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PHARMACOLOGICAL CONCENTRATIONS OF ZN: THE EFFECTS ON PRODUCTION, TISSUE AND BLOOD CONCENTRATIONS, ANTIOXIDANT STATUS AND METALLOTHIONEIN CONCENTRATIONS IN GROWING PIGS

By

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ABSTRACT

PHARMACOLOGICAL CONCENTRATIONS OF ZINC: THE EFFECTS ON PRODUCTION, TISSUE AND BLOOD CONCENTRATIONS, ANTIOXIDANT STATUS AND METALLOTHIONEIN CONCENTRATIONS IN GROWING PIGS

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Growth performance determines whether swine producers makes a profit. The development of nutritional practices that enhance swine growth performance are of interest. Previous studies reported that an improvement in growth performance occurred when pharmacological concentrations of Zn (3,000 ppm) were fed to nursery pigs. However, the mechanism of action for the enhanced growth is unknown and needs to justify the use of 3,000 ppm Zn in the nursery pig diet. Thus, experiments were conducted to determine a possible mode of action behind the observed increase in growth performance when nursery pigs were fed high concentrations of Zn. Average daily gain (ADG), feed intake, plasma and tissue concentrations of Zn, Cu and Fe, RBC Cu/Zn superoxide dismutase activity,metallothionein (MT) concentrations, and intestinal morphology of the duodenum were evalutated. The results of these studies indicates that 3,000 ppm Zn as Zn oxide should always be fed for at least the first 2 wks post-weaning in order to enhance growth performance in early- and traditionally-weaned nursery pigs. The mechansim of action by which feeding high Zn may enhance growth is the increase of intestinal MT by maintaining Zn homeostasis and the alteration of the duodenal intestinal architecture which improves nutrient absorption.

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INTRODUCTION

The use of pharmacological concentrations of zinc (Zn) and (or) copper (Cu) in nursery pig diets to enhance growth has been widely accepted in the swine industry. A routine recommendation is to add 2000 to 3000 ppm Zn in the form of zinc oxide (ZnO) and (or) to add 250 ppm Cu as copper sulfate ($CuSO_4$) to the diet. Some swine producers use these concentrations of Zn and Cu for the entire nursery period of up to eight weeks. While the effects of longer term use of pharmacological concentrations of Zn and Cu on soil nutrient management are not known, intuitively the suspicion is that the effects will be negative and disposal of lagoon effluent on crop land may have a negative impact. This is particularly important in situations where a nursery production system is the only source of nutrients for a land mass.

Pharmacological mineral supplementation has been practiced for many years. Dietary Cu research started about seventy years ago when Hart et. al. in 1928 found Cu to play a role in hematopoiesis in rats. Arnon and Stout (1939) reported Cu to be an essential nutrient for plant and animal life. In 1955 the minerals, Cu and Zn, were recognized as being important in applied swine nutrition. In that year, Barber et al. (1955) reported an enhancement in growth performance of growing pigs when fed 250 ppm Cu as copper sulfate. In the same year, Tucker and Salmon (1955) reported that feeding Zn to growing pigs prevented and cured parakeratosis. Subsequent Cu research (Bunch et al., 1961 and Stahly et al.,

1980) showed a similar response over a wider range of supplementation: 125 ppm to 250 ppm Cu. These are pharmacological concentrations because the Cu requirement for swine is 5 to 6 ppm for the weanling pig (NRC, 1988). Copper is relatively toxic essential trace mineral. Since dietary Cu requirements vary greatly among species, the recommended Cu intake for one specie may cause Cu toxicity in another specie.

Copper and Zn are currently receiving significant attention in the swine industry for their roles in improving growth and immunity. Both Cu and Zn function as essential elements in enzymes which are necessary for normal body metabolism. Research from Europe (Poulsen, 1989 and 1995) showed that pharmacological concentrations of ZnO in nursery diets reduces the incidence of diarrhea from stress and from the proliferation of enterotoxigenic *Escherichia coli* (E. coli) in the newly weaned pig. In the United States, feeding Zn at pharmacological concentrations has been shown to improve growth performance (Hahn and Baker, 1993; Hill et al., 1996; Smith et al., 1997). However, no studies have investigated the mechanism by which pharmacological concentrations of Cu and (or) Zn improves growth of the nursery pig.

The purpose of this research is to evaluate the use of pharmacological concentrations of Zn and (or) Cu in the growing pig. The results may provide a better understanding of the mechanism underlying the improved growth observed as a result of feeding high concentrations of Zn and Cu and may alleviate some environmental concerns.

Chapter 1:

Literature Review

Zinc - An Essential Nutrient

Zinc has been known to be an essential nutrient for more than 100 years. Because Day and Skidmore (1947) found the Zn requirement for mice to be less than 5 ppm, it was generally assumed for many years that a Zn deficiency would not occur in farm animals. However, today Zn deficiency is a well established disorder reported in several species including farm animals. The initial clinical signs of a Zn deficiency are characterized by a reduction in feed intake causing a decrease in growth rate and an increased incidence of diarrhea (Hambidge et al., 1986). These symptoms are followed by a skin disorder, parakeratosis, that has been reported in pigs, humans, poultry, ruminants and lab animals. When parakeratosis was first reported in swine by Tucker and Salmon (1955), they observed hyperkeratinization or lesions of the skin. This skin disorder can be cured and prevented by the supplementation of dietary Zn. Each species has symptoms that vary, but Zn deficiency is most commonly characterized by an unthrifty appearance. Other symptoms of a Zn deficiency include: anorexia, impaired immunity, diminished gonadal function, slow wound healing, and impaired taste/smell functions, and a significant alteration in development and behavior (Sandstead et al., 1967; Whitenack et al., 1978; Wada and King, 1986). In comparison, excessive Zn intake (5,000 ppm) by swine causes growth depression, arthritis, unthriftiness, low birth weights and death (Brink et al., 1959; Hill et al., 1983).

Zinc deficiency has many different effects on the overall body metabolism. It is required in over 200 proteins and enzyme systems. Roles include enzyme co-factor and maintenance of membrane structure. Keilin and Mann (1940) discovered that carbonic anhydrase contained Zn, and the activity was dependent on the presence of Zn. The activities of lactic dehydrogenase, malic dehydrogenase, alcohol dehydrogenase, and alkaline phosphatase in either testis, bone, esophagus or kidneys have been reduced in Zn deficient rats (Prasad et al., 1967). Other influences of Zn deficiency include a reduction in the amount of insulin (Quarterman et al., 1966), superoxide dismutase activity (Bettger et al., 1978), and many of the enzymes involved in DNA and RNA replication (Scott and Turnlund, 1994). A Zn deficiency may increase the susceptibility of tissue to oxidative damage and increase RBC fragility (Bray and Bettger, 1990). Cousins (1985) reported that Zn is involved in the synthesis and degradation of metallothionein. The function will be reviewed in a later section.

Zinc Metabolism

Zinc is absorbed primarily through the small intestine of monogastric animals and to a lesser extent from the stomach, the abomasum of cattle and the proventriculus of chicks (Cousins, 1985). But, studies of intestinal Zn absorption have yielded conflicting results. In some studies, the duodenum has been the site of maximal absorption (Davis, 1980), while in others, maximum uptake has occurred in jejunum and ileum (Emes and Arthur, 1975; Antonson et al., 1979). A variety of

factors influence the availability of Zn for absorption. Dietary components which are the main source of reduction in Zn absorption, are phytate, fiber, calcium (Ca), phosphorus (P), Cu, cadmium (Cd), and ferrous iron (Miller et al., 1979).

The association of Zn in the intestine with the large number of low molecular weight ligands such as pancreatic secretions proceeds transport into the mucosal cell (Hempe and Cousins, 1989). These ligands, which include citrate, picolinate, EDTA and the amino acids histidine and glutamic acid have been reported to enhance mucosal uptake and absorption of Zn under experimental conditions (Hambidge et al., 1986). Dietary Zn is retained in the intestinal mucosa cells. Within the intestinal mucosal cell, Zn transfer appears to be regulated by metallothionein (MT), a metal-binding protein (Cousins, 1979). The absorbed Zn is then transported into portal circulation bound to albumin (Cousins, 1985).

Following absorption, about two thirds of Zn in plasma is loosely bound to albumin suggesting that albumin transports Zn from the intestinal cells to the liver (Evans and Hahn, 1974). However, there is some evidence that transferrin transports Zn from the intestine through the portal vein to the liver (Evans and Winter, 1975). Therefore, it appears that Zn absorbed across intestinal mucosal cells is carried to the liver in portal plasma by transferrin and venous plasma by albumin.

One control mechanism for Zn absorption is the individual's need for Zn. Over a range of Zn intakes, homeostatic adaption occurs regulating the amounts of Zn absorbed (Hambidge et al., 1986). Thus, high Zn status decreases Zn

absorption; where as, low Zn status increases Zn absorption. High concentrations of Zn in the body reduces Cu body stores by decreasing the amount of Cu that is absorbed (Hill et al., 1983a). Zn absorption increases when higher concentrations of Cu are fed. There is an increase in urinary Zn excretion resulting in a decrease in Zn retention (Scott and Turnlund., 1994). Whanger et. al. (1981) found the accumulation of Zn in the hepatic tissue of sheep and cattle is rapidly depleted from metallothionein (MT) when the animals are transferred to a diet containing no supplemental Zn. Zinc supplementation induces intestinal MT synthesis (Bremner and Mehra, 1985).

Zinc is widely distributed throughout the body (Underwood, 1977). However, it appears that the liver is a major storage organ of Zn. Both dietary or parenteral administration of Zn results in accumulation of Zn in the liver and MT acts as the major storage form of Zn in the liver (Bremner and Davies, 1975; Richards and Cousins, 1976). Many minerals are stored in body tissue for use during periods of high nutritional demands or limited dietary supply. Body stores of Zn may be relatively large, but many tissue sources must be considered as sinks because the Zn retained is unavailable (e.g. hair) or only slowly available for biological purposes (e.g. bone) compared to more easily mobilized pools such as those in the liver (Bremner and Davies, 1975; Feldman and Cousins, 1976; Richards and Cousins, 1976; Bobilya et al.,1994).

The primary route of Zn excretion is via the feces, which account for about 90% of the total Zn excreted (Pekas, 1966). However, fecal Zn is mainly the

unabsorbed dietary Zn. There is endogenous fecal Zn that is derived from gastrointestinal, pancreatic and biliary secretions and this increases with an increase in Zn intake (Weigand and Kirchgessner, 1978). Therefore, the amount of fecal Zn is highly related to dietary Zn concentrations. Other routes of Zn excretion include urine, losses through body surfaces such as sweat, skin, hair and seminal fluid (Underwood, 1977). However, these losses of Zn account for less than 10 % (Weigand and Kirchgessner, 1978).

Metallothionein Protein

Metallothionein (MT) is characterized as a family of proteins with an asymmetric structure, a low molecular weight (6,000 kD), a high metal binding capacity (7-10 g atoms/ mole) for Zn, Cd and Cu, a cysteine-rich content (about 30% of amino acid residues) and a lack of aromatic amino acids (Kagi et al., 1974). This widely distributed Cu and Zn-binding protein is believed to play important roles in the control and metabolism of several minerals (Bremner and Mehra, 1985). Metallothionein is found in variable amounts in a wide range of tissues but is particularly rich in the liver, kidneys and the cytosol of intestinal mucosa cells (Richards and Cousins, 1976).

The functions of MT proteins have been debated for at least two decades. However, it seems unlikely that MT proteins only protect cells against physiologically harmful heavy metals such as mercury (Hg) and Cd by handling the intracellular and extracellular transport of these metals. Therefore, MT may have

roles in scavenging reactive intermediates, regulating Zn and Cu metabolism, controlling the transfer of Zn to transcription factors, cellular detoxification and storage of Zn (Richards and Cousins, 1976; Hall et al., 1979).

Metallothionein is unique in that the metals that bind to the protein (Cu, Cd, and Zn) also induce MT synthesis by increasing the rate of MT gene transcription (Blalock et al., 1988). The degradation of the MT protein with the turnover of metals bound to MT can occur as a complex or by the exchanging bound metals with other free metals. The half-life of MT varies according to the metal bound, such as the Cu-MT complex is 12 to 17 h, the Zn-MT complex is 18 to 20 h, and the Cd-MT complex is 84 h (Bremner et al., 1978; Feldman et al., 1978; Feldman and Cousins, 1976). Each molecule of metallothionein can bind up to 7 atoms of Zn and 12 atoms of Cu (Kagi and Schaffer, 1988). Therefore, the MT protein appears to have a regulatory function in Zn and Cu metabolism. Metallothionein synthesis is influenced both by dietary Zn concentration and by plasma Zn concentration (Bremner and Davies, 1975; Richards and Cousins, 1976) and can regulate the quantity of Zn entering the body, thus playing a major role in Zn homeostasis (Cousins, 1979). Hall et al. (1979) reported that the amount of intestinal mucosa MT is linearly related to the amount of Zn being fed. Blalock et al. (1988) observed similar results and noted that Zn stimulates MT concentrations in tissues. Starcher et al. (1980) concluded that MT plays a role in Zn absorption, when the observed amount of Zn absorbed is correlated with intestinal MT concentrations. The dietary Zn absorbed in the intestine is regulated by MT, which controls the amount of Zn

that is transferred across the basolateral membrane into circulation (Richards and Cousins, 1976). Intestinal mucosal MT can be found within four hrs after Zn administration and reaches a maximum by 20 h in rats (Starcher et al., 1980; Menard et al., 1981). The increase in intestinal MT provides a temporary storage of Zn, since the Zn-MT complex is eventually sloughed with the mucosal cell and prevents the animal from being overloaded with Zn (Cousins, 1985).

Zinc has lower binding affinity for MT than Cu (Bremner and Davies, 1975). Therefore, Zn is readily displaced by Cu. Copper is tightly bound to MT in the intestinal cells and is not available for transport across the basolateral membrane into plasma (Fischer et al., 1981 and 1983). Since the turnover of the MT is longer than that of intestinal cells in which the MT complex is held, the bound Cu is lost from the body through sloughing off of the intestinal epithelial cells. The Cu-MT complex ends up in the feces and resulting in a decrease of Cu uptake from the intestine and (or) body stores. Thus, a reduction in Cu absorption is observed when high concentrations of Zn are fed (VanCampen and Scaife, 1967). The high binding affinity of Cu for MT and ability to displace Zn from the protein are partly responsible for the inhibitory effect of Zn on Cu absorption. This mechanism has some clinical significance for example: Zn induced hypocupraemia in sickle-cell patients (Prasad et al., 1978) and control of Cu accumulation in Wilson's disease (Brewer et al., 1983) by the administration of pharmacological concentrations of Zn.

Copper - An Essential Nutrient

Copper has been acknowledged for many years as an essential nutrient for humans and animals. Dietary Cu research began about seventy years ago when Hart et al. in 1928 found Cu to play a role in normal hemoglobin synthesis of rabbits and rats. Copper serves as a co-factor for more than thirty enzymes, more specifically, the activity of oxidative enzymes for normal metabolism (O'Dell et al., 1961). When the enzymes are impaired, many of the symptoms of Cu deficiency occur . The absence of Cu or a deficiency of Cu in the diet is known to cause microcytic hypochromic anemia due to poor iron mobilization; diarrhea; bone disorders such as bowing of the legs and spontaneous fractures; neonatal ataxia; hair and wool depigmentation; poor keratinization; decreased synthesis of collagen, elastin, and myelin; infertility, cardiovascular disorders; impaired glucose and lipid metabolism and a depressed immune system (Hart et al., 1928; O'Dell et al., 1961). Copper deficiency in humans was first observed in 1964 by Cordano et al. They reported that infants fed milk diets, which are low in Cu, developed neutropenia which is usually followed by anemia.

Some of the Cu requiring enzyme systems include superoxide dismutase in the cytosol, a member of the antioxidant enzyme family that provides the cell with a major defense against oxygen toxicity and will be discussed further in a later section. Copper is required for intracellular cytochrome c oxidase activity. It has the functional role in electron transport of being the terminal oxidase in the respiratory chain of mitochondria (Hsieh and Frieden, 1975). If Cu is deficient,

cytochrome c oxidase activity decreases in the liver and heart. Copper aids in the formation of extracellular lysyl oxidase which is involved in the cross-linking of connective tissue proteins, collagen and elastin (O'Dell et al., 1961). One of the most dramatic signs of Cu deficiency is sudden death associated with spontaneous rupture of blood vessels or the heart due to decreased cross-linking of connective proteins. Dopamine *B*-hydroxylase in the central nervous system is necessary for catecholamine production and requires Cu to function.

Hsieh and Frieden (1975) determined that ceruloplasmin (Cp), a Cu containing protein that accounts for 90 to 95 % of the plasma Cu, plays a major role in transport of protein to Cu containing proteins. Ceruloplasmin may be the molecular link between iron (Fe) and Cu metabolism due to ceruloplasmin's ability to promote Fe mobilization. However, anemia is often a sign of Cu deficiency (Lahey et al., 1952; Cartwright et al., 1956), not all individuals with low Cp have impaired Fe metabolism. Ceruloplasmin also serves as an extracellular scavenger for oxygen free radicals to protect the extracellular surface of cell membranes (Cousins, 1985). Repletion of Cu after a Cu deficiency, alleviates anemia, increases the liver cytochrome c oxidase activity, and restores growth rate.

Pigs are more tolerant of high concentrations of Cu than ruminants (sheep and cattle), but there appears to be a narrow margin between the growth enhancing and toxicity concentrations. Copper is toxic when concentrations exceed 250 ppm in the diet fed to pigs for several months (Wallace et al., 1960). When the dietary Cu concentrations are in excess of the requirement (NRC, 1988), Cu accumulates

in body tissues, especially in the liver. Hepatic Cu storage varies among species and among individual animals. Also, the concentration of Zn and Fe in the diet are influential in determining the concentration of Cu that can be tolerated by the pig. Copper toxicity signs include decreased hemoglobin concentrations, arthritis, jaundice, reduced performance and ultimately death. However, if concentrations of Zn and Fe are decreased in the diet or Ca concentrations are increased, the Cu toxicity symptoms can be eliminated (Suttle and Mills, 1966a,b; Prince et al., 1984).

Copper Metabolism

Researchers documented that Cu is absorbed from the upper small intestine in the monogastric animal (Tompsett, 1940; VanCampen and Scaife, 1967). Copper absorption can vary from zero to as high as 75% depending on a number of factors. The factors that affect the absorption of Cu are the species of animal, the form of Cu consumed, the Cu status of the animal and the presence of other dietary compounds which tend to chelate Cu. Underwood (1977) reported that 10 to 33 % of dietary Cu is absorbed and depending on dietary factors such as phytates, ascorbic acid, Ca, Zn, Fe, lead, silver, molybdenum, sulfur, selenium, Cd, cobalt and manganese.

The amount of Cu available in a typical feedstuff is less the 1 ppm and more than half of the dietary Cu is absorbed in conjunction with endogenous copper secreted into the gastrointestinal tract from various endogenous fluids (Cousins, 1985). Copper is first absorbed in the intestinal mucosa cells and then transported

into the bloodstream, where Cu is loosely bound to serum albumin and amino acids. The rate of Cu absorption is regulated by MT on the serosal surface in the intestine to maintain body pools (Hall et al., 1979). The uptake of Cu across the basolateral membrane of mucosal cell is by simple diffusion and controlled by mass action (Fischer and L'Abbe, 1985). In the blood plasma, Cu immediately binds to serum albumin and is delivered to the liver and kidney. The liver is the site where dietary Cu is first deposited and is the major storage organ for Cu (Owen, 1965). From there, some of the Cu is used for production of required liver proteins, other Cu is incorporated into Cp for resecretion into the blood and some enters the bile, usually as a breakdown product of Cp Therefore, Cp is probably the main source of Cu for the other tissues, delivering via specific receptors at various organs (Harris and Percival, 1988). It is synthesized by the liver and reaches the maximum concentration in the blood within 24 hrs after a Cu infusion and has a half life of about 12 hrs (Frieden and Hsieh, 1976). Excretion of Cu occurs mainly via the bile and ultimately the feces (Butler and Newman, 1956). Only traces of Cu appear in the urine accounting for about 4 % of the Cu intake (Walter, 1964). Copper is present in skin, sweat, hair and nails and accounts for about 1-2 % of the total body Cu. The amount of Cu excreted depends on sex, temperature, environment and daily Cu intake.

Menkes' and Wilsons' genetic diseases are characterized by low plasma Cu and Cp, and inappropriate management of Cu. Both diseases result in brain damage and neurological disorders. With Wilsons' disease, copper accumulates

in the liver, kidney, and brain resulting in Cu toxicity (Bewer et al., 1983). The Wilsons' disease patients benefit from an increase of intestinal MT production during Zn therapy to decrease Cu absorption (Bettger et al., 1978 and 1979; Lee et al., 1989; Brewer et al., 1990). Menkes' disease includes depigmented hair, fragile blood vessels, and defective bone structure which is typical for Cu deficiency. However, with Menkes' disease, Zn therapy does not alleviate the problems caused by the genetic error (Danks et al., 1973).

Zinc - Copper Interactions

The theoretical framework for the interaction between minerals and trace elements was set by the studies of Hill and Matrone (1970). These investigators postulated that many elements with similar physical and chemical properties act antagonistically to each other biologically. The implication is that such metals could compete for binding sites on transport proteins or on enzymes requiring metals as co-factors. Therefore, Zn and Cu, with similar orbitals, configurations and coordination numbers, have been recognized as potential antagonists. Early work by O'Dell et al. (1976) showed that plasma Cu is elevated in a Zn deficiency when plasma Zn is depressed. Copper deficiency had no effect on plasma Zn concentrations when plasma Cu was reduced. High dietary concentrations of Zn induce a Cu deficiency indicating an interaction does exist between Cu and Zn (VanCampen, 1966). Therefore, Zn has a greater effect on Cu metabolism than Cu exerts on Zn (VanCampen and Scaife, 1967). Many aspects of the Zn and Cu

interaction are known to involve MT. This is evident at the absorption level, where the decrease in Cu absorption in Zn supplemented animals is related to the induction of MT synthesis in the intestinal mucosa by Zn (Hall et al., 1979). They observed that feeding 900 ppm Zn diet to rats decreased Cu absorption by 40% and associated this reduction with the increase in intestinal mucosal MT concentration.

Hill et al. (1983b,c) reported that feeding 5000 ppm Zn through two parities reduced litter size and weights at weaning. Offspring from these sows fed high concentrations of Zn, as ZnO, had lower tissue (liver, heart, pancreas, esophagus, aorta and testes) Cu and hemoglobin when fed a low Cu diet after weaning (Hill et al., 1983d). Therefore, feeding high concentrations of Zn results in reduced liver Cu concentrations (Hill et al., 1983a; Hurley et al., 1985). These studies indicate a Cu deficiency can be induced with high concentrations of dietary Zn. Until recently, most swine producers fed 100 to 150 ppm of Zn to nursery pigs to meet NRC (1988) recommendations. However, feeding 2000 to 3000 ppm Zn to nursery pigs in order to combat post-weaning stress and diarrhea has become common (Poulsen, 1989 and 1995). Current studies have shown that pigs fed pharmacological concentrations of Zn have highly variable growth (Hahn and Baker, 1993; Hill et al., 1996; Smith et al., 1997).

Bremner and Mehra (1985) stated that high dietary Zn intake decreased Cu concentrations and activities of Cu-dependent enzymes in tissues, while high dietary Cu had no effect on Zn absorption. Suttle and Mills (1966b) reported that

the addition of large quantities of Cu to swine diets could create an imbalance within the animal and impair body functions. They fed diets supplemented with 750 ppm Cu and observed toxicity symptoms such as jaundice, decreased hemoglobin concentrations and increased liver Cu concentrations. The toxicity signs were eliminated by decreasing plasma Cu concentrations when 500 ppm Zn or 750 ppm Fe was added to the swine diets containing 750 ppm Cu. They concluded that the addition of Fe and Zn to swine diets containing supplemental Cu to improve growth rate and feed efficiency would reduce the incidence of Cu toxicity.

Superoxide Dismutase - Zinc/Copper Antioxidant Enzyme

Superoxide dismutase enzymes are members of the antioxidant family that provides defense against oxygen toxicity in the cell. Members of the family include Cu/Zn superoxide dismutase (Cu/Zn SOD) in the cytosol and manganese superoxide dismutase (MnSOD) in the mitochondria. Each superoxide dismutase enzyme works as an antioxidant that scavenges and detoxifies superoxide radicals that are produced by normal metabolic processes, such as the production of ATP (McCord and Fridovich, 1969). This involves metabolism of superoxide anion (peroxyl radical) to produce hydrogen peroxide and oxygen. The Cu/Zn superoxide dismutase has been of interest scientifically due to the cytosolic location, dual metal requirement and role in disease. Two atoms of Cu and two atoms of Zn are present per molecule of protein and all four atoms are essential for superoxide dismutase activity (Mann and Keilin, 1938). Both metals are relatively tightly bound,

but Cu appears to regulate the amount of superoxide dismutase activity (Paynter et al., 1979). The superoxide dismutase enzyme is the first line of defense against the superoxide radical (peroxyl anion). While other antioxidants exist, the Cu/Zn SOD enzyme is crucial for the protection of cells by shielding intracellular components from oxidative damage.

Paynter et al. (1979) observed that a Cu deficiency causes a reduction in superoxide dismutase activity in the liver, kidney and red blood cells of a rat. The liver superoxide dismutase activity was most sensitive to changes in dietary Cu. Therefore, Cu availability affects superoxide dismutase synthesis and turnover, but not when Cu is in excess. Pigs fed Cu deficient diets had decreased superoxide dismutase in the liver and red blood cells. The investigators concluded that superoxide dismutase activity can be modulated by dietary Cu (Williams et al., 1975). However, a reduction in superoxide dismutase activity due to a Zn deficiency or Zn toxicity in the growing pig has not been reported. Bettger et al. (1979) reported that Zn supplementation (100 ppm) in chicks does not affect the superoxide dismutase activity in the red blood cells. They concluded that superoxide dismutase activity is dependent on Cu status, but not Zn status.

Intestinal Morphology

During the early postnatal period, the newborn piglet's small intestinal tissue develops at a greater rate than other body tissues (Widdowson, 1984). Smith and Jarvis (1978) reported that the absorptive area of the small intestine doubles within

10 d postnatally in the baby pig. The more luminal surface area in the small intestine (longer villi and deeper crypts), the greater the number of mature enterocytes allowing for maximum digestive and absorptive capability. Therefore, any adverse conditions that influence the intestinal morphology could impact growth performance.

When pigs are weaned, the initial period after weaning has been called post-weaning lag because of the reduced feed intake and body weight gain associated with stress. These stress factors include loss of maternal contact, introduction into unfamiliar surroundings (pen and pigs) and a dramatic change of diet. Signs of post-weaning lag stress include reduced feed intake, diarrhea, villus atrophy with lower digestive and absorptive capacity and ultimately a reduction in growth performance. Powell (1987) stated that because small intestinal enterocytes absorb water together with nutrients, villus height and crypt depth may influence the homeostasis of fluids and cause diarrhea. After weaning, the net absorption of fluid and electrolytes in the small intestine of pigs is decreased, which could cause diarrhea (Nabuurs et al., 1994). Cera et al. (1988) observed that reduced villus length is linked to post-weaning lag and a reduction in feed intake when pigs are weaned from the sow to a starter diet.

Hampson (1986) reported that weaned pigs experience some alterations in intestinal morphology. The changes in intestinal morphology created by weaning are commonly reported as increases in the ratio of crypt depth to villus height, thus resulting in post-weaning diarrhea in newly weaned pigs (Nabuurs et al., 1993). Li

et al. (1991) found that pigs fed soybean meal diet had shorter villi and deeper crypts than pigs fed a milk based diet after weaning. With increasing villus length, growth performance was improved 14 d post-weaning. Therefore, Li et al. (1991) concluded that a reduction in villus length could decrease intestinal absorptive capacity and could result in less digestive enzymes and (or) a decrease in the absorption of nutrients at the villus surface. Hampson (1986) concluded that changes in absorption capacity by increasing depth and number of crypts, which is associated with an increase in enterocyte proliferation. The appearance of less mature enterocyte population explains the increased susceptibility of the pig to diarrhea and depressed growth in the post-weaning period (Hampson, 1986).

Many nutritional changes and regimens have been investigated to lessen post-weaning lag and diarrhea. Initial reports from Europe (Poulsen, 1989), showed that pharmacological concentrations of Zn fed to newly weaned pigs reduced the incidence of diarrhea. Zarling et al. (1985) reported that Zn deficiency had no effect on intestinal villus length or crypt depth. Radecki et al. (1992) found that dietary supplementations of Cu had no effect on intestinal villus length or crypt depth. However, Shurson et al. (1990) reported that traditionally-weaned pigs (> 21 d) fed 250 ppm Cu had an increase in villus height and crypt depth. Therefore, changes in intestinal morphology could be affected by Cu and (or) Zn supplementation by accelerating intestinal cell turnover, but alterations after Zn supplementation have not been reported.

Environmental Implications

In the swine industry, it is a common nutritional practice to formulate swine diets with excessive concentrations of trace minerals. Many swine nursery diets can contain more than twenty or fifty times the NRC recommendation (1988) for Zn and Cu concentrations. Swine producers are becoming increasingly aware of the environmental concerns of high concentrations of minerals added to swine diets. Environmentalists have blamed high concentrations of minerals in animal diets and manure for polluting water and poisoning wildlife. Thus, elevated concentrations of Zn and (or) Cu in animal diets are at risk of being a contaminate to land and water creating potential EPA regulations in the future.

The problem associated with feeding pharmacological concentrations of trace minerals involves the method of incorporation of animal manure nutrients back into the environment as fertilizer on crops and forages. Zn accumulation in the soil has been implicated in the reduction of plant growth (Chaney, 1993). The reduction in plant growth is thought to occur by the alteration in soil pH. Application of manure from nursery pigs fed pharmacological concentrations of Zn to soil over an extended period of time (10 years or more) can result in higher soil Zn concentrations, especially in livestock populated regions. The excess application of high mineral concentrated swine waste causes the soil pH to drop, resulting in an increase in Zn solubitity allowing the plant to take up the Zn in the soil. The excess Zn in the plant results in the reduction of crop yields.

Chapter 2:

Early- and Traditionally Weaned Nursery Pigs Benefit from Phase-Feeding Pharmacological Concentrations of Zinc Oxide : Effect on Metallothionein and Mineral Concentrations

ABSTRACT

Benefits of feeding pharmacological concentrations of zinc (Zn) provided by Zn oxide (ZnO) to 21 d conventionally-weaned pigs in the nursery have been documented; however, several management questions remain. We conducted two experiments to evaluate the effect on growth of feeding 3,000 ppm Zn as ZnO during different weeks of the nursery period. In Experiment 1 (n = 138, d 11.5, 3.8 kg BW) and Exp. 2 (n = 246, d 24.5, 7.2 kg BW), pigs were fed diets containing either 150 ppm Zn (adequate) or 3,000 ppm Zn (high) supplemented as ZnO to the basic ingredients and premix. Pigs were fed a two (Exp. 1) or four (Exp. 2) dietary phase regimen to better meet the physiological needs of the pig for a 28-d nursery period. Dietary Zn regimens were (1) adequate Zn fed wk 1 to 4, (2) high Zn fed wk 1, (3) high Zn fed wk 2, (4) high Zn fed wk 1 and 2, (5) high Zn fed wk 2 and 3, and (6) high Zn fed wk 1 to 4. In both Exp. 1 (P = .04) and Exp. 2 (P = .001), pigs fed high Zn for wk 1 and 2 or the entire 28 d nursery period had the greatest ADG. During any week, pigs fed high Zn had greater concentrations of metallothionein (MT) and Zn in plasma, liver, and kidney than those pigs fed adequate Zn (P < .05). In summary, pharmacological concentrations of Zn provided as ZnO should be fed for a minimum of two weeks immediately after weaning to enhance ADG.

Keywords: Nursery Pig, Zinc, Growth, Metallothionein

INTRODUCTION

Previous research has shown that traditionally weaned nursery pigs have an increase in growth performance when fed pharmacological concentrations of Zn as ZnO; (Hahn and Baker, 1993; Hill et al., 1996; Smith et al., 1997). Limited research is available examining the effects of pharmacological Zn concentrations fed to early weaned pigs, as well as the duration which high dietary Zn needs to be fed. A problem of feeding pharmacological concentrations of Zn is that manure application on soil causes environmental concerns. For example, Zn accumulation in the soil has been implicated in reduced plant growth (Chaney, 1993). A mode of action for the enhanced growth observed when pigs are fed high concentrations of Zn should be established to justify the use of pharmacological concentrations of Zn in nursery pigs to producers and environmentalists. Other possible contributors to the mechanism of increased growth including the protein metallothionein (MT), which is involved in maintaining Zn homeostasis (Richards and Cousins, 1975) and physiological changes in the gastrointestinal tract, which may enhance nutrient absorption by altering intestinal morphology (Hampson,1986).

The objectives of these experiments were 1) to determine whether the early-weaned nursery pig would respond with increased growth performance when fed 3,000 ppm Zn as ZnO, 2) to determine in which period post-weaning high concentrations of Zn need to be fed to enhance growth and 3) to determine a possible mode of action for increased growth performance with the feeding of pharmacological Zn as ZnO.

MATERIALS AND METHODS

Animal Use and Care: The experimental protocol used in this study was approved by the All-University Committee on Animal Use and Care at Michigan State University (AUF number: 03/94-059-03).

Animals and Diets: Two experiments were conducted with (Yorkshire X Landrace) X Hampshire cross bred pigs at the Michigan State University Swine Teaching and Research Farm. All pigs (Exp. 1 and Exp. 2) were allotted to dietary treatment by initial weight and equalized for ancestry and sex in a randomized complete block design. Six dietary Zn regimens were utilized with two dietary Zn concentrations as follows: 1) adequate Zn (150 ppm Zn) the entire 28-d nursery period; 2) high Zn (3,000 ppm Zn as ZnO) only during wk 1; 3) high Zn only during wk 2; 4) high Zn during wk 1 and 2; 5) high Zn during wk 2 and 3; and 6) high Zn for the entire nursery period. Zinc treatments were obtained by substituting ZnO(feed grade, 78% Zn) for corn. All diets were formulated to meet or exceed estimated nutrient recommendations for the 10 to 20 kg pig (NRC, 1988). Diet complexity and phases were different for the two experiments because of the average initial weaning weights. Diet compositions and chemical analysis are shown in Tables 1 and 2.

In Exp. 1, 138 pigs with an average initial weight of 3.8 kg (11.5 d of age) were housed in an isolated on-site nursery with 13 pigs per pen (1.75 x 1.22 m) in one replication and 10 pigs per pen in another replication of each treatment. The replication with 13 pigs per pen had one pig removed each week of the 28-d study

to obtain organ samples. The same nursery rooms were used with two weaning groups. Dietary phases were changed weekly (Table 1) during the 28-d nursery period. The segregated early-weaned (SEW) and transition (Trans) diets were pelleted. At the time of weaning, the early-weaned pigs were administered .5 cc of LA-200 (Liquamycin; Pfizer Animal Heath, Inc.) im, and fed six times per day in a pan on the floor during the first week to stimulate feed intake.

In Exp. 2, 246 pigs with an average initial weight of 7.2 kg (24.5 d of age) were used in four replications and housed in 2.44 x 1.22 m pens with 12 pigs per pen in one replication (one pig per pen per trt was killed each wk), 9 pigs per pen in another replication and 10 pigs per pen in the final two replications. The diets used were phase 3 fed during wk 1 and phase 4 fed during wk 2 to 4.

Performance, Blood and Tissue Collection: Pig weights and feed disappearance were determined weekly for the duration of the experiments. These data were used to calculate average daily gain (ADG), feed intake, and feed efficiency (Gain to Feed). Blood was collected by venapuncture from the anterior vena cava each week into 10 mL heparinized (143 units of sodium heparin/tube) vacutainer tubes with 20 gauge, 1 inch needles. Blood was immediately centrifuged at 4^{0}C, 2,000 x *g*, for 10 min (Beckman GS-6KR, Palo Alto, CA). Plasma was collected into polypropylene tubes and stored at -80^{0}C until mineral analysis could be performed. Pigs were killed using a lethal injection of pentobarbital (80 mg/kg, iv) to obtain liver, kidney, and intestine tissue from one pig per dietary regimen per week. Tissue samples were weighed and stored in Whirl-Pak bags (Nasco, Fort

Atkinson,WI) at -80°C until analysis could be performed.

Superoxide Dismutase Analysis: Red blood cell Cu/Zn superoxide dismutase (SOD; EC 1.15.1.1) activity was determined (Hill et al., 1997) by a modification of the method of Marklund and Marklund (1974). Red blood cells obtained from centrifugation were washed three times in two volumes of ice-cold .9% saline. Between each wash, cells were centrifuged (as above) and saline removed by aspiration. After the third and final wash, cells were hemolyzed in an equal volume of ice-cold deionized distilled water and frozen at -80°C until analysis. Liver tissue (approximately 1 g), for SOD, was homogenized with a tissumizer probe (S25N-10G, 10mm diameter) in 10 X volumes of ice-cold potassium phosphate buffer (pH 7.2, .05 *M* phosphate, .24 *M* sucrose) using an Ultra Turrax T25 homogenizer (Tekmar-Dohrmann Corp., Cincinnati, OH). Red blood cell hemolysates or tissue homogenates were extracted with .6 volumes of ice-cold ethanol-chloroform (25:15) to inactivate the manganese dependent SOD. After ethanol-chloroform addition, samples were gently mixed and centrifuged (4°C, 5,000 x *g*,15 min). Aliquots of clear supernatant from red blood cells and tissue were diluted 1:10 or 1:25, respectively, with 50 m*M* Tris-HCL, 1.0 m*M* diethylenetriamine pentaacetic acid (DTPA) buffer. The reaction mixture in five cuvettes of the DU 7400 spectrophotometer (Beckman, Palo Alto, CA) contained Tris-HCL-DTPA buffer and varying amounts of supernatant to equal 900 µL, plus 50 µL of 10 m*M* sodium azide. Cuvettes were preincubated at 25°C for 5 min, and finally 50 µL of 4 m*M* pyrogallol was added to initiate the dismutation of the peroxyl radical (final volume,

1.0 mL). Protein concentrations of the supernatant were determined by the method of Lowry et al. (1951).

Mineral Analysis: Tissue samples (approximately 1 g) were sliced from the same area within a frozen tissue for determination of organ mineral concentrations. Tissue samples were wet-ashed (Hill et al., 1983) in a mixture of 10 M perchloric acid (3 mL) and 14 M nitric acid (20 mL). Digested samples were diluted with deionized distilled water as necessary to determine copper (Cu), Zn and iron (Fe) concentrations by flame atomic absorption spectrophotometry (Smith-Heiftje 4000, Thermo Jarrell Ash Corporation, Franklin, MA). Dry matter of liver and kidney were determined by drying approximately 12 hrs in a vacuum oven (Heinicke; Portland, OR).

Plasma was diluted 1:7 with deionized distilled water for analysis of Zn and Cu by flame atomic absorption spectrophotometry. Plasma Fe was determined by a method (Olson and Hamlin, 1969) utilizing .5 mL plasma deproteinized with 1 mL of 20% trichloroacetic acid (TCA), incubated at 90^{0}C for 15 min and centrifuged (2,000 x *g*, 10 min). The supernatant was subsequently read on a flame atomic absorption spectrophotometer.

All mineral analysis were determined using glassware that had been washed in 30% nitric acid and rinsed with deionized distilled water. Bovine liver standard (1577b; NIST: National Institute of Standards and Technology, Gaithersburg, MD) was used to establish accuracy of instrument analysis. Variation was accepted within the specified limits of NIST. Zinc, Cu and Fe concentrations were calculated

using an exogenous calibration curve.

Intestinal Mucosa Analysis: The small intestine was ligated at the ligament of Treitz to remove a 15 cm section of the duodenum. The intestinal sample was weighed and rinsed three times in ice-cold .9% saline. The section of duodenum was cut longitudinally to expose mucosa and rinsed again with cold saline. The intestinal mucosa was scraped using a glass microscope slide. Scrapings were weighed and homogenized (Tissumizer Ultra Turrax T25) for 1 min at 4^0C in 1:4 (wt : vol) ice-cold buffer (Tris-HCL, 10 mmol/L; NaCl, 154 mmol/L; NaN_3, .2 g/L; phenylmethylsulfonyl fluoride, .2 mmol/L; leupeptin, .6 mg/L; pepstatin A, .9 mg/L; pH 8.6). Homogenates were centrifuged (4^0C, 9,000 x *g*, 20 min) in an induction drive centrifuge (Beckman J2-21M; Palo Alto, CA) to remove cell debris and nuclei. Supernatant was collected and recentrifuged for 60 min (4^0C, 105,000 x *g*) in an ultracentrifuge (Beckman L8-80M; Palo Alto, CA) using a SW50.1 rotor and 5 mL thin wall polyallomer tubes. Again, supernatant was collected and frozen at -80^0C until analysis could be performed.

Proteins in the intestinal homogenate supernatant were separated by gravity-flow gel filtration chromatography, to determine the approximate molecular weight and size. Supernatant (2.5 mL) from intestinal mucosa scrapings was filtered through both a .8 µm (Corning Glass Works; Corning, NY) and a .45 µm (Millipore; Bedford, MA) syringe filter, then applied to a 1.6 x 100 cm Sephadex G75 column (separation in MW range of 10,000 - 60,000 kD) followed by 2 mL of .1 *M* phosphate buffer. Fractions (5mL) were collected using a Retriever 500 Fraction

Collector (ISCO Inc., Lincoln, NE). Protein peaks were determined by reading absorbance (280 nm) of individually collected fractions (DU 7400 Spectrophotometer; Beckman, Palo Alto, CA). The molecular size of protein peaks was determined after column calibration with the following protein standards (Sigma Chemical, St Louis,MO) : cytochrome c (11,700 kD), ribonuclease A (13,700 kD), chymotrypsinogen A (25,000 kD), ovalbumin (43,000 kD), and albumin (67,000 kD). Fractions were also analyzed on a flame atomic absorption spectrophotometer to determine Zn and Cu concentrations at protein peaks.

Metallothionein Assay: Approximately 1 gram of tissue (liver, kidney or intestine) from each pig was homogenized in 4 mL of .5 mol/L glycine buffer (pH 8.3) and heated for 2 min at 100^{0}C. The homogenized and heated samples were centrifuged at 25,000 *g* for 2 min, and the supernatant solutions were used for measurement of MT. The MT concentration was determined using a non-radioactive silver binding assay (Lee et al., 1989). The amount of silver in the final supernatant fraction was assumed to be proportional to the amount of metallothionein present. The concentration of silver was measured by a flame atomic absorption spectrophotometry.

Statistical Analysis: Data in both experiments were analyzed as a randomized complete block design using the general linear model procedure of Statistical Analysis System (SAS,1985). Pigs were blocked by initial weight and equalized for ancestry and sex. Pen was the experimental unit for analysis of performance data (growth and feed efficiency). For blood and tissue analysis, individual pig was the

experimental unit. The mean differences between the treatments were detected by comparison of the least square means. Differences were considered significant at the level of $P < 0.05$.

RESULTS

Growth Performance: In Exp. 1, early-weaned pigs fed pharmacological concentrations of Zn during either wk 1 and 2 or the entire 4-wk nursery period had the greatest ADG for the entire 28-d compared ($P = .04$) to pigs fed adequate Zn the entire period, or pharmacological ZnO during a single week (Table 3). Those pigs fed high concentrations of Zn during wk 2 and 3 had the lowest ADG indicating that the early-weaned pig responds most favorably to pharmacological concentrations of Zn during wk 1 and 2 post-weaning. Traditionally weaned pigs, in Exp. 2, had the greatest overall ADG when fed 3,000 ppm Zn during wk 1 and 2 or the entire 28-d nursery period ($P = .001$) while pigs fed adequate Zn during the study had the poorest overall growth performance (395 and 420 vs 340 g/d). Other dietary regimens were intermediate in growth performance. Feed intake was similar for all dietary Zn treatments and regimens within experiments ($P > .05$). However, as expected, traditionally weaned pigs ate more than early-weaned pigs (Table 3). Feed efficiency did not differ among dietary Zn treatments or regimens ($P > .05$).

Blood Characteristics: Results from Exp. 1 and Exp. 2 indicate that pharmacological concentrations of Zn increase plasma Zn concentration ($P =$

.0001), and plasma Zn concentrations are reflective of duration of dietary Zn regimen (Table 4).

At the end of Exp. 1, the early-weaned pigs fed 3,000 ppm Zn the entire nursery period had the greatest plasma Zn concentrations (P = .0001) compared to pigs fed adequate Zn or pharmacological concentrations of ZnO during any week combination of the study (Table 4). Pigs fed 3000 ppm Zn the entire 28-d period had a peak plasma Zn concentration at wk 3 of the study. Plasma Cu concentrations were lower when pigs were fed 3000 ppm Zn the entire nursery period (P = .0001) compared with the other dietary regimens (Table 4). At the end of the study, pigs fed high Zn either the entire 28-d nursery period or during wk 2 and 3 tended (P = .06) to have higher plasma Fe concentrations compared to pigs fed adequate Zn or pharmacological Zn during wk 1, wk 2 or wk 1 and 2 (2.43 and 2.28 µg/mL vs .87, .82, .95, and 1.06 µg/mL, respectively).

At the termination of Exp. 2, the traditionally weaned pigs fed 3,000 ppm Zn the entire 28-d nursery period had the greatest plasma Zn concentration (P = .0001; Table 4). Similar to the early weaned pigs, these pigs had a peak plasma Zn concentration at wk 3 of the study. Plasma Cu concentrations averaged 1.05 µg/mL and did not differ between dietary Zn treatments and regimens (P = .61). At the end of wk 3 of the study, plasma Fe concentrations were greatest when pigs were fed high concentrations of Zn during wk 2 and 3 or the entire nursery period (P = .01) compared to pigs fed adequate Zn the entire 28-d study, or fed 3,000 ppm Zn during wk 1 only, wk 2 only and wk 1 and 2 (.66 or .68 µg/mL vs .59, .61, .60,

and .60 μg/mL, respectively). Plasma Fe concentrations did not differ between dietary Zn regimens at any other week (data not shown, $P > .05$).

Red blood cell Cu/Zn SOD activity was not affected ($P = .87$) by dietary Zn treatments and regimens in the traditionally weaned nursery pig (data not shown). At the end of the study, early weaned pigs fed either 3,000 ppm Zn the entire 28-d period or during wk 2 and 3 had less SOD activity ($P = .005$) than pigs fed adequate Zn, and pharmacological concentrations of Zn during wk 1, wk 2 or wk 1 and 2 (125 and 130 U/mg protein vs 141, 143, 140 and 137 U/mg protein, respectively).

Liver and Kidney Characteristics: In the early weaned pig, liver Cu and Fe concentrations (Table 5) were not affected by dietary Zn treatments and regimens ($P = .28$ and .96, respectively). Liver Zn concentrations reflected dietary Zn regimen. Similar to plasma Zn concentrations, pigs fed 3,000 ppm Zn the entire 28-d study had a peak liver Zn concentration during wk 3 (baseline = 92.5, wk 1 = 192.4, wk 2 = 180.5, wk 3 = 280 and wk 4 = 194.3 μg/g). Kidney Fe concentrations were not different between dietary Zn regimens ($P = .95$). However, kidney Cu and Zn concentrations were greater when pigs were fed 3000 ppm Zn the entire nursery period ($P = .05$). Liver and kidney weights (data not shown) relative to BW did not differ among dietary Zn treatments or regimens.

In Exp. 2, the pattern of liver Zn and Cu concentrations (Table 4) were similar to results observed in Exp. 1. Liver concentrations of Cu and Fe (Table 5) did not differ between dietary Zn treatments and regimens ($P = .37$ and .28, respectively). Kidney concentrations of Cu and Fe did not differ between dietary

regimens (P = .24 and .94, respectively). However, liver Zn concentrations reflected dietary Zn treatments and regimens (P = .02) as pigs fed 3,000 ppm Zn the entire 28-d period had the greatest concentration of Zn in the liver compared to pigs fed any other high Zn regimen or adequate Zn the entire 28-d period. Pigs fed pharmacological concentrations of Zn for wk 1 and 2 had greater concentrations of Zn in the liver than those pigs fed high Zn during wk 1 or wk 2. Kidney Zn concentrations followed a similar pattern. Pigs fed high Zn the entire 28-d period had the greatest Zn concentration in the kidney (P = .002) compared pigs fed any other high Zn regimen or adequate Zn the entire 28-d period (Table 5). The weights of liver and kidney relative to BW did not differ (P = .67 and .89, respectively) between dietary Zn treatments (2.9%, SEM .39 and .49%, SEM .04, respectively). In both experiments, liver and kidney dry matters did not differ (P = .38 and .49, respectively) between dietary Zn treatments (24.8%, SEM .27 and 18.5%, SEM .38 respectively).

Liver SOD activity (data not shown) did not differ (P = .23) in the traditionally-weaned pig (Exp. 2). However, the early-weaned pigs fed 3,000 ppm Zn the entire nursery period had lower liver SOD activity (P = .02) than any other treatment (43.8 U/mg protein vs 50.2, 52.9, 47.6, 59.8 and 48.8 U/mg protein for treatments wk 0,1,2, 1-2 and 2-3, respectively).

Intestinal Protein and Metallothionein: In both Exp. 1 and 2, pigs fed 3,000 ppm Zn as ZnO had greater Zn concentrations in the protein peak at 12,000 kD, which has been established as MT (Richards and Cousins, 1975). Copper

concentrations in MT protein fraction were not affected by dietary Zn treatments (data not shown). The intestinal mucosa cells from pigs fed pharmacological concentrations of Zn as ZnO the entire 28-d nursery period had the greatest concentration of MT (Table 6). Liver and kidney MT concentrations were reflective of duration 3,000 ppm Zn was fed in both experiments.

In Exp. 1, early weaned pigs fed 3,000 ppm Zn for wk 1 and 2 had greater concentrations of MT in the liver than pigs fed adequate Zn or high Zn during wk 1 or wk 2. Pigs fed high Zn during wk 1 and 2 had greater concentrations of MT than pigs fed 3,000 ppm Zn during wk 2 and 3. Liver MT concentrations were greater in Exp. 1 than in Exp. 2 indicating differences could be due to age and perhaps weaning stress. In Exp. 1, kidney MT concentrations were greater in pigs fed 3,000 ppm Zn wk 2 and 3 or wk 1 to 4 than the other dietary regimens which were similar. Renal MT concentration, in Exp. 2, was similar between pigs fed high Zn during wk 1 and 2 or wk 2 and 3, but was less than that of pigs fed high Zn the entire 28-d study. Pigs fed high Zn diets had greater intestinal mucosa MT concentrations in both experiments than those fed adequate Zn for 4-wks. Patterns for intestinal mucosa MT concentrations were similar between experiments. In Exp. 1 and Exp. 2, pigs fed adequate Zn diets the 28-d nursery period had the lowest MT concentrations in liver, kidney or intestinal mucosa.

DISCUSSION

Enhanced growth with pharmacological concentrations of Zn has been shown previously (Hahn and Baker, 1993; Hill et al., 1996; Smith et al., 1997). Here we showed for the first time that feeding 3,000 ppm Zn during only the first 2 wk post-weaning in the early- or traditionally weaned pig produces an ADG equivalent to that achieved with pharmacological concentrations of ZnO fed for the entire 28-d nursery period. These studies show that pharmacological Zn supplementation does not enhance growth if fed only during wk 1. It is possible that the newly weaned pig does not consume enough feed during the first week post-weaning to maximize MT production. However, the low daily feed intake during wk 1 might also contribute to the need for greater concentrations of Zn in the phase 1 nursery diet. Zinc supplementation during wk 2 to 3 alone does not enhance growth performance in the nursery pig probably due to the need for early preloading of Zn and stimulation of MT production to enhance Zn absorption in the small intestine. Starcher et al. (1980) reported that an increase in Zn absorption was observed with an induction of intestinal mucosa MT which supports our findings.

In Exp. 2, there was a trend ($P = .08$) for the traditionally-weaned pigs fed pharmacological concentrations of Zn to have greater overall daily feed intake. However, the enhanced growth response is not solely due to increased voluntary feed intake. Other researchers (Poulsen, 1995; Carlson et al., 1995; Smith et al., 1997) have reported a range of 10 to 26% increase in growth performance when nursery pigs are fed pharmacological (2,500 to 4,000 ppm) concentrations of Zn

with either no or small improvements in feed intake. These results do not support the findings of Hahn and Baker (1993), who observed an increase in feed intake by 14% when pigs were supplemented with 3000 ppm Zn as ZnO. Recently researchers from 10 universities in the United States cooperated in a study to investigate the impact of pharmacological dietary Zn and (or) Cu on performance of weanling pigs (n=1156) with diverse health status and housing conditions. Improved daily growth (15%), and feed intake (11%) were found with 3000 ppm Zn in the diet (Hill et al., 1996).

Plasma Zn concentrations were reflective of dietary Zn regimen fed in this study. Hahn and Baker (1993) reported a positive relationship between plasma Zn and ADG when plasma Zn concentrations were below 2.5 mg/L. In our study, plasma Zn concentrations were below 2.5 mg/L at all times, and ADG was enhanced which supports Hahn and Baker (1993) and Poulsen's (1989, 1996) conclusions that plasma Zn concentrations need to be enhanced (around 2.5 mg/L) to observe better growth performance. Pigs fed diets with adequate Zn, through the entire nursery period, had an average plasma Zn concentration of .63 mg/L in the early-weaned study and .48 mg/L in the traditionally-weaned study. However, by wk 4 in the early-weaned study, pigs had plasma Zn concentrations similar to the traditionally-weaned pigs at weaning. The traditionally-weaned (24.5 d) pigs fed adequate Zn in this study had slightly lower plasma Zn concentrations than pigs weaned at 21-d (.50 vs .61 mg/L) reported by Hill et al. (1983), but both were in the normal range of .5 to 1.5 mg/L (Kaneko, 1989). Plasma Zn concentrations

appeared to reach a saturation point after pharmacological concentrations of ZnO were fed for 2 wks. This is probably tied to the role MT has in Zn homeostasis which is known to be controlled by intestinal mucosa cells regulating the amount of Zn transferred to plasma (Richards and Cousins, 1975).

Plasma Cu concentration was unaffected in the traditionally-weaned pigs, however, the early-weaned pig seems to be more affected when pharmacological concentrations of Zn are fed. This could be due to reduction in pre-nursery Cu status because the pigs were allowed fewer suckling days prior to weaning. Milne and Matrone (1970) observed that newborn pigs have high concentrations of liver Cu and low ceruloplasmin activity, however, ceruloplasmin activity rises to adult concentrations in about 2 wks. This could also explain why plasma Cu concentrations were only altered in the early-weaned pigs (Exp. 1). Therefore, it appears that Cu status is altered by Zn supplementation as well as age.

Red blood cell SOD activity did not differ until d 28 of Exp. 1, when early-weaned pigs fed 3000 ppm Zn the entire period had the lowest SOD activity in liver and RBC, an observation which has not previously been reported. The reduction in SOD activity prior to a decrease in liver Cu may give a better understanding of Cu bioavailability. At the same time, renal Cu concentrations increased indicating that Cu may be shuttled away from hepatic Cu stores. Copper and Zn are known components of SOD (Mann and Keilin, 1938) and Cu appears to regulate the enzyme's activity (Paynter et al., 1979). Superoxide dismutase activity can be modulated by diet as Williams et al. (1975) observed. When pigs were fed a

copper deficient diet, SOD activity was decreased in liver and red blood cells. In our study, early-weaned pigs showed a decrease in plasma Cu concentration as soon as wk 2, however, pigs fed 3,000 ppm Zn the entire nursery period had a dramatic reduction in plasma Cu concentration by d 28. At that time (d 28), RBC SOD activity was also reduced. Early-weaned pigs had a slightly reduced hepatic Cu storage and a greater concentration of Cu in the kidney which supports the reduction in SOD activity in the liver due to removal of Cu from the liver via renal function. The amount of time required to observe a reduction in SOD activity may be related to red cell turnover rate and therefore affected by RBC half life (62 d).

Liver and kidney Zn concentrations were reflective of the duration that high Zn was fed. In those pigs fed high Zn the entire 28-d, kidney Zn concentration plateaued around d 14 of the study. This represents a potential renal homeostatic role. Previous work (Poulsen, 1995) indicates that the kidneys and (or) gastrointestinal tract are involved in homeostatic regulation as observed in these experiments . However, intestinal MT concentration and the amount of Zn associated with MT did not plateau when pigs were fed 3000 ppm ZnO the entire 28 d nursery period.

Within the intestinal mucosal cell, dietary Zn absorption is regulated by MT, which competes with intestinal ligands involved in Zn absorption and controls the amount of Zn that is transferred across the basolateral membrane into circulation (Richards and Cousins, 1975). Metallothionein regulates the quantity of Zn entering the body by binding dietary Zn in the intestine. Excess Zn-MT complex

is not absorbed, and is later sloughed off in the epithelial cells (Cousins, 1985). Zinc transported into the plasma is deposited in the liver, where MT functions in its release and storage. Tissue concentrations of MT are stimulated by the amount of Zn being fed (Blalock et al., 1988). Changes in dietary Zn concentration influence MT mRNA expression in the intestine and liver (Lee et al., 1989; Cousins and Lee-Ambrose, 1992). In our study, pigs fed 3000 ppm Zn for 4-wks had increased hepatic MT concentration approximately 10 times and 4 times that of pigs fed adequate Zn for 4-wks or high Zn for 1 and 2 wks, respectively. Similar results were observed in the intestinal mucosa. In this study, the concentration of Zn in liver, kidney and intestine mirrored MT concentration in the same tissues, therefore, Zn concentration increased, MT concentration increased. This is important relative to MT's role in maintaining Zn homeostasis. As dietary Zn concentration increases, the amount of Zn bound to intestinal MT increases. The induction of MT in the intestine could be a possible mode of action for enhanced growth performance observed in nursery pigs fed pharmacological concentrations of Zn.

Intestinal MT can be found within 4 h after Zn administration and reaches a maximum by 20 h in rats (Starcher et al., 1980; Menard et al., 1981). The increase in intestinal MT provides a temporary storage of Zn which is eventually sloughed and prevents the animal from being overwhelmed until the Zn load is reduced.

Metallothionein gene expression is elevated in tissues during fetal development (Mercer and Grimes, 1986) suggesting a role in growth and development. Zinc has a role in RNA-DNA cell proliferation which suggests that an

increase in Zn held in the intestinal cell via MT may aid in improving gut health for increased protein synthesis and cell proliferation.

In conclusion, these experiments support the hypothesis that early-weaned pigs (< 14 d) have an improvement in growth performance when fed 3,000 ppm Zn as ZnO in the nursery period. Both early-weaned and traditionally weaned pigs must be fed 3,000 ppm Zn for at least the first 2 wks post-weaning to enhance growth. Pigs fed pharmacological concentrations of Zn have elevated plasma and tissue Zn concentrations resulting in an increase in MT concentration in liver, kidney and intestinal mucosa. Finally, dietary Zn stimulates MT production in liver, kidney, and intestinal mucosa and alters Cu status, perhaps by inducing MT which Zn induced MT binds Cu in the intestinal mucosa.

IMPLICATION

Both traditionally-weaned (> 21 d) and early-weaned (< 14 d) pigs benefit with greater weight gain when fed pharmacological (3,000 ppm) concentrations of zinc as zinc oxide during at least the first two weeks post-weaning. Pharmacological concentrations of Zn stimulate metallothionein synthesis in the mucosal cell which regulates zinc uptake into the body and perhaps improves cell proliferation in the intestine which may be related to the improved growth observed in the weanling pig.

Table 1: Percentage composition of experimental diets[a] (as-fed basis)

Item	SEW[b]	Trans[b]	Phase 3[c]	Phase 4[c]
Corn, dent yellow	34.65	44.65	45.6	43.9
Whey, dried	25	20	20	20
Skim milk, dried	10	2.5	10	-
Fish meal, menhaden	10	10	-	-
Spray-dried animal plasma	7.5	5	7.5	-
Soybean meal, dehull	5	10	5.08	29.4
Choice white grease	5	5	-	-
Oat groats	-	-	5	-
Soybean oil	-	-	3	3
Dical Phosphate, 21%	.6	.6	1.2	1.1
Limestone	.45	.45	.87	.85
Vitamin Premix[d]	.6	.6	.6	.6
Mineral Premix[e]	.23	.23	.6	.6
Salt, NaCl	.5	.5	.3	.3
Mecadox-10[f]	.25	.25	.25	.25
DL-Methionine	.15	.15	-	-
L-lysine HCL, 78.8%	.07	.07	-	-
Zinc Oxide[g]	0 or .41	0 or .41	0 or .41	0 or .41

[a]Formulated to contained .8% Ca, and .65% P.

[b]Diets were pelleted and fed in EXP 1 only; Contained 23%CP and 1.7% lysine in SEW diet (fed wk 1) and 21% CP and 1.45% lysine in Trans diet (fed wk 2).

[c]Phase 3 (fed wk 3 in Exp. 1 and wk 1 in Exp. 2) and Phase 4 (fed wk 4 in Exp. 1 and wk 2 to 4 in Exp. 2) diets contained 18% CP and 1.25% lysine.

[d]Provided per kg of complete diet: 2500 IU vitamin A, 250 IU vitamin D_3, 30 IU vitamin E, 2 mg riboflavin, 12 mg niacin, 15 µg vitamin B_{12}, 2 mg vitamin K_3, 0.5 mg thiamin, 0.45 mg pyridoxine and 8 mg pantothenic acid.

[e]Provided per kg of complete diet: 100 mg Zn, 100 mg Fe, 10 mg Cu, 10 mg Mn, 150 µg I and 300 µg Se.

[f]Antibacterial agent, 2.2% carbadox (Animal Health Co., NY).

[g]For the 3,000 ppm Zn diet, corn was removed for the addition of .41 % Zn oxide.

Table 2: Chemical analysis of experimental diets, ppm

Diet and Phase	Zinc	Copper	Iron
Exp 1:			
SEW			
Low Zn	161.9	35.3	322.6
High Zn	2902.2	53.2	469.6
Trans			
Low Zn	58.2	33.4	336.7
High Zn	3283.5	42.7	397.2
Phase 3			
Low Zn	71	21.3	246.7
High Zn	3505.6	40.4	481.2
Phase 4			
Low Zn	61.1	21.2	239.8
High Zn	3113.7	37.3	456.4
Exp. 2:			
Phase 3			
Low Zn	149.6	12.8	197.2
High Zn	2943.6	13.1	245.5
Phase 4			
Low Zn	153.2	10.9	192.4
High Zn	3013.3	12.1	234.1

Table 3. Effect of phase-feeding pharmacological Zn on growth performance, g/d

Variable[f]	Weeks Fed High Zn (3,000 ppm) 0	1	2	1-2	2-3	1-4	SEM	P Value
EXP1: Early-Wean								
Avg. Daily Gain, g/d								
Week 1	128[b]	130[b]	120[bc]	147[a]	98[c]	125[b]	11	.04
Week 2	234[c]	260[b]	273[b]	310[a]	237[c]	315[a]	17	.005
Week 3	354[a]	320[b]	320[b]	350[a]	287[c]	335[ab]	16	.05
Week 4	280[a]	278[a]	250[b]	260[a]	230[c]	295[a]	20	.03
Overall ADG	250[b]	255[b]	245[bc]	270[a]	220[c]	275[a]	13	.04
Avg. Daily Intake, g/d								
Overall ADFI	360	352	354	370	351	367	16	.41
EXP2:Traditional								
Avg. Daily Gain, g/d								
Week 1	155[c]	216[a]	175[b]	235[a]	176[b]	210[a]	16	.005
Week 2	252[e]	285[d]	326[c]	350[b]	351[b]	416[a]	17	.0001
Week 3	436[b]	428[b]	471[a]	453[a]	455[a]	480[a]	20	.05
Week 4	511[c]	510[c]	495[c]	530[b]	497[c]	575[a]	15	.02
Overall ADG	340[c]	350[bc]	360[bc]	395[a]	375[b]	420[a]	12	.001
Avg. Daily Intake, g/d								
Overall ADFI	472	506	484	518	510	548	30	.08

[abcde]Means in the same row lacking a common superscript differ significantly.

[f] Least square means are reported. In Exp. 1, wk 1 n = 138, wk 2 n = 132, wk 3 n = 126 and wk 4 n = 120 in 2 replications per trt. In Exp 2, wk 1 n = 246, wk 2 n = 240, wk 3 n = 234 and wk 4 n = 228 in 4 replications per trt.

Table 4. Effect of phase-feeding pharmacological Zn on plasma mineral concentration, mg/L

	Weeks Fed High Zn (3,000 ppm)							
Variable[d]	0	1	2	1-2	2-3	1-4	SEM	P Value
EXP1: Early-Wean								
Zinc, mg/L								
Baseline	.85	.92	.87	.89	.89	.91	.10	.62
Week 1	.59[c]	.79[b]	.67[c]	.99[a]	.58[c]	.94[a]	.03	.0001
Week 2	.65[c]	.70[c]	1.54[b]	1.74[a]	1.57[b]	1.77[a]	.07	.0001
Week 3	.59[c]	.71[c]	.95[b]	1.06[b]	2.28[a]	2.43[a]	.06	.0001
Week 4	.47[d]	.49[d]	.58[cd]	.73[c]	.83[b]	1.25[a]	.04	.0001
Copper, mg/L								
Baseline	1.85	1.93	1.62	1.78	1.84	1.78	.08	.18
Week 1	.97	.94	.94	.87	.92	.89	.04	.59
Week 2	.98[a]	.98[a]	.79[bc]	.73[c]	.85[bc]	.81[bc]	.04	.0003
Week 3	1.14[a]	1.09[ab]	.98[ab]	.98[ab]	.83[b]	.82[b]	.05	.0001
Week 4	1.13[a]	1.09[ab]	1.04[ab]	1.03[ab]	.96[b]	.73[c]	.05	.0001
EXP2:Traditional								
Zinc, mg/L								
Baseline	.45	.50	.49	.50	.51	.53	.02	.10
Week 1	.48[b]	.78[a]	.47[b]	.77[a]	.47[b]	.73[a]	.03	.0001
Week 2	.48[c]	.55[c]	.80[b]	1.01[a]	.79[b]	1.08[a]	.05	.0001
Week 3	.49[c]	.49[c]	.52[bc]	.56[b]	.86[ab]	1.30[a]	.04	.0001
Week 4	.50[c]	.51[c]	.54[c]	.57[c]	.73[b]	1.08[a]	.03	.0001

[abc]Means in the same row lacking a common superscript differ significantly.

[d] Least square means are reported. In Exp. 1, baseline and wk 1 n = 138, wk 2 n = 132, wk 3 n = 126 and wk 4 n = 120 in 2 replications per trt. In Exp 2, baseline and wk 1 n = 246, wk 2 n = 240, wk 3 n = 234 and wk 4 n = 228 in 4 replications per trt.

Table 5. Effect of phase-feeding pharmacological Zn on organ mineral concentration (wet basis)

	Weeks Fed High Zn (3,000 ppm)							
Variable[d]	0	1	2	1-2	2-3	1-4	SEM	P Value
EXP1: Early-Wean								
Liver, µg/g								
Zinc	46.6[d]	138[c]	149[c]	234[b]	295[b]	194[a]	33	.0002
Copper	59.4	63.2	64.4	53.4	58.2	48.3	12	.28
Iron	357	421	375	358	426	334	108	.96
Kidney, µg/g								
Zinc	17.5[c]	23[c]	25.6[c]	32.1[bc]	35.1[b]	43[a]	2	.05
Copper	11[b]	17[b]	14.3[b]	19.1[b]	15.8[b]	27.8[a]	5	.05
Iron	53.2	51.3	47	47	50.3	49.3	7	.95
EXP2:Traditional								
Liver, µg/g								
Zinc	28.4[d]	77.5[c]	54[c]	103[b]	104[b]	218[a]	35	.02
Copper	71	58	57	59	67	60	6	.37
Iron	158	95	170	154	127	170	25	.28
Kidney, µg/g								
Zinc	16[c]	18.5[bc]	17.4[c]	28.4[ab]	21.6[b]	34.3[a]	1	.002
Copper	10	8.8	9	10.5	11.7	15.7	2	.24
Iron	38	37.8	32.7	38.1	38.4	39.1	4	.94

[abc]Means in the same row lacking a common superscript differ significantly.

[d] Least square means are reported. In Exp. 1 and Exp. 2, n = 4 per trt and organ.

Table 6. Effect of phase-feeding pharmacological Zn on organ metallothionein concentration

	Weeks Fed High Zn (3,000 ppm)							
Variable[f]	0	1	2	1-2	2-3	1-4	SEM	P Value
EXP1: Early-Wean								
Liver MT, µg/g	137[e]	412[d]	468[d]	1786[b]	1397[c]	2988[a]	229	.0001
Kidney MT, µg/g	83.2[b]	149[b]	137[b]	169[b]	442[a]	416[a]	76	.001
Intestinal MT, µg/g	5.9[d]	15.6[c]	16.1[c]	17[c]	43.4[b]	118[a]	9	.0009
EXP2:Traditional								
Liver MT, µg/g	189[e]	585[cd]	338[d]	887[b]	695[c]	2208[a]	167	.0001
Kidney MT, µg/g	25.7[e]	55.5[d]	77.4[c]	94.8[b]	95.6[b]	349[a]	12	.02
Intestinal MT, µg/g	6.5[d]	18.1[c]	20.8[c]	27.7[c]	52.4[b]	137[a]	18	.001

[abcde] Means in the same row lacking a common superscript differ significantly.

[f] Least square means are reported. In Exp. 1 and Exp. 2, n = 4 per trt and organ.

Chapter 3:

Impact of pharmacological concentrations of Cu sulfate and various forms of Zn on growth, mineral status, metallothionein and superoxide dismutase activity in the growing pig

ABSTRACT

Three experiments were conducted to investigate the use of pharmacological concentrations of various forms of zinc (Zn) either with or without copper (Cu) on growth promotion. In Exp. 1, pigs (n = 100, d 24 of age) were fed either Zn carbonate ($ZnCO_2$), Zn acetate [$Zn(C_2H_3O_2)_2$] , Zn oxide (ZnO) or Zn sulfate ($ZnSO_4$) each at 3000 ppm Zn or 150 ppm ZnO. A 2 x 2 factorial design in Exp. 2 and 3 was: 1) 11 ppm Cu, 150 ppm Zn (LCuLZn), 2) 250 ppm Cu, 150 ppm Zn (HCuLZn), 3) 11 ppm Cu, 3000 ppm Zn (LCuHZn), 4) 250 ppm Cu, 3000 ppm Zn (HCuHZn). In Exp. 1, pigs fed 3,000 ppm Zn from ZnO had the greatest growth performance during wk 2 and 3 of the nursery period while pigs fed all other Zn forms at 3,000 ppm performed similar to pigs fed 150 ppm ZnO (P = .003). In contrast, pigs fed high Zn had greater ADG (P = .008) for the entire study than pigs fed low Zn. In Exp. 2, pigs (n=80) fed 3,000 ppm Zn with either 11 ppm or 250 ppm Cu from Cu sulfate ($CuSO_4$) had greater overall ADG (P = .0001) than pigs fed 150 ppm Zn with or without high Cu. In Exp. 3, pigs (n = 40) were fed the four dietary treatments from weaning to finish (154 d). During the 5 wk nursery phase, pigs fed the LCuHZn diet had a greater ADG (P = .0006) than pigs fed the LCuLZn, HCuLZn or HCuHZn diets (527 g/d vs 347, 439 and 477 g/d, respectively). Pigs fed pharmacological minerals past the nursery phase gained similarly (P > .05).

However, pigs fed LCuHZn diets remained heavier through the entire study with overall ADG for LCuLZn = 656 g/d, HCuLZn = 657 g/d, LCuHZn = 718 g/d and HCuHZn = 698 g/d. In Exp. 3, blood was collected on d 0, 35, 70, 112, 154 for determination of plasma Cu and Zn concentrations, ceruloplasmin (Cp) and red blood cell Cu/Zn superoxide dismutase (SOD) activity. At 154 d, dietary treatments had no effect ($P > .05$) on pigs' plasma Cu concentration and Cp activity. Pigs fed 3000 ppm Zn had greater plasma Zn concentrations ($P = .0001$). Pigs fed LCuHZn diets had lower RBC Cu/Zn SOD activity ($P = .0001$). At slaughter (154-d), pigs fed HCuLZn diets had greater liver SOD activity ($P = .01$). Liver Fe concentrations were higher in pigs fed 11 ppm Cu compared to pigs fed 250 ppm Cu diets ($P = .0001$). Pigs fed the 3000 ppm Zn had greater liver Zn and metallothionein (MT) concentrations than pigs fed 150 ppm Zn ($P = .0001$). In conclusion, 3,000 ppm Zn in the form of ZnO improves pig performance during the nursery period with or without 250 ppm Cu. While no overt signs of Cu deficiency were observed when 3,000 ppm Zn was fed through the 154 d period, RBC Cu/Zn SOD activity was depressed.

Key words: Zinc, Copper, Pigs, Metallothionein, Superoxide Dismutase

INTRODUCTION

Swine producers face many challenges in raising hogs to market weight. Weaning causes stress to the pigs, reduces feed intake, increases the incidence of diarrhea and hence decreases growth rate.

Recently, much attention has been focused on the addition of pharmacological concentrations of minerals in swine diets to enhance growth (Hahn and Baker, 1993; Hill et al., 1996; Smith et al., 1997). For years, British researchers have observed that pigs fed 250 ppm Cu stimulated daily gain and feed efficiency when compared to pigs receiving no supplemental Cu (Braude, 1967). Of the Cu sources, $CuSO_4$ gave the best response (Braude and Ryder, 1973). Subsequent research in the United States (Barber et al., 1960; Bunch et al., 1961; Stahly et al., 1980) showed a similar response over a wider range of supplementation (125 ppm to 250 ppm Cu). However, the Cu requirement for swine is 5 to 6 ppm Cu for the weanling pig (NCR, 1988). The growth response to Cu in swine is independent of and in addition to the growth response to antibacterial agents (Stahly et al., 1980). Copper may be toxic when dietary concentrations in excess of 250 ppm are fed for several months.

It was observed from European research (Poulsen, 1989 and 1995) that higher concentrations of ZnO in nursery diets prevented diarrhea from stress or E. Coli in the newly weaned pig. While feeding these pharmacological concentrations of Zn, a growth promoting response was observed. In the United States, further research documented the observation that pharmacological concentrations of Zn fed during the nursery period improved growth performance (Hahn and Baker, 1993; Carlson et al., 1995; Hill et al., 1996). This improvement in daily gain occurred with or without the supplementation of Cu (Carlson et al., 1995; Hill et al., 1996; Smith et al., 1997). A Cu deficiency was induced in offspring of sows fed

5000 ppm Zn from ZnO indicating a possible detrimental effect of long term feeding of high dietary Zn (Hill et al., 1983a). Therefore, the objectives of our experiments were to: 1) determine what forms of Zn would enhance growth 2) evaluate the potential interactive or additive effects between Cu and Zn supplementation on growth performance during the nursery period 3) determine if there are further benefits from pharmacological concentrations of Zn and Cu fed past the nursery period and 4) investigate if pharmacological Zn supplementation affects Cu status of the growing pig.

MATERIALS AND METHODS

Animal Care and Use: The experimental protocol used in this study was approved by the All-University Committee on Animal Use and Care at Michigan State University (AUF number: 03/94-059-03).

Animals and Diets: All experiments were conducted at the Michigan State University Swine Teaching and Research Farm utilizing (Yorkshire X Landrace) X Hampshire crossbred pigs. In Exp. 1, 100 nursery pigs with an average initial weight of 6.8 kg (23.5 d of age) were used in two replications to measure growth performance only. Pigs were allocated by initial weight and equalized by ancestry and sex to five dietary treatments with 10 pigs per pen (2.44 x 1.22 m). The five dietary treatments were 1) Zn carbonate ($ZnCO_2$), 2) Zn acetate [$Zn(C_2H_3O_2)_2$] , 3) Zn oxide (ZnO) 4) Zn sulfate ($ZnSO_4$) each at 3000 ppm Zn or 5) 150 ppm ZnO. All Zn forms were reagent grade and the forms used were selected based upon feed

industry availability. All diets were formulated to meet or exceed the estimated nutrient requirements for swine (NRC, 1988).

In Exp. 2, 80 nursery pigs with an average initial weight of 7.2 kg (24 d of age) were used in two replications for a 3 wk nursery trial to measure growth performance only. These pigs were exposed to rotavirus in the farrowing house and hence represented compromised health status nursery pigs. Exp. 3 was a continuation of Exp. 2 and utilized one replication of pigs (n = 40) from Exp. 2. During the nursery period, pigs (Exp. 2 and 3) were housed in 2.44 x 1.22 m pens with 10 pigs for a 3 wk nursery period. After the nursery period, pigs in Exp. 3 (n = 40) remained in their penned group but were moved to a grow/finish pen (1.32 x 4.19 m) for the remainder of the study.

Exp. 2 and 3 utilized a 2 x 2 factorial design with four dietary treatments 1) 11 ppm Cu, 150 ppm Zn (LCuLZn), 2) 250 ppm Cu from $CuSO_4$, 150 ppm Zn (HCuLZn), 3) 11 ppm Cu, 3000 ppm Zn from ZnO (LCuHZn) and 4) 250 ppm Cu from $CuSO_4$, 3000 ppm Zn from ZnO (HCuHZn). All nursery periods in Exp. 1 and 2 used two dietary phases (Table 1); phase 1 (1.5 % lysine) was fed during wk 1 and phase 2 (1.25 % lysine) was fed during wk 2 and 3 (Exp. 1 and 2) or wk 2 to 5 (Exp. 3). In Exp. 3, to determine effects of long term pharmacological mineral supplementation, the same Cu and Zn treatments were fed through the grow-finish period which included: grower 1 diet was fed for 5 wks, grower 2 diet was fed for 6 wks and finisher diet was fed for 6 wks (Table 1).

Pigs were weighed weekly during the nursery period and bi-weekly through

the grow-finisher phase during Exp. 3 (154 d study). No feed intake data was collected. In Exp. 3, blood was collected by venapuncture from the anterior vena cava on d 0, 35, 70, 112 and 154 of the experiment into 10 ml heparinized (143 units of sodium heparin/tube) vacutainer tubes with 20 gauge, 2.54 cm needles. Blood was immediately centrifuged at 4°C, 2000 x *g*, for 10 min (Beckman GS-6KR centrifuge, Palo Alto, CA). Plasma, serum and red blood cells (RBC) were collected into polypropylene tubes and stored at -80°C for determination of RBC SOD activity, serum Cp activity and plasma Cu and Zn concentration analysis could be performed. Twenty pigs were randomly selected (5 pigs/trt) to be slaughtered on d 154 to obtain liver tissue for determination of SOD activity, metallothionein (MT) concentration and iron (Fe), Cu, and Zn concentrations. Duodenum sections were collected to measure MT concentration and the Zn and Cu concentration associated with the MT protein peak.

Superoxide Dismutase Analysis: Red blood cell Cu/Zn superoxide dismutase (EC 1.15.1.1) activity was determined (Hill et al., 1997) by a modification of the method of Marklund and Marklund (1974). Red blood cells obtained from centrifugation were washed three times in two volumes of ice-cold .9% saline. Between each wash, cells were centrifuged (as above) and saline removed by aspiration. After the third and final wash, cells were hemolyzed in an equal volume of ice-cold deionized distilled water and frozen at - 80°C until analysis. Liver tissue (approximately 1 g), for SOD, was homogenized with a tissumizer probe (S25N-10G, 10 mm diameter) in 10 X volumes of ice-cold potassium phosphate buffer (pH

7.2, .05 *M* phosphate, .24 *M* sucrose) using an Ultra Turrax T25 homogenizer (Tekmar-Dohrmann Corp., Cincinnati, OH). Red blood cell hemolysates or tissue homogenates were extracted with .6 volumes ice-cold ethanol:chloroform (25:15) to inactivate the manganese dependent SOD. After ethanol:chloroform addition, samples were gently mixed and centrifuged (4^0C, 5000 x *g*, 15 min). Aliquots of clear supernatant from red blood cells and tissue were diluted 1:10 or 1:25, respectively, with 50 m*M* Tris-HCL, 1.0 m*M* diethylenetriamine pentaacetic acid (DTPA) buffer. The reaction mixture in five cuvets of the DU 7400 spectrophotometer (Beckman, Palo Alto, CA) contained Tris-HCL-DTPA buffer and varying amounts of supernatant together to equal 900 µL, and 50 µL of 10 m*M* sodium azide. Cuvet were preincubated at 25^0C for 5 min, and 50 µL of 4 m*M* pyrogallol to initiate the dimutation of the peroxyl radical (final volume, 1.0 ml). Protein concentration of the supernatant was determined by the method of Lowry et al. (1951).

Ceruloplasmin Analysis: Ceruloplasmin (EC 1.12.3.1) activity was determined with a Beckman DU 7400 spectrophotometer to measure color development at 540 nm wavelength in a semimicro cuvet with a 1 cm light path using deionized distilled water as a blank. Ceruloplasmin oxidase activity was determined by absorbance at 15 min minus absorbance at 5 min * 6.25 *10-1 U/ml with Cp activity expressed in international units (Schosinsky et al., 1974; Lehmann et al.,1974).

Mineral Analysis: Tissue samples (1 g) were sliced from same area of

frozen tissue for determination of mineral concentrations. Samples were wet-ashed (Hill et al., 1983b) in a mixture of 10 *M* perchloric acid (3 mL) and 14 *M* nitric acid (20 mL). Digested samples were diluted with deionized distilled water as necessary to determine Cu, Zn and Fe concentrations by flame atomic absorption spectrophotometry (Smith-Heiftje 4000, Thermo Jarrell Ash Corporation, Franklin, MA). Dry matter of liver was determined by drying to a constant weight after approximately 12 h in a vacuum oven (Heinicke; Portland,OR).

Plasma was diluted 1:7 with deionized distilled water for analysis of Zn and Cu by flame atomic absorption spectrophotometry. Plasma Fe was determined by a method (Olson and Hamlin, 1969) utilizing .5 mL plasma deproteinized with 1 mL of 20% trichloroacetic acid (TCA), incubated at 90^{o}C for 15 min and centrifuged (2000 x *g*, 10 min). The supernatant was subsequently read on a flame atomic absorption spectrophotometer.

All mineral analyses were determined using glassware that had been washed in 30% nitric acid and rinsed with deionized distilled water. Bovine liver standard (1577b; NIST: National Institute of Standards and Technology, Gaitherburg, MD) was used to establish accuracy of instrument analysis. Variation was accepted within the specified limits of NIST. Zinc, Cu and Fe concentrations were calculated using an exogenous calibration curve.

Intestinal Mucosa Analysis: The small intestine was collected on the slaughter floor prior to refrigeration and ligated at the ligament of Treitz to remove a 15 cm section of the duodenum. The intestinal sample was weighed and rinsed

three times in ice-cold .9% saline. The section of duodenum was cut longitudinally to expose mucosa and rinsed again with cold saline. The intestinal mucosa was scraped using a glass microscope slide. Scrapings were weighed and homogenized (Tissumizer Ultra Turrax T25) for 1 min at 4°C in 1:4 (wt : volume) ice-cold buffer (Tris-HCL, 10mmol/L; NaCl, 154 mmol/L; NaN_3, .2 g/L; phenylmethylsulfonyl fluoride, .2 mmol/L; leupeptin, .6 mg/L; pepstatin A, .9 mg/L; pH 8.6). Homogenates were centrifuged (4°C, 9000 x *g*,20 min) in an induction drive centrifuge (Beckman J2-21M; Palo Alto, CA) to remove cell debris and nuclei. Supernatant was collected and recentrifuged for 60 minutes (4°C, 105,000 x *g*) in an ultracentrifuge (Beckman L8-80M, Palo Alto, CA) using a SW50.1 rotor and 5 mL thinwall polyallomer tubes. Again, supernatant was collected and frozen at -80°C until analysis could be performed.

Proteins in the intestinal homogenate supernatant were separated by gravity-flow gel filtration chromatography to determine the approximate molecular weight and size. Supernatant (2.5 mL), from intestinal mucosa scrapings, were filtered through both a .8 µm (Corning Glass Works; Corning, NY) and a .45 µm (Millipore; Bedford, MA) syringe filter, then applied to a 1.6 x 100 cm Sephadex G75 column (separation in MWt range of 10,000 to 60,000 kD) followed by 2 mL of .1 *M* phosphate buffer. Fractions (5mL) were collected using a Retriever 500 Fraction Collector (ISCO Inc., Lincoln, NE). Protein peaks were determined by reading absorbance (280 nm) of individual collected fractions (DU 7400 Spectrophotometer, Beckman, Palo Alto, CA). The molecular size of protein peak was determined after

column calibration with the following protein standards (Sigma Chemical, St Louis,MO): cytochrome c (11,700 kD), ribonuclease A (13,700 kD), chymotrypsinogen A (25,000 kD), ovalbumin (43,000 kD), and albumin (67,000 kD). Fractions were also analyzed on a flame atomic absorption spectrophotometer to determine Zn and Cu concentrations at protein peaks. The first component containing Cu and Zn elutes in the void volume, indicating a molecular weight exceeding 70,000 D. The second component elutes around cytochrome c at 12,000 D and contains the protein metallohtionein in the di-molar form (Richards and Cousins, 1975).

Metallothionein Assay: Liver (1 g) from each pig was homogenized in 4 mL of .5 mol/L glycine buffer (pH 8.3) and heated for 2 min at 100^{0}C. The homogenized and heated samples were centrifuged at 25,000 *g* for 2 min, and the supernatant solutions were used for measurement of liver cytosolic MT. The MT concentration was determined using non-radioactive silver binding assay (Lee et al., 1989). The amount of silver in the final supernatant fraction was assumed to be proportional to the amount of MT present. The concentration of silver was measured by a flame atomic absorption spectrophotometer.

Statistical Analysis: All statistical analysis were performed by the general linear model procedure of Statistical Analysis System (SAS,1985). Pigs were blocked by initial weight and equalized for ancestry and sex to dietary treatments. Data were analyzed as a randomized complete block design with main effects being dietary treatment and replication. Pen was the experimental unit for analysis of all

performance data. For blood and tissue analysis, individual pig was the experimental unit. The mean differences between the treatments were detected by comparison of least square means. Differences were considered significant at the level of $P < 0.05$.

RESULTS

Growth Performance: In Exp. 1, contrasting high versus low Zn diets from ZnO, ADG did not differ in phase 1 (Table 3) but was greater in phase 2 (476 vs 380 g/d, respectively) and overall ADG was 15% greater ($P = .008$) when pigs were fed pharmacological Zn as ZnO. During wk 1, pigs fed 3000 ppm Zn in the form of acetate were slower growing than the control (150 ppm ZnO) and Zn carbonate ($P = .02$). No differences were observed on growth performance between the other dietary Zn forms (oxide, carbonate, and sulfate). However, in phase 2 (wk 2 and 3), pigs fed 3000 ppm of ZnO had the greatest growth performance ($P = .006$) with the other forms at 3000 ppm being intermediate and ZnO at 150 ppm having the poorest ADG (Table 3). For the entire nursery phase, pigs fed 3000 ppm Zn as ZnO had greatest growth performance compared to other dietary Zn forms (3,000 ppm) and ZnO at 150 ppm ($P = .008$).

In Exp. 2 during the 21-d nursery study, pigs which were of reduced health status due to rotavirus exposure in farrowing house responded similarly as pigs in Exp. 1 to pharmacological concentrations of ZnO. Overall growth performance improved ($P = .0001$) by 37% or 45% with 3,000 ppm Zn and either 11 ppm or 250

ppm Cu, respectively (Table 4). During wk 2 and 3, pigs fed pharmacological concentrations of both Zn and Cu (HCuHZn) had the greatest ADG, with pigs fed high Zn (LCuHZn) being second, pigs fed high Cu (HCuLZn) third and pigs fed at or above NRC (1988) requirements (LCuLZn) having the poorest ADG ($P = .0001$). The growth pattern for the overall nursery period ($P = .0001$) was a mirror image of wk 2 and 3.

In Exp. 3, the nursery phase was extended for two more weeks and the pigs fed 3,000 ppm Zn (LCuHZn, HCuHZn) had increased growth performance (Table 5) in this 5-wk nursery phase ($P = .0006$) of the study. Pigs fed 3000 ppm Zn with either 11 ppm (LCuHZn) or 250 ppm Cu (HCuHZn) gained similarly ($P > .05$). Also, pigs fed 250 ppm Cu with either 150 ppm (HCuLZn) or 3000 ppm Zn (HCuHZn) had similar growth performance ($P > .05$). During nursery and grower 1 phases, pigs fed the LCuLZn diet (at or above NCR requirements) had the poorest ADG compared to pigs supplemented with either 250 ppm Cu or 3000 ppm Zn (Table 5). In grower 2 phase, pigs responded similarly to dietary treatments, but there was a trend for pigs fed LCuHZn to have greater growth performance ($P = .08$). However, during finisher phase, some apparent compensatory gain or loss of effectiveness from long term pharmacological doses of Cu and (or) Zn occurred in pigs fed the LCuLZn diets when they gained significantly faster than pigs on the HZn and HCu diets but not greater than HCuHZn combination (Table 5).

Laboratory Observations: Ceruloplasmin (Cp) activity did not differ ($P > .05$) by dietary treatment at d 0, 35, 70 and 154 (Table 6). However, on d 112, pigs fed

HCuHZn had greater Cp activity than pigs on the other dietary treatments. Plasma Cu concentrations (Table 7) of pigs fed 250 ppm Cu were higher (P = .04) only during the nursery period (d 35) with pigs fed HCuHZn being numerically greater than pigs fed HCuLZn diet. At d 154, pigs fed HCuLZn had the greatest plasma Cu concentration (P = .05) compared to pigs fed LCuLZn, LCuHZn and HCuHZn treatments. At all bleeding times following d 0, plasma Zn concentrations increased (P = .0001 and .02) when pigs were fed diets containing HZn with or without HCu (Table 8). However, d 112, pigs fed HCuHZn diets had higher plasma Zn concentrations than pigs fed LCuHZn diets.

Pigs fed HCu with either 150 or 3000 ppm Zn had greater RBC SOD activity (Table 9) than pigs fed LCu with or without HZn at d 35 and 70. However, d 112 and d 154, pigs fed LCuHZn diets had lower RBC SOD activity (P = .05 and .02, respectively) than pigs fed LCuLZn, HCuLZn or HCuHZn treatments.

Liver SOD activity (Table 10) did not follow the same pattern as the RBC SOD activity, but mirrors that of hepatic Cu concentrations. After 154-d of receiving pharmacological concentrations of Zn and (or) Cu, pigs fed HCu (250 ppm) diets had the greatest SOD activity in the liver (P =.04) compared to pigs fed the other dietary treatments.

Liver Cu concentrations were reflective of liver SOD activity, as pigs fed diets containing high Cu but low Zn (HCuLZn) had increased hepatic Cu storage while pigs fed high Cu with high Zn (HCuHZn) had hepatic Cu concentrations not significantly greater than pigs fed 11 ppm Cu. Also, at slaughter, two of five pigs

fed 3,000 ppm Zn as ZnO with adequate Cu (11 ppm) for the 154 d experimental period, had broken lower hind legs in the dehairer machine indicating a possible Cu deficiency as evidence by decreased bone health. Liver Zn concentrations reflect the plasma Zn patterns with pigs fed 3,000 ppm Zn having greater (P = .0001) Zn concentrations than pigs fed diets containing 150 ppm Zn.

Liver Fe concentrations were lowest (P = .0001) in pigs fed the diet containing 250 ppm Cu with adequate Zn (HCuLZn), but intermediate (Table 10) when Zn was increased to 3000 ppm (HCuHZn). This demonstrates the three-way interaction between Fe, Cu, and Zn.

In Exp. 3, at slaughter following d 154 d of the trial, intestinal mucosa cells were collected and analyzed for metallothionein concentration and then were separated into protein fractions. Pigs fed pharmacological concentrations of Zn (3,000 ppm) with or without copper (LCuHZn and HCuLZn) had greater concentrations of MT (P = .005) than pigs fed LCuLZn and HCuLZn diets (5.1 and 5.5 µg/mL vs .08 and 1.4 µg/mL, respectively). However, the protein fractions from the sephadex G-75 column did not follow the same pattern observed in MT concentration with the intestinal mucosa cells. Instead, pigs fed pharmacological concentrations of Cu and (or) Zn had greater concentrations (P = .0001) of these nutrients (Figure 1) in the protein fraction at 12,000 kD (area between 125 to 150 mL), established to be MT by Richards and Cousins (1975). Pigs fed the LCuLZn diet had almost an undetectable MT peak that contained Cu and Zn concentrations that were on an average less than .05 mg/L (Figure1: Graph A). Copper

concentrations in the MT fraction were greater (avg .25 mg/L) when pigs were fed 250 ppm Cu and adequate Zn than pigs fed LCuLZn diets (Figure 1: Graph B). Pigs fed 3000 ppm Zn and adequate Cu (LCuHZn) had an average Zn concentration of .88 mg/L (Figure 1: Graph C). However, when pharmacological concentrations of Cu and Zn were fed (HCuHZn), pigs had much greater concentrations (avg 1.2 and 1.7 mg/L, respectively) of both minerals in the MT fraction than pigs fed the adequate Cu and Zn diet (Figure 1: Graph D).

Metallothionein concentrations in the liver (Table 10) are reflective of dietary Zn supplementation and intestinal MT, as pigs fed 3,000 ppm Zn with or without 250 ppm Cu had the greatest hepatic MT concentration ($P = .001$). Pigs fed 250 ppm Cu with adequate Zn (HCuLZn) had MT concentrations that were only slightly numerically greater than LCuLZn pigs. However, when 250 ppm Cu was fed with 3000 ppm Zn no depression or addition in MT concentration was observed.

DISCUSSION

Feeding pharmacological concentrations of Zn in the form of ZnO improved growth performance when fed to traditionally-weaned nursery pigs of varied health status. Other Zn forms were not effective. Previous observations have indicated that traditionally-weaned nursery pigs fed pharmacological concentrations of Zn from ZnO (3,000 ppm) had enhanced growth performance than pigs fed 150 ppm Zn with or without the addition of 250 ppm Cu (Hahn & Baker, 1993; Hill et al., 1996 and Smith et al., 1997). Thus, 3,000 ppm Zn induces a growth response in nursery

pigs without an additive response from Cu. No differences were observed in growth performance during the first week of the nursery period between dietary treatments in all experiments indicating that Zn saturation may need to occur before a growth response can occur in weeks 2 thru 5. When the Zn supplemental feeding was extended beyond the nursery to market weight, the ZnO no longer stimulated growth performance after 10 weeks. Pigs fed 3,000 ppm Zn remained heavier at d 154, thus indicating a benefit or carry over effect from Zn supplementation past the nursery phase. Pigs fed HZn were an average of 10 to 11 kg heavier than pigs fed LCuLZn which could result in reaching market weight about 10.5 d earlier.

Plasma Zn concentrations were reflective of dietary Zn fed regardless of Cu concentration in the diet. It has been established that Zn is a more effective antagonizer of Cu than Cu is of Zn (Cousins, 1985). Pigs fed diets with adequate Zn, through the entire 154-d study, had an average plasma Zn concentration of .81 mg/L. After the nursery period, it appears that plasma Zn concentration of pigs fed 3,000 ppm Zn reach a threshold at about 2.0 mg/L. The continuation of pharmacological Zn feeding did not increase plasma Zn concentration during the remainder of the study. This is due to the role of intestinal MT in Zn uptake and homeostasis as previously observed by Richards and Cousins (1975).

Pigs fed adequate Cu with or without the addition of high Zn have a reduced plasma and liver Cu concentrations. The first indication of altered Cu status was the decreased RBC SOD activity on d 35 and 70 of the study. However, by d 112 and 154, pigs fed diets with 3000 ppm Zn and adequate Cu (LCuHZn) had the

lowest RBC SOD activity. Pigs fed adequate Zn and Cu (LCuLZn) RBC SOD activity could not be distinguished from pigs fed high Cu (250 ppm) with or without high Zn (HCuLZn and HCuHZn). This reduced SOD activity indicates that the pharmacological concentrations of Zn have stimulated MT, which preferentially binds Cu over Zn (Bremner, 1976) or competed with the Cu for intestinal uptake. Hence, less Cu is available for the SOD enzyme, an indication of lowered Cu status due to the interaction between Cu and Zn (Fischer et al., 1983). The Cu,Zn SOD enzyme requires these minerals for catalytic function (Mann and Keilin, 1938), but Cu regulates the functional activity of shielding intracellular components from oxidative damage (McCord and Fridovich, 1969). The reduction in RBC SOD activity by supplementation of Zn in the growing pig has not been reported, but SOD can be modulated by diet (Williams et al., 1975). A Cu deficiency results in a reduction in SOD activity in rat tissue specifically liver, kidney and RBC (Paynter et al., 1979). Therefore, Cu availability affects synthesis and turnover of the SOD enzyme. A similar observation was made in swine (Williams et al., 1975), when SOD activity in both RBC and liver decreased in Cu deficient swine. Bettger et al. (1978 and 1979) observed that a low Zn diet (100 ppm) or a severe Zn deficiency has little effect on RBC SOD activity in the rat and chick. Therefore, they concluded that RBC SOD activity was dependent on Cu status but not Zn status. Superoxide dismutase activity may be regulated by other metabolic factors (hormonal influences, aging, genetics, and cell differentiation), but these have not been studied. The slight increase in RBC SOD activity observed in pigs fed adequate Cu

and Zn (LcuLZn) during the entire 154 d study may indicate that age alters this Cu parameter.

Ceruloplasmin (Cp) and plasma Cu concentrations were not affected by the Zn supplementation indicating their lack of responsiveness to Cu status since hepatic Cu was significantly reduced. Williams et al. (1975) reported that Cp activity declined more rapidly than SOD activity in Cu deficient swine. The Cp activity and Cu status was not altered in this study. These two parameters are not independent since 90-95% of plasma Cu is associated with Cp (Hsieh and Frieden, 1975). However, at d 112, since pigs fed HCuHZn diets had greater Cp activity than pigs fed HCuLZn diets, another regulator of Cp activity may be involved.

In the liver tissue, SOD activity did not follow the same pattern that was observed in the RBC. Pigs fed HCuHZn had RBC SOD activity similar to the pigs fed HCuLZn, however, in the liver, only pigs fed 250 ppm Cu with adequate Zn (HCuLZn) had an increase in liver SOD activity, possibly indicating that Zn suppresses SOD activity by decreasing Cu status (VanCampen and Scaife, 1967) resulting in less Cu available for hepatic SOD synthesis (Paynter et al., 1979). Panemangalore and Bebe (1996) reported that high dietaty Zn had no effect on RBC SOD activity in rats during a 4 wk study. However, neither RBC or liver SOD activity have not been investigated when pharmacological Zn is fed for an extended period of time. And even though SOD activity decreases in a Cu deficiency, the decreases are usually not as severe as those observed in other enzymes (Prohaska, 1983). Therefore, it appears SOD activity is essential to the cell and

highly regulated.

Within the intestinal mucosal cells of the duodenum, nursery pigs supplemented with $CuSO_4$ and ZnO had higher concentrations of Cu and Zn associated with the MT protein fraction compared to pigs receiving adequate dietary Cu and (or) Zn, however, only intestinal MT concentration increased when 3000 ppm ZnO was fed independent of Cu additions. This supports the work by Bremner and Davies (1975) that found MT has greater binding affinity for Cu than for Zn and will preferentially bind Cu, but the Zn bound to MT stimulates MT synthesis. As dietary Zn concentration increases, the amount of Zn bound to the intestinal MT protein increases resulting in a subsequent stimulation of tissue MT concentration (Blalock et al., 1988). Intestinal mucosa Cu concentrations decrease when MT was induced by high concentrations of Zn (Reeves et al., 1994).

It has been documented that high concentrations of Zn will depress Cu status and is thought to occur in the intestine (Richards and Cousins, 1975). This happens via Cu binding tightly to Zn-induced MT preventing Cu transport across the basolateral membrane into circulation (Cousins, 1985). The MT bound to Cu is lost through the intestinal tract as epithelial cell slough off. In this study, Zn elevated intestinal MT, providing the trap that captures Cu in transit and the high binding affinity of Cu for MT may or may not displace Zn from MT due to the differences in Cu and Zn concentrations in the diet. In conclusion, the higher hepatic SOD activity observed when pigs were fed the HCuLZn diet could be the result of greater Cu available to initiate the SOD activity (Paynter et al., 1979). And

pigs fed the LCuHZn diet had lower RBC SOD activity due to the inhibitory effect of Zn on Cu absorption (Cousins, 1985) resulting in less Cu available in circulation to stimulate RBC SOD activity. Therefore, MT functions as a storage and metal detoxification protein, rather than the SOD enzyme, because SOD (MW 32,000) utilizes Cu and Zn at an even ratio of 2 each while MT (MW 6,500) binds 7 atoms of Zn and 10 atoms of Cu (Kagi and Schaffer, 1988).

IMPLICATION

These data provide support for the reason that there is not an additive growth promotant response when both 250 ppm copper and 3,000 ppm zinc are fed to the growing pig. The main reason is that copper and zinc act as antagonists due to the similar chemical properties (Hill and Matrone, 1970). Metallothionein induced by zinc in the intestinal epithelial cells binds copper and inhibits copper absorption (Cousins, 1985) possible making the copper unavailable to act as a growth promotant. Overall, nursery diets supplemented with 3,000 ppm zinc in the form of Zn oxide improved average daily gain. The improvement in growth performance from feeding high concentrations of zinc does not occur past 10 weeks post-weaning, but the weight gained during the nursery phase is maintained to market weight. However, it appears that the growth response observed when pigs are fed 3,000 ppm Zn occurs with or without 250 ppm copper in the diet. Therefore, feeding 3,000 ppm zinc as zinc oxide would be beneficial but should never be fed to replacement gilts, after the nursery phase, since copper storage and utilization is depressed.

Table 1: Percentage composition of experimental diets[a] (as-fed basis)

Ingredient	Phase 1[b]	Phase 2[b]	Grower 1[c]	Grower 2[c]	Finisher[d]
Corn, dent yellow	36.5	38.1	60.5	70.5	80.5
Whey, dried	20.0	22.5	-	-	-
Skim milk, dried	10.0	-	-	-	-
Spray-dried animal plasma	7.5	-	-	-	-
Soybean meal, dehull	14.5	32.5	35.3	25.4	16.5
Oat groats	5.0	-	-	-	-
Soybean oil	3.0	3.0	-	-	-
Dical Phosphate, 21%	1.0	1.48	1.5	1.5	.75
Limestone	.9	.87	1.1	1.1	1.0
Vitamin Premix[e]	.5	.5	.5	.5	.5
Mineral Premix[f]	.5	.5	.5	.5	.5
Salt, NaCl	.3	.3	.5	.5	.25
Mecadox-10[g]	.25	.25	-	-	-
Banminth-48[h]	-	-	.1	-	-
Copper sulfate[i]	-	-	-	-	-
DL-methionine	.05	-	-	-	-
Zinc[j]	-	-	-	-	-

[a] Formulated to contain at least .8% Ca and .65% P.

[b] Diets fed during the nursery phase of Exp. 1-3 and contained either 1.5% or 1.25% lysine and 20% or 18% crude protein. Phase 1 fed during wk 1 and Phase 2 fed during wk 2 to 5.

[c] Diets fed in Exp. 3 (wk 6 to 11 and 12 to 18) and contained .86% lysine and 16% crude protein.

[d] Diet fed in Exp. 3 (wk 19 to 25) and contained .68% lysine and 14% crude protein.

[e] Provided per kg of complete diet: 2500 IU vitamin A, 250 IU vitamin D_3, 30 IU vitamin E, 2 mg riboflavin, 12 mg niacin, 15 µg vitamin B_{12}, 2 mg vitamin K_3, .5 mg thiamin, .45 mg pyridoxine and 8 mg pantothenic acid.

[f] Provided per kg of complete diet: 100 mg Zn, 100 mg Fe, 10 mg Cu, 10 mg Mn, 150 µg I and 300 µg Se.

[g] Antibacterial agent, 2.2% carbadox (Animal Health Co., NY).

[h] Provides 48g of pyrantel tartrate per lb.

[i] Copper sulfate was added in at .1% in Exp. 3.

[j] For the 3000 ppm Zn diets, ZnO = .41%, $Zn(C_2H_3O_2)_2$ = .84%, $ZnCO_2$ = .57%, and $ZnSO_4$ = .85%. (substituted in the place of corn).

Table 2: Chemical analysis of experimental diets[a] in Exp. 3, ppm (as-fed basis)

Nutrient	Phase 1	Phase 2	Grower 1	Grower 2	Finisher
Exp. 1					
Low Zinc					
Zinc	106.2	192.9			
Copper	250.5	240			
Iron	219.5	301.2			
Zn Acetate					
Zinc	2535.0	2601.0			
Copper	234.3	229.8			
Iron	234.9	299.1			
Zn Carbonate					
Zinc	3421.0	3090.5			
Copper	266.1	242.4			
Iron	212	261.7			
Zn Oxide					
Zinc	2962.7	3285.0			
Copper	235.2	267.1			
Iron	226.5	298.5			
Zn Sulfate					
Zinc	3145.0	2884.4			
Copper	251.1	277.6			
Iron	275.9	285.7			
Exp. 3					
LCuLZn					
Zinc	276.7	185.7	254.5	228.3	233
Copper	16.8	7.3	21.9	17.9	14.9
Iron	271.1	228.2	316.2	315.4	251
HCuLZn					
Zinc	251.4	262.8	309.6	241.4	290.5
Copper	334.3	313.6	250.8	251	312.1
Iron	231.7	257.2	295.1	304.2	249
LCuHZn					
Zinc	2601	2669	2746	2862	2774
Copper	17.7	18.8	17.2	16.6	14.8
Iron	268.7	282.8	279.8	268.7	266.5
HCuHZn					
Zinc	2633	2709	2822	2747	2663
Copper	291.8	267.9	279.3	290	308.5
Iron	279.3	286.4	325.4	340.1	323.1

[a] Analysis state that diets contained .9 ppm Ca , .75 ppm P and 4.0 kcal/g GE.

Table 3. Effect of dietary Zn source on ADG during the nursery period in Exp. 1, g/d

	Source and Dietary Zn Concentration (ppm)						
Parameter[d]	ZnO 150	$Zn(C_2H_3O_2)_2$ 3000	$ZnCO_2$ 3000	ZnO 3000	$ZnSO_4$ 3000	SEM	P Value
Phase 1 (wk 1)	248	193	248	231	220	10	.14
Phase 2 (wk 2-3)	380[c]	434[b]	416[bc]	476[a]	409[bc]	17	.006
Overall	336[c]	354[b]	360[b]	395[a]	346[b]	15	.008

[abc] Means in the same row lacking a common superscript differ significantly.
[d] Least square means are reported from one hundred weanling pigs with 10 pigs per pen and two pens per treatment.

Table 4. Effect of Cu and Zn supplementation on ADG during the nursery period in Exp. 2, g/d

	Dietary Treatments					
Parameter[e]	LCuLZn	HCuLZn	LCuHZn	HCuHZn	SEM	P Value
Phase 1 (wk 1)	391	534	524	536	54	.18
Phase 2 (wk 2-3)	425[d]	500[c]	729[b]	864[a]	40	.0001
Overall	414[d]	511[c]	661[b]	754[a]	31	.0001

[abcd] Means in the same row lacking a common superscript differ significantly.
[e] Least square means are reported from eighty weanling pigs with 10 pigs per pen and two pens per treatment.

Table 5. Effect of Cu and Zn supplementation on ADG in Exp.3, g/d

	Dietary Treatments					
Parameter[c] (wk)	LCuLZn	HCuLZn	LCuHZn	HCuHZn	SEM	P Value
Nursery (5)	347[c]	439[b]	527[a]	477[ab]	28	.0006
Grower 1 (5)	493[b]	704[a]	744[a]	769[a]	44	.0003
Grower 2 (6)	843	764	871	770	47	.08
Finisher (6)	880[a]	724[b]	736[b]	767[ab]	41	.04
Overall (22)	656	657	718	697	24	.2
Final BW (kg)	107	108	118	114	10	.14

[ab] Means in the same row lacking a common superscript differ significantly.
[c] Numbers in parathesis indicate weeks fed dietary phase.
[d] Least square means are reported from forty weanling pigs with 10 pigs per treatment.

Table 6. Effect of Cu and Zn supplementation on ceruloplasmin activity in Exp.3, U/mg serum

	Dietary Treatments					
Parameter[c]	LCuLZn	HCuLZn	LCuHZn	HCuHZn	SEM	P Value
Day 0	.14	.18	.13	.15	.014	.07
Day 35	.13	.16	.16	.18	.015	.21
Day 70	.18	.21	.20	.20	.015	.61
Day 112	.27[b]	.27[b]	.27[b]	.36[a]	.022	.01
Day 154	.18	.21	.21	.22	.019	.56

[ab] Means in the same row lacking a common superscript differ significantly.
[c] Least square means are reported from five pigs per treatment.

Table 7. Effect of Cu and Zn supplementation on plasma Cu concentration in Exp.3, mg/L

	Dietary Treatments					
Parameter[d]	LCuLZn	HCuLZn	LCuHZn	HCuHZn	SEM	P Value
Day 0	1.91	1.94	1.86	1.81	.11	.84
Day 35	1.59[b]	1.87[ab]	1.68[b]	2.06[a]	.12	.04
Day 70	1.72	1.93	1.69	1.82	.12	.22
Day 112	1.81	1.92	1.80	2.12	.17	.18
Day 154	1.73[c]	2.20[a]	1.79[c]	1.96[b]	.13	.05

[abc] Means in the same row lacking a common superscript differ significantly.
[d] Least square means are reported from five pigs per treatment.

Table 8. Effect of Cu and Zn supplementation on plasma Zn concentration in Exp.3, mg/L

	Dietary Treatments					
Parameter[d]	LCuLZn	HCuLZn	LCuHZn	HCuHZn	SEM	P Value
Day 0	.78	.78	.77	.73	.04	.74
Day 35	.92[b]	.97[b]	2.05[a]	2.16[a]	.11	.0001
Day 70	.89[b]	.79[b]	1.17[a]	1.30[a]	.12	.02
Day 112	.93[c]	.89[c]	1.6[b]	2.10[a]	.16	.0001
Day 154	.90[b]	.89[b]	1.70[a]	1.83[a]	.12	.0001

[abc] Means in the same row lacking a common superscript differ significantly.
[d] Least square means are reported from five pigs per treatment.

Table 9. Effect of Cu and Zn supplementation on RBC SOD activity in Exp. 3, U/mg protein

	Dietary Treatments					
Parameter[c]	LCuLZn	HCuLZn	LCuHZn	HCuHZn	SEM	P Value
Day 0	77.5	81.2	77.3	73.4	5.4	.79
Day 35	91.1[b]	100.7[a]	90.8[b]	109.5[a]	3.6	.002
Day 70	82.7[b]	96.5[a]	80.2[b]	92.5[a]	3.7	.01
Day 112	105.3[a]	111.4[a]	96.2[b]	112.8[a]	4.4	.05
Day 154	105.7[a]	112.3[a]	93.1[b]	111.0[a]	4.6	.02

[ab] Means in the same row lacking a common superscript differ significantly.
[c] Least square means are reported from five pigs per treatment.

Table 10. Effect of Cu and Zn supplementation on hepatic parameters in Exp. 3 after 154 days (wet basis)

	Dietary Treatments					
Parameter[d]	LCuLZn	HCuLZn	LCuHZn	HCuHZn	SEM	P Value
SOD[c]	76.2[b]	112.7[a]	67.9[b]	68.6[b]	11	.04
Cu (µg/g)	5.1[b]	467.6[a]	8.4[b]	36.3[b]	30.5	.0001
Fe (µg/g)	166.9[a]	55.1[c]	153.3[a]	88.8[b]	8.3	.0001
Zn (µg/g)	95.0[b]	107.7[b]	588.0[a]	636.4[a]	43.4	.0001
MT (µg/g)	37.7[b]	80.7[b]	387.9[a]	383.8[a]	39	.001

[ab] Means in the same row lacking a common superscript differ significantly.
[c] Activity units per mg protein.
[d] Least square means are reported from five pigs per treatment.

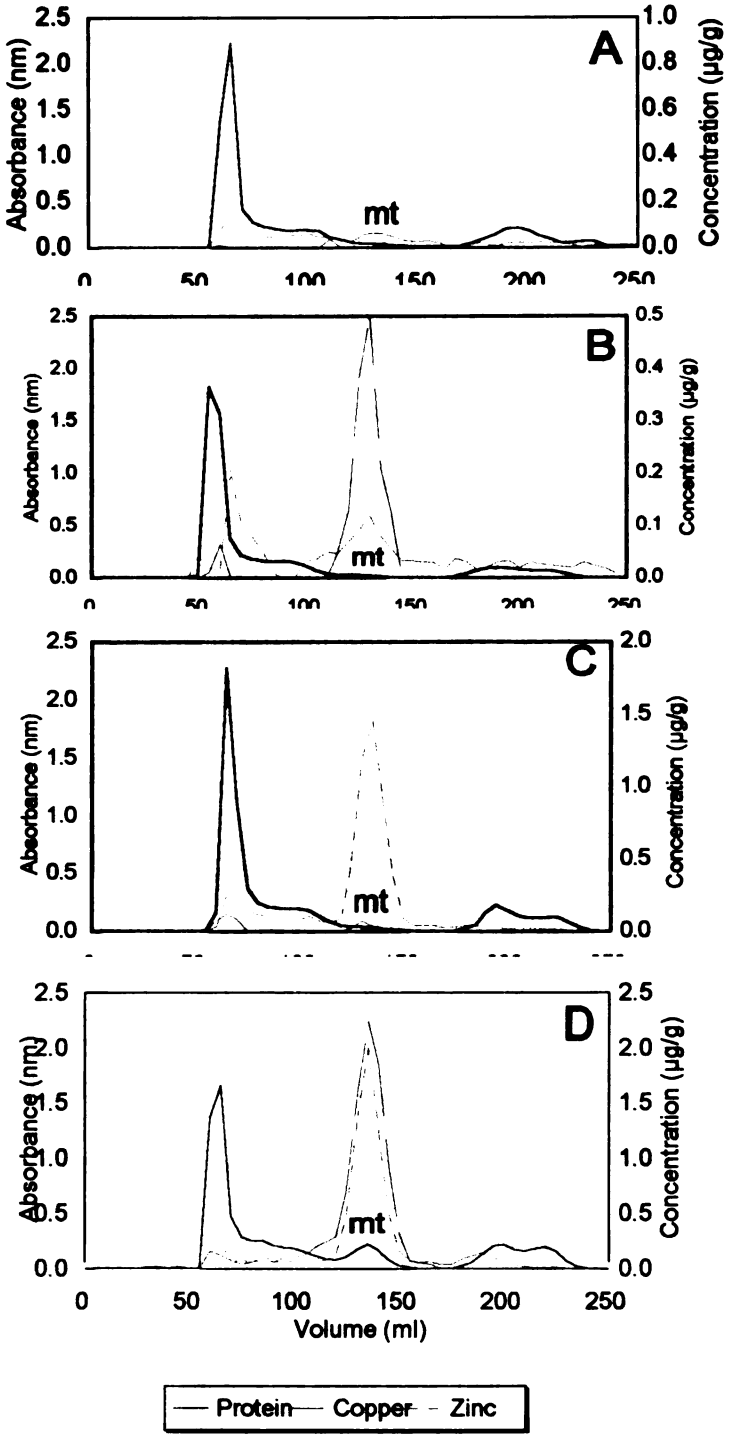

Figure 1: The effects of Cu and Zn supplementation on pig intestinal mucosa proteins after separation on a Sephadex G-75 column. A. LCuLZn (11 ppm Cu and 150 ppm Zn) B. HCuLZn (250 ppm Cu and 150 ppm Zn) C. LcuHZn (11 ppm Cu and 3000 ppm Zn) D. HCuHZn (250 ppm Cu and 3000 ppm Zn). (n = 5)

Chapter 4:

The effect of pharmacological concentrations of organic and inorganic zinc on metallothionein concentration and intestinal morphology in the nursery pig

ABSTRACT

Previous research has shown that pharmacological concentrations of organic and inorganic zinc (Zn) improve growth performance in the nursery pig. The objective of these experiments was to determine a possible mode of action explaining the growth enhancement. Two experiments were conducted to determine the impact of pharmacological concentration of Zn on intestinal morphology and metallothionein (MT) concentration in the nursery pig. In Exp.1, twenty-four pigs (avg 21 d and 6.1 kg) were fed one of two dietary treatments: 1) 150 ppm Zn as Zn oxide (ZnO) or 2) 3000 ppm Zn as ZnO in two phases. On d 28 of the study, six pigs from each treatment were killed to obtain intestinal tissue. Pigs fed the high Zn (3,000 ppm) had deeper duodenal crypts (P = .01) and greater total thickness from villus tip to serosa (P = .001) than pigs fed the adequate (150 ppm) Zn diet (40.8 and 165 µm vs 28 and 131.8 µm, respectively). There was a trend for longer villi (77.4 vs 64.5 µm) and lower villus:crypt ratio (VCR, 1.93 vs 2.39) when pigs were fed the 3000 ppm Zn compared to pigs fed 150 ppm Zn diet (P = .14 and .18, respectively). Pigs fed the 3000 ppm Zn had greater intestinal MT concentration (P = .01) than pigs fed the adequate Zn diet (1831 vs 240.7 µg/g). There was a trend for ADG to be greater (P = .27) for pigs fed the high Zn diet (345 vs 308 g/d). In Exp.2, twelve barrows (avg 19 d and 6.68 kg) were allotted by litter

to one of four dietary treatments as follows: 1) basal (250 ppm Zn as Zn sulfate), 2) basal + 250 ppm Zn methionine complex (ZnMet), 3) basal + 250 ppm Zn amino acid complex (ZnAA), and 4) basal + 3,000 ppm Zn as ZnO. Zinc concentrations in the intestinal MT fractions of the pigs fed the ZnO diet were greater (P = .02) compared to pigs fed the basal, ZnMet and ZnAA diets (.79 µg/mL vs .27, .22, and .29 µg/mL). Copper concentrations in MT fractions were not affected (P > .05) by dietary treatment. Intestinal mucosa cell MT concentrations were greater (P = .02) in basal and ZnO fed pigs compared to pigs fed the ZnMet and ZnAA diets (98 and 116 µg/mL vs 56.6 and 71.3 µg/mL, respectively). Therefore, intestinal MT responds differently to organic and inorganic forms of Zn. These results are consistent with a possible mode of action for improved growth performance from pharmacological Zn that involves increased metallothionein in the intestine and (or) altered duodenal morphology.

Key words: Nursery Pig, Organic and Inorganic Zinc, Metallothionein, Intestinal Morphology,

INTRODUCTION

In the swine industry, producers routinely add 2,000 to 3,000 ppm of inorganic Zn to nursery diets to improve growth performance. However, the mode of growth promotion is unknown. Interest in using organic mineral complexes has increased because of the reported potential of higher bioavailability than from inorganic mineral sources (Hahn and Baker, 1993). Ward et al. (1996) observed

improved growth performance when pigs were fed either 250 ppm Zn from Zn methionine complex (organic) or 2,000 ppm Zn from ZnO (inorganic). If there is no reduction in growth by feeding a lower concentration of organic Zn and greater amounts are available to the body. Therefore, less Zn would be excreted during the nursery phase. One hypothesis is that Zn supplementation stimulates the induction of the protein metallothionein (MT), which has a role in metal detoxification and Zn homeostasis (Richards and Cousins, 1975). A second hypothesis is that Zn stimulates intestinal proliferation and improves nutrient absorption during the post-weaning phase.

Therefore, the objectives of these experiments were: 1) to determine a possible mode of action for improved growth performance when nursery pigs are fed pharmacological concentrations of Zn, 2) to determine if pharmacological concentrations of Zn affects the intestinal morphology of the newly weaned pig and 3) to determine if MT concentration in the intestinal mucosa is affected differently by organic and inorganic Zn sources.

MATERIALS AND METHODS

Animal Use and Care: The experimental protocol used in this study was approved by the All-University Committee on Animal Use and Care at Michigan State University (AUF number: 03/94-059-03).

Animals and Diets: Experiment 1 was conducted with (Yorkshire X Landrace) X Hampshire crossbred pigs at the Michigan State University Swine

Teaching and Research Farm. Twenty-four pigs with an average initial weight of 6.1 kg (21 d of age) were housed in a nursery with 12 pigs per pen (2.44 x 1.22 m) in one replication. Phase 1 diet was fed during wk 1 and phase 2 diet was fed during wk 2 to 4. Pigs were allotted to dietary treatment and regimen by weight, sex and ancestry in a randomized complete block design. The two dietary Zn treatments were 1) 150 ppm Zn (adequate) and 2) 3,000 ppm Zn as ZnO (high). On 28-d post-weaning, pigs were killed using a lethal injection of pentobarbital (80 mg/kg, iv) to obtain liver, kidney and intestine tissue for metallothionein concentration analysis and determine intestinal morphology.

In Exp.2, twelve PIC barrows were weaned (19 d and 6.68 kg) and allotted by litter to one of four treatments fed in one dietary phase. The dietary treatments were 1) basal (250 ppm Zn from Zn sulfate), 2) basal + 250 ppm Zn from Zn methionine complex (ZnMet), 3) basal + 250 ppm Zn from Zn amino acid complex (ZnAA) and 4) basal + 3,000 ppm Zn from ZnO. These are typical pelleted commerical nursery diets fed in the swine industry. Pigs were assigned to individual stainless steel pens equipped with stainless steel feeders and waters in a temperature controlled room. For the first 14-d, pigs were given ad libitum access to feed and distilled water. On d 15 through d 20, pigs were fed 90% of daily ad libitum intake divided into three feedings per day. At d 20, pigs were killed by electric-immobilization and exsanguination to obtain liver, kidney and intestine tissue for metallothionein concentration analysis.

Dietary Zn treatments were obtained by substituting Zn for corn. All diets

were formulated to meet or exceeded estimated nutrient recommendations for the 10 to 20 kg pig (NRC, 1988). All dietary treatment compositions and chemical analysis are represented in Tables 1 and 2.

Intestinal Mucosa Analysis: A small Intestinal segment was obtained at the ligament of treitz to remove a 15 cm section of the duodenum. The intestinal sample was weighed and rinsed three times in ice-cold .9% saline. The section of duodenum was cut longitudinally to expose mucosa and rinsed again with cold saline. The intestinal mucosa was scraped using a glass microscope slide. Scrapings were weighed and homogenized (Tissumizer Ultra Turrax T25) for 1 min at 4^{0}C in 1:4 (wt : volume) ice-cold buffer (Tris-HCL, 10mmol/L; NaCl, 154 mmol/L; NaN_3, 0.2 g/L; phenylmethylsulfonyl fluoride, 0.2 mmol/L; leupeptin, 0.6 mg/L; pepstatin A, 0.9 mg/L; pH 8.6). Homogenates were centrifuged (4^{0}C, 9000 x *g*, 20 min) in an induction drive centrifuge (Beckman J2-21M; Palo Alto, CA) to remove cell debris and nuclei. Supernatant was collected and recentrifuged for 60 min (4^{0}C, 105,000 x *g*) in an ultracentrifuge (Beckman L8-80M, Palo Alto, CA) using a SW50.1 rotor and 5 mL thinwall polyallomer tubes. Again, supernatant was collected and frozen at -80^{0}C until analysis could be performed.

Proteins in the intestinal homogenate supernatant were separated by gravity-flow gel filtration chromatography to determine the approximate molecular weight and size. Supernatant (2.5 mL), from intestinal mucosa scrapings, were filtered through both a .8 µm (Corning Glass Works; Corning, NY) and a .45 µm (Millipore; Bedford, MA) syringe filter, then applied to a 1.6 x 100 cm Sephadex G75 column

(separation in MWt range of 10,000 to 60,000 kD) followed by 2 mL .1 *M* phosphate buffer. Fractions (5mL) were collected (Retriever 500 Fraction Collector; ISCO Inc., Lincoln, NE). Protein peaks were determined by reading absorbance (280 nm) of individual collected fractions (DU 7400 Spectrophotometer, Beckman, Palo Alto, CA). The molecular size of protein peak was determined after column calibration with the following protein standards (Sigma Chemical, St Louis,MO) : cytochrome c (11,700 kD), ribonuclease A (13,700 kD), chymotrypsinogen A (25,000 kD), ovalbumin (43,000 kD), and albumin (67,000 kD). Fractions were also analyzed on a flame atomic absorption spectrophotometer (Smith-Heiftje 4000, Thermo Jarrell Ash Corporation, Franklin, MA) to determine Zn and Cu concentrations in fractions.

Metallothionein Assay: Duodenal mucosa, liver and kidney tissue (approximately 1 g, each) from each pig was homogenized in 4 mL of .5 mol/L glycine buffer (pH 8.3) and heated for 2 min at 100^0C. The homogenized, heated samples were centrifuged at 25,000 *g* for 2 min, and MT was determinedin the supernatant using a non-radioactive silver binding assay (Lee et al., 1989). The amount of silver in the final supernatant fraction was determined to be proportional to the amount of metallothionein present. The concentration of silver was measured by a flame atomic absorption spectrophotometry.

Intestinal Morphology: In Exp. 1, intestinal segments (duodenum, ileum and colon; 10 cm each) were obtained from twelve traditionally weaned pigs fed either 150 ppm or 3,000 ppm Zn from ZnO after a 28-d nursery period. Intestinal

segments were collected, rinsed with phosphate buffered saline until ran clear, blotted dry, and weighed. Each intestinal segment was sutured closed at the distal end, distended with a constant volume of 10% neutral buffered formalin, then the proximal end was closed and the segment was submerged in 20 mL of formalin. Slides of intestinal sections were stained with hematoxylin and eosin stain according to Moxley and Olson (1989) and Coussement et al. (1982). The length of the villi (from the tip of the villus to the crypt orifice), depth of crypt (junction of villus/crypt to base of crypt), villus:crypt ratio (number of crypts to each villus), brunner's gland thickness, peyers galnd thickness, circumferential muscle thickness, longitudinal smooth muscle thickness and total thickness from villus tip to serosa surface (accounts for interstitial space) were quantified by an Olympus BX40 microscope with an ocular micrometer. Each intestinal segment, on a minimum of seven different areas, was measured and means calculated using an image analysis computer system.

Statistical Analysis: These experiments were analyzed using SAS software for GLM procedure and least squares of means by treatment (SAS, 1985). Pigs were blocked by initial weight and equalized for ancestry and sex. Individual pig was the experimental unit for analysis of growth performance and tissue. Data were analyzed as a randomized complete block design with either two dietary treatments (Exp 1) or four dietary treatments (Exp. 2). The mean differences between the treatments were detected by comparison of least square means. Differences were considered significant at the level of $P < 0.05$.

RESULTS

Growth Performance: In Exp. 1, growth performance was slightly increased (345 vs 308 g/d) when pigs were fed 3,000 ppm Zn during the 28-d nursery period ($P = .27$) compared to pigs fed the 150 ppm Zn diet (Table 3). Growth performance was not determined in Exp. 2.

Intestinal Morphology: In Exp. 1, histopathology determinations were performed on traditionally-weaned pigs that received either diets with adequate Zn (150 ppm) or pharmacological Zn as ZnO (3,000 ppm) for a 28-d study (Table 4). Pigs fed pharmacological concentrations of Zn had deeper crypts ($P = .01$) and greater total thickness from villus tip to serosa ($P = .001$) when compared to pigs fed adequate Zn diets (40.8 and 165 µm vs 28 and 131.8 µm, respectively). There was a trend for longer villi ($P = .14$) and a lower villus:crypt ratio ($P = .18$) when pigs were fed pharmacological concentrations of Zn for 28-d compared to pigs fed adequate Zn diets (77.4 and 1.93 vs 64.5 µm and 2.39, respectively). Ileum and colon intestinal segments were unaffected ($P > .05$) by dietary Zn treatment (Table 4).

Metallothionein Characteristics: In Exp. 1, pigs fed the 3000 ppm ZnO diet had a greater amount of MT in the intestinal mucosa, liver and kidney compared to the pigs fed the 150 ppm Zn diet (Table 5). Those pigs fed the 3000 ppm ZnO diet had greater hepatic MT concentrations ($P = .0001$) than pigs fed the adequate Zn diet (2528.5 µg/g vs 247.2 µg/g). Renal MT concentrations were higher ($P = .0001$) in pigs fed the 3000 ppm Zn than pigs fed the 150 ppm Zn diet (462.4 µg/g

vs 65.5 µg/g). And intestinal mucosa cell MT concentrations followed the exact same pattern as the liver and kidney tissue, as pigs fed the high ZnO diet were greater (P = .001) than pigs fed the adequate Zn diet (131.1 µg/g vs 20.7 µg/g).

In Exp. 2, metallothionein concentrations were determined in the liver and kidney as well as the amount of Cu and Zn associated with MT protein in the intestinal mucosa (Table 6). Hepatic MT concentrations were greater (P = .0001) for those pigs fed the 3000 ppm ZnO diet compared to pigs fed the basal, ZnMet and ZnAA diets (5338.7 µg/g vs 797.6, 940.3, and 900.4 µg/g, respectively). Pigs fed the 3,000 ppm ZnO diet had greater (P =.0001) than pigs fed the basal , ZnMet and ZnAA diets (527.4 µg/g vs 162.2, 144.1 and 160.3 µg/g, respectively). Pigs fed the basal (250 ppm Zn) diet had similar MT concentrations in the liver and kidney as the pigs fed the ZnMet or ZnAA (500 ppm Zn) diets (P > .05).

Pigs fed either 3000 ppm Zn from ZnO or 250 ppm Zn from Zn sulfate (both inorganic Zn forms) had the greatest intestinal mucosa MT concentrations (P = .02) compared to the organic Zn treatments of ZnMet and ZnAA at a total of 500 ppm Zn (116 and 98 µg/g vs 56.6 and 71.3 µg/g, respectively). After protein separation of the intestinal mucosa cells by a Sephadex G-75 column, pigs fed the 3,000 ppm ZnO had the greatest Zn concentration (P = .02) in the metallothionein protein fraction. Pigs fed the basal diet (250 ppm $ZnSO_4$) had similar MT concentrations in the intestinal mucosa as pigs fed the 3,000 ppm ZnO diet, but had less Zn associated with the MT protein fraction (.267 mg/L vs .792 mg/L). There was a trend for the pigs fed the 3000 ppm ZnO diet to have a greater (P = .25) amount of

Cu associated with the MT protein fraction.

DISCUSSION

In this study, intestinal morphology was evaluated as a possible mode of action for enhancement of growth by pharmacological concentrations of Zn. The data support that a pharmacological concentration of Zn alters the intestinal absorption surface area. Therefore, Cera et al. (1988) observations that reduced villus height is linked to post-weaning lag and reduced feed intake when pigs were weaned from the sow to a starter diet could be eleviated by pharmacological zinc. Hampson (1986) reported that deeper crypts are associated with an increase in enterocyte proliferation. This proliferation will increase the small intestine absorption area and provide more mature cells, thus possibly helping to explain the decrease in diarrhea and improved growth observed when feeding 3,000 ppm Zn as ZnO. The appearance of less mature enterocyte population help to explain the increased susceptibility of the pig to diarrhea and growth check in the post-weaning period (Hampson, 1986).

Researchers have established a relationship between intestinal MT and dietary Zn in rats. Starcher et al. (1980) observed that zinc absorption is directly related to intestinal metallothionein concentration. However, Richards and Cousins (1975) and Cousins (1979) reported that zinc absorption is inversely related to intestinal metallothionein. The theory is that MT binds Zn and limits Zn transfer into plasma, thus MT plays a role in Zn homeostasis. These two research groups

might appear to contridict each other, but Cousin's work was done when dietary Zn was in excess.

Therefore, pigs fed either the basal (250 ppm $ZnSO_4$) or 3,000 ppm ZnO diets had similar MT content in the intestinal mucosa , but there was less Zn associated with the MT protein fraction of pigs fed the basal diet than pigs fed the 3,000 ppm ZnO indicating that possibly some other mechanism beside the stimulation of MT production by Zn since it appears that MT is not saturated with Zn in the pigs fed the basal diet. Both these treatments are the inorganic Zn forms. This indicates that inorganic and organic forms of Zn are absorbed differently in the intestine. These results suggest that inorganic forms of Zn stimulate and organic Zn forms depress MT production in the intestinal mucosa cells.

The improved growth performance of nursery pigs due to feeding pharmacological concentrations of Zn is consistent with an increased intestinal surface area allowing for more nutrient absorption and with an elevated concentration of MT in the intestinal mucosa cells.

IMPLICATION

The mechanism for the enhancement in growth performance is consistent with an increase in gastro-intestinal cell proliferation during pharmacological zinc feeding creating less mature cells allowing for greater nutrient absorption. Stimulation of metallothionein in the mucosal cell to regulate zinc uptake into the body and perhaps improved cell proliferation in the intestine may be related to the improved growth observed in the weanling pig.

Table 1: Percentage composition of experimental diets[a], % (as-fed basis)

Ingredient	Phase 1[b]- (Exp. 1)	Phase 2[b]- (Exp. 1)	Phase 1& 2[c]- (Exp. 2)
Corn, dent yellow	45.6	43.9	21.05
Whey, dried	20	20	23.57
Fat, whey	-	-	10.89
Skim milk, dried	10	-	-
Fish meal	-	-	10
Spray-dreid animal plasma	7.5	-	4.5
Soybean meal, dehull	5.08	29.4	5.0
Oat groats	5	-	21.93
Soybean oil	3	3	-
Dical Phosphate, 21%	1.22	1.12	.05
Limestone	.89	.86	.64
Vitamin Premix[d]	.6	.6	-
Mineral Premix[e]	.6	.6	-
Salt, NaCl	.3	.3	-
Mecadox-10[f]	.25	.25	-
Oat flour	-	-	.5
L-lysine	-	-	.01
Flavor	-	-	.4
Cu-lysine	-	-	.1
Antibiotic[g]	-	-	.5
Microingredients[h]	-	-	.86
Zinc Oxide[i]	-	-	-

[a]Formulated to contained .8% Ca, and .65% P.
[b]Exp 1. diets contained in phase 1: 21% CP and 1.45% lysine and phase 2: 18% CP and 1.25 % lysine.
[c]Exp 2. diet contained 21% CP and 1.5% lysine.
[d]Provided per kg of complete diet: 2500 IU vitamin A, 250 IU vitamin D_3, 30 IU vitamin E, 2 mg riboflavin, 12 mg niacin, 15 µg vitamin B_{12}, 2 mg vitamin K_3, 0.5 mg thiamin, 0.45 mg pyridoxine and 8 mg pantothenic acid.
[e]Provided per kg of complete diet: 100 mg Zn, 100 mg Fe, 10 mg Cu, 10 mg Mn, 150 µg I and 300 µg Se.
[f]Antibacterial agent, 2.2% carbadox (Animal Health Co., NY).
[g]Antibiotic provided 100g chlortetracycline, 100g sulfathiazole and 50g penicillin per ton of feed.
[h] Microingredients included vitamin and trace mineral premix per kg, 11.36 IU vitamin A, 1.36 IU vitamin D_3, 50 IU vitamin E, 13.75 IU vitamin K, 4.54 mg menadione, .1 mg biotin, 3 mg folic acid, 28.4 pantothenic acid, 6.8 mg riboflavin, 25 mg vitamin B_{12}, 1 mg iodine, 160 mg Fe, 25 mg Mn, .3 mg Se and 160 mg Zn.
[i]For the 3000 ppm Zn diet, Zn was added at .41% and corn was removed for the addition of Zn.

Table 2. Chemical analysis of experimental diets (Exp. 1), ppm (as-fed basis)

Treatment and Phase	[Zn]	[Cu]	[Fe]
Exp 1: Phase 1			
LZn (150 ppm)	228.7	9.04	211.7
HZn (3000 ppm)	3050.5	6.37	232.9
Exp 1: Phase 2			
LZn (150 ppm)	240.3	5.89	214.0
HZn (3000 ppm)	2970	7.01	240.0

Table 3. Effect of pharmacological concentration of Zn on growth performance (Exp. 1)[a], g/d

	Dietary Treatments			
Parameter	LZn	HZn	SEM	P Value
d 0, kg	9.42	9.43	.36	.97
d 28, kg	18.14	19.2	.62	.37
ADG, g/d	308	345	40	.27

[a] Least square means are reported from 12 pigs per treatment.

Table 4. Effect of pharmacological concentrations of Zn on intestinal morphology (Exp. 1), micron (μm)

	Dietary Treatments			
Parameter[c]	LZn	HZn	SEM	P Value
Duodenum				
Villus length	64.5	77.4	5.4	.14
Crypt depth	28.0[b]	40.8[a]	2.7	.01
VCR	2.39	1.93	.22	.18
Brunner's gland	17.3	23.6	3.1	.19
Circular muscle	8.5	9.5	1.1	.57
Longitudinal muscle	4.67	5.5	1.0	.55
Total thickness	131.8[b]	165[a]	4.9	.001
Ileum				
Villus length	50.3	39.5	4.5	.12
Crypt depth	27.5	29.5	3.0	.65
Peyer's gland	93.8	92.0	7.6	.86
Circular muscle	17.3	19.2	1.6	.45
Longitudinal muscle	8.7	8.8	.71	.87
Total thickness	216.8	209.5	11	.67
Colon				
Mucosal thickness	47.5	52.4	3.7	.39
Circular muscle	9.7	12.8	1.6	.22
Longitudinal muscle	4.2	5.4	.73	.26
Total thickness	74.2	86.2	4.3	.07

[ab] Means in the same row lacking a common superscript differ significantly.

[c] Least square means are reported from six pigs per treatment.

Table 5. Effect of pharmacological concentrations of Zn on metallothionein concentration (Exp. 1), µg/g

	Dietary Treatments			
Parameter[c]	LZn	HZn	SEM	P Value
Liver	247.2[b]	2528.5[a]	50.7	.0001
Kidney	65.5[b]	462.4[a]	21.9	.0001
Intestine	20.7[b]	131.1[a]	14.1	.001

[ab] Means in the same row lacking a common superscript differ significantly.
[c] Least square means are reported from six pigs per treatment.

Table 6. Effect of pharmacological concentrations of organic and inorganic Zn on organ parameters (Exp. 2), µg/g (wet basis)

	Dietary Treatments					
Parameter[c]	Basal	ZnMet	ZnAA	ZnO	SEM	P Value
Intestine						
Zn	.267[b]	.217[b]	.288[b]	.792[a]	.16	.02
Cu	.068	.045	.103	.177	.045	.25
MT	98[a]	56.6[b]	71.3[b]	116[a]	11	.02
Liver						
MT	797.6[b]	940.3[b]	900.4[b]	5338.7[a]	283	.0001
Kidney						
MT	162.2[b]	144.1[b]	160.3[b]	527.4[a]	14.9	.0001

[ab] Means in the same row lacking a common superscript differ significantly.
[c] Least square means are reported from three pigs per treatment.

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