intestinal mucosal cells so that dietary Zn supplement did not improve liver Zn storage and growth.

There is little information available on the possible nutritional interventions to counter-balance the detrimental effects of long-term DEX therapy on nutrient metabolism and growth in human premature infants. This study explored one such possibility. Our results suggest that a dietary Zn supplement is beneficial but not enough to normalize Zn and Cu metabolism in the DEX treated piglet model.

6.2 Proposed future research

Although the present studies have answered some of the questions regarding the ontogeny of intestinal Zn and Cu absorptive functions and the influence of dietary Zn and exogenous glucocorticoid hormone on intestinal Zn and Cu absorption in early life, further areas remain to be explored:

1. We studied the functional maturation in the mechanisms for Zn and Cu transport across the intestinal BBM, but the molecular basis of these maturational processes is undetermined. One or more Zn and Cu channel(s) or transport protein(s) must be involved and the induction of their gene expression by diet or hormone could be the molecular basis for the functional maturation. To understand this molecular basis, we should isolate and purify Zn and Cu transport proteins from the intestinal BBM. The determination of their N- or C-

terminal amino acid sequences will lead to the synthesis of the specific RNA probes to screen cDNA library of small intestinal enterocytes at various developmental stages. The corresponding genes should be cloned and sequenced. To determine if these proteins and their genes are functionally involved in membrane Zn or Cu transport, one could transfect the cultured intestinal enterocytes with the constructed vector containing the promoter for MT gene and the newly identified genes for the supposed Zn or Cu transport proteins. The involvement of the cloned genes in Zn and Cu transport is demonstrated by the up-regulated Zn or Cu uptake by transfected cells when Cd is added into cell culture medium. The inhibitory effects on Zn or Cu uptake by the intestinal BBMV of the specific antibodies against the cloned membrane proteins may further confirm the involvement of these proteins in Zn or Cu transport. If we cloned these proteins and obtained specific RNA probes and antibodies, the molecular basis of this ontogenic process could be determined by *in-situ* hybridization, northern blot analysis and immunocytochemistry methods. In addition, to further understand the mechanisms of what on BBM, additional studies should be done on the Zn and Cu transport proteins in BLM. A similar strategy as described above could be used in these studies.

In situ intestinal perfusion is a useful tool to assess the functional maturation for Zn or Cu absorption. If

a dual perfusion of intestinal lumen and its blood vessels were performed in piglets at various developmental stages, one could further understand more completely the ontogeny of functional process for intestinal Zn and Cu absorption.

2. Dietary Zn supplementation did not change Zn transport across BBM but abolished the effect of DEX treatment on it. The regulation of Zn transport protein(s) on BBM by these treatments should be investigated. If the gene for Zn transport protein on the intestinal BBM was cloned, it would not be difficult to observe the regulation in the gene expression by dietary Zn supplement or DEX treatment or both treatments. It is possible that there are Zn responsive and glucocorticoid hormone responsive elements in the flanking region of the proposed gene for the Zn transporter on BBM like MT gene; however, the binding of Zn to the element reverses the effect of glucocorticoids on the induction of the gene expression. Because high dietary Zn intake was found to suppress Zn uptake by intestinal BBM in adult animals but not in young animals, the changes in the regulation of the gene expression by Zn with development should be explored. The regulation of the gene expression for a Cu transporter on BBM by these treatments may be similar to those proposed for the Zn transporter. Thus, we may use the same research strategy to study this aspect.

3. There has been some controversy about the induction of intestinal MT by DEX. To my knowledge, our study is the first *in-vivo* experiment to clearly demonstrate that intestinal MT can be induced by DEX. Short-term high dose DEX treatment did not induce intestinal MT but long-term low dose DEX treatment did. Whether the positive result in the long-term treatment is due to a longer period of treatment or because DEX was administered orally rather than intramuscularly is unknown. The results of a short-term oral high dose DEX treatment and a short-term intramuscular low dose DEX treatment in piglets may clarify this question.

Another question concerns the down-regulation of liver MT synthesis by long-term DEX treatment. We postulate that the suppressed liver MT concentration in DEX-treated piglets is due to Zn deficiency because of the important role of Zn fingers in the binding of glucocorticoid receptor to GRE. To verify this hypothesis, a lower expression of MT gene in the liver of these piglets should be confirmed. If the gene expression is lower, the capability of glucocorticoid receptor for binding to GRE in the piglets receiving long-term DEX treatment should be investigated. One could use a DNAase 1 protection method to reveal the amount of GRE bound to the purified glucocorticoid receptors in DEX treated piglets and the control piglets.

The third question concerns the higher plasma Cu level

in response to long-term DEX treatment. We proposed that it was due to the release of tissue Cu store. In the present study we only measured the Cu content in liver and intestine. A more complete assessment of Cu content in various tissues including bone and muscle in the piglets treated with DEX for 15 d will confirm this hypothesis.

Finally, the effects of DEX treatment on Zn and Cu transport across intestinal BLM in developing animals has not been studied. We hypothesised that the down-regulated Zn transport across BLM by DEX is one of the mechanisms for Zn accumulation in intestinal mucosa. A similar strategy for cloning Zn and Cu transporters on BBM could be used to clone Zn and Cu transporter on BLM and study the regulation of the gene expression by development, drug and diet.

In summary, this study investigated basic physiological mechanisms of Zn and Cu absorption and the impact of development, drug and dietary factors on these processes in piglets. Neonatal piglets appear to be an excellent model of human premature infants as they respond to the interventions in a way that parallels what is known to occur in premature infants. This similarity suggests that our results may be applicable to the clinical population of human premature infants.

This research has provided new knowledge on the mechanisms for Cu transport across BBMV, Cu-Fe interaction

(in-vitro), ontogeny of processes of Zn and Cu absorption, and the impact of long-term DEX+Zn on Zn and Cu metabolism. Future studies should be directed to further knowledge of the mechanisms by which DEX and dietary minerals alter Zn and Cu metabolism. One such area would be identification of protein(s) responsible for Zn and Cu transport across intestinal BBM and BLM. The cloning of these proteins will greatly facilitate the study on the regulation of Zn and Cu absorption by developmental, drug and dietary factors.

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APPENDIX I

Pig Diet Composition:

Nutrient	per litre
Energy	796 kcal
Carbohydrate [1]	78 g
Fat[2]	36 g
Protein	42 g
Whey[3]	12 g
Skim milk powder[4]	30 g
Calcium[5]	67 mmol
Phosphorus[5]	46.5 mmol
Zinc[6]	95 μmol
Iron[7]	35 μmol
Vit D	50.4 IU
Vit A	4056 IU
Vit E	2.5 IU
Folic Acid	1.1 μmol
Thiamine	3.6 μmol
Riboflavin	10.4 μmol
Pyridoxine	49.4 μmol
panthothenic acid	26.5 μmol
Niacin	71.5 μmol

Manganese[8]	3.1 μmol
Magnesium[9]	7.4 mmol
Copper[10]	21.1 μmol
Sodium[11]	38 mmol
Potassium[12]	66.4 mmol
Chloride	35.6 mmol
Methylcellulose[13]	4 g

[1] Lactose from skim milk powder and added powered lactose, Nutrimax, Maximum Nutrition Ltd., Toronto, ON.

[2] Mazola Corn oil (30%) and Canola oil(70%), Best Foods Canada Inc., Etobicoke, ON.

[3] Electrodialysed whey, Ross Laboratories, Columbus, OH.

[4] Skim milk powder, Wyeth Canada Ltd., Windsor, ON, Canada

[5] Calcium glycerophosphate, Paul Lohmann, Emmerthal, FRG

[6] Zinc from skim milk powder plus added zinc sulphate

[7] Iron from skim milk powder plus added iron sulphate

[8] Manganese from skim milk powder plus manganese sulphate

[9] Magnesium sulphate

[10] Copper from skim milk powder plus added copper sulphate

[11] Sodium chloride

[12] Potassium chloride

[13] Methyl cellulose, BDH Chemicals Ltd., Prole, UK.

APPENDIX II

BBMV preparation:

This method was modified from the procedure described in Davidson and Lonnerdal (1988) and Menard and Cousins (1983).

Buffer A: for tissue preparation

0.1 mol/L D-mannitol
1 mmol/L Tris-HEPES
pH7.5

Buffer B: for BBMV resuspension and Zn and Cu transport study

0.3 mol/L D-Mannitol
10 mmol/L Tris-HEPES
pH 6.7

Sample: 1.5-2 g intestinal mucosa

ALL STEPS CARRIED OUT AT 4°C.

1. Homogenize mucosa in 10 volumes (10 x sample wt) of Buffer A at set 5 for 30 seconds with a Polytron homogenizer (Kinematica GMBH, Lucern, SUI). Homogenize in centrifuge tube placed in beaker packed in ice.

2. Save 500 μl aliquot for enzyme assays for pre/post check on protein recovery and sucrase activity.

3. Centrifuge - 10 000g for 10 min., save supernatant, resuspend pellet in 5 volumes Buffer A. Homogenize at set 5 for 60 sec. Combine with the supernatant.

4. Add solid anhydrous $MgCl_2$ to 20 mmol/L concentration to precipitate cellular material and basolateral membrane.

5. Stir on ice for 30 min gently.

6. Centrifuge at 4000g for 10 min. Discard pellet.

7. Supernatant: centrifuge at 30 000g for 20 min.

8. Resuspend pellet in 10 vol of Buffer A.

9. Homogenize with Caframo Stirrer (Wiarton, ON, Canada) in centrifuge tubes; teflon pestle - 6 stroke up and down.

10. Add $MgSO_4$ to 0.1 mmol/L as protease inhibitor

11. Centrifuge at 4000g for 10 min. Discard pellet. Decant supernatant into another tube.

12. Centrifuge the supernatant at 48 000g for 15 min and resuspend in 0.5 ml of buffer B (pH 6.7) in a plastic Eppendorf centrifuge tube.

APPENDIX III

Zn^{2+} Transport study:

Buffer C:

0.3 mol/L D-mannitol
10 mmol/L Tris-HEPES
1 mmol/L $ZnCl_2$

The millipore MultiScreen Filtration System was used for transport
studies (Millipore, Bedford, MA, USA)

PROCEDURES

3a: measuring Zn transport kinetics

1. A 65Zn stock solution is prepared with Buffer C (radioactivity is diluted with constant concentration of Zn^{2+})(ie 3 nCi/μl) (ie 1 mmol/L Zn^{2+}solution).

2. BBMV solution: 50-100 μg protein/50 μL.

Adding the following solution into the wells on Millipore filtration plate. All samples are triplicated.

Zn^{2+}(mmol)	BBMV solution	1 mmol/L Zn2++Zn^{65}	Buffer B
0.05	50 uL	5 uL	45 uL
0.1	50 uL	10 uL	40 uL
0.125	50 uL	12.5 uL	37.5 uL
0.15	50 uL	15 uL	35 uL
0.175	50 uL	17.5 uL	32.5 uL
0.2	50 uL	20 uL	30 uL
0.225	50 uL	22.5 uL	27.5 uL

0.25	50 uL	25 uL	25 uL
0.275	50 u	27.5 uL	22.5 uL
0.3	50 uL	30 uL	20 uL
0.35	50 uL	35 uL	15 uL
0.24	50 uL	40 uL	10 uL
0.45	50 uL	45 uL	5 uL
0.5	50 uL	50 uL	0 uL

Blank: 50 ul Buffer B instead of the BBMV solution. One blank for each concentration.

Incubate the above solution at room temperature for 1 min.

Stop the incubation by filtration of the solution through 0.22 μm nitrocellulose filters.

rinsing the filters with ice cold buffer B.

The filters are punched off the plate and placed in a test tube for counting the radioactivity.

The test tubes are counted on a gamma counter (Beckman Gamma 5500, Irivine, CA). The cpm data are averaged and corrected using the blank.

The velocities (nmol Zn^{2+} min/mg vesicular protein) are calculated and plotted against Zn2+ concentrations.

Kinetic data (V_{max} and K_m are determined from the velocities using Eadie/Hofstee plots (Eadie, 1942)(Hofstee, 1952)

3b. <u>Time Curve</u>

Each solution contained 50 μL BBMV + 50 μL 0.2 mM Zn^{2+} + Zn^{65} solution is incubated at 37°C or 4°C for the following time periods:

0.25, 0.5, 1, 1.5, 2, 5, 10, 15, 20 min.

Procedure 4-9 followed as described above.

3c. Determining the rate of Zn^{2+} efflux across BBMV

150 uL BBMV solution (BBMV preparation resuspended in Buffer B) was incubated with 350 uL Buffer B containing 0.3 mmol/L ZnCl2 and 2 uCi ^{65}Zn for 60 min at 37°C. After taking triplicate 20 ul samples to examine the amount of ^{65}Zn loaded in BBMV, the incubation solution was diluted 19 fold with Buffer B containing 10 mmol/L EGTA. At different time intervals after the dilution, the efflux of ^{65}Zn across BBMV was terminated by rapid filtration. The filters were washed twice with Buffer B containing 10 mmol/L EGTA and counted for ^{65}Zn radioactivity (Beckman Gamma 5500).

The loading of BBMV with ^{65}Zn was also performed at 4°C for 60 min to determine whether BBMV Zn influx occurred via active uptake or passive diffusion and binding to the membrane.

A blank was included (no BBMV) to correct for the nonspecific binding of ^{65}Zn to the filter.

APPENDIX IV

Cu^{2+} transport study in BBMV

Buffer C:

0.3 mol/L D-Mannitol
10 mmol/L Tris- HEPES pH 6.7
1 mmol/L $CuCl_2$

Reagents:

Buffer B and Buffer C
^{64}Cu is diluted with buffer C
Buffer B containing 5 mmol/L EDTA

The Millipore MultiScreen Filtration System was used for transport studies (Millipore, Bedford, MA, USA)

PROCEDURES:

4a. measurement of Cu transport kinetics:

All samples were done in duplicate.

Buffer B and C are incubated in a plastic plate (Costar, Cambridge, Mass., USA) according to the following concentrations:

Buffer B (uL)	1 mmol/L Cu^{2+} + ^{65}Cu
156	4
152	8
148	12
146	14
144	16
142	18
140	20
136	24
132	28
128	32
124	36

120	40
100	60
80	80
40	120
0	160

Blanks: 40 ul of Buffer C is used instead of the BBMV solution. One blank for each concentration.

40 ul of BBMV preparation is added, one raw at a time using a Ranin Multi-Channel Pipette (Woburn, MA, USA). After 1 min incubation, 100 ul of the BBMV, Cu^{2+} and buffer mixture is transferred to a 0.22 μm filter plate (Millipore, Bedford, MA, USA).

With full vacuum, the filter was washed 2 times with 100 uL Buffer B containing 5 mmol/L EDTA solution.

When filters are dry they are punched off the plate and placed in a glass counting tube. The tube was then counted on a gamma counter
(Beckman Gamma 5500, Irvine, CA).

The cpm were averaged and corrected for the nonspecific binding with the blank. The velocities (nmol/min/mg protein) were calculated and plotted against Cu^{2+} concentrations.

4b. <u>Determining the time course of Cu^{2+} uptake by BBMV.</u>

5 uCi ^{64}Cu was added into 0.75 ml incubation buffer containing 0.2 mmol/L $CuCl_2$. After taking three 20 ul aliquot of this isotope solution to filters to measure nonspecific binding, about 700 ug BBMV protein was added into the solution incubated at 37°C. At different time intervals triplicate 20 ul samples were filtered to terminate the incubation.

4c. <u>Measurement and calculation of the decay factor for ^{64}Cu.</u>

Because of the short half life of ^{64}Cu (12.7 h), the decay of ^{64}Cu should be corrected for all ^{64}Cu radioactivity measurements. A ^{64}Cu decay curve (the percentage of radioactivity remaining as a function of time) was established (see the following table). The ^{64}Cu radioactivity corrected for decay by this curve was compared to the radioactivity corrected by the general decay equation ($A_t = A_0 e^{-\Omega t}$ and

$\lambda=0.693/T_{1/2}$) (Isserstedt and Christian 1989). The result shows that the half life of our ^{64}Cu preparation was about 23 h, longer than the reported value.

Measured data for the decay curve of ^{64}Cu radioactivity

elapsed time in hours	remaining activity	
	cpm	%
0	63950	100
1.62	61584	96.3
2.75	59988	93.8
3.75	58277	91.1
5.42	57091	89.3
6.33	51921	84.3

APPENDIX V

Calculation of uptake velocity

Velocity of Zn or Cu transport across BBMV was calculated from the results of gamma ray counting of filters.

The radioactivity of ^{65}Zn stock solution (of specific volume) was measured on the gamma counter before being diluted for the transport study. The specific activity of the ^{65}Zn stock solution was calculated according to this measurement and the chemical quantity of elemental Zn and was expressed as cpm/nmol Zn.

The specific activity is necessary to determine total Zn^{2+} transported as opposed to ^{65}Zn transported - which is the value represented by the results of gamma ray counting. The velocity also accounts for the time incubated, and the relative amount of BBMV present in the 50 ul aliquot. This relative amount is represented by total protein measurements of the BBMV solution.

(cpm of filter - cpm of blank)/specific radioactivity
= cpm/(cpm/nmol Zn^{2+}) = nmol Zn^{2+}

nmmol Zn^{2+}/ time incubated = nmol Zn^{2+}/min

(nmol Zn^{2+}/min)/total protein in BBMV aliquot
= nmol Zn^{2+}/min/mg protein

Velocity is expressed as nmol Zn^{2+}/min/mg protein

Once the velocity of Zn^{2+} transport across BBMV was calculated, the kinetics of Zn transport was determined by Eadie-Hofstee plot which converts the rectangular saturation curve of Zn transport to a straight line. The y axis represents velocity in nmol Cu/min/mg protein and the x axis is represented as velocity/[Cu]. A straight line was fitted by linear regression using computer program "Minitab" (Minitab Inc., College Station, MA). The intercept of the line at y axis indicates the value of Vmax and the slope of the line indicates the value of Km.

The kinetics of the transport was also determined by fitting the data into the equation describing the combined functions of carrier mediated transport and passive diffusion:

$$V = \frac{V^{max} \times S}{K_m + S} + P \times S$$

APPENDIX VI

Metallothionein Assay

The method was developed by Ms. L.M.J. Smith in Dr. E. Nieboer's laboratory, Department of Biochemistry, McMaster University.

1. Solutions

Stock ^{109}Cd solution
100 μL pure ^{109}Cd (1.3 μCi/μL)
100 μL distilled, doubly deionized water

Working ^{109}Cd solution
20 μL stock solution
10 μL $CaCl_2$ (10 μg/μl)
970 μL saline

2% hemoglobin (w/v), made up in saline

10 mmol/L Tris-HCl, pH 8.5

500 mmol/L Tris-HCl, pH 8.5

50 μmol/L Tris-HCl, pH 8.5

2. Sample preparation

0.2 gram homogenized liver or intestinal mucosa sample plus 0.8 mL 10 mmol/L Tris-HCl was homogenized using a Polytron homogenizer (Kinematica GMBH, Lucern, SUI) at set 5 for 1 min. The homogenate was centrifuged at 10 000g for 20 min at 4°C. The supernatant was heated in a boiling water beth for 2 min and then centrifuged again at 10 000g for 2 min. 200 μl supernatant was stored at - 70°C freezer for MT assay.

3. MT assay

1) Add 100 μL sample or blank (50 μmol/L Tris-HCl, pH 8.5) to an Eppendorf tube.

2) Add 9 μL Tris-HCl (500 mmol, pH 8.5).

3) Add 30 μL working solution, swirl and incubate at room temperature for 15 min.

4) Add 50 μL 2% hemoglobin solution.

5) Swirl and boil for 1.5 min.

6) Cool on ice.

7) Microfuge at 10 000g for 3 min.

8) Transfer 150 μL to a clean Eppendorf tube (record exact volume).

9) Add 5.7 μL Tris-HCl and 50 μL 2% hemoglobin.

10) Repeat steps 5 to 9.

11) Repeat steps 5 to 8.

12) Make a 1/10 dilution of the working solution, in saline.

13) Pipet 20 μL of the 1/10 dilution and 100 μL of each sample into gamma-counting tubes.

14) Counting these samples on a gamma counter.

4. Calculations

1) ^{109}Cd specifications:

a μCi ^{109}Cd/μL

b μg Cd/μL of pure label (calculated directly from the bottle of pure ^{109}Cd)

2) Working label solution concentration:

20 μL stock A + 10 μL $CdCl_2$ + 970 μL NaCl (0.87%) = 1000 μL working solution, yields: c. μCi ^{109}Cd/1000 μg Cd/1000 μL working solution.

3) μmol/Cd/μCi:

(1000 μg $CdCl_2$/ d μg ^{109}Cd)*(1 μmol $CdCl_2$/228.3 μg $CdCl_2$) = e μmol Cd/μCi ^{109}Cd

4) Given that 20 μL(1/10 x diluted working solution) = 2 μL working solution

5) μmol Cd/cpm:
   (e μmol Cd/μCi ^{109}Cd) x (a μCi ^{109}Cd/μL) x (2 μL working solution/cpm) = f μmol Cd/cpm

6) μmol MT/cpm:
   f μmol Cd/cpm x 1 μmol MT/ 7 μmol Cd= h μmol MT/cpm

7) nmol MT/g wet wt:
   nmol MT/g wet wt =3x5x10x(cpm/g wet wt)x(i nmol MT/cpm)

3 accounts for the fact that 1/3 of the sample remains. 5 accounts for the fact that the sample (wet tissue) is initially diluted with buffer by a factor of five. 10 accounts for the fact that only 1/10 of the final solution to be counted.

APPENDIX VII

Sucrase and Lactase Assay

These methods are modified from Dahlqvist (1968).

Sucrase Assay

Reagents:

1. Glucose standard: 18 mg glucose in 10 mL 0.1 mol/L phosphate buffer as stock. Diluting the stock 10 times for assay.

2. Peroxidase solution: dissolve 12 mg peroxidase in 10 mL DDI and freeze in aliquots of 1.1 mL.

3. PGO solution: 0.3 mL glucose oxidase
   1.0 mL peroxidase
   0.2 mL O-dianisidine solution (0.1 g in 10 mL of 95% ethanol and store in dark bottle and cupboard)

   Make up these three reagents in a brown bottle up to a total volume of 40 mL with Tris buffer, pH7. This solution is stable at 4°C for 2-3 weeks.

4. Substrate: 56 mmol/L sucrose in 0.1 mol/L phosphate buffer, pH 6.1.

5. Set up standard curve:

nmol/L glucose	μL glucose standard	μL phosphate buffer
10	10	190
20	20	180
40	40	160
60	60	140
100	100	100

150	150	50
200	200	0

6. Procedure:

1) Using 200 μL of phosphate buffer as blank.

2) Set up glucose standard curve according to the chart above.

3) In triplicate, pipette 10 μL of the prepared mucosa homogenate into a glass test tube. Add 100 μL of sucrose substrate and 90 μL of phosphate buffer (0.1molL).

4) Incubate blank and sample at 37 °C for 15 min. Add 1 mL of PGO to each tube. Incubate at 37 °C for 30 min. Read the absorbance at 475 nm.

Lactase Assay

Substrate: 6 mmol/L lactose in 0.1 mol/L phosphate buffer, pH 6.1

1) blank - same as above

2) Set up glucose standard as above

3) In triplicate, pipette 20 μL of the prepared mucosa homogenate into a glass test tube. Add 100 μL lactose substrate and 80 μL of phosphate buffer (0.1 mol/L)

4) Incubate blank and samples at 37°C for 15 min, adding PGO and read the absorbance at 475 nm as above.

APPENDIX VIII

Sample preparation for atomic absorption spectrometry (AAS)

A. Tissue samples

1. Pre-weigh acid -washed crucibles
2. In triplicate, weigh out 1 gram of tissue in a crucible. Cover with a lid.

   For liver standard reference material (NBS, #1577b) weigh out 2.5 g of sample in a crucible.

   For nofat milk powder reference (NBS, #,1549), weigh out 2 g of sample in a crucible.
3. Place covered crucibles in a muffle furnace at 80 °C for 12 hours to evaporate moisture from the sample in order to obtain dry weight.
4. Reweigh the crucibles and record the dry weight when the crucibles are completely cool.
5. Place the crucibles back to the muffle furnace and heat at 500 °C for 4 days or until ash becomes white.
6. Let the crucible cool and record the weight of ash.
7. Dissolve mucosal and SMA samples in 5 mL volumetric flasks with 1.25 N nitric acid. Dissolve liver and liver reference material in 10 mL of 1.25 N nitric acid.
8. Cover the volumetric flask with parafilm and invert the flask three times.
9. Transfer the sample from the flask to a acid washed glass tube. Cover the test tube with parafilm.
10. Set up AA using specific Zn or Cu lamp and ensuring that the lamp, flame and detector are aligned.
11. Establish standard curve by measuring prepared standards

(The standards are prepared by diluting the purchased Zn or Cu standards with 1.25 N nitric acid).

12. measuring standard reference samples and tissue samples.

B. Plasma samples

Plasma samples were directly measured by AAS after a 1:5 dilution with 1.25 N nitric acid. The standards for plasma sample determination were prepared in 5% glycerol rather than 1.25 N nitric acid. This is because 5% glycerol more closely matches the viscosity of plasma.

C. Urine samples

Urine samples were directly measured for Zn and Cu by AAS. The standards for urine Zn and Cu determination were prepared in urine diluting fluid (a fluid has similar mineral composition as urine).

DATA FOR CHAPTER 3

The ontogeny of intestinal absorption of Zn and Cu and body Zn and Cu distribution in the piglet model

The Ontogeny of Cu Uptake

	Cu uptake nmol/mg.min^{-1}			
Cu (mmol/L)	32 h (n=4)	52 h (n=7)	10 d (n=7)	20 d (n=6)
0.02	0.9±0.2	0.1±0.1	2.0±0.5	3.0±0.1
0.04	0.9±0.2	0.16±0.1	3.0±0.9	3.9±0.5
0.06	0.9±0.2	0.16±0.03	2.2±0.07	4.5±0.6
0.07	1.1±0.1	0.19±0.03	3.5±0.6	5.5±0.9
0.08	1.2±0.2	0.17±0.1	3.3±0.7	5.6±0.8
0.09	1.2±0.2	0.2±0.1	3.5±0.8	5.9±0.9
0.1	1.4±0.2	0.24±0.1	3.4±0.6	5.5±0.9
0.12	1.7±0.2	0.26±0.1	3.4±0.9	6.8±0.3
0.14	1.8±0.3	0.28±0.1	3.2±0.6	6.9±1.0
0.16	2.1±0.3	0.3±0.1	4.3±1.2	7.0±1.0
0.18	2.2±0.3	0.3±0.1	4.7±0.9	7.3±1.0
0.2	2.6±0.3	0.3±0.1	4.7±0.9	7.0±1.0
0.3	3.8±0.8	0.4±0.1	5.2±0.9	8.1±0.5
0.4	3.8±0.9	0.6±0.2	5.9±1.3	8.8±0.5
0.6	4.0±1.4	1.0±0.3	5.6±1.7	9.7±1.8

The Ontogeny of Zn Uptake by BBMV

Zn (mmol/L)	Zn uptake nmol/mg.min^{-1}			
	32 h (n=4)	52 h (n=7)	10 d (n=7)	20 d (n=6)
0.1	16.7±2.0	7.3±2.0	7.4±2.5	9.0±1.0
0.15	23.2±2.0	9.6±3.0	10.4±1.0	11.0±1.0
0.2	29.4±3.0	13.2±4.0	13.4±2.0	15.5±6.0
0.25	36.7±5.0	16.2±6.0	14.0±3.0	18.6±2.0
0.3	51.4±10	19.2±5.0	17.0±3.0	20.4±2.0
0.35	72.2±20	20.2±4.0	18.5±2.2	23.0±3.0
0.4	82.3±22	27.6±8.0		22.7±2.0
0.45	117.6±24	33.2±8.0	19.0±2.0	25.3±3.0
0.5	119±28	34.9±11	19.5±3.0	24.3±2.5

Intestinal Mucosa Cu (μg/g dry wt)

pig No.	32 h	pig No.	52 h	pig No.	10 d	pig No.	20 d
146	139	145	112	152	13.5	100	15
147	101	150	133	153	13.6	104	20
148	133	151	125	157	11.0	106	16.3
149	138.4			158	13.9	110	12.7
				161	10.3	114	15.1
				163	11.7	117	21.6
						120	14.0
mean±SD	127.9±18		123.2±6.2		12.3±1.6		16.4±3.3

Intestinal Mucosa Zn (μg/g dry wt)

pig No.	32 h	pig No.	52 h	pig No.	10 d	pig No.	20 d
146	61.1	145	84.7	152	76.9	100	115.5
147	56.6	150	78.6	153	82.7	104	101.8
148	79.5	151	97.2	157	81.2	106	120.2
149	93.2	188	56.5	159	93.5	110	97.8
		189	63.7	161	94.3	114	120.6
				163	77.2	117	118.9
						120	130.9
mean±SD	72.6±17		76.1±16.3		84.29±7.8		115.1±11.5

Liver Zn (µg/g dry wt)

pig No.	32 h	pig No.	52 h	pig No.	10 d	pig No.	20 d
146	555.8	145	372.2	157	178.6	100	136.9
147	351.1	151	395.7	158	148.9	104	197.1
148	317.2	188	292.1	161	69.7	106	209.8
149	377.1	189	251.2	163	78.1	110	168.1
				152	119.3	114	118.7
				153	158.4	117	152.9
						120	158.9
mean±SD	400.3±106.5		327.8±67.6		125.5±44.4		163.2±32

Liver Cu (µg/g dry wt)

pig No	32 h	pig No	52 h	pig No	10 d	pig No	20 d
146	547.1	145	359.0	153	223.7	100	236.6
147	370.3	150	265.1	157	262.6	104	348.6
148	362.9	151	300.7	158	301.6	106	331.8
149	221.4			161	207.1	110	262.9
				163	178.3		
mean±SD	377±163		280±52		234.6±48.3		295±53.8

Plasma Zn (μmol/L)

pig No.	32 h	pig No.	52 h	pig No.	10 d	pig No.	20 d
146	3.4	145	4.6	152	13.1	100	15.9
147	5.0	150	4.2	157	13.5	104	11.3
148	3.3	151	3.9	158	11.1	106	14.3
149	3.9			161	13.8	110	17.3
				163	11.75	117	14.95
				153	11.31	120	13.76
mean±SD	3.9±0.7		4.2±0.4		12.4±1.2		13.9±2.5

Plasma Cu (μg/g dry wt)

pig No.	32 h	pig No.	52 h	pig No.	10 d	pig No.	20 d
146	10.9	145	12.8	152	6.1	100	30.3
147	12.8	150	8.5	153	6.7	104	23.5
148	10.9	151	12.4	157	7.7	106	22.6
149	11.1			158	6.9	114	26.5
				161	12.8	117	24.8
				163	11.2	120	21.5
mean±SD	11.4±1.0		11.2±2.4		8.6±2.8		24.9±32

Liver MT (nmol/g wet wt)

pig No.	32 h	pig No.	52h	pig No.	10 d	pig No.	20 d
146	203.3	145	196.3	153	15.4	100	4.3
147	196.7	150	198.6	158	18.2	104	8.5
148	201.4	151	200	161	9.9	106	8.5
149	191.2			163	7.8	114	7.0
						117	6.7
						120	7.0
mean±SD	198.2±5.4		198.3±2.0		13.0±5.0		7.0±2.0

Intestinal Mucosa MT (nmol/g wet wt)

pig No.	32 h	pig No.	52 h	pig No.	10 d	pig No.	20 d
146	2.4	145	2.5	153	1.6	100	3.6
147	1.9	150	2.1	157	1.6	104	4.4
148	4.4	151	2.0	158	1.7	106	3.2
149	3.2			161	2.9	114	5.2
				163	1.9	117	4.5
						120	6.3
mean±SD	3.0±1.0		2.2±0.3		1.9±0.6		4.5±1

DATA FOR CHAPTER 4

The interactive effects of dietary Zn and Fe on Cu uptake by intestinal BBMV in the developing piglet

The Time Course of Cu Uptake and the Effects of Zn

	Cu uptake nmol/mg.min^{-1}		
Time (min)	Control (n=3)	Zn(1 mmol/L) (n=3)	Zn(2 mmol/L) (n=3)
0.5	5.0±1.8	3.1±1.0	3.9±1.0
1	5.9±1.2	3.4±1.4	4.1±1.3
2	7.0±1.4	4.5±0.5	4.3±1.2
3	7.4±1.7	4.2±0.9	4.3±0.4
4	7.0±1.6	4.6±1.0	5.5±0.1
5	7.0±1.8	4.3±0.8	5.4±0.6
10	8.1±1.3	4.5±0.7	5.6±0.1
12	7.4±1.7	5.0±0.1	5.5±0.2
15	8.1±1.7		
17	7.7±1.9	5.4±0.4	5.3±0.7
20	7.4±1.7	5.2±0.13	5.4±0.6

Time Course of Cu Uptake and the Effects of Fe

Time(min)	Control (n=5)	Fe (1 mmol/L) (n=5)	Fe (2 mmol/L) (n=5)
	Cu uptake nmol/mg.min^{-1}		
0.5	5.0±1.8	17.0±1.3	14.2±0.2
1	5.9±1.2	18.9±2.1	16.4±1.4
2	7.0±1.5	18.2±2.0	18.1±1.0
3	7.4±1.7	19.6±2.0	18.5±1.4
5	7.0±1.6	20.5±2.6	21.4±1.4
7.5	7.0±1.8	20.5±2.1	23.9±1.5
10	8.1±1.3	21.1±3.0	23.8±1.4
12	7.4±1.7	20.5±2.9	26.2±2.0
17	8.1±1.7	18.2±4.0	26.1±1.6
20	7.7±1.9	21.0±2.0	26.9±2.4

The Effect of Fe on Cu binding to BBMV

	Cu uptake nmol/mg.min^{-1}	
Osmo^{-1}	Control (n=3)	Fe(1 mmol/L) (n=3)
1.25	4.1±1.0	14.7±3.7
2	5.0±1.3	16.8±1.7
3.33	6.8±1.0	20.2±4.0
5	7.7±2.0	21.5±4.0

Cu uptake by BBMV in response to 5 d high Zn feeding versus placebo treatment

	Cu uptake nmol/mg.min^{-1}	
Cu(mmol/L)	High Zn (n=5)	Control (n=5)
0.02	3.0±0.1	1.3±0.1
0.04	3.9±0.5	1.5±0.1
0.06	4.5±0.6	1.9±0.4
0.07	5.5±0.9	1.9±0.3
0.08	5.6±0.8	2.0±0.4
0.09	5.9±0.9	2.0±0.5
0.1	5.5±0.9	2.4±0.3
0.12	6.8±0.3	2.1±0.4
0.14	6.9±1.0	2.3±0.5
0.16	7.0±1.0	2.2±0.5
0.18	7.3±1.0	3.9±1.3
0.2	7.0±1.0	6.9±2.2
0.3	8.1±0.4	14.4±6.4
0.4	8.8±0.5	93.8±35.2
0.5	9.7±1.8	264±97.4
0.8	12.6±3.4	465±156

Liver Cu (μg/g dry wt)

pig No.	Control	pig No.	High Zn
100	294.2	108	308.3
104	506.0	111	93.0
117	219.5	112	203.4
120	250.4	113	424.2
		116	202.0
		122	228.0
mean±SD	317.5±129		243±112.4

Intestinal Mucosa Cu (μg/g dry wt)

pig No.	Control	pig No.	High Zn
104	20.3	108	23.5
106	9.6	113	13.6
114	27.2	122	15.1
120	21.3	123	27.1
117	15.1	116	16.6
mean±SD	18.7±6.7		19.2±5.8

Plasma Cu (nmol/L)

pig No.	Control	pig No.	High Zn
100	30.3	108	23.4
104	23.5	113	29.0
106	22.5	116	19.5
114	26.5	122	33.5
120	21.5	123	20.0
117	24.8		
mean±SD	25.0±3.2		25.0±6.0

DATA FOR CHAPTER 5

Alterations in intestinal uptake and compartmentalization of Zn in response to short-term DEX therapy or excess dietary Zn in piglets

The velocities of Zn uptake by BBMV at different extravesicluar Zn concentrations

	Zn uptake nmol/mg.min^{-1}		
Cu(mmol/L)	Control (n=6)	DEX (n=5)	High Zn (n=6)
0.1	9.0±1.0	14.2±2.0	9.5±1.7
0.15	11.4±1.4	22.0±3.4	13.7±2.3
0.2	15.5±1.8	28.0±3.5	17.6±3.0
0.25	18.6±2.2	33.9±4.6	20.8±3.3
0.3	20.4±1.8	38.1±4.6	21.6±3.3
0.35	23.0±3.0	41.2±5.6	24.5±3.9
0.4	22.7±2.5	45.3±6.7	24.8±3.8
0.45	25.3±3.2	47.8±7.2	27.6±4.0
0.5	24.3±2.5	51.4±5.7	28.0±4.2

The time Course of Zn Efflux across BBMV

Time(min)	% of Zn remaining in BBMV	
	Control (n=5)	DEX (n=5)
0.5	7.2±1.4	31.0±4.7
1	5.4±0.1	28.4±4.7
1.5	4.8±0.9	27.0±4.4
2	4.5±0.9	25.0±4.8
2.5	4.2±0.9	23.8±5.0
3	3.9±1.0	20.6±5.0
4	3.9±1.0	19.1±5.2
5	3.6±1.0	17.9±5.0

Intestinal Mucosa Zn (μg/g dry wt)

pig No.	Control	pig No.	DEX	pig No.	High Zn
100	115.4	99	126.0	108	160.8
104	101.8	105	140.3	111	199.4
106	120.2	107	127.3	112	205.5
110	97.8	109	117.2	113	203.9
114	120.6	115	124.6	116	149.7
117	118.9	118	147.7	122	152.4
120	131.0	121	124.0	123	104.0
mean±SD	115±11.5		129.6±10.6		167.9±37.5

Liver Zn (μg/g dry wt)

Pig No.	Control	pig No.	DEX	pig No.	High Zn
100	137.0	99	120.2	108	350.6
104	197.0	105	210.3	111	567.8
106	209.8	107	138.5	112	224.4
110	168.0	109	159.9	113	541.2
114	118.7	115	106.6	116	342.0
117	153.0	118	83.7	122	263.0
120	159.0	121	54.0	123	151.7
mean±SD	163.2±32		124.7±51		349±156

Plasma Zn (μmol/L)

pig No.	Control	pig No.	DEX	pig No.	High Zn
100	16.0	99	12.7	108	11.7
104	11.3	105	11.7	111	11.0.
106	14.3	107	10.2	112	7.7
110	17.3	109	9.0	113	21.0
114	10.2	115	11.5	116	12.0
117	15.0	118	12.3	122	8.7
120	13.8	121	10.0	123	10.2
mean±SD	14.0±2.5		11.0±1.4		11.7±4.3

Liver MT (nmol/g wt)

pig No.	Control	pig No.	DEX	pig No.	High Zn
100	4.3	99	6.5	108	43.2
104	8.5	105	9.3	111	102.5
106	8.5	109	5.6	112	63.2
114	7.0	115	7.0	113	113.0
117	6.7	118	7.1	116	73.3
mean±SD	7.0±1.6		7.1±1.4		79.0±28.7

Intestinal Mucosa MT (nmol/g wet wt)

pig No.	Control	pig No.	DEX	pig No.	High Zn
100	3.6	99	5.3	108	4.1
104	4.4	105	3.6	111	2.5
106	3.2	109	2.1	112	7.7
114	5.2	115	2.0	113	6.3
117	4.5	118	5.4	116	2.5
120	6.3				
mean±SD	4.5±1.1		3.7±1.7		4.6±2.3

DATA FOR CHAPTER 6

Long-term consequence of chronic DEX therapy ± High dietaryZn on growth and Zn, Cu metabolism in the piglet model

Time Course of ^{65}Zn Uptake by Intestine

Time(min)	%^{65}Zn remaining in the perfusate		
	Control (N=5)	DEX (N=4)	DEX±Zn (N=6)
0	100	100	100
5	50.4±2.5	35.8±2.7	46.6±5.1
10	48.2±2.3	34.3±2.7	45.3±4.8
15	45.4±3.5	34.3±2.4	43.0 ±4.7
20	49.5±3.4	34.1±2.9	42.8±5.5
25	47.0±3.5	32.7±2.9	42.4±5.8
30	44.3±3.5	32.7±2.7	42.0±6.5

Cu Uptake by BBMV and the Effects of DEX and DEX+Zn

Cu (mmol/L)	Cu uptake nmol/mg . min^{-1}		
	Control (N=6)	DEX (N=6)	DEX+Zn (N=6)
0.02	0.4±0.1	0.5±0.2	0.5±0.2
0.04	0.5±0.2	0.7±0.2	0.6±0.1
0.06	0.6±0.2	1.0±0.3	0.8±0.2
0.07	0.8±0.2	1.3±0.4	1.0±0.3
0.08	0.8±0.3	1.2±0.4	1.2±0.4
0.09	0.8±0.3	1.3±0.4	1.3±0.4
0.1	1.1±0.3	1.5±0.4	1.3±0.4
0.12	1.1±0.3	1.5±0.4	1.4±0.4
0.14	1.1±0.4	1.8±0.4	1.5±0.4
0.16	1.2±0.3	2.0±0.2	1.7±0.2
0.18	1.1±0.4	1.9±0.3	1.9±0.3

The Appearance of ^{65}Zn Tracer in Portal Blood During and Following Intestinal Perfusion

Time(min)	cpm per gram of blood		
	Control (N=5)	DEX (N=4)	DEX+Zn (N=6)
0	0	0	0
8	37±19	32±4	40±12
16	107±36	38±2	81±20
24	118±51	58±22	91±21
32	141±39	96±24	97±20
40	129±39	97±34	104±17
48	106±9	87±42	97±20
56	51±16	65±33	84±28
60	40±26	51±18	48±9

Blood Glucose at d 16 of Treatment (mmol/L)

pig No.	Control	pig No.	DEX	pig No.	DEX+Zn
174	2	176	2	178	2
175	2	177	3	179	2
184	3	187	10	182	8
185	4	186	9	183	10
196	4	192	2	194	10
197	3	193	7	195	10
mean±SD	3±1		5.5±3.6		7±4

The Amount of ^{65}Zn Tracer in Liver After Intestinal Perfusion

	cpm per gram of liver homogenate				
pig No.	Control	pig No.	DEX	pig No.	DEX+Zn
174	335	176	187	178	505
175	623	177	397	179	260
184	438	187	253	182	186
185	325	193	539	183	173
196	1260			194	333
				195	377
mean±SD	596±390		344±157		306±126

Intestinal Accumulation of ^{65}Zn After Intestinal Accumulation

cpm per gram of intestinal mucosa

pig No.	Control	pig No.	DEX	pig No.	DEX+Zn
174	45263	176	218832	178	93507
175	54123	177	85115	179	267229
184	52088	187	111839	182	148469
185	26479	193	131090	183	146747
196	71524			194	138129
				195	446222
mean±SD	49895±16281		136719±57897		206717±130793

Total Elemental Zn uptake by Intestine During Perfusion

μg Zn/g dry wt

pig No.	Control	pig No.	DEX	pig No.	DEX+Zn
174	89.6	176	140.0	178	74.2
175	85.6	177	192.6	179	82.3
184	69.6	187	174.7	182	131.6
185	128.3	193	236.9	183	186.4
196	166.4			195	230.8
Total	108±39		186±40		141±67

Wet Weights of spleen and kidney

pig No.	Control	pig No.	DEX	pig No.	DEX+Zn
Spleen (g wet)					
184	21.7	187	11.8	182	12.1
185	19.2	193	7.8	183	11.6
196	12.3	186	9.9	194	2.8
197	12.6	192	3.2	195	3.2
Kidney L (g wet)					
184	25.3	186	28.8	182	30.7
185	27.5	187	22.0	183	22.3
196	21.2	192	14.4	194	12.2
197	25.6	193	13.6	195	12.9
Kidney R (g wet)					
184	27.2	186	28.6	182	27.7
185	28.5	187	23.5	183	18.6
196	19.3	192	13.2	194	11.4
197	24.3	193	13.7	195	12.9

Plasma Zn (μg/ml) at d 0, d 7 and d 16 of Treatment

Control

pig No.	d 0	d 7	d 16
174	0.7	0.5	0.7
175	0.7	0.6	0.5
184	1.0	1.4	0.6
185	0.8	0.6	0.8
196	1.0	0.5	0.9
197	0.8	0.6	0.8
mean±SD	0.9±0.1	0.7±0.4	0.7±0.2

DEX

pig No.	d 0	d 7	d 16
176	0.8	0.7	0.7
177	0.7	0.6	0.5
186	0.7	0.6	0.8
187	0.6	0.6	0.6
192	0.9	0.6	0.4
193	1.0	0.5	0.7
mean±SD	0.8±0.1	0.6±0.1	0.6±0.2

DEX+Zn

pig No.	d 0	d 7	d 16
178	0.5	0.6	0.5
179	0.6	0.6	0.6
182	0.6	1.0	0.9
183	1.1	0.9	0.7
194	0.7	0.6	0.6
195	0.6	0.6	0.6
mean±SD	0.7±0.2	0.7±0.2	0.6±0.1

Plasma Cu (μg/ml) at d 0, d 7 and d 16 of treatment

Control

pig No.	d 0	d 7	d 16
174	0.9	0.9	0.8
175	0.6	0.8	0.9
184	1.5	1.4	1.0
185	1.1	0.9	0.9
196	0.7	1.0	0.9
197	1.0	0.9	0.9
mean±SD	1.0±0.3	1.0±0.2	0.9±0.1

DEX

pig No.	d 0	d 7	d 16
176	1.0	1.3	1.4
177	0.7	0.9	1.1
186	1.4	1.6	1.6
187	1.2	1.4	1.4
192	0.7	1.8	1.7
193	0.8	1.4	1.2
mean±SD	1.0±0.3	1.4±0.3	1.4±0.1

DEX+Zn

pig No.	d 0	d 7	d 16
178	1.3	1.1	1.6
179	0.7	1.1	1.3
182		1.1	1.3
183	1.3	1.8	1.6
194	0.6	1.5	1.1
195	1.0	1.6	1.5
mean±SD	1.0±0.3	1.4±0.3	1.4±0.2

Intestinal Mucosa Zn (μg/g wet wt)

Pig No.	Control	pig No.	DEX	pig No.	DEX+Zn
174	14.2	176	15.6	178	15.6
175	14.6	177	15.9	179	14.8
184	14.3	187	16.5	182	16.7
185	16.9	186	16.8	183	19.1
196	15.5	192	23.8	194	16.0
197	16.1	193	17.5	195	16.0
mean±SD	15.3±1.1		17.7±3.1		16.4±1.5

Intestinal Cu (μg/g wet wt)

pig No.	Control	pig No.	DEX	pig No.	DEX+Zn
174	3.7	176	2.8	178	7.4
175	3.9	177	6.7	179	3.9
184	3.8	187	2.6	182	3.7
185	3.5	186	4.0	183	3.8
186	3.9	192	3.7	194	4.1
197	3.1	193	3.9	195	3.4
mean±SD	3.6±0.3		4.0±1.5		4.4±1.5

Liver Zn (µg/g wet wt)

pig No.	Control	pig No.	DEX	pig No.	DEX+Zn
175	77.2	176	39.0	178	45.4
184	43.4	177	35.5	179	29.5
185	42.3	187	32.3	182	29.5
196	59.9	186	36.5	183	50.5
197	71.0	192	34.5	194	24.0
		193	36.2	195	30.6
mean±SD	58.7±16		35.7±2.2		34.9±10.5

Liver Cu (µg/g wet wt)

pig No.	Control	pig No.	DEX	pig No.	DEX+Zn
175	15.7	176	17.0	178	21.6
184	6.4	177	20.9	179	15.6
185	5.7	186	9.0	182	11.1
196	14.2	187	9.3	183	7.7
197	16.3	192	22.2	194	11.8
		193	21.9	195	14.1
mean±SD	11.7±5.2		16.7±6.2		13.7±4.8

Liver MT (nmol/g wet wt)

pig No.	Control	pig No.	DEX	pig No.	DEX+Zn
174	4.0	176	1.4	178	5.3
175	9.1	177	2.7	179	2.3
184	4.9	186	5.4	182	5.5
185	5.4	187	1.9	183	5.7
196	10.4	192	2.7	194	0.9
197	9.9			195	1.4
mean±SD	7.3±2.8		2.8±1.5		3.5±2.2

Intestinal Mucosa MT (μg/g wet wt)

pig No.	Control	pig No.	DEX	pig No.	DEX+Zn
174	2.6	176	14.0	178	16.2
175	3.2	177	13.7	179	8.5
184	5.5	186	7.7	182	9.3
185	6.8	187	7.6	183	11.3
186	4.5	192	16.9	194	13.3
197	5.0	193	10.0	195	11.0
mean±SD	4.6±1.5		11.7±3.8		11.6±2.8