Aus dem Institut für Veterinär-Physiologie
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Interactions of zinc with the intestinal epithelium - effects on transport properties and zinc homeostasis

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der Freien Universität Berlin

vorgelegt von
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Tierärztin aus Kassel

Berlin 2015

Journal-Nr.: 3813

Gefördert durch die Deutsche Forschungsgemeinschaft und die H.W. Schaumann Stiftung

Gedruckt mit Genehmigung des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Dekan:	Univ.-Prof. Dr. Jürgen Zentek
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Deskriptoren (nach CAB-Thesaurus):
pigs, weaning, zinc, intestines, epithelium, jejunum, ion transport

Tag der Promotion: 15.09.2015

Bibliografische Information der *Deutschen Nationalbibliothek*
Die Deutsche Nationalbibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie; detaillierte bibliografische Daten sind im Internet über <http://dnb.ddb.de> abrufbar.

ISBN: 978-3-86387-656-2
Zugl.: Berlin, Freie Univ., Diss., 2015
Dissertation, Freie Universität Berlin
D 188

Choriner Str. 85 - 10119 Berlin
verlag@menschundbuch.de – www.menschundbuch.de

Für Oliver, Kilian und Elena

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List of Abbreviations

AE	Acrodermatitis Enteropathica
AGP	Antibiotic Growth Promoters
ASC	Amino Acid Transporter
ATP	Adenosine Triphosphate
Caco-2	Human Epithelial Colorectal Adenocarcinoma Cells
cAMP	Cyclic Adenosine Monophosphate
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
cGMP	Cyclic Guanosine Monophosphate
CXCL2α	Macrophage Inflammatory Protein-2-alpha
ETEC	Enterotoxigenic *E. coli*
GLUT2	Glucose Transporter 2
HeLa	Cervical Cancer Cells (derived from Henrietta Lacks)
Hsp	Heatshock Protein
IPEC-J2	Intestinal Porcine Epithelial Cell Line J2
LDH	Lactate Dehydrogenase
MT	Metallothionein
MTF-1	Metal-response Element Binding Transcription Factor-1
MUC4	Mucin 4
NHE3	Sodium-Hydrogen Exchanger 3
NKCC1	Sodium-Potassium-2 Chloride Cotransporter
PWD	Post-Weaning Diarrhoea
SCFA	Short Chain Fatty Acids
SGLT1	Sodium-/Glucose-Linked Co-Transporter 1
ST_p	*E. coli* heat-stable Enterotoxin
VIP	Vasoactive Intestinal Peptide
ZIP	Zrt-, Irt-like Protein Family (SLC39A)
ZIP4	Zinc Transporter SLC39A4
ZnT	Zinc Transport Protein Family (SLC30A)
ZnT1	Zinc Transporter SLC30A1
5-HT	Serotonin

Chapter 1: Introduction

Weaning is a crucial phase in modern pig production systems and a stressful event for the piglets (Martinez-Puig et al. 2007). The separation from the sow and the switch of the diet from milk to cereal-based diets are serious stress factors for the animals (Pluske et al. 1997). The intestinal immunological protection, previously generated by the sow's milk, fades away while the piglet's own immunity is not yet completely established (Blecha 1998). Regarding the intestinal barrier function, it is disturbed after weaning as the villous surface is reduced also implying a reduction in the absorptive capacity of the intestine (Wijtten et al. 2011). Thus, the piglet is temporarily affected by malabsorption and a nutritional undersupply (Nabuurs et al. 1996; Nabuurs 1998). This frequently results in infections by intestinal pathogens and the emergence of "*post-weaning diarrhoea"* (PWD). Decreased daily weight gain attributable to serious losses of electrolytes and water and increased piglet mortality are the consequences (Heo et al. 2013).

For many years, antibiotic growth promoters (AGP) have been used because of their positive effects on piglet performance, such as daily feed intake and weight gain (Broom et al. 2006). Since their ban within the EU in January 2006, the search for alternatives in the prevention of PWD has become essential and natural feed additives have been introduced with the expectation of evolving similar effects to those of AGP previously. In addition to specific enzymes, organic acids or probiotic compositions, zinc has particularly been used as an in-feed alternative to AGP. It is frequently applied as zinc oxide (ZnO), zinc sulfate ($ZnSO_4$) or zinc chloride ($ZnCl_2$) in high doses above the dietary requirements during the postweaning phase. The effects of these pharmacological zinc concentrations on piglet performance (Hill et al. 2001; Broom et al. 2006; Martin et al. 2013b), intestinal barrier functions (Rohweder et al. 1998; Sturniolo et al. 2002; Zhang and Guo 2009b), and bacterial populations (Fiteau and Tomkins 1994; Walsh et al. 1994; Bednorz et al. 2013) have widely been studied, whereas investigations concerning its immediate effects at the intestinal epithelium, in particular on absorptive and secretory transport processes, have mostly resulted in contradictory findings (Lee et al. 1989; Rodriguez-Yoldi et al. 1994; Carlson et al. 2004; Hoque et al. 2005; Feng et al. 2006; Carlson et al. 2008). Furthermore, the available results are difficult to compare because of differences in the applied zinc concentration, the age of the piglets/maturity of the intestinal cells or the duration of the zinc supplementation. Knowledge concerning the effects of zinc on absorptive transport properties is, on the one hand, important with regard to the uptake of nutrients into the organism, especially during weaning when absorption is generally reduced (Wijtten et al. 2011).

On the other hand, the influence of zinc on the secretory capacity of the intestinal epithelium is of similar importance because secretion essentially determines the severity of diarrheal disease. Thus, this thesis has focussed on the effects of dietary zinc administration on absorptive and secretory transport properties in the small intestinal epithelium of weaned piglets *ex vivo* after chronic or acute application of zinc and, furthermore, on the homeostatic mechanisms, barrier effects, and potential toxic effects that occur in intestinal epithelial cells *in vitro* when the extracellular zinc concentration is elevated.

Chapter 2: Literature review

Physiological and pathophysiological aspects of zinc:

Zinc is an essential trace element in the body of mammals and is involved in a variety of general cellular functions (Vallee and Falchuk 1993) and in the metabolism of nutrients (Hutterer 2006). It has multitudinous effects on the growth and development of the organism and on immune and nervous functions (Semrad 1999). As a regulatory, catalytic, and structural component, it regulates the activity of enzymes, transcription factors, and signaling molecules (Yamasaki et al. 2007). It modulates the stability of cell membranes by reducing peroxidative damage (Srivastava et al. 1995) and gives protection against the disruption of cells (Hennig et al. 1993) by maintaining the structure and function of the membrane barrier in several tissues (Rodriguez et al. 1996; Sturniolo et al. 2001; Lambert et al. 2004; Bao and Knoell 2006). The ubiquitous functions of zinc within the organism suggests that a deficiency or excess of zinc might lead to a generalized impairment of various metabolic functions (Hambidge 2000).

In zinc-deficient states, animals and humans have shown an increased susceptibility towards the structural and functional impairment of several organ systems, such as the epidermal, central nervous, and immune systems and, not at least, the intestinal tract (Hambidge and Walravens 1982). Clinical symptoms include anaemia, loss of appetite, reduced growth, immune dysfunction, developmental problems, and teratogenesis (MacDiarmid et al. 2000).

Severe zinc deficiency can be observed in humans suffering from *Acrodermatitis enteropathica (AE)*, a disease that has its origin in a mutation in the ZIP4 gene, leading to reduced intestinal zinc absorption. Thereby, symptoms such as dermatitis, alopecia (Braun et al. 1976; Danbolt 1979; Van Wouwe 1989), ophthalmological diseases, growth retardation, hypogonadism, alteration of immunological functions, and neuropsychiatric disorders (Braun et al. 1976; Danbolt 1979; Sehgal and Jain 2000) are evoked.

With regard to the intestinal tract, zinc deficiency often leads to diarrheal diseases and pathogenic shifts within the intestinal microbiota (Wapnir 2000). The stability of the intestinal microbiota has a tremendous impact on the non-specific resilience of an organism towards infectious diseases. Disturbances in the balance of the microbiota can lead to an excessive proliferation of indigenous and exogenous pathogenic bacteria, one consequence of which can be gastrointestinal disorders (Katouli et al. 1999). In addition, zinc depletion can lead to the disruption of the intestinal barrier integrity *in vitro* because of the delocalization of compounds of the intestinal tight junction, such as zonula occludens, occludin, beta-catenin, and E-cadherin (Finamore et al. 2008)

On the other hand, dietary zinc supplementation has been established to have beneficial properties on gut health, not only in human patients with AE (Mills 1989), but also in animals (see below). In *in vivo* studies with rats, zinc supplementation protects the colonic mucosa from acidic damage via the upregulation of heatshock protein (Hsp) 72 (Odashima et al. 2006), which belongs to a class of proteins that protect intestinal tight junctions from disintegration. Li et al. (2001) have further shown that high levels of dietary zinc increase the thickness of the small intestinal mucosa and the length of the microvilli by 25 to 50 %, thereby enhancing the mechanic und functional integrity of the intestinal barrier (Zhang and Guo 2009b). As a consequence of these properties, zinc is useful in the therapy of diarrheal diseases. In developing countries, the treatment of diarrhea in children has shown to be more effective if oral rehydration solutions are supplemented with zinc compared with those without this supplement (Sachdev et al. 1988; Dutta et al. 2000; Bahl et al. 2002).

As numerous as the essential positive functions of zinc are within the organism, so are the diverse possibilities regarding the way that zinc can interfere in biological processes and cause adverse negative effects when it is chronically administered in pharmacological concentrations (Maret and Sandstead 2006). As a consequence of zinc intake beyond the requirements, negative effects, especially on the immune system, have been recognized such as decreased cytotoxicity in natural killer cells and reduced phagocytosis in neutrophil granulocytes (Ibs and Rink 2003). Furthermore, the normal functions of T cells can be impaired, and B cells can undergo increased apoptosis (Telford and Fraker 1995; Ibs and Rink 2003). However, other organ systems are also afflicted with symptoms that are similar to those of zinc deficiency. Zinc can also induce neuronal death *in vivo* (Koh et al. 1996; Sensi and Jeng 2004) and distinctly decreases the viability of intestinal cells *in vitro* (Lodemann et al. 2013), indicating a cytotoxic potential at high concentrations (Cario et al. 2000; Zodl et al. 2003). The chronic intake of high dosages of zinc can induce a copper deficiency accompanied by a reduction of copper-dependent enzymes (Maret and Sandstead 2006). Insights such as these have gradually restrained the unrestrained use of pharmacological zinc concentrations, especially in human nutrition (Haase et al. 2008), and concerns have been raised regarding the unsolicited effects of zinc supplementation.

Zinc in piglet nutrition:

In the nutrition of piglets, zinc has been used as a feed supplement for many years because of its beneficial properties (see also the previous section) within several organ systems. Positive effects on the immune system (Fischer Walker and Black 2004) and skin health (Agren 1999) have been reported. The beneficial effects of zinc are especially considered around weaning

and with a focus on the gastrointestinal tract. Dietary zinc supplementation has positive effects on performance parameters of weaned pigs such as feed intake, feed conversion, and daily weight gain (Hahn and Baker 1993; Hill et al. 2001; Zhang and Guo 2009) and on the intestinal barrier and microbiota (Broom et al. 2006). High dietary zinc levels have been shown to reduce the incidence and severity of post-weaning diarrhoea and mortality attributed to enterotoxigenic *E. coli* (ETEC) infections in piglets (Poulsen 1995; Holm and Poulsen 1996; Huang et al. 1999; Owusu-Asiedu et al. 2003; Fairbrother et al. 2005). The last-mentioned might be correlated with the fact that the damage to intestinal cells by pathogenic bacteria, such as enteropathogenic *E. coli*, is reduced by zinc *in vitro* (Roselli et al. 2003). Under normal conditions, the intestinal microbiota is stable and composed of diverse groups of bacterial strains (Katouli et al. 1999). Zinc has proven "antibiotic" properties as it is able to inhibit many strains of *Staphylococcus aureus* (Walsh et al. 1994), endotoxins from *Salmonella typhi*, and hemolysins of *Aeromonas hydrophilia* (Fiteau and Tomkins 1994). However, zinc has the disadvantage of promoting an excessive growth of opportunistic and antibiotic-resistant populations of pathogens in the gut (Bednorz et al. 2013).

On the other hand, high levels of zinc supplementation can reduce the translocation of bacteria themselves from the small intestine into the gut-associated lymph nodes (Huang et al. 1999). Furthermore, the expression of inflammatory genes that are related to diarrhea induced by *E. coli*, such as macrophage inflammatory protein-2-alpha (CXCL2α), is reduced. Sargeant et al. (2010) have found that mucin 4 (MUC4) also shows lower expression in piglets fed a ZnO-supplemented diet. This is of crucial interest as MUC4 is strongly believed to act as a gene for one of the ETEC K88 receptors that are the site for fimbrial adhesion of ETEC strains (Jin and Zhao 2000) in pigs.

Several potential mechanisms exist for the role of zinc in improving post-weaning performance by reducing the severity of intestinal infections and supporting the innate immune system (Sargeant et al. 2010). Modes of action might involve effects on the microbiota of the gut (Li et al. 2001; Broom et al. 2006; Hedemann et al. 2006) or the intestinal barrier (Zhang and Guo 2009b) and its transport mechanisms (Carlson et al. 2004; Carlson et al. 2006). Despite this knowledge of zinc effects on gut health and performance in weaned pigs, the underlying mechanisms have not as yet been completely elucidated.

The mentioned beneficial effects result from zinc concentrations of 2500 ppm or more in the diet, despite the recommended zinc concentration in the feed of piglets being 80 to 100 ppm (NRC 1998). The high zinc concentrations used in such diets can be considered as a pharmacological level of zinc supply, as 150 ppm is the maximal concentration legally allowed within the EU

(EC.1334 2003). This limit has been set because of concerns regarding the potential for ecological damage attributable to an increased emission of zinc into the environment (European Union Register of Feed Additives pursuant to Regulation (EC) No. 1831/2003). Zinc might be an environmental threat to aquatic organisms and is one of the elements that frequently exceeds the water quality criteria in Europe and USA (Bodar et al., 2005).
The overall positive character of zinc is furthermore impaired because of recent studies establishing unsolicited effects of zinc supplementation on the organism itself, such as its cytotoxic potential and immunological alterations (see also the previous paragraph).

The tolerable level of dietary zinc is, amongst other factors, dependent on the source of this element (Jensen-Waern et al. 1998). In weaning pigs, dietary supplementation with 2000 ppm of zinc to the feed, supplied as zinc carbonate, leads to symptoms of zinc toxicosis accompanied by depressed feed intake and performance (Brink et al. 1959), whereas no such effects have been observed after feeding the same levels of zinc as zinc oxide (Hsu et al. 1975; Damgaard Poulsen 1989). Today, the common zinc source for supplementing diets is zinc oxide.
Although Holm and Poulsen (1996) have not observed toxic effects in piglets until a concentration of 3000 ppm zinc oxide in the diet, increased mortality rates occur in feed containing 4000 to 8000 ppm zinc (Suttle 2010). Furthermore, Lodemann et al. (2013) have elucidated detrimental effects of zinc on cell viability as measured by lactate dehydrogenase (LDH) leakage, ATP production, and the conversion of the cell proliferation agent WST-1 *in vitro*. These effects primarily occur at high zinc concentrations of 200 µM $ZnSO_4$, which is a concentration comparable with that present at the apical side of small intestinal cells after feeding with extremely high zinc levels. However, studies assessing the exact concentration of zinc after feeding pharmacological zinc levels are very rare. For comparison, Dintzis et al. (1995) have determined the concentration of free zinc in the human jejunum to range between 50 and 80 µM. Cragg et al. (2002) have calculated the concentration of zinc after a meal containing an average amount of zinc to be 100 µM in the human intestinal lumen. In piglets supplemented with high zinc doses (2425 ppm), jejunal concentrations of 152 µM zinc after 6 days of exposure (32 d old piglets) and 232 µM zinc after 28 days of exposure (54 d old piglets) have been observed (Pieper et al. 2013).
In turn, the plasma levels of zinc are less variable, even when piglets are fed pharmacological concentrations of zinc. When fed with a diet containing 2000 ppm zinc (Carlson et al. 1999; Carlson et al. 2007) or 2500 ppm zinc (Pieper et al. 2012), piglets show a plasma zinc concentration of 23 µM, whereas 100 ppm zinc in the diet leads to values of 15.3 µM zinc in the plasma of piglets (Feng et al. 2006).

Although the zinc plasma pool is very small, it is highly mobile and immunologically very important (Favier and Favier 1990). However, an assessment of the actual zinc status is complex even in human nutrition, as measurement techniques are limited in sensitivity and specificity (Maret and Sandstead 2006). Nevertheless, what applies to human nutrition is also valid for the zinc supplementation of pigs. Zinc supplementation studies are difficult to compare because of many reasons. Individuals with a different zinc status will probably react differently to supplementation as the zinc demand also depends on sex and age. The chemical composition of the supplement also plays a role, as zinc interacts with other nutrients within the diet. Therefore, the exact ranges between a safe or an unprofitable high zinc intake remain narrow (Maret and Sandstead 2006).

Effect of zinc on intestinal epithelial transport properties:

Apart from effects on barrier function or the intestinal microbiota, zinc can also have effects on transport properties such as secretion or absorption in the intestine.

- **Effect of zinc on chloride secretion in the intestine and diarrhea:**

The secretion of water and electrolytes is a central physiological function of the intestine (Barrett and Keely 2000); for example, a large amount of bicarbonate can be secreted into the proximal duodenum to protect it from gastric acid and pepsin (Hogan et al. 1994). When diarrhea begins, the secretion of ions and water into the intestinal lumen constitutes an important unspecific defence mechanism against pathogens (El Asmar et al. 2002). Under physiological conditions, absorption predominates the endogenous secretion (Montrose et al. 1999). Both absorption and secretion occur along the crypt-villus axis, with absorption mainly in villi, and with secretion mainly in crypts (Thiagarajah and Verkman 2012). Although bicarbonate and potassium are secreted along the whole intestine, the predominant electrolyte that drives fluid secretion is chloride (Barrett and Keely 2000). It is transported from the basolateral to the apical side of enterocytes. The basolateral uptake of chloride into the cell occurs electroneutrally *via* the sodium-potassium-2 chloride cotransporter (NKCC1). The sodium gradient driving the cotransporter is maintained by the sodium-potassium ATPase (Blikslager et al. 1999b) and is the basis for the transport of chloride *via* the NKCC1. Secretory epithelia store chloride far above the electrochemical equilibrium (Foskett 1990). The chloride transport *via* the apical membrane is predominantly enabled by the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP- and cGMP-dependent chloride channel (Loffing et al. 1998; Riordan 2005). Upon the opening of the CFTR channel, the transcellular secretion of chloride ions is followed by

paracellular sodium ion secretion (Dawson 1991) and, because of the resulting osmotic gradient, by water secretion into the gut (Pappenheimer and Reiss 1987; Agre 2004).

Chloride secretion can be activated by at least three different pathways:
a) by activating adenylate cyclase and increasing the cAMP level (Blikslager et al. 2001b),
b) by activating the cGMP system (Field 2003),
c) by increasing the intracellular calcium level (Leonhard-Marek et al. 2009).

Whereas cAMP and cGMP can act directly on CFTR to increase chloride secretion (Golin-Bisello et al. 2005), elevations in the intracellular Ca^{2+} concentration stimulate chloride secretion predominantly indirectly *via* the activation of epithelial K^+ conductances, thus resulting in an increased electrical driving force for chloride secretion (Schultheiss and Diener 1997). Diarrhea can occur if intestinal electrolyte absorption is impaired and/ or secretion is enhanced ("secretory diarrhea") or if osmotically active substances such as glucose cannot be absorbed because of the destruction of epithelial cells, as is the case for rotavirus infections ("osmotic diarrhea") (Schiller 1999). The "post-weaning diarrhea" of piglets is an acute process and is mainly attributable to the first mechanism. Secretory diarrhea can result from bacterial toxins (e.g. *E. coli* heat-stable enterotoxin (ST_p)), a reduced absorptive surface area caused by villus atrophy, or luminal or circulating secretagogues (such as various hormones (e.g. 5-HT), drugs, and poisons) (Schiller 1999).
As a possible explanation for the antidiarrheal effect of zinc, decreased secretory responses to various secretagogues (e.g. serotonin, theophylline, and vasoactive intestinal peptide) have been assessed in previous studies *in vitro*, when zinc was applied at the basolateral side of intestinal epithelia taken from weaned piglets (Feng et al. 2006; Carlson et al. 2008). However, these results are not reproducible or are contradictory (Carlson et al. 2004; Feng et al. 2006) and difficult to compare, as variable results might be the consequence of different experimental setups (e.g., age of the piglets, zinc source, zinc concentration, time of exposure, and stimulating secretagogues *in vitro*).

- **Effect of zinc on absorptive transport in the intestine:**

The absorption of nutrients within the small intestine occurs *via* various transport systems. The apical absorption of glucose is mediated electrogenically *via* the sodium-glucose cotransporter 1 (SGLT1) (Breves et al. 2001; Wright et al. 2004). The SGLT1 is the major glucose transporter in the small intestine and is located apically (Wright 1993). This transport mechanism is sodium-coupled (Schultz and Curran 1970; Reuss 2000), so that two sodium ions are transported

together with one molecule of glucose with Michaelis-Menten kinetics (Holtug and Skadhauge 1991; Mackenzie et al. 1994; Breves et al. 2000).

Predominantly, the absorption of glucose occurs within the villus cells (Stewart and Turnberg 1987). With the aid of facilitated diffusion, glucose is then discharged basolaterally by the glucose transporter 2 (GLUT 2) out of the cell (Wright 1993; Au et al. 2002; Wright et al. 2004). Glucose is transported electroneutrally by GLUT 2 without a coupled ion (Thorens 1993).

For a short time, e.g., after a meal rich in carbohydrates, the glucose transported by SGLT1 also promotes the integration of GLUT2 into the apical membrane and thereby enhances absorption up to three-fold (Kellett and Brot-Laroche 2005).

Studies investigating the effect of zinc on the absorption of glucose are contradictory. With increasing concentrations of zinc, a decreased glucose absorption has been observed in the intestine of rats and pigs in previous *in vitro* and *in vivo* studies (Lyall et al. 1979; Watkins et al. 1989; Yoldi et al. 1992; Rodriguez-Yoldi et al. 1994). In accordance, Southon et al. (1984b) have observed increased glucose absorption in zinc-deficient rats. In contrast, Lee et al. (1989) have shown that zinc absorption is enhanced by glucose, and conversely, that zinc addition raises glucose uptake in the human small intestine. However, some studies have been unable to show any effect on this transport mechanism (Carlson et al. 2004; Hoque et al. 2005).

The second transport mechanism examined within this thesis is the absorption of the amino acid L-glutamine. Amino acids, such as L-glutamine, are also absorbed in a sodium-coupled manner in the small intestine (Rhoads et al. 1994; Kandil et al. 1995; Blikslager et al. 1999a; Blikslager et al. 2001a). The major part of L-glutamine is absorbed across the apical membrane *via* the sodium-dependent amino acid transporter (ASC) (Munck et al. 2000), and the rest *via* two further transport systems called N and B_0 (Bode 2001). Different from SGLT 1, ASC is found not only in the villus cells, but also in the crypts (Blikslager et al. 2001a). The discharge of L-glutamine at the basolateral membrane of the cell occurs sodium-independently, probably *via* facilitated diffusion (Blikslager et al. 2001a).

With regard to glucose, studies investigating the intestinal absorption of L-glutamine after zinc supplementation have delivered contradictory results in other species (Boldizsar and Simon 1981; Monteilh-Zoller et al. 1999), but to date no studies have been conducted on this topic in the pig.

Zinc transport and mechanisms to regulate zinc homeostasis:

In continuation to the first part of the thesis, the second part focusses on the mechanisms by which intestinal epithelial cells maintain zinc homoeostasis when increasing concentrations of zinc are applied and the way that zinc transporters and factors that regulate zinc levels within the cell are influenced.

Physiological cell functions are incompatible with both excessive zinc accumulation and zinc deficiency. Therefore, in mammals, zinc homeostasis involves the regulation of zinc uptake from the ingesta, the distribution of zinc within the organism, and the secretion of surplus zinc through the bile and the urine (Krebs and Hambidge 2001).

The main sites of zinc absorption are the duodenum and the jejunum. The bioavailibility of the zinc source plays a decisive role for the efficiency of absorption. Zinc oxide is usually used for dietary zinc supplementation in farm animals. At neutral pH, it is extremely insoluble, but when pH decreases, as is the case in the stomach and the proximal small intestine, its solubility is increased. Zinc in the intestinal lumen is derived from two sources: the diet (about 10 mg/d) and the digestive juices (about 3 mg/d). The pancreatic juice contains the highest concentration of zinc and might possess a ligand that promotes the absorption of zinc in the intestine (Semrad 1999). The gastrointestinal tract plays an important role in the exchange of zinc between the organism and the environment (Wang and Zhou 2010) and is placed at a critical position to control zinc homeostasis (Roselli et al. 2005). Thus, enterocytes are exposed to remarkably high extracellular zinc concentrations, leading to the necessity of tightly regulated intestinal influx and efflux mechanisms for maintaining zinc homeostasis.

Common markers for assessing the zinc status in animals are, for example, the zinc plasma concentration or the intracellular zinc concentration in specific organs (liver, pancreas, bone, kidney, or hair). However, these contents are strongly variable because of homeostatic regulatory mechanisms; thus, in order to acquire a more precise zinc status, several laboratory values should be combined (Suttle 2010). **The intracellular zinc concentration** is often used to assess the zinc status of the cell. Up to certain threshold values of extracellular zinc concentration (100 µM) and time of exposure, the intracellular zinc concentration is essentially unchanged *in vitro* (Haase and Beyersmann 1999). At higher zinc concentrations, increased levels in intracellular zinc have been measured, but they do not necessarily have toxic effects on the cells. Only when extracellular zinc is raised above 200 µM has a loss in viability of rats glioma cells been observed (Haase et al. (1999). These observations suggest the existence of a regulated system being responsible for maintaining zinc homeostasis within the cell.

Zinc homeostasis is achieved by the coordinated regulation of zinc uptake into the cell, intracellular compartmentalization, and zinc efflux, whereby several zinc transporters are responsible. Mammalian zinc transporters are mainly found within two gene families that traffic zinc actively across biological membranes:
a) the ZnT proteins (solute-linked carrier family 30 (SLC 30)) and
b) the ZIP (Zrt- and Irt-like proteins) family (solute-linked carrier family 39 (SLC 39))

ZnT and ZIP proteins appear to have opposite roles for zinc homeostasis (Liuzzi and Cousins 2004), as ZnT transporters mediate zinc efflux out of the cytosol, and the ZIP transporter family mobilizes zinc to regulate its influx, for example from the intestinal lumen (Eide 2004; Palmiter and Huang 2004). Up to now, many studies have been conducted investigating the regulation of zinc transporters in rodent models, but porcine studies are still rare (Hill and Link 2009; Martin et al. 2013a). The functional analysis of zinc transport is crucial in elucidating the underlying mechanisms of zinc homeostasis. In addition to cell membrane-associated zinc transporters, mobile ligands such as metallothioneins exist that, together with the zinc transporters, form an integrated system of zinc homeostasis aimed at preventing toxic zinc accumulation within the cell.

Zinc transporter 1 (ZnT1, SLC30A1) is located at the basolateral membrane of tissues involved in zinc acquisition or recycling, such as intestinal epithelial cells (McMahon and Cousins 1998; Yu et al. 2007). In addition, ZnT1 is also found in the renal tubular epithelium, placenta (Liuzzi and Cousins 2004), and tonsils (Tran et al. 2009). ZnT1 lowers intracellular zinc concentrations by mediating zinc efflux from cells or influx into intracellular vesicles (Lichten and Cousins 2009). It is more abundant in the intestinal villus than in crypt cells, and its abundance is highest in the small intestine, where it can participate in the transfer of zinc to the circulation (McMahon and Cousins 1998). ZnT1 distribution can, however, vary within enterocytes (Liuzzi and Cousins 2004). For example, in lactating rats, intestinal ZnT1 is found in vesicles at the apical surface at day 1 of lactation, whereas at day 14 of lactation, ZnT1-containing vesicles are equally distributed at the apical and basolateral side of enterocytes (Liuzzi et al. 2003). This variation in localization might be a reflection of transporter-mediated zinc trafficking responding to physiological stimuli (Liuzzi and Cousins 2004).
In states of zinc deficiency, contradictory results have been obtained regarding ZnT1 mRNA expression. Whereas it is not affected in the small intestine of rats (McMahon and Cousins 1998; Liuzzi et al. 2001), it is upregulated in rat colon (Pfaffl and Windisch 2003). By contrast, ZnT1 mRNA expression is reduced in the pancreas and small intestine of zinc-deficient mice (Liuzzi et

al. 2004). These results suggest organ-specific differences in the regulation of zinc balance. When the zinc supply is high, transcript levels of ZnT1 have been shown to be upregulated in rat small intestine and kidney (Liuzzi et al. 2001) and in porcine jejunum (Martin et al. 2013a). This type of zinc responsiveness is similar to that of metallothionein, although ZnT1 shows a smaller extent of changes in mRNA expression (Cousins et al. 2003).
Cells overexpressing the ZnT1 gene are able to grow and differentiate in the presence of high extracellular zinc concentrations by promoting zinc efflux activity, i.e., the overexpression of ZnT1 allows zinc resistance in an otherwise zinc-sensitive cell line (Palmiter and Findley 1995). Conversely, embryonic lethality has been observed in ZnT1-knockout mice. The latter suggests that ZnT1-mediated zinc transport is crucial for normal development (Cousins and McMahon 2000; Andrews et al. 2004).

Zinc transporter 4 (ZIP4, SLC39A4) is localized at the apical membrane, most notably in the small intestine (Wang et al. 2002; Dufner-Beattie et al. 2003). ZIP4 is the opponent of ZnT1 that is involved in the transport direction of zinc, as it mediates zinc influx into the cell with high specificity and saturable kinetics (Dufner-Beattie et al. 2003). Its expression is regulated at both transcriptional and post-transcriptional levels, and activation leads to an increase in the intracellular zinc concentration (Liuzzi and Cousins 2004). In zinc-deficient states, the expression of ZIP4 has been shown to be upregulated in the small intestine of mice (Dufner-Beattie et al. 2003; Liuzzi et al. 2004), whereas zinc supplementation leads to its downregulation in the small intestine of rats (Fujimura et al. 2012). Previous studies in mouse intestine and mouse hepatoma (Hepa) cells have provided an insight into the zinc responsiveness of ZIP4 expression. For example, Weaver et al. (2007) have shown that the zinc status has only a limited impact on the transcription of ZIP4 but instead affects the stability of ZIP4 mRNA. At the protein level, ZIP4 is degraded at high extracellular zinc levels (Mao et al. 2007).
ZIP4 has been localized to the same chromosomal region as the disease acrodermatitis enteropathica (AE) (Wang et al. 2002). As several mutations in this gene have been found in patients with AE (Wang et al. 2001; Kury et al. 2002; Wang et al. 2002), it has been identified as the responsible gene for this rare, human, zinc-malabsorption disease (Bleck et al. 2001; Kury et al. 2001; Nakano et al. 2002). The supplementation of oral zinc to those patients has effectively been established to overcome their zinc deficiency (Mills 1989). A corresponding disorder attributable to a hereditary ZIP4 mutation has been observed in cattle (Weismann and Flagstad 1976; Krametter-Froetscher et al. 2005; Yuzbasiyan-Gurkan and Bartlett 2006; Siebert et al. 2013), but an acquired form of this disorder also exists (Moynahan 1974). Prompt treatment with

oral zinc alleviates all clinical symptoms and leads to normal development (Yuzbasiyan-Gurkan and Bartlett 2006).

An additional zinc-homeostatic mechanism is **metallothionein (MT)**, a protein with a very high content of cysteine. Metallothionein is a mobile ligand and can be induced when the extracellular zinc level exceeds a threshold in concentration or time of exposure. All vertebrates contain two or more MT isoforms designated MT-1 through MT-4 (Hijova 2004). MT regulates the level of free intracellular zinc by strongly binding and buffering zinc molecules in the cytosol (Jiang et al. 1998; Colvin et al. 2008). It is increased after zinc supplementation (Martinez et al. 2004; Martin et al. 2013a). MT can bind up to seven zinc atoms, and when isolated from tissues, it is saturated with zinc or with zinc in combination with other metals (Muraina 2013). The metal-binding activity of MT also enables protection against oxidative stress and heavy-metal induced toxicity (Cousins et al. 2006). However, as has been shown *in vivo* and *in vitro*, cells that are not able to synthesize any MTs and mice that cannot build MT-1 or MT-2 show only marginally increased sensitivity towards zinc toxicity (Kelly et al. 1996). Nonetheless, MTs take a critical position within zinc metabolism. Studies with transgenic mice have revealed that MT first protects cells from toxic zinc accumulation by producing complexes with zinc, and that, second, MT provides a driving force for zinc-uptake by the transient production of apo-MT (Suhy et al. 1999). This indicates that MT possesses functions as a chaperone for synthesizing metalloproteins (Hijova 2004). The promoter region of MT contains metal-response elements (MREs) and the **MRE- binding transcription factor-1 (MTF-1)** has been shown to bind to these MREs as a transcriptional factor (Stuart et al. 1985; Radtke et al. 1993).
The binding of the MTF-1 is required for both the basal and the heavy metal-induced expression of MT (Heuchel et al. 1994) and for ZnT1 gene transcription (Palmiter and Findley 1995; Langmade et al. 2000).
MTF-1 is a key regulator of zinc in higher eukaryotes and has been suggested to act as a cytoplasmic metal sensor (Gunther et al. 2012) based on the observation that its DNA-binding activity responds to changes in free zinc levels within the cell (Dalton et al. 1997; Bittel et al. 1998). Zinc seems to stimulate the nuclear translocation of MTF-1 in reticulocytes of mice (Smirnova et al. 2000) and in human cervical cancer (HeLa) cells (Otsuka et al. 2000). Furthermore, evidence has been provided that MTF-1 plays a central role in the detoxification of heavy metals (Egli et al. 2003). However, the exact mechanisms by which it senses elevated metals to activate the transcription of genes related to zinc homeostasis control are not completely understood (Palmiter and Huang 2004).

Chapter 3: Aims and objectives of the thesis

High oral zinc doses have been shown to have specific effects within the intestinal tract of humans and animals. Its beneficial effects during piglet weaning have been investigated in several studies. However, the positive effects on the transport and barrier functions of the intestinal epithelium have only been descriptively characterized, and the results are sometimes contradictory. The underlying mechanisms of zinc homeostasis and signaling pathways mediating the effects of zinc are still unknown or only partially characterized in porcine tissues or cells.

The main goal of the present studies has been the characterization of the effects of increasing concentrations of zinc on epithelial functions and underlying mechanisms. The present thesis presents a comprehensive approach investigating the direct effects of zinc on absorptive and secretory transport in the porcine jejunum and on processes and signaling pathways that regulate zinc homeostasis in intestinal epithelial cells *in vitro*.

The following specific issues have been examined in relation to increasing zinc concentrations:

- secretory functions (e.g., Cl^- secretion) and signaling pathways
- absorptive functions (e.g., glucose transport, amino acid transport)
- paracellular permeability *in vitro* (in cooperation with S. Zakrzewski)
- effects on viability
- mechanisms regulating zinc homeostasis

In addition to increasing zinc doses, the influence of various maturation status or the age of the piglets and the chronic versus the acute application of zinc have been considered. The effects of high concentrations of zinc on intestinal epithelial transport and barrier properties have been examined in tissues from control piglets and from piglets supplemented with zinc at various doses during the first two weeks after weaning and in a porcine and a human intestinal cell culture model.

Experiments have been conducted

(1) to assess the effect of three increasing dietary zinc supplementation levels on both absorptive and secretory transport properties at the porcine jejunal epithelium of weaned piglets aged 32, 39, 46, or 53 days.

In Ussing chamber experiments, the secretory capacity of the intestinal epithelium in response to various secretagogues has been tested, each stimulating secretion *via* a different mode of

activation. The effect of zinc on L-glutamine and D-glucose absorption has been assessed in absorptive studies. As the absorption of nutrients at the intestinal epithelium has been shown to be generally decreased after weaning, a potentially increasing effect on absorption might compensate the transient nutritional undersupply of the pig, whereas a reducing effect might aggravate it.
To dissociate the long-term effects of zinc feeding from short-term effects, isolated intestinal epithelia from all zinc supplementation levels and age groups have additionally been tested for the effects of an acute serosal application of zinc *in vitro*.

(2) to study the effect of increasing zinc concentrations on the intracellular zinc concentration of porcine (IPEC-J2) and human (Caco-2) intestinal epithelial cells. As transport processes were influenced by the age of the animal, i.e. different states of intestinal differentiation in the first study, the question was addressed as to whether cells of different maturation (preconfluent vs. postconfluent) are differentially affected, and whether the time of exposure (6h or 24 h) or application side (apical vs. basolateral) of zinc exert an impact.
The intracellular zinc concentration, viability, barrier function, the mRNA and protein expression of the homeostatic zinc transporters 1 (ZnT1) and 4 (ZIP4) and of metallothionein (MT), and the mRNA expression of the metal-response element-binding transcription factor 1 (MTF-1) and the chaperone heat shock-protein 70 have been measured.

Chapter 4: Effects of Age and Zinc Supplementation on Transport Properties in the Jejunum of Piglets

This chapter has been published in: Journal of Animal Physiology and Animal Nutrition
Manuscript received at the Journal of Animal Physiology and Animal Nutrition: April 26, 2013
Initial review completed: November 9, 2013
Revision accepted: June 30, 2014

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http://www.ncbi.nlm.nih.gov/pubmed/25039419
DOI: 10.1111/jpn.12232

Chapter 5: Regulation of intracellular Zinc homeostasis in two intestinal epithelial cell models at various maturation time points

This chapter has been published in: *The Journal of Physiological Sciences*
Manuscript received at the Journal of Physiological Sciences: November 14, 2014
Initial review completed: December 15, 2014
Revision accepted: February 20, 2015

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http://www.ncbi.nlm.nih.gov/pubmed/25757458
DOI: 10.1007/s12576-015-0369-4

Chapter 6: Discussion

Weaning represents a challenging period for young piglets. The animals are, on the one hand, separated from the sow and mixed with piglets from other litters, a change that involves social stress, and, on the other hand, the rapid switch in the dietary composition from milk to cereals frequently overstrains their physiological capacity of adaptation (Pluske et al. 1997; Blecha 1998). Increased susceptibility towards intestinal infections, decreased feed intake, and impaired immune functions are the consequences. Therefore, weaning is associated with a high incidence of PWD, which leads to high economic losses and issues of animal welfare. After the ban of AGPs in 2006 because of the increasing rates of antibiotic-resistant bacterial strains, manifold attempts have been made to find alternatives that exert assimilable effects on performance parameters and diarrhea incidence in piglets. Dietary zinc supplementation has been one of the promising approaches. As an in-feed ingredient, it has proven to improve the daily feed intake and daily weight gain and to decrease the occurrence and severity of PWD in piglets (Poulsen 1995; Owusu-Asiedu et al. 2003; Fairbrother et al. 2005). However, these positive effects are subject to restrictions as, for example, growth-promoting effects can only be observed within a time period of two weeks of administration (Martin et al. 2013a). Many studies have been conducted to gain further knowledge about the underlying mechanisms of these zinc effects. However, the ways by which zinc influences gut health directly at the intestinal epithelium and intracellular zinc homeostasis is maintained, even under pharmacological supplementation levels, are not fully understood.

In the feeding trial of the present study, the effects of zinc supplementation were examined in three different concentrations in piglets up to four weeks after weaning. The focus was on the absorptive and secretory capacities in the intestine. Because the former studies showed contradictory effects, we chose a broad approach that assessed absorption with L-glutamine and D-glucose and three different modes of activated Cl secretion. To consider the influences of intestinal maturity further, all effects were evaluated in four different age groups.

No sustained effect of zinc feeding was observed on the absorptive and secretory capacity of the porcine jejunum; however, the effects on transport properties were shown to depend instead on the acute presence of zinc. Previous studies that examined the antisecretory effects had shown that these effects were mainly seen with the basolateral addition of zinc (Hoque et al. 2005; Carlson et al. 2008; Bzik et al. 2012). Therefore, an unpublished work not included in the present thesis, also aimed at elucidating the effects of an apical or basolateral application of zinc *in vitro* (Lodemann et al. 2015). Barrier function and viability decreased, and metallothionein and

HSP70 expression increased, most prominently when $ZnSO_4$ was added in high concentrations at the basolateral side. Thus, higher zinc concentrations elicit effects on absorption, secretion, viability, and epithelial integrity predominantly when added acutely at the basolateral side compared with apical addition.

To limit the negative effects on viability and tissue integrity, the zinc concentration for the animal and within the cells has to be regulated. Thus, mechanisms regulating zinc homeostasis are necessary, including zinc transporters for uptake and efflux and zinc-binding proteins such as the metallothioneins. In a comparison manuscript coauthored by the doctoral candidate Eva-Maria Näser (not included in the present thesis), the expression of the efflux zinc transporter 1 has been shown to be upregulated, both in animals supplemented with 2500 ppm of zinc and in intestinal epithelial cells at 200 µM of zinc (corresponding to zinc levels in gut lumen after supplementing piglets 2500 ppm zinc) (Martin et al. 2013a). Zinc uptake by zinc transporter 4 was concomitantly downregulated. Metallothionein was also increased at the mRNA and protein level in either animals or epithelial cells by high zinc concentrations. However, the homeostatic mechanisms could not prevent an accumulation of zinc in either jejunal tissues, bones, liver, or intestinal epithelial cells at the highest zinc concentrations applied; this confirms the conclusion that such treatment might impair performance during a longer supply. The latter is supported by the fact that the feeding of pharmacological zinc levels increases weight gain only during the first two weeks, whereas performance is lower in the third week of zinc supplementation (Martin et al. 2013a; Martin et al. 2013b).

As the project was part of a collaborative research centre (SFB 852), many different aspects of zinc supplementation have been assessed and allow a broader picture to be integrated with our own results. Although positive effects on barrier function have been shown *in vitro* (Lodemann et al. 2015) and *in vivo* with human and rat small and large intestine (Rohweder et al. 1998; Sturniolo et al. 2001; Sturniolo et al. 2002), the piglets of the present collaborative feeding trial exhibited no changes in jejunal or colonic barrier function (Silke Zakrzewski, personal communication). In pancreas, a long-term supply of 2500 ppm ZnO increased zinc and metallothionein concentration and digestive enzyme activity but also triggered oxidative stress reactions, indicating the unbalancing of zinc homeostasis (Bondzio et al. 2012; Bondzio et al. 2013).

The supplementation with pharmacological zinc levels improved fecal scores (Chai et al. 2014) and weight gain during the first two weeks, but performance was lower in the third week (Martin et al. 2013a; Martin et al. 2013b). Regarding morphological aspects, villus height and crypt depth were not influenced by the dietary concentrations of zinc. However, the zinc

supplementation affected the innate and adaptive gut-associated immune system of piglets. High dietary zinc reduced the sulfomucins and increased the sialomucins in the crypts of the distal jejunum. It also led to a reduced abundance of CD8(+) γδ T-cells, resulted in the reduced gene expression of β-defensin 3, but did not affect the expression of TFF3 (Liu et al. 2014a; Liu et al. 2014b). Janczyk et al. (2013) further showed zinc supplementation at a pharmacological level (2500 ppm) provided no protection towards *Salmonella enterica* infections. Moreover, increased proportions of antibiotic resistant *E. coli* strains were observed by Bednorz et al. (2013).

Within the broader context of the large collaborative feeding trial, the experiments of the present thesis were intended to characterize the absorptive and secretory transport properties across the jejunal epithelium of piglets aged 32, 39, 46, or 53 days fed with either a marginal (50 ppm), a medium (150 ppm), or a pharmacological (2500 ppm) concentration of zinc oxide. Both absorption and secretion were stimulated with various substances; D-glucose and L-glutamine were used to study absorptive capacities, and secretion was stimulated by prostaglandin E_2, carbachol, or *E. coli* heat-stable enterotoxin.

The magnitude of the glucose-stimulated ΔI_{sc} in jejunal epithelia from control pigs was in accordance with other electrophysiological studies (Lodemann et al. 2006; Lodemann et al. 2008). No such reference data are available for glutamine in porcine jejunum as yet. The acute presence of 23 µM $ZnSO_4$ on the serosal side induced a small but significant reduction in both glucose- and glutamine-stimulated currents. In general, the L-glutamine-stimulated transport and the effect of zinc on this transport mechanism were weaker but comparable with those after stimulation with D-glucose.

Since the effect of chronic zinc feeding on glucose and glutamine transport processes was not statistically significant, the findings of Carlson et al. (2004) have been confirmed in which the supplementation of the diet of piglets with increasing concentrations of zinc had no effect on Na^+-dependent glucose transport. However, these results are in contrast to the observations of Lyall et al. (1979) who have revealed that the feeding of 200 mg $ZnCl_2$/kg bodyweight decreases the absorption of glucose in rat small intestine.

To date, no information is available regarding the effects of zinc feeding on L-glutamine transport, either in porcine tissues or in any other species. However, Lyall et al. (1979) have stated that zinc feeding has no effect on alanine transport in rats. The present results extend these observations and also confirm no persistent effect of chronic zinc feeding on L-glutamine transport.

As zinc deficiency might potentially also affect glucose and glutamine absorption, the lowest zinc level tested in the present study (50 ppm) was obviously sufficient to maintain a stable Na^+-dependent glucose and amino acid absorption. A generally higher absorptive reaction was observed in the Zn_{50} group after stimulation with D-glucose at day 32; this might be attributable to the proximity to weaning during which piglets might be more sensitive towards a slight zinc deficiency than at later time points. Accordingly, zinc deficiency has been described to increase glucose absorption *in vivo* (Southon et al. 1984a).

Different from the results of chronic dietary zinc administration, the acute addition of $ZnSO_4$ *in vitro* led to a significant decrease in glucose and glutamine absorption. This observation is in agreement with *in vitro* studies of small intestinal epithelia of pigs (Watkins et al. 1989), rats (Lyall et al. 1979), and rabbits (Yoldi et al. 1992; Rodriguez-Yoldi et al. 1994). By contrast, Lee et al. (1989) conducted perfusion studies on the small intestine and showed that glucose absorption in the human small intestine was increased by zinc, whereas Hoque et al. (2005) did not find any effect of zinc on glucose absorption in an *in vitro* study with ileal tissues of rats.

Similar results had been previously obtained by Lyall et al. (1979) with regard to the amino acid L-alanine, as its absorption is not affected by the administration of zinc *in vitro*. However, this is not reflected by our findings, which have shown that L-glutamine transport is significantly reduced by acute zinc addition *in vitro*, albeit the changes are very small.

The reasons for the partially contradictory observations in previous investigations and the present study remain unclear. As one possible explanation, however, the previous studies involved the use of millimolar zinc concentrations; this is far above the concentration that was used in the present study. The mechanisms of the inhibitory effect of short-term zinc application *in vitro* on absorptive transport processes are not as yet elucidated. Heavy metals have previously been hypothesized to decrease intestinal sugar transport by binding to chemical groups of the membrane protein and thereby influencing the cotransport system (Rodriguez-Yoldi et al. 1989; Yoldi et al. 1992; Mesonero et al. 1993). Since the sugar transport is driven by the transmembrane Na^+ gradient, zinc might also reduces the sugar uptake by decreasing the activity of the Na^+-/K^+-ATPase (Rodriguez-Yoldi et al. 1994). As decreasing effects on absorption have been observed after acute zinc addition *in vitro* but not after chronic zinc supplementation *in vivo*, zinc might influence the mechanisms controlling absorptive transport within the small intestine only as long as it is actually present. The reducing effect of zinc on absorptive transport processes appears initially to be contra-productive for the animal, as Wijtten et al. (2011) have observed a general decrease in absorptive transport after weaning; this might be amplified by the effect of zinc. However, over the total length of the porcine intestine, this might also be compensated in distal parts of the gut. Herrmann et al. (2012) have suggested

that the porcine ileum plays a more important role in glucose absorption than the porcine jejunum. On the further assumption that the decreased small intestinal absorption increases the concentration of non-absorbed carbohydrates in the large intestine, the production of short-chain fatty acids (SCFA) by hindgut bacteria might also increase. Ramakrishna et al. (2000) have observed that an increased production of SCFA in the colon after a surplus supply of non-absorbed carbohydrates enhances colonic fluid absorption and, thereby, makes oral rehydration solutions more efficient in the therapy of acute diarrhea. These results suggest, in turn, that the reducing effect of zinc on absorptive transport might have an indirectly diarrhoea-preventive effect by stimulating colonic SCFA production.

Similar to the absorptive transport properties, no significant effect of chronic zinc supplementation has been observed on secretory transport processes. Only *E. coli* toxin-induced secretion tends to be slightly decreased with higher levels of zinc supply, but a significant feeding effect has not been established. The missing effect of chronic zinc supplementation on the secretory capacity of porcine jejunum is in contradiction to the data of Carlson et al. (2004) who have found significantly reduced secretory responses towards theophylline and serotonin (5-HT) when piglets were fed a diet containing 2500 ppm ZnO compared with 100 ppm ZnO. Unfortunately, no information is available in the literature concerning the effect of dietary zinc on PGE_2-stimulated Cl secretion. Secretion induced by the *E. coli* heat-stable enterotoxin is numerically lowest in the piglets supplemented with the highest zinc concentration (Zn_{2500}), especially in the three younger age groups (32 d, 39 d, and 46 d). This effect was not statistically significant but seems to be in agreement with results of the infection experiments of Owusu-Asiedu et al. (2003). Piglets that were inoculated with *E. coli* K88 strains and simultaneously treated with high doses of dietary zinc needed less time to recover from diarrhoea than the control group. As such, the decreased ST_p-induced secretion during high zinc supply in our study might plausibly explain the anti-diarrheal efficacy of zinc in the challenge trial of Owusu-Asiedu et al. (2003). Alternatively, the tendency for reduced secretion might also be explained by the ability of zinc to decrease the permeability of the intestinal tissue by enhancing the expression of zonula occludens protein-1 and occludin (Zhang and Guo 2009a). The latter might prevent bacterial toxins permeating paracellularly through the intestinal epithelium and, therefore, might diminish ion and water secretion into the intestinal lumen.

The results after the acute zinc addition *in vitro* gave a clearer picture than those after chronic zinc feeding. Basolateral zinc significantly reduced Cl secretion stimulated by PGE_2 and *E. coli*

heat-stable enterotoxin, whereas it only numerically reduced carbachol-elicited secretion. This inhibitory effect might play a decisive role in the effectiveness of zinc as an anti-diarrhoeal agent. Previous studies have shown that the presence of zinc at the basolateral side of the epithelium reduces secretion induced by secretagogues such as 5-HT, vasoactive intestinal peptide (VIP), and theophylline in pigs *in vitro* (Carlson et al. 2006). Furthermore, Canani et al. (2005) have found that zinc at a concentration of 35 µmol/l reduces cholera toxin-induced secretion and cAMP concentrations in Caco-2 cells. However, in contrast to our results, Canani et al. (2005) have not observed a reducing effect of zinc on ST_p-induced ion secretion and cGMP levels in Caco-2 cells. Nevertheless, this does not really imply a contrast to our results, as the relative inhibition of secretion was very weak, even though statistical significance was reached in the present study.

With regard to carbachol, acute treatment with zinc resulted in a numerically reduced secretion by about 9 %, but this effect was not significant. This is in contrast with the previous findings that serosal zinc *in vitro* leads to a significant reduction of carbachol-induced ion secretion in the small intestinal epithelium of piglets (Carlson et al. 2006) and in Caco-2 cells (Berni Canani et al. 2010), although secretion is at least numerically reduced in the present study. However, the results of the present study are in accordance with a study of the intestinal tissues of rats; this suggests that zinc has a significantly inhibitory effect only on cAMP-activated chloride secretion but not on calcium-mediated secretion. In a previous study, serosal zinc application significantly decreased the response to forskolin and 8-BrcAMP but not to carbachol (Hoque et al. 2005). This agrees with our results, since PGE_2-induced secretion (cAMP-activated) is significantly reduced by zinc; this is not the case for carbachol (calcium-mediated). However, observations in human colonic mucosa have established that zinc inhibits PGE_2-mediated and also carbachol-induced secretion *in vitro* (Medani et al. 2012).

The mechanisms behind the reduced secretory responses caused by zinc administration *in vitro* might rely on the finding that zinc selectively inhibits K^+ channels that are activated by cAMP (Hoque et al. 2005), indicating that this effect is independent of the production of cAMP itself (Medani et al. 2012). Indeed, the magnitude of secretory inhibition after stimulation with diverse secretagogues differed between the several studies, but, ultimately, each study showed reduced responses to all kinds of secretory transduction pathways. This kind of non-specific inhibition is comparable with other K^+ channel-blocking substances such as loperamide (Taylor and Baird 1995) or clotrimazole (Rufo et al. 1997). These drugs inhibit Cl secretion independently of the stimulus.

The results of the *ex vivo* studies confirmed but also contradicted results of previous studies, both regarding absorptive and secretory transport properties. An explanation for the lack of chronic effects of dietary zinc supplementation on the transport properties might, on the one hand, be that the high variability between animals masked potential effects. On the other hand, zinc might exert its effects in dependence on the duration of administration. When also considering the observations of Martin et al. (2013a), it is proposed that zinc effects might only be present for short-term periods.

With regard to the contradictory results after acute serosal application of zinc in the previous and the present *in vitro* studies, one possible explanation might be the wide range of zinc concentrations used. For example, in some secretory studies, zinc concentrations of 23 µmol (Carlson et al. 2006; Carlson et al. 2008) have been used as in our study, but higher concentrations from 35 µmol/l (Berni Canani et al. 2010) up to even 1 mmol/l (Hoque et al. 2005) have also been applied. A further point might be the choice of the various secretagogues and secretagogue concentrations, which can cause diverse effects, even when acting *via* the same activation pathway.

In addition, most studies (including the present one) have focussed on zinc effects on electrogenic pathways, but electroneutral mechanisms might also be involved in the reducing effect of zinc on secretory responses and its positive effects on diarrhea. In the emergence of diarrhea, not only chloride ions are secreted, but also the electroneutral NaCl absorption is inhibited (Donowitz and Welsh 1986). The Na^+/H^+ exchanger 3 (NHE3) takes on an important role in electroneutral NaCl absorption. Hoque et al. (2009) have shown that zinc increases NHE3 activity, and conversely, the inhibitory effect of cAMP on NHE3 is decreased by zinc.

A better understanding of the mechanisms of action *via* which zinc exerts its effects will be necessary to find an explanation for the missing effects of zinc feeding on intestinal transport properties in piglets after long-term supplementation periods with zinc. In the same piglets that we have used for our studies, Janczyk et al. (2013) have conducted infection studies with *Salmonella enterica*, but no protective effect of zinc administration had been observed. Furthermore, increased proportions of antibiotic-resistant *E. coli* strains had been found in the highly zinc supplemented pigs by Bednorz et al. (2013). Therefore, a definitive statement as to whether high levels of zinc supplementation predominantly cause positive or negative effects on the animal cannot be provided. Martin et al. (2013b) showed that high doses of zinc lead to its accumulation in jejunal tissues and in bones, the liver, kidney, pancreas, and spleen. These observations were accompanied by the fact that the growth-promoting effect of zinc was even reversed in the high supplementation group after long-term administration, a finding that is

supported by several similar results (Martinez et al. 2005; Broom et al. 2006; Janczyk et al. 2013). Furthermore, the mRNA expression of zinc transporter (ZnT1, ZIP4) and metallothionein (MT) in the jejunal tissues of these piglets showed an adaptation to the high dietary zinc levels (Martin et al. 2013a). This suggests that these long-term effects are a consequence of an imbalance of zinc homeostasis within the organism and is furthermore supported by the findings of Bondzio et al. (2012; 2013) who had shown an increased expression of stress proteins in pancreatic and hepatic tissues in the same piglets. The project therefore aimed at conducting further investigations regarding the mechanisms regulating zinc homeostasis in intestinal epithelial cells, as summarized in the second manuscript of the present thesis.

Given the results from the feeding trial indicating the need for deeper insights into the effects of zinc supplementation on cellular zinc metabolism, the second part of the thesis was focused on the mechanisms by which intracellular zinc homeostasis is regulated and maintained in the intestinal epithelial cells of piglets (IPEC-J2) and of humans (Caco-2). In cell culture experiments, increasing concentrations of zinc (0-200 µM $ZnSO_4$) were applied to the cells, and the mRNA and protein expression of the zinc efflux transporter ZnT1, the influx transporter ZIP4, of metallothionein (MT), and of metal-response element binding transcription factor-1 (MTF-1) were assessed. Furthermore, the potential influence of the maturation status of the cells was taken into account. Based on the question as to whether the time of exposure to zinc has an impact on its effects and regulatory mechanisms, the cells were incubated for either 6 h or 24 h.

The applied zinc concentrations (0, 50, 100, 200 µM) were chosen with the aim of using similar concentrations to those calculated in the jejunum after supplementation with high doses of zinc *in vivo* in pigs (Pieper et al. 2013) and humans (Cragg et al. 2002). However, zinc supplementation can lead to values as high as the millimolar range within the ingesta (Zemann et al. 2011).

Cellular zinc uptake was measured by atomic absorption spectrometry (AAS), and the amount was normalized to the total protein content within the cells.

In both cell lines, the intracellular zinc content rose dose-dependently, but this increase was only numerical in the concentration range between 0 – 100 µM zinc. A strong and significant increase occurred at 200 µM of extracellular zinc. With regard to the relative changes in intracellular zinc values after the application of increasing zinc concentrations, our own data are consistent with those from the studies of Zodl et al. (2003) in Caco-2 cells.

The observation that high zinc concentrations are tolerated only up to a certain limit is also in accordance with a previous study of rat glioma cells by Haase and Beyersmann (1999) who

have found that the intracellular zinc level is relatively stable when the extracellular zinc concentration is raised within a certain range, because of the compensation by zinc transporters (Haase 2001; Eide 2004; Palmiter and Huang 2004) and binding proteins such as MTs (Vallee 1995), which control the intracellular zinc level. However, if a certain threshold value of about 100 µM is exceeded, increasing intracellular zinc contents can be observed; this might indicate that the capacity of homeostatic mechanisms is overstrained. Accordingly, distinct effects of high zinc levels on cell viability have been observed in IPEC-J2 cells in previous experiments (Lodemann et al. 2013).

An increase in intracellular zinc does not necessarily evoke toxic effects on the cells, as zinc can also be bound to MT within the cell. The induction of MTs is one of the protective mechanisms that occur when cells are exposed to high zinc levels (Hamer 1986; Takahashi 2012). MT binds free zinc with high affinity (Beyersmann and Haase 2001) and can be used as an indicator of the intracellular zinc level (Cheng et al. 2012). In previous experiments *in vivo*, a high dietary zinc supply led to an elevated expression of MT in porcine or rat small intestinal tissue (Tran et al. 1999; Martinez et al. 2004; Martin et al. 2013a). The assumption of zinc being bound to MT as a function of detoxification has been confirmed by the present study, as increased zinc levels were also accompanied by an increase in MT levels.

In addition to the applied concentration, other factors can be assumed to have additional impacts on the transcription of homeostatic transport proteins following excess zinc exposure (and thereby can evoke potentially toxic effects of zinc within the cell). The maturation status of the cells is one of these factors, at least when considering the way that zinc homeostasis is maintained within the cell. In the present study, proliferating preconfluent IPEC-J2 cells showed the highest increase in intracellular zinc levels during excess extracellular zinc supplementation. Correspondingly, MT1A expression was numerically upregulated, and MTF-1, which regulates the transcription of MT1A and ZnT1, was significantly increased in preconfluent IPEC-J2 cells compared with postconfluent ones. With regard to MT, Beyersmann and Haase (Beyersmann and Haase 2001) have found MT to be overexpressed in proliferating tissues. Therefore, the binding of zinc *via* increased induction of MT might play a predominant role in detoxification in preconfluent IPEC-J2 cells. In contrast, postconfluent IPEC-J2 cells primarily seemed to utilize a transport-dominated way to regulate zinc homeostasis *via* the induction of ZnT1. These cells efficiently induced ZnT1 upon exposure to high extracellular zinc concentrations leading to lower intracellular zinc contents compared with those of preconfluent IPEC-J2 cells.

Jou et al. (2010) suggest that the cellular mechanisms by which the absorption of zinc is controlled underlie developmental regulation. Therefore, the expression pattern of zinc

transporters, such as ZnT1 in IPEC-J2 cells, can be proposed to change during the maturation process of the small intestine.

In Caco-2 cells, the present data suggest a different way of regulating zinc homeostasis. In contrast to IPEC-J2 cells, higher intracellular zinc levels were observed with advancing maturation. When the extracellular zinc supply was increased, the intracellular zinc content also rose in preconfluent cells, but to a smaller extent. This was associated with significantly higher increases in ZnT1 mRNA levels in preconfluent Caco-2 cells compared with postconfluent cells. It is concluded that proliferating Caco-2 cells pursue the pathway *via* the induction of ZnT1 to discharge intracellular amounts of zinc that exceed a physiological threshold.

As a high basal MT protein expression has been measured in postconfluent Caco-2 cells, being accompanied by a high increase in intracellular zinc contents, postconfluent cells are proposed to use a more storage-dominated way of disposing of increased intracellular zinc levels.

In addition to the impact of the applied zinc concentration and the differentiation status of the cells, the time of exposure to zinc seems also to exert an effect. Previous studies with a variety of zinc concentrations in porcine (Tran et al. 2009) or human intestinal cells (Jou et al. 2010; Zemann et al. 2011) have shown that ZnT1 is upregulated during a high zinc supply when the cells are incubated for time periods of 1 h to 24 h. Conversely, Cragg et al. (2005) have shown reduced ZnT1 mRNA and protein expression in Caco-2 cells after a long-term study for three days. Linking the present findings with studies in porcine brain capillary endothelial cells (Bobilya et al. 2008) and Caco-2 cells (Shen et al. 2008), the increase in ZnT1 mRNA levels during the high zinc supply seems to occur within a time frame of 6-12 h after the application of the zinc. Exposure of the cells for longer than 24 h might lead to lower or even no detectable increases in the mRNA level of ZnT1 (Bobilya et al. 2008).

The induction of ZnT1 gene transcription depends on the metal-response element binding transcription factor-1 (MTF-1) (Palmiter and Findley 1995; McMahon and Cousins 1998; Langmade et al. 2000). The binding of MTF-1 is also required for the induction of MT expression (Heuchel et al. 1994). MTF-1 is a key player in zinc metabolism of higher eukaryotes and is proposed to be a cytoplasmic metal sensor (Gunther et al. 2012). Furthermore, MTF-1 plays a crucial role in the detoxification of heavy metals (Egli et al. 2003). Nevertheless, the underlying mechanisms of the sensing of elevated concentrations of metals and the activation of genes related to zinc homeostasis have not as yet been elucidated (Palmiter and Huang 2004).

In the present study, increasing zinc concentrations caused only minor changes in MTF-1 mRNA levels. A significant effect on MTF-1 mRNA expression was only observed in IPEC-J2 cells after incubation with the highest zinc concentration (200 µM). To our knowledge, the effect of zinc on MTF mRNA expression has not been previously investigated in porcine small intestinal cells,

although studies of human prostate, breast cancer, and cervical carcinoma cells have been published (Otsuka et al. 2000; Hasumi et al. 2003; Ostrakhovitch et al. 2007) and are consistent with our results. Although only small, the changes in MTF-1 mRNA abundance in the presence of high extracellular zinc levels are correlated with those in ZnT1 expression. This supports the assumption that an elevated induction of MTF-1 controls the expression of ZnT1. Therefore, a useful approach to obtain a deeper insight into the activity of MTF-1 might be to carry out electrophoretic mobility shift assays in future studies. Such studies are suitable for demonstrating the nuclear translocation and binding of MTF-1 to the promotor region of the respective target genes (Smirnova et al. 2000).

ZIP4 is a transport protein that is abundantly expressed in tissues involved in zinc absorption, such as the intestine (Lichten and Cousins 2009). It is located in the apical membrane of small intestinal epithelial cells (Wang et al. 2002). Its expression is regulated at both the transcriptional and post-transcriptional levels. It increases the cellular zinc concentration by enhancing the zinc influx into the cell or by stimulating the release of zinc from intracellular vesicles (Liuzzi and Cousins 2004). The current results have revealed that ZIP4 mRNA is downregulated at 200 µM $ZnSO_4$ in both tested cell lines. In previous studies with rat and porcine intestinal tissue, ZIP4 mRNA abundance was also downregulated when the animals were supplemented with high dietary zinc concentrations (Fujimura et al. 2012; Martin et al. 2013a). However, in several cell culture studies with Caco-2 cells, no changes in the mRNA level of ZIP4 were observed after zinc application at high concentrations (Cragg et al. 2005; Zemann et al. 2011).
No previous studies in the literature have examined the changes in ZIP4 protein expression in porcine tissue or a porcine cell line in response to zinc supplementation. The present study has shown that the downregulation of ZIP4 mRNA is accompanied by a respective downregulation of ZIP4 protein expression only in Caco-2 cells after application of high extracellular zinc concentrations. However, this was not evident for IPEC-J2 cells. The reasons for these divergent responses have not been elucidated so far. The results indicate post-transcriptional regulation that might include translational regulation or a different turnover of the ZIP4 protein. As has previously been shown, zinc not only regulates the abundance of ZIP4 mRNA, but also induces a rapid loss of the ZIP4 protein from the apical surface of the cell after the application of zinc (Cragg et al. 2005; Jou et al. 2010). The latter indicates that the integration of this protein into the cell membrane is a metal-regulated process (Dufner-Beattie et al. 2003).

Conclusion

To prevent PWD, piglets are often fed high doses of dietary zinc during the post-weaning period. The two present studies have investigated the effects of zinc feeding *in vivo* on the transport of nutrients and electrolytes across the small intestinal epithelium and have aimed at elucidating the ways by which intestinal epithelial cells maintain zinc homeostasis when increasing zinc concentrations are applied *in vitro*.

Dietary zinc supplementation shows only minor long-term effects on absorptive or secretory properties within the jejunum of piglets. By contrast, acute treatment with zinc *in vitro* leads to a significant reduction of absorptive and secretory responses. This supports the assumption that the effects of zinc on epithelial functions are mainly mediated by its acute presence at the intestinal epithelium. Intestinal epithelial cells themselves show corresponding adaptation of zinc transporter expression when exposed to increasing extracellular zinc concentrations applied *in vitro*. An upregulation of the zinc efflux transporter ZnT1 and factors associated with zinc homeostasis (MT, MTF-1) and the respective downregulation of the zinc influx transporter ZIP4 have been observed; albeit at high zinc concentrations, the induction of the these compensatory mechanisms does not prevent significant increases in intracellular zinc contents. Furthermore, the maturation status of the cells and the application time exhibit significant effects *in vitro*. Together with the knowledge from previous studies, it is concluded that zinc supplementation in weaning piglets can be used successfully as an alternative for AGP. However, the long-term administration of high zinc levels might induce imbalances within the zinc homeostasis of the organism. Thus, the duration of zinc administration in the feed is critical, especially with view to the continuing maturation of the gut. As such, the results of the present study support the conclusions of a comparison study within the SFB 852 (Martin et al. 2013a) proposing that the supply of high zinc concentrations to piglets for periods longer than two weeks might have toxic effects on the animal.

Chapter 7: Summary/ Zusammenfassung

Summary of the PhD thesis:

Interactions of zinc with the intestinal epithelium - effects on transport properties and zinc homeostasis.

In modern piglet nutrition, diets are often supplemented with high doses of zinc oxide to decrease the incidence of weaning-associated diseases such as post-weaning diarrhea. However, the underlying mechanisms by which high zinc concentrations exert their effects within the organism are not sufficiently elucidated and concerns exist regarding the environmental pollution by zinc-loaded liquid manure.

The present thesis investigated the effect of zinc on transport properties in the small intestine of piglets initially in a feeding trial. Zinc effects seemed to depend on its acute application at the intestinal epithelium and furthermore on the side of application *in vitro*. In association with previous observations of Lodemann et al. (2013) showing a distinct influence of high zinc levels on viability and barrier properties of intestinal epithelial cells, the present thesis further aimed at elucidating the homeostatic mechanisms that may prevent toxic effects of high zinc concentrations in the intestine of piglets.

The first study involved a feeding trial with piglets being fed marginal (50 ppm), normal (150 ppm), or pharmacological concentrations (2500 ppm) of zinc oxide and an investigation of its impact on jejunal transport properties. Ussing chamber experiments with jejunal isolated epithelia were performed with several substances stimulating either absorptive (L-glutamine, D-glucose) or secretory (prostaglandin E_2, carbachol, *E. coli* heat-stable enterotoxin) transport. In addition to chronic dietary zinc supplementation, the effect of acute zinc application was tested *in vitro*. The dietary zinc supplementation had no significant influence on absorptive and secretory responses. However, with an exception for carbachol, the acute zinc treatment *in vitro* led to small but significant decreases in both absorptive and secretory capacities. In conclusion, chronic zinc supplementation in the post-weaning phase sustainably affected neither the absorptive nor the secretory transport properties within the jejunum. However, as the jejunal transport was influenced by acute zinc addition *in vitro*, it is proposed that the potential epithelial effects of zinc depend on the acute presence of this ion at the intestinal epithelium.

In the second part of this thesis, cell culture experiments with the porcine cell line IPEC-J2 and the human cancer cell line Caco-2 were performed. On the assumption that the tight regulation of intracellular zinc homeostasis in enterocytes of weaned piglets is a crucial necessity, the aim

of this study was to elucidate the way that porcine intestinal epithelial cells regulate intracellular zinc homeostasis and maintain it during a challenge with increasing extracellular zinc concentrations. A further question was whether the differentiation status of the cells (preconfluent/ postconfluent) or the duration of zinc exposure (6 or 24 h) affected the response to increasing zinc concentrations.

The intracellular zinc content rose dose-dependently in both cell lines with increasing extracellular zinc concentrations. Correspondingly, the expression of the zinc efflux transporter 1 (ZnT1) and of metallothionein (MT1A) were upregulated, whereas the zinc influx transporter 4 (ZIP4) was downregulated. The mRNA expression of the metal response element-binding transcription factor 1 (MTF-1) remained largely unchanged. However, a higher MTF-1 abundance was detected in IPEC-J2 cells after incubation with the highest zinc concentration (200 µM).

The effects of increasing zinc concentrations were partly different between cell lines and maturation status. The time of exposure to zinc also evoked an impact.

In conclusion, the second study showed that an adaptation of the tested target genes was responsible for regulating zinc homeostasis in response to increasing zinc concentrations. However, despite the induction of compensatory mechanisms, an increase in intracellular zinc levels was observed after high extracellular zinc levels had been applied, indicating that high zinc levels and a longer incubation time might exceed the capacity of homeostatic regulation. Toxic effects, as previously assessed by Lodemann et al. (2013), can occur because of an imbalance of zinc homeostasis. Therefore, long-term supplementation with high levels of dietary zinc cannot be recommended, as it may induce homeostatic imbalances with negative effects on the animal.

Zusammenfassung der Dissertation zum Thema:

Wechselwirkungen von Zink mit dem intestinalen Epithel – Effekte auf Transporteigenschaften und Zinkhomöostase.

In der modernen Ernährung von Ferkeln wird das Futter der Tiere häufig mit hohen Dosen an Zinkoxid supplementiert, um das Auftreten mit dem Absetzen assoziierter Erkrankungen, wie zum Beispiel Durchfallerkrankungen, zu vermindern. Allerdings sind die zugrundeliegenden Mechanismen, über welche die hohen Zinkkonzentrationen ihre Effekte innerhalb des Organismus entfalten, unzureichend erforscht und es existieren Bedenken bezüglich der Umweltbelastung durch zinkhaltige Gülle.
Die vorliegende Arbeit untersuchte zunächst in einem Fütterungsversuch den Effekt von Zink auf Transporteigenschaften im Dünndarm von Ferkeln. Zinkeffekte zeigten sich *in vitro* in Abhängigkeit von seiner akuten Applikation am intestinalen Epithel und von der Applikationsseite. In Verbindung mit vorherigen Untersuchungen von Lodemann et al. (2013), welche einen deutlichen Einfluss hoher Zinkkonzentrationen auf die Vitalität und Barriereeigenschaften von intestinalen, epithelialen Zellen zeigten, verfolgte die vorliegende Arbeit weiterhin das Ziel, die homeostatischen Mechanismen aufzuklären, welche toxische Effekte hoher Zinkkonzentrationen im Verdauungstrakt von Ferkeln vermeiden könnten.
In der ersten Studie wurde ein Fütterungsversuch durchgeführt, in dem Ferkel nach dem Absetzen niedrige (50 ppm), normale (150 ppm) oder pharmakologische Konzentrationen (2500 ppm) an Zinkoxid mit dem Futter erhielten und in dem nachfolgend die Effekte der Fütterung auf die jejunalen Transporteigenschaften untersucht wurden. Hierfür wurden Ussing-Kammer Versuche mit isolierten jejunalen Epithelien durchgeführt, wobei durch verschiedene Substanzen der sekretorische (Prostaglandin E_2, Carbachol, *E. coli* Enterotoxin) und absorptive (L-Glutamin, D-Glukose) Transport am Epithel stimuliert wurde. In Ergänzung zur chronischen Zinkfütterung wurde auch der Effekt einer akuten Zinkzugabe *in vitro* untersucht. Die diätetische Zinksupplementierung hatte weder auf den absorptiven noch auf den sekretorischen Transport einen signifikanten Effekt. Allerdings führte die akute Zinkapplikation *in vitro* zu einer schwachen aber signifikanten Senkung der absorptiven und auch sekretorischen Kapazität. Folgende Schlussfolgerungen lassen sich deshalb ziehen: Die chronische Zinksupplementierung in der Phase nach dem Absetzen hat weder die absorptiven noch die sekretorischen Transporteigenschaften des Jejunums nachhaltig beeinflusst. Da der jejunale Transport allerdings durch die akute Zinkapplikation *in vitro* verändert wurde, nehmen wir an, dass

potentielle, epitheliale Zinkeffekte von der akuten Präsenz dieses Ions am intestinalen Epithel abhängen.

Im zweiten Teil dieser Dissertation wurden Zellkulturversuche mit der intestinalen, epithelialen porcinen Zelllinie IPEC-J2 und der humanen Tumorzelllinie Caco-2 durchgeführt. Basierend auf der Annahme, dass die strenge Regulation der intrazellulären Zinkhomöostase in Enterozyten von Absatzferkeln von entscheidender Bedeutung ist, war das Ziel dieser Studie, die Mechanismen aufzuklären, welcher sich porcine intestinale Zellen bedienen, um die intrazelluläre Zinkhomöostase zu regulieren und während der Einwirkung hoher, extrazellulärer Zinkkonzentrationen aufrecht zu erhalten. Eine weitere Frage war, ob der Differenzierungsgrad der Zellen (prekonfluent/ postkonfluent) und die Einwirkdauer (6 h vs. 24 h) die Reaktion auf steigende Zinkkonzentrationen beeinflusst.

Der intrazelluläre Zinkgehalt stieg mit ansteigenden extrazellulären Zinkkonzentrationen dosisabhängig bei beiden Zelllinien. Damit verbunden war auch eine Heraufregulation des Efflux-Zinktransporters 1 (ZnT1) und von Metallothionein (MT1A), während der Influx-Zinktransporter 4 (ZIP4) herunterreguliert war. Die Expression des Metall-responsiven Transkriptionsfaktors (MTF-1) zeigte sich weitgehend unverändert. Allerdings konnte eine höhere Abundanz von MTF-1 in IPEC-J2 Zellen nach Inkubation mit der höchsten Zinkkonzentration (200 µM) festgestellt werden.

Zusätzlich zu den konzentrationsabhängigen Effekten konnten auch Unterschiede in Abhängigkeit der Zelllinie und des Differenzierungsgrades der Zellen festgestellt werden. Desweiteren spielte auch die Inkubationszeit eine Rolle.

Schlussfolgernd zeigte die zweite Studie eine Anpassung der untersuchten, für die Regulation der Zinkhomöostase verantwortlichen Zielgene als Reaktion auf die Inkubation mit ansteigenden Zinkkonzentrationen. Allerdings wurde trotz der Induktion kompensatorischer Mechanismen ein Anstieg des intrazellulären Zinkgehaltes in beiden Zelllinien nach der Inkubation mit hohen Zinkkonzentrationen beobachtet, was darauf hindeuten könnte, dass diese hohen Konzentrationen und lange Inkubationszeiten die Kapazität der homöostatischen Regulation übersteigt. Toxische Effekte, wie sie zuvor von Lodemann et al. (2013) gezeigt wurden, können aufgrund eines Ungleichgewichtes der Zinkhomöostase entstehen. Eine Langzeitsupplementierung des Futters von Ferkeln mit hohen Zinkdosen ist daher nicht zu empfehlen, da dies ein Ungleichgewicht innerhalb der Zinkhomöostase mit darauf resultierenden negativen Effekten auf das Tier verursachen kann.

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Regulation der intrazellulären Zinkhomöostase in porzinen und humanen Darmepithelzellen.

30. Jahrestagung der Gesellschaft für Mineralstoffe und Spurenelemente e.V, München-Weihenstephan, Abstracts p. 19

Lodemann, U, **Gefeller, EM**, Aschenbach, J.R., Martens, H., Einspanier, R., Bondzio, A. Einfluss apikaler und basolateraler Zinksupplementierung auf die Integrität, Vitalität und die Expression von Metallothionein in intestinalen epithelialen Zellen. *21. Tagung der Fachgruppe Physiologie und Biochemie der Deutschen Veterinärmedizinischen Gesellschaft*, Zürich, 13.02. - 15.02.2014

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Eva-Maria Gefeller, Holger Martens, Jörg R. Aschenbach, Shanti Klingspor, Ulrike Lodemann Effekte von unterschiedlichen Zinkkonzentrationen im Futter auf absorptive und sekretorische Eigenschaften im Jejunumepithel von Ferkeln. 20. *Tagung der Fachgruppe Physiologie und Biochemie der Deutschen Veterinärmedizinischen Gesellschaft*, München, 16.02. -18.02.2012, Abstracts p.75

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Danksagung

Professor Aschenbach: Mein erster Dank gilt Ihnen! Zunächst für die Überlassung dieses spannenden Dissertationsthemas, welches mir die Möglichkeit vielseitigen, wissenschaftlichen Arbeitens gegeben hat. Die Verbindung dieser Arbeit mit dem Besuch der Dahlem Research School hat mir einen herausfordernden, aber horizonterweiternden Blick in die Forschungswelt ermöglicht. Für Ihre stets konstruktive Kritik, das Wissen, Sie jederzeit um Rat fragen zu können und Ihre fortwährende Unterstützung bis zum Ziel danke ich Ihnen sehr!!

Professor Martens: Sie haben mich schon durch die Zeit des Studiums und nun durch die Promotion begleitet, wobei Sie mir zu jeder Zeit ein sehr angenehmer und wohlwollender Gesprächspartner, Ratgeber und Unterstützer waren. Ich danke Ihnen für die Unterstützung und das Interesse, welches Sie meiner Arbeit entgegen gebracht haben und dass Sie mich immer wieder an Ihrem reichen Erfahrungsschatz teilhaben ließen!!

Ulrike Lodemann: Ohne Dich hätte ich so manches Mal in einem Moment unkontrollierten Temperaments das Handtuch geworfen! Für Dein immer offenes Ohr, Deine stetige Unterstützung, zu Tages- wie Nachtzeiten, die weit über das Fachliche hinausging, möchte ich dir von ganzem Herzen danken. Ich hätte mir keine bessere Betreuerin wünschen können; niemand kann den Spagat zwischen erfolgreicher Arbeit und Kindern besser nachempfinden als Du! Dafür bist Du mir immer ein Vorbild! Danke, dass ich mit Dir zusammen arbeiten und lachen konnte, dass ich von Deiner unbeirrbaren, professionellen und akribischen Einstellung lernen durfte!!

Institut für Physiologie: Ein großes Dankeschön an die Mitarbeiter und Kollegen der Physiologie für ihre stetige Hilfe und den Spaß, den wir gemeinsam hatten. Ich habe die Zeit am Institut sehr genossen und werde mich immer gern daran zurück erinnern. Daran habt ihr alle euren Anteil. Mein besonderer Dank gilt hier Katharina Wolf und Uwe Tietjen für ihre ermunternde und unterstützende Art, ihr Engagement und ihre Hilfe im Labor. Ihr hattet immer ein offenes Ohr für mich! Danke **Uwe**, für viele Schoko-Croissants und Feierabend-Runden im Keller der Ussing-Kammer und danke **Katharina**, dass du notfalls sogar zum Kellerfenster herein kamst, wenn Kito vor dem Labor saß.
Außerdem danke ich **Shanti** für die wunderbare Büro-WG, die wir zusammen hatten, für viele, gemeinsame Erlebnisse bei denen wir Tränen gelacht haben oder uns gegenseitig über die

Stolpersteine der Promotion hinweg geholfen haben. Ich hoffe, wir bleiben weiterhin, auch über die Zeit in der Physio hinaus, in Verbindung.

Meiner Familie: Ohne Euch hätte ich dieses Ziel sowie viele andere zuvor nicht erreicht. Danke für viele, viele aufmunternde Worte und Taten! **Olli**, danke für deinen unerschütterlichen Rückhalt ohne viele Worte und deinen immerwährenden Glauben an mich und meine tausend Projekte. **Mama**, für deine aufopfernde Liebe und Zuneigung und deinen Optimismus, in schweren wie schönen Zeiten, ich kann stolz sein, dass alle sagen, ich sei Dir so ähnlich. **Robert** für den besten Bruder der Welt, der immer da ist, wenn ich ihn brauche, für mich und von ganz klein an auch für Kilian. Deine Sicht auf die Welt ist anders und eröffnet mir neue Perspektiven. Du machst Deinen Weg!
Ihr habt wieder und wieder die Kinder „mitgeschaukelt“, so dass ich Zeit und Ruhe zum Schreiben finden konnte und habt immer an mich geglaubt! Ein „Danke“ allein reicht dafür nicht aus!!

Lukas: Ein optisch ansprechendes Gesamtpaket ist diese Arbeit nur durch Dein Zutun geworden. Dass Du dir dafür von jetzt auf gleich Zeit genommen hast, hat mir sehr geholfen. Für unser Treffen auf Augenhöhe bin ich mehr als nur dankbar!

Darüber hinaus danke ich dem **Sonderforschungsbereich 852/1** sowie der **H.-W.-Schaumann Stiftung** für ihre ein- bzw. zweijährige, finanzielle Unterstützung, welche mir das Arbeiten an diesem umfangreichen und zeitaufwendigen Projekt sehr viel leichter gemacht hat!

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet zu haben. Die Arbeit ist in dieser Form noch keiner anderen Prüfungsbehörde vorgelegt worden.

Berlin, den 14. April 2015

Eva-Maria Näser