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Effects of zinc supplementation on virus infection in pigs

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Dedication

Dedicated to my Mother's soul, to my Father, to my sister, to my dearest wife

Weidong

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List of Abbreviations

CFU	Colony forming units
CPE	Cytopathic effect
CTL	Cytotoxic T lymphocytes
DMEM	Dulbecco's modified Eagle's medium
dpi	Days post infection
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
H&E	Hematoxylin and eosin
HIV	Human immunodeficiency virus
IFN	Interferon
IFN-γ SC	Interferon gamma secreting cells
IL	Interleukin
MT1	Metallothionein-1
NA	Neutralizing antibodies
NK cells	Natural killer cells
NO	Nitric oxide
OAS	Oligoadenylate synthetase
OD	Optical density
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PRRS	Porcine reproductive and respiratory syndrome
PRRSV	PRRS virus
RPL13	60S ribosomal protein L13
RT	Reverse transcription

SDHA	Succinate dehydrogenase subunit A
SIV	Swine influenza virus
ST cells	Swine testicle cells
TCID50	50% tissue culture infective doses
TER	Transepithelial resistance
TGE	Transmissible gastroenteritis
TGEV	TGE virus
Th	T-helper
TNF	Tumor necrosis factor
VNT	Viral neutralization test
ZIP4	Zn transporter SLC39A4
Zn	Zinc
ZnO	Zinc oxide
ZnT1	Zn transporter SLC30A1
ZnT2	Zn transporter SLC30A2
ZnT5	Zn transporter SLC30A5

Zusammenfassung

Effekte der Zink supplementierung auf Infektionen mit virus bei Schweinen

Die therapeutische Nutzung von Zink (Zn) zur Behandlung und zum Schutz von Nutztieren (wie dem Schwein) vor verschiedenen mikrobiellen Pathogenen unter differenziellen Bedingungen war in der Vergangenheit bereits Gegenstand zahlreicher Untersuchungen. Dagegen ist bisher über die Wirkung von Zink auf virale Infektionen und den Verlauf einer Virusinfektion beim Schwein kaum berichtet worden. In der vorliegenden Arbeit wurde der potenzielle Nutzen der Futtersupplementierung mit Zinkoxid (ZnO) für den Schutz des Schweins vor Infektion mit zwei wirtschaftlich bedeutsamen viralen Pathogenen, dem Porcinen Reproduktiven und Respiratorischen Syndrom Virus (PRRSV) und dem Transmissiblen Gastroenteritis Virus (TGEV) untersucht.

In einem ersten Challenge-Experiment wurde geprüft, welchen Einfluss die Futtersupplementierung mit ZnO auf die Impfung der Tiere gegen PRRSV hat. Hierzu erhielten Schweine Futter, dem entweder kein ZnO (natürlicher Zn-Gehalt: 50 – 80 ppm), Zn in mittlerer Dosis (150 ppm) oder hoher Konzentration (2500 ppm) zugesetzt war. Die Tiere ohne Zinkergänzung dienten als Kontrollgruppe (Zn^{low}). Die beiden anderen Fütterungsgruppen repräsentieren die experimentellen Fütterungsgruppen (Zn^{med} und Zn^{high}). Die Hälfte aller Tiere jeder Fütterungsgruppe wurde einmalig mit einer experimentellen, inaktivierten Vakzine geimpft und anschließend mit PRRSV infiziert. Verglichen mit der Kontrollgruppe (Zn^{low}) fand sich bei den Tieren der experimentellen Fütterungsgruppen (Zn^{med} and Zn^{high}) keine Veränderung des klinischen Verlaufs der PRRSV-Infektion. Auch die Impfung beeinflusste den Infektionsverlauf in keiner der drei Fütterungsgruppen. Allerdings wurde in den experimentellen Fütterungsgruppen nach PRRSV-Infektion eine im Vergleich zur Kontrollgruppe gesteigerte Gewichtszunahme verzeichnet. In den Fütterungsgruppen mit höheren ZnO-Dosierungen wurden zudem 4, 7 und 21 Tage nach der Infektion leicht erhöhte Konzentrationen an PRRSV-spezifischen Antikörpern. festgestellt. Gleiches gilt für den Nachweis virus-neutralisierender Antikörper 28-35 Tage nach der Infektion. Eine Steigerung der durch die PRRSV-Infektion induzierten, spezifischen antiviralen IFN-γ Produktion konnte in den experimentellen Fütterungsgruppen weder in ungeimpften noch geimpften Tieren gemessen werden. Dagegen führte die erhöhte Zufütterung von Zink zu einer Steigerung des prozentualen Anteils der γδ T-Zellen im peripheren Blut. Die

Fütterung der erhöhten ZnO-Dosen hatte keinen Einfluss auf die Viruslast der Tiere. Auch die Ausscheidung von Viren war im Vergleich zur Kontrollgruppe nicht verändert. Die erzielten Ergebnisse legen den Schluss nahe, dass die Fütterung höherer Zinkdosen beim Schwein nur einen leichten Effekt auf die humorale und zelluläre Immunantwort hat. Dieser Einfluss führt in den Tieren aber nicht zu einer Reduktion der Virustiter im Tier oder in deren Ausscheidungen.

Da bekannt ist, dass Zinkzusatz im Futter die Durchfallhäufigkeit in der Absetzphase reduziert, wurde in einem weiteren Fütterungsexperiment unter dem gleichen, für PRRSV beschriebenen Diätregime, der Einfluss von unterschiedlichen ZnO-Dosierungen auf die Infektion mit TGEV, einem wichtigen enterischen Schweinevirus, untersucht. Ziel war es zu ermitteln, ob höhere Zinkdosierungen intestinale Virusinfektionen günstig beeinflussen können. Hierzu wurden Absatzferkel im Alter von 28 Tagen eine Woche nach der Fütterung mit den drei oben beschriebenen Diäten mit TGEV oral infiziert und dann nach einem Tag oder nach 18 Tage getötet und vergleichend untersucht.

In den mit erhöhten Zink Konzentrationen gefütterten Tieren konnte eine Verbesserung der fäkalen Konsistenz sowie ein gesteigerter Zuwachs an Körpergewicht verzeichnet werden. Ein Einfluss der Fütterung auf die mukosale Abwehr ließ sich nicht nachweisen, aber im Hinblick auf die humorale Immunität wurde in der Fütterungsgruppe mit der höchsten Zink-Konzentration eine frühere und höhere Produktion spezifischer Antikörper gegen TGEV gemessen. In der Zn^{high} Gruppe wurde im jejunalen Epithel eine Hemmung der Caspase-3-induzierten Apoptose und der Villusathrophie beobachtet. Ebenfalls herunterreguliert waren Interferon (IFN)-α, die Oligoadenylatsynthetase (OAS) und der Zink-Transporter SLC39A4 (*ZIP4*). Hochreguliert waren dagegen Metallothionein-1 (MT) sowie die Zink-Transporter SLC30A1 (*ZnT1*) und SLC30A5 (*ZnT5*). Schließlich wurde festgestellt, dass die Forskolin-induzierte Chloridsekretion und der epitheliale Widerstand ausschließlich in der Zn^{high}-Gruppe auf der physiologischen Ebene reguliert werden. Die Ergebnisse des TGEV-Experiments weisen darauf hin, dass eine Diät mit hoher Zn-Supplementierung einen erhöhten Schutz vor einer TGEV-Infektion des Darmtraktes verleihen kann.Auf der Basis der erzielten Ergebnisse der Fütterungsversuche mit PRRSV und TEGV können für den Effekt einer erhöhten Zinkfütterung folgende Schlussfolgerungen gezogen werden:

1. Eine erhöhte Konzentration von ZnO im Futter verbessert stellenweise den klinischen Status von Schweinen während der Infektion mit PRRSV und mit TGEV.

2. Zn-Supplemetierung des Futters hatte keinen Einfluss auf die Viruslast und die Virusausscheidung der Tiere.

3. Die Fütterung der hohen Zink-Konzentration hatte bei beiden Virus-Infektionen nur einen gering positiven Effekt auf die systemisch humorale Immunreaktion der Tiere. Die mukosale Immunantwort war im Infektionsversuch mit TGEV durch die Fütterung nicht beeinflusst.

4. Die Supplementierung mit hohen Dosen ZnO unterdrückte die Caspaseaktivierung und die Apoptose im intestinalen Epithel. Sie verhinderte auch den durch das TGEV bedingten Kollaps der intestinalen Transportfunktionen.

5. Die Fütterung von erhöhtem ZnO hatte bei der Impfung mit experimentellem, inaktiviertem PRRSV-Impfstoff keinen Adjuvanseffekt.

Summary

Effect of zinc supplementation on virus infection in pigs

The therapeutic application of zinc (Zn) to treat or prevent various pathogens has been investigated in different experimental settings, but there is no report on the proposed effects of Zn treatment on virus infection in pigs. We investigated the potential benefits of Zn oxide (ZnO) in protecting pigs from infections with two important porcine viral pathogens: porcine reproductive and respiratory syndrome virus (PRRSV), which causes respiratory tract and persistent systemic infections and transmissible gastroenteritis virus (TGEV), which causes enteric infections.

In a first challenge experiment, we determined the influence of dietary ZnO supplementation on vaccination and challenge infection with PRRSV. Pigs were fed with three different diets containing no additional ZnO (natural Zn content: 50 ppm; Zn^{low} = control group), supplemented with medium levels of ZnO (150 ppm, Zn^{med}), or high levels (2,500 ppm, Zn^{high}) and half of the pigs were prime-only vaccinated with an experimental inactivated vaccine.

We found no influence of either diets or vaccination on the clinical course of PRRS, apart from an increased weight gain after infection in Zn^{med} and Zn^{high} groups, when compared to Zn^{low} group. Treatment with higher doses of ZnO resulted in slightly higher levels of PRRSV-specific antibody at 4, 7 and 21 dpi as well as neutralizing antibodies at 35 dpi. Virus-specific IFN-γ responses were not altered by vaccination and diets, whereas Zn^{high} supplementation increased the percentage of $\gamma\delta$ T cells in both vaccinated and non-vaccinated animals. Feeding higher Zn diets had no effect on viral loads and/or shedding. The results in this work suggested that feeding high levels of Zn had marginal effects on humoral and cellular immune responses, but did not positively affect clinical disease and virus loads, and did not improve immune responses to vaccination with an inactivated vaccine.

Since Zn supplementation has been shown to reduce the incidence of diarrhea in the weaning period, we evaluate the protective effect of the same three diets, as used in the PRRSV challenge trial, against an important porcine enteric virus, TGEV, in a second experimental challenge experiment. Weaned piglets were orally infected with TGEV at the age of 28 days, when the piglets had been fed for 1 week with the respective diets. Piglets were sacrificed on

day 1 and day 18 after challenge infection. Fecal consistency was improved and body weights were increased in the Zn^{high} group when compared to the other groups, but there was no effect of Zn treatments on mucosal immune responses. In the Zn^{high} group we also observed an earlier and higher TGEV-specific antibody response and decreased caspase-3-mediated apoptosis of jejunal epithelium and a prevention of villus atrophy, as well as down-regulation of interferon (IFN)-α, oligoadenylate synthetase (OAS), Zn transporter *SLC39A4 (ZIP4)*, but up-regulation of metallothionein-1 (MT), Zn transporters *SLC30A1 (ZnT1)* and *SLC30A5 (ZnT5)*. Finally, forskolin-induced chloride secretion and epithelial resistance were controlled at a physiological level in the Zn^{high} but not the other groups. The results from this TGEV challenge trials indicated that high dietary Zn could provide enhanced protection against TGEV infection in the intestinal tract.

Based on these two challenge trials in pigs, we concluded the following regarding the effects of elevated Zn levels in the diet:

1. High doses of dietary ZnO could partly improve clinical performance in both PRRSV and TGEV challenge infections;
2. ZnO supplementation had no effect on viral loads and shedding in PRRSV challenge trial;
3. Feeding high levels of ZnO had only marginal effects on systemic humoral immune responses in both challenge trials, but had no effect on mucosal immune response in the TGEV challenge trial;
4. Supplementation of diet with high levels of ZnO could suppress apoptosis in intestinal epithelial cells and prevent the disruption of the intestinal barrier integrity in TGEV challenge infection;
5. Feeding higher ZnO had no adjuvant effect of vaccination with an experimental inactivated PRRSV vaccine.

Chapter 1: General introduction

1.1 Zinc

1.1.1 Overview of zinc

Zn is a component of more than 300 enzymes from all six enzyme classes (Vallee and Falchuk, 1993), where Zn has diverse biological functions in DNA replication (Wu and Wu, 1987), RNA transcription (Dreosti, 2001; Falchuk, 1993; Wu and Wu, 1987), cellular signal transduction (Beyersmann and Haase, 2001), enzymatic catalysis (Auld, 2001), redox regulation (Maret, 2006), cell proliferation (Bohnsack and Hirschi, 2004; MacDonald, 2000; Prasad et al., 1996), cell differentiation (Petrie et al., 1991), and apoptosis (Sunderman, 1995; Truong-Tran et al., 2001).

In humans, Zn deficiency leads to growth and development retardation, thymic atrophy, hypogonadism, infertility, dermatitis, delayed wound healing, alopecia, poor pregnancy outcomes, teratology, anorexia, diarrhea, and decreased function of immune system (Maret and Sandstead, 2006; Salgueiro et al., 2000).

Recognition that Zn deficiency may be a problem for swine did not occur until 1955, when Tucker and Salmon reported that Zn supplementation prevented and cured parakeratosis in swine fed a diet low in Zn (Tucker and Salmon, 1955). In the 1960s, it was found that feeding 250 mg Cu/kg diet as a growth stimulant for swine increased mortality and resulted in a parakeratosis that was overcome by increasing dietary Zn (Suttle and Mills, 1966).

Zn requirements for growing pigs were stated by National Research Council recommendations (NRC) (NRC, 1998) as 50 mg/kg diet or 80 - 100 mg/kg dry matter of feed according to the German Association for Nutrition Physiology (GfE, 2006) and ZnO is often added as a supplement to the feed to cover these requirements. With Zn poorly absorbed, it can become highly concentrated in manure. To minimize the risk of environmental pollution, European regulations have reduced the maximal Zn concentration authorized in pig diets to 150 mg/kg diet irrespectively of Zn origin (Commission Regulation (EC), 2003). In the North American swine industry, however, dietary ZnO is frequently added at high dose of 2,000-3,000 mg/kg diet of weaned pigs since this was shown to reduce non-specific post-weaning diarrhea and improve the performance of piglets (Hu et al., 2012; Janczyk et al., 2013; Martin et al., 2013; Molist et al., 2011; Poulsen, 1995; Sales, 2013). Following this rationale, over the years several hundred Zn

supplementation studies have been conducted, investigating the effects of nutritional Zn supplementation on different diseases.

1.1.2 Zn supplementation for disease prevention and therapy

1.1.2.1 Vaccination

Vaccination is the administration of vaccine to induce protection against infections by stimulating the development of long-lived effector cells and memory cells (Abbas, 2005). Most vaccines in routine use today attempt to induce humoral immunity, and to stimulate cell-mediated immune responses. Vaccination is the most effective method of preventing infectious diseases, and widespread immunity due to vaccination is largely responsible for the worldwide eradication of smallpox and the restriction of diseases such as polio, measles, and tetanus from much of the world.

The antibody production during immune response is disturbed by Zn deficiency (Fraker et al., 1986; Fraker et al., 1987), suggesting that Zn supplementation could improve vaccination results. It had been reported that Zn supplementation increased IgG titers after tetanus vaccination and the number of circulating T lymphocytes (Duchateau et al., 1981). Kreft et al. found that hemodialysis patients who did not respond to diphtheria vaccination have lower serum Zn levels than responders and age-matched controls, possibly indicating a correlation between Zn status and vaccination response (Kreft et al., 2000). Tipu et al. also showed that Zn treated rabbits have a higher anti-newcastle disease virus antibody titer than non-Zn feeding control group after vaccination and Zn also has the ability to counteract methotrexate-induced immunosuppression (Tipu et al., 2012).

However, the verification of this effect of Zn for other vaccinations is not always successful (Table 1). Some studies investigated the effect of Zn supplementation on influenza vaccination in the elderly and hemodialysis patients which have a high risk for being Zn deficient, but no effect of Zn on influenza vaccination was found (Provinciali et al., 1998; Turk et al., 1998). The influence of Zn on cholera vaccination was also investigated, but with inconsistent results. Zn treatment lead to an increase of vibriocidal antibody titers (Albert et al., 2003; Karlsen et al., 2003), while a suppression of antibody formation against cholera toxin was found in other study (Karlsen et al., 2003; Qadri et al., 2004). The reason for these contradictory results remains unclear.

Table 1. Effect of Zn supplementation on vaccination. (modified from Overbeck et al., 2008)

Vaccination	Zn Species	Zn dosage	Period	Participants	Effect of Zn	Reference
Cholera	Zn acetate	20 mg, daily, elemental	6 w, starting 3 w before vacc.	human, 125 (Zn), 124 (P)	increased serum Zn levels and vibriocidal antibody titer	(Albert et al., 2003)
	Zn acetate	20 mg, daily, elemental	6 w, starting 3 w before vacc.	human, 125 (Zn), 124 (P)	lower increase in antibody titer (IgA and IgG)	(Qadri et al., 2004)
	Zn sulfate	200 mg, daily in 3 doses	9 d, starting 2 d before vacc.	human, 15 (Zn), 15 (P)	lower increase in antibody titers (IgA and IgG); increased fecal antibody titer (IgA) and vibriocidal antibody titer	(Karlsen et al., 2003)
Diphtheria				human, 16	Significantly decreased serum Zn levels could be detectd in non-responders after vaccination	(Kreft et al., 2000)
Influenza	Zn sulfate	220 mg, daily in 2 doses	4 w starting 7 d before vacc.	human, 43 (Zn), 41 (P)	no effect on vaccination	(Remarque et al., 1993)
	Zn sulfate	220 mg, daily in 2 doses	60 d, starting 15 d before vacc.	human, 194 (Zn), 190 (P)	increased plasma Zn levels; no effect on vaccination	(Provinciali et al., 1998)
	Zn sulfate	120 mg, 2-3 times per week after hemodialysis	1 mo	human, 13 (Zn), 13 (P), 13 (C)	increased serum Zn levels; no effect on vaccination	(Turk et al., 1998)
Newcastle	Zn	1 mg/kg feed	2 w, starting 7 d after first vacc and ending before second vacc	rabbits, 6 in each group	Zn and (Zn + methotrexate) treated rabbits have a higher anti-newcastle disease virus antibody titer than control group after vaccination	(Tipu et al., 2012)
Tetanus	Zn sulfate	220 mg, daily in 2 doses	1 mo prior to vacc.	human, 11 (Zn), 11 (P)	Increased anti-tetanus toxin IgG titer	(Duchateau et al., 1981)

1.1.2.2 Diarrhea

Diarrhea in piglets is a complex problem resulting from interaction between management procedures, host immunity, and infective agents. The most common causes of diarrhea in piglets are coronavirus (including transmissible gastroenteritis virus and porcine epidemic diarrhea virus), rotavirus, enterotoxigenic *Escherichia coli* (ETEC), *Clostridium perfringens*, and coccidia (including *Isospora suis* and *Cryptosporidium parvum*) (Katsuda et al., 2006). In addition, the piglet at weaning is subjected to momentous nutritional and environmental changes that can dramatically alter the quilibrium of the gastrointestinal microbiota, which could lead to post-weaning diarrhea. Because there seem to be no obvious differences in the effects of Zn treatment, all forms of diarrhea will be discussed together. On the one hand, diarrhea leads to increased intestinal loss of micronutrients, including Zn, which can be corrected by Zn supplementation (Hoque and Binder, 2006). On the other hand, extensive studies show that Zn can reduce the duration, severity, and incidence of diarrhea (Fischer Walker and Black, 2004; Galvao et al., 2013; Hoque and Binder, 2006; Lazzerini and Ronfani, 2012; Walker and Black, 2010).

In human, many studies confirmed that Zn supplementation has a substantial benefit effect for the treatment of both acute and persistent diarrhea in children (Bhutta et al., 2000; Karamyyar et al., 2013). However, this may not be the case for infants younger than six months of age (Walker et al., 2007). In children aged less than six months, the available evidence suggests zinc supplementation may have no effect on mean diarrhea duration, and may even increase the proportion of children whose diarrhea persists until day seven (Lazzerini and Ronfani, 2012, 2013).

In swine industry, it was demonstrated that Zn dietary supplementation reduced the incidence of diarrhea (Jensen-Waern et al., 1998; Owusu-Asiedu et al., 2003; Peace et al., 2011), even when weaned piglets were exposed to pathogenic strains of *E. coli* via their environment (Melin and Wallgren, 2002). It had been suggested that the diarrhea-reducing effect of Zn could contribute to its ability to inhibit bacterial adhesion and internalization, prevent the disruption of barrier integrity and modulate cytokine expression (Roselli et al., 2003). Zhang and Guo reported that diet supplemented with 2000 mg Zn/kg from ZnO reduced intestinal permeability by enhancing occludin and zonula occludens protein-1 expression in weaning piglets (Zhang and Guo, 2009). Some authors also suggest that the mechanism of this activity involves the stabilization of the

membrane structure (O'Dell, 2000) or the displacement of redox-active metals to prevent free-radical oxidative damage (Canali et al., 2000; Powell, 2000).

1.1.2.3 Virus infection

Effect of Zn supplementation on virus infection has been investigated in different experimental settings (Table 2). One viral infection for which the use of Zn has been extensively investigated is the common cold, which is a syndrome caused by a multitude of different viruses and many of them belonging to the rhino- and coronaviruses. The results have already been summarized in detail elsewhere (Hulisz, 2004; Jackson et al., 2000; Singh and Das, 2011), and were found to contradictory to each other. Chelated Zn given in a dose of 15 mg once a day for 3 months failed to reduce the incidence of the common cold in 8 to 13-year-old school children, but decreased the number of days on which children suffered from cough, rhinorrhoea and the likelihood of having two or more symptoms of the common cold (Rerksuppaphol and Rerksuppaphol, 2013). However, two meta-analyses did not confirm the effect of Zn in reducing the symptoms of the common cold (Jackson et al., 2000; Jackson et al., 1997). By using 4.5-23.7 mg elemental Zn per single dose, the results from those studies are as inconsistent as the treatment conditions.

Overall, it can be concluded that Zn is effective in shortening the duration of common cold on the condition that it is administered no later than 24 h within the onset of its symptoms (Hulisz, 2004; Singh and Das, 2011, 2013). But it is difficult to make firm conclusion about the dose, formulation and duration of Zn supplementation that should be used in future, because of the recorded differences in study populations, dosages, formulations and duration applied.

Given the particular importance of Zn for T cell development (Fraker and King, 2004; Wellinghausen et al., 1997a), it seems to be a sensible approach to use it as a supporting therapeutic intervention for human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS). The initial studies reported that short term supplementation of Zn to a relatively small group of 5 patients led to an increase in HLA-DR positive cells, a stimulation of lymphocyte transformation by phytohemagglutinin and concanavalin A, and increased phagocytosis by polymorphonuclear neutrophils (Zazzo et al., 1989). And long-term (18-month) Zn supplementation at nutritional levels delayed immunological failure and decreased diarrhea over time (Baum et al., 2010). However, in contrast with these results, lots of studies didn’t find the effect of Zn on immune response to tuberculosis, lymphocyte subsets, CD4/CD8 ratio, viral load, or antibody response to a pneumococcal conjugate vaccine (Bobat et al., 2005; Deloria-Knoll et al., 2006; Green et al., 2005). Furthermore, increased intake of Zn in HIV-1 infected

patients led to an augmented risk for the progression to AIDS and a lower survival (Tang et al., 1993; Tang et al., 1996). The possible reason for these contradictory results could be the different Zn status of the patients. While moderate Zn treatment to Zn-deficient patients can help stabilize their immune system, supplementation to Zn-sufficient subjects may accelerate disease progression and increase mortality (Overbeck et al., 2008).

The effect of Zn supplementation on hepatitis C, which is induced by an infection with hepatitis C virus (HCV), was also investigated. Ko et al. (Ko et al., 2005) reported that no effect of Zn in virological responses, but decreases in the incidence of gastrointestinal disturbances, body weights loss, and mild anemia were found after Zn treatment in patients with chronic hepatitis C. In addition, Zn supplementation was more effective against chronic hepatitis C when given in combination with IFN-α than a therapy with IFN-α alone (Takagi et al., 2001).

Herpes simplex virus (HSV) infections are extremely widespread in human population. Mahajan showed that 4% $ZnSO_4$ is an effective therapeutic modality not only for treatment but also for prolonging remissions in herpes genitalis (Mahajan et al., 2013). Two studies designed multifunctional Zn oxide (ZnO) micro-nano structures (MNSs) capped with nanoscopic filopodia-like spikes which mimic the cellular filopodial structure with a partial negative charge due to oxygen vacancies present on them to against the infection of HSV *in vitro* (Antoine et al., 2012; Mishra et al., 2011). The partially negatively charged MNSs could efficiently trap the virions via a novel virostatic mechanism rendering them unable to enter into human corneal fibroblasts – a natural target cell for HSV-1 infection (Mishra et al., 2011). This result was further confirmed by Antoine, who showed that ZnO tetrapods (ZnOTs) has the ability to prevent, neutralize or reduce Herpes simplex virus type-2 (HSV-2) infection, which is one of the most frequent sexually transmitted infections worldwide, bases on their ability to bind the HSV-2 virions *in vitro* (Antoine et al., 2012).

In addition, many *in vitro* studies demonstrated that high Zn^{2+} concentrations could inhibit the replication of various viruses, including influenza virus (Uchide et al., 2002), respiratory syncytial virus (Suara and Crowe, 2004), SARS-coronavirus (te Velthuis et al., 2010), equine arteritis virus(te Velthuis et al., 2010), and transmissible gastroenteritis virus (Wei et al., 2012). However, the *in vivo* relevance of an inhibition of these virus infections remains to be demonstrated.

Table 2. Effect of Zn supplementation on virus infection. (modified from Overbeck et al., 2008)

Diseases	Zn species	Zn dosage	Period	Participants	Effect of Zn supplementation	References
Acute lower respiratory infection	Zn gluconate	10 mg, daily, elemental	6 mo	298 (Zn), 311 (P)	Increased plasma Zn, decreased episodes of infection	(Sazawal et al., 1998)
	Zn acetate	20 mg, daily in 2 doses, elemental	5 d	76 (Zn), 74 (P)	Increase in serum Zn and recovery rates from illness and fever in boys	(Mahalanabis et al., 2004)
Chronic hepatitis C	Zn gluconate	78 mg, 5 times daily	6 mo	18 (Zn), 20 (P), 20 (C)	Increased serum Zn levels; decreased incidences of gastrointestinal disturbances, body weight loss, and mild animia	(Ko et al., 2005)
	polaprezinc	75 mg, 2 times daily	24 w	35 (Zn), 40 (C)	Zn supplementation increases serum Zn levels and improves the response to IFN-α therapy	(Takagi et al., 2001)
Common cold	>12 different studies				Variable results, Zn reduces duration of symptoms if administered within 24h of onset	(Hulisz, 2004; Singh and Das, 2011)
Diarrhea	Different studies with different design				Decrease duration, severity and occurrence of diarrha	(Hoque and Binder, 2006)
HIV/AIDS	Zn gluconate	45 mg, 3 times daily	15 d	5 (Zn), 5 (C)	Increased Zn in red blood cells and number of HLA-DR+ cells, stimulation of lymphocyte transformation and phagocytosis of opsonized zymosan by PMN	(Zazzo et al., 1989)
	Zn gluconate	50 mg, daily	6 d	44 (Zn), 45 (P)	No improvements in antibody responses to a pneumococcal conjugate vaccine	(Deloria-Knoll et al., 2006)
	Zn sulfate	200 mg, daily	1 mo	29 (Zn), 28 (P)	Increase or stabilization in body weight; increase in plasma Zn levels, CD4+ T cells and plasma active Zn-bound thymulin; reduced or delayed frequency of opportunistic infections due to *Pneumocystis jiroveci* and *Candida*, not to *Cytomegalovirus* and *Toxoplasma*	(Mocchegiani et al., 1995)
	Zn sulfate	10 mg, daily, elemental	6 mo	44 (Zn), 41 (P)	No effect on HIV-1 viral load, but reduction of morbidity from diarrhea	(Bobat et al., 2005)
	Zn sulfate	220 mg, daily	1 mo	31 (Zn), 34 (P)	No effects on immune response to tuberculosis, CD4/CD8 ratio, lymphocyte subsets and viral load	(Green et al., 2005)
	Zn sulfate	25 mg, daily	6 mo	200 (Zn), 200 (P)	When supplemented to pregnant HIV-positive women, no effect on birth outcomes or T-lymphocyte counts, and negative effects on hematological indicators	(Fawzi et al., 2005)
	Zn sulfate	25 mg, daily	6 mo	200 (Zn), 200 (P)	Increased risk of wasting	(Villamor et al., 2006)
	Zn	12 mg daily element for women, 15 mg daily element Zn for men	18 mo	210 (Zn), 21 (P)	Long-term (18-month) Zn supplementation at nutritional levels delayed immunological failure and decreased diarrhea over time	(Baum et al., 2010)
HSV	Zn sulfate	in concentrations of 1%, 2% and 4%	3 mo	90 (Zn), 10 (C)	4% $ZnSO_4$ has been found to be an effective therapeutic modality not only for treatment but also for prolonging remissions in herpes genitalis	(Lazzerini and Ronfani, 2013; Mahajan et al., 2013)
	Zn Acetate/ Carrageenan	10-30 µg Zn released from the gel	10 min before infection		The prototype gel comprising Zn acetate in protected mice against vaginal and rectal HSV-2 challenge	(Fernandez-Romero et al., 2012)
Viral warts	Zn sulfate	600 mg daily	2 mo	26 (Zn)	Oral zinc sulfate was found to be a good option in the treatment of viral warts, as it was safe and effective without important side-effects	(Mun et al., 2011)

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1.1.3 Mechanisms involved in the beneficial effects of Zn

The beneficial effects of Zn have been linked to many potential mechanisms, which include the inhibition effects of Zn on viral infection and replication, and the modulation of immune system, in particular cytokine production and the activation of immune cells (Overbeck et al., 2008).

1.1.3.1 Zn and virus replication

Zn^{2+} is an important cofactor of many viral proteins, and Zn ions could change the activities of different transcription factors and thus the expression patterns of cellular and viral genes (Lazarczyk and Favre, 2008).

Although it has not been demonstrated *in vivo*, Zn inhibits the formation of viral capsid proteins and the rhinovirus 3C protease, thus preventing the replication of rhinoviruses *in vitro* (Geist et al., 1987; Turner, 2001). Rhinoviruses, by attaching to the nasal epithelium via the intracellular adhesion molecule-1 (ICAM-1) receptor, cause most colds. The positively charged Zn ions can bind to the negatively charged regions at the carboxyl termini of rhinovirus coat proteins and inactivate the virus, thereby preventing the binding to ICAM-1 receptor (Hulisz, 2004; Novick et al., 1996). This explanation is in line with the inhibition of polyprotein processing by Zn ions that was observed in cells infected with human rihinovirus and coxsackievirus B3 (Krenn et al., 2009).

An inhibitory effect of Zn^{2+} on the activity of purified RNA-dependent RNA polymerase (RdRps) from rhinoviruses and hepatitis C virus was noted, and detailed investigation was confirm by te Velthuis (te Velthuis et al., 2010), who not only showed that corona- and arterivirus replication can be inhibited by increased Zn^{2+} levels, but also used both isolated replication complexes and purified recombinant RdRps to demonstrate that this effect may be based on direct inhibition of nidovirus RdRps.

1.1.3.2 Zn and immune system

The immune system consists of a non-specific, prompt component without immunological memory (innate immunity), and an antigen-specific, slowly developing but long lasting component (adaptive immunity). The innate immune response is very fast but lacks specificity and a memory. The adaptive immune system reacts specifically against consists of specialized cells a humoral part (B cells) and cellular immunity (cytotoxic T cells) are produced as

precursors and educated to recognize their specific antigen in the thymus (T cells) or bone marrow (B cells) (Abbas, 2005).

The function of the innate immune system can be modulated by Zn supplementation. Zn treatment can trigger events required for the recruitment of leukocytes to the site of infection *in vitro*. For example, high Zn concentrations induce chemotaxis of polymorphonuclear cells (Hujanen et al., 1995), and Zn promotes the adhesion of myelomonocytic cells (Chavakis et al., 1999).

Zn is also essential for adaptive immunity. It was reported that Zn deficiency not only affects B cells, but it has also been shown to lead to a reduction in antibody-mediated immune defense (Fraker and King, 2004), indicating the necessity of Zn for optimal results during antiviral actions. Thymulin is essential for differentiation and function of T cells. Zn deprivation reduces the level of biologically active thymulin in the circulation (Iwata et al., 1979). After supplementation of the serum with Zn in vitro, thymulin activity could be restored (Dardenne et al., 1984), indicating that T cell function is directly dependent on Zn. Furthermore, Zn plays an important role in the clearance of viral infections, since the cytotoxic T lymphocytes are highly dependent on the presence of it (Ibs and Rink, 2003).

In addition, Zn may counteract infections through its influence on the synthesis of cytokines. It was reported that Zn supplementation leads to an increased production of the IL-6, IL-1β, and TNF-α (Wellinghausen et al., 1997a) and IFN-α as well as IFN-γ (Berg et al., 2001; Cakman et al., 1997). In T cells, cytokine secretion is indirectly affected by Zn. Zn induced the production of IFN-γ and the soluble IL-2 receptor depends on the presence of monocytes, and is based on direct cell to cell contact and Zn-mediated production of the monokines IL-1 and IL-6 (Wellinghausen et al., 1997a).

1.1.3.3 Zn and epithelial cells

The integrity of the barrier is fundamental to the proper functioning of the epithelial cells and to preventing the entry of pathogens. Zn can protect proteins and nucleic acids from degradation, while stabilizing the microtubular cytoskeleton and cellular membranes, which indicates that Zn may be vital for maintaining the integrity and function of epithelial cells. Roselli et.al proved that Zn may protect or stabilize the cell membrane (Roselli et al., 2003), which could contribute to an inhibition of the entry of pathogens into cells. In addition, Zn also has been shown to be vital as anti-inflammatory agent in epithelial cells. For example, Sturniolo et.al demonstrated that high-

dose Zn regulated tight-junction permeability, with possible implications for healing processes in inflammatory diseases (Sturniolo et al., 2002).

1.2 Porcine reproductive and respiratory syndrome virus

1.2.1 Overview of PRRSV

Since the first emergence in late 1980s, porcine reproductive and respiratory syndrome virus (PRRSV) is still one of the most economically important infectious agents for the swine industry worldwide. PRRSV belongs to the family Arteriviridae and it possesses a positive sense RNA genome. The virus is known for frequent mutations resulting in constant emergence of genetic variants. Based on its genetic diversity, the virus was divided into two genotypes, Type1 (European) and Type 2 (North American). Lelystad virus (LV) and VR2332 are the prototype wildtype parental strains of Type 1 and Type 2 PRRSV, respectively. Each genotype contains several subtypes and strains, which are genetically highly diverse and display significant differences in their virulence and pathogenicity (Kim et al., 2007). PRRS is a major component of the porcine respiratory disease complex (Chand et al., 2012), and the clinical symptoms are characterized by developing high fever, anorexia, tachypnea or dyspnoea, reddening of the skin, chemosis, edema of the eyelids, and unusually high morbidity (50% - 100%) and mortality (20% - 100%) in pigs of all ages (Xiao et al., 2010). When the virus infects sows, late term abortion is often observed (Meulenberg, 2000; Rossow, 1998).

1.2.3 PRRS pathogenesis

PRRSV infects macrophages and dendritic cells, whose abilities to secrete inflammatory and immunomodulatory cytokines, and to present antigens to T cells, are essential for the induction of effective adaptive immune responses (Murtaugh and Genzow, 2011). Infection of a susceptible pig, leads to an acute infection and results in a viremia that lasts approximately 3–5 weeks (Xiao et al., 2004). PRRSV has the ability to subvert porcine innate and adaptive immune responses, which results in persistent infections. Persistent infection is characterized by the cessation of viremia and restricted viral replication in lymphoid tissues (Beyer et al., 2000; Rowland et al., 2003). Virus shedding from persistently infected pigs is one of the main factors that hinder the control of PRRSV infection in the farm. PRRSV is known to dampen immune system as early as 2 days post-infection (Dwivedi et al., 2012), and it continues to do so for several weeks post-infection (Mateu and Diaz, 2008). This immunosuppression leads to

increased susceptibility to infections with secondary pathogens (Done and Paton, 1995; Renukaradhya et al., 2012).

1.2.3 PRRS prevention and treatment

Vaccination is the principal means used to control and fight against PRRSV infection. Currently, these vaccines include products containing live virus derived by cell culture attenuation of virulent field isolates (e.g. Ingelvac® PRRS MLV and Porcilis® PRRS), inactivated preparations of virulent isolates expanded by *in vitro* cell culture for use in the same herd (autogenous vaccines), inactivated preparations of attenuated PRRSV strains (e.g. Progressis® and PRRomiSe®), inactivated preparation of multiple virulent isolates enriched with viral antigens (e.g. MJPRRS), and subunit vaccines expressing selected proteins (e.g. PRRSV-RS) (Murtaugh and Genzow, 2011). However, only for few of these products, their immunological properties and capacities to elicit protective immunity have been documented. All current live and/or killed PRRSV vaccines are ineffective in inducing a complete protection because of the diversity of PRRSV genotypes and strains within a given genotype (Labarque et al., 2004; Mengeling et al., 2003), and also have certain drawbacks concerning safety (Botner et al., 1997; Nielsen et al., 2001; Scortti et al., 2006). For example, the efficacy of available killed PRRSV vaccines is inadequate to protect pigs against even genetically closely related PRRSV, and none of the current vaccines prevent respiratory infection and pig-to-pig transmission of PRRSV (Kimman et al., 2009). Administration of either field isolates of PRRSV or vaccine including MLV-PRRS by the parenteral or intranasal route suppresses the innate natural killer cell cytotoxic function and IFN-α production (Albina et al., 1998; Dwivedi et al., 2012; Renukaradhya et al., 2010).

There are several substantial barriers to new PRRSV vaccine development. For example, uncertainty about the viral targets of protective immunity prevents a research focus on individual viral structures and proteins (Murtaugh and Genzow, 2011). More importantly, the great genetic diversity of PRRSV means that immunity to the initial infecting genotype of the PRRSV may therefore provide partial to or no protection against reinfection, reflecting not only the complexity of the genetics but also immunological variation among strains (Botner et al., 1997; Kimman et al., 2009; Martelli et al., 2009; Murtaugh and Genzow, 2011). Therefore, there is an urgent demand for novel strategies to control PRRSV infection, and these strategies need to provide broadly cross-reactive protection against challenge with genetically dissimilar, heterologous, strains.

1.3 Transmissible gastroenteritis virus

1.3.1 Overview of TGEV

Transmissible gastroenteritis (TGE) is a severe enteric disease causing devastating economic consequences for swine industry (Sestak et al., 1996; Ren et al., 2011). The etiologic agent responsible for this disease is the coronavirus TGEV, belonging to the family *Coronaviridae*. It was isolated for the first time in 1946 (Doyle and Hutchings, 1946). TGEV is an enveloped virus with a positive-stranded RNA genome approximately 28.5-kb in size, and it consists of four structural proteins: the spike (S), the integral membrane (M) glycoprotein, and the nucleocapsid (N) protein (Penzes et al., 2001; Schwegmann-Wessels et al., 2002). The glycoprotein S is primarily responsible for inducing neutralizing antibodies and for initiating infection. Neonatal piglets infected with TGEV may suffer from gastroenteritis within 20 h post-infection and death may happen in 1 - 4 days (Schwegmann-Wessels and Herrler, 2006). Survived elder pigs usually show growth retardation and low rate of reward to feeding. TGEV infects and destroys villous enterocytes of the small intestine leading to vomiting, severe malabsorptive diarrhea, dehydration, and high morbidity in animals of all ages as well as high mortality rate (up to 100%) in seronegative suckling piglets (Laude et al., 1993; Ren et al., 2011).

1.3.2 TGE prevention and treatment

Protective lactogenic immunity to TGEV could be conferred to suckling piglets through sIgA antibodies that are secreted into the milk (De Diego et al., 1994; VanCott et al., 1994). But it seems that the parentally applied inactivated viruses do not induce the local immune response in the small intestine that is required for protection. Commercially available vaccines, either inactivated or attenuated, have failed to provide full protection to piglets (Tuboly et al., 2000). Although closely related TGEV variant porcine respiratory coronavirus (PRCoV) has been found beneficial in preventing TGEV infections, possibly through induction of neutralizing antibodies that can provide cross-protection against TGEV infection (Schwegmann-Wessels and Herrler, 2006; Wesley and Lager, 2003), TGE prevalence is still being reported, and some TGEV strains have been isolated from domestic pigs in different parts of the world (Ren et al., 2011).

Many potential treatments were studied to control and treat TGEV infection. By identifying two shRNA-expressing plasmids pEGFP-U6/P1 and pEGFP-U6/P2 that target RNA-dependent RNA polymerase (RdRP) gene of TGEV, Zhou et al. found that the shRNA could inhibit TGEV replication both *in vitro* and *in vivo* (Zhou et al., 2010). But the shRNA have potential toxicity to

mini-pigs used in their studies, given that some damages could be found in the liver, kidney and lymphocytes proliferation in intestinal lamina propria. Although the combined administration of the swIFN-α and swIL-18 cytokines using attenuated Salmonella enterica serovar Typhimurium as an oral carrier provides enhanced protection against intestinal tract infection with TGEV(Lee et al., 2011), the optimized combination of cytokines need further compared and the use of attenuated Salmonella vector should always be cautious when it applied as a vaccine to pigs. Several novel and potent anti-coronavirus agents, like Lithium chloride, tylophorine compounds, and benzothiazolium $3CL^{pro}$ inhibitors were also reported (Ren et al., 2011; Yang et al., 2010; Yang et al., 2007), but the *in vivo* relevance of the inhibition effect of these agents remains to be demonstrated.

Since the lack of quick and potent anti-TGEV therapeutic agents (Brim et al., 1995; Saif, 2004; Zhou et al., 2010), the discovery and development of new, highly potent anti-TGEV agents and effective approaches for controlling the emergence of TGEV infection remains an important mission.

1.4 Aim of study

The worldwide trend towards a reduced reliance on in-feed antibiotics has increased the pressure to develop alternative strategies for the management of infectious diseases in the livestock industry. Extensive research into the beneficial effects of Zn has revealed its important role in host defense and immune responses, but there have been hardly any reports concerning the effects of Zn against virus infections in pigs yet.

Since the current vaccines or therapies are not successfully providing full protection against PRRSV and TGEV infections, the overall goals of this thesis were to investigate the systemic diet ZnO effects on an acute and persistent PRRSV infection and the local diet effects on a mild and self-limiting TGEV infection in pigs, and to explore the potentials of Zn as a single approach for multiple diseases. Therefore, in the PRRSV challenge infection trial, we tried to find out if high levels of Zn could influence the success of vaccination with an experimental inactivated vaccine and if it could affect the level of protection in vaccinated and/or non-vaccinated pigs challenged with a heterologous PRRSV strain. And in the trial of TGEV infection, which causes serious gastroenteritis in piglets, we sought to determine if Zn supplementation could improve systemic and/or mucosal immune responses after infection, and the possible protection mechanisms involved in the antiviral potential of Zn were investigated.

The specific aims of this study are:

1. To detect if high level of Zn could improve the clinical outcome after virus infections in pigs;
2. To evaluate whether Zn supplementation has an effect on the innate and adaptive immune responses to virus infections;
3. To explore potential of Zn treatment on the immunogenicity of a PRRSV vaccine;
4. To investigate the mechanisms responsible for a protective effect of Zn against virus infections *in vivo*.

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Chapter 2: Effect of dietary zinc oxide on porcine reproductive and respiratory syndrome virus (PPRSV) infection in pigs

2.1 Abstract

Background: Porcine reproductive and respiratory syndrome (PRRSV) is still one of the most economically important infectious agents for the swine industry worldwide. Therapeutic application of zinc (Zn) to treat or prevent pathogens has been demonstrated. The purpose of this study was to determine the influence of dietary zinc oxide supplementation on vaccination and challenge infection with PRRSV.

Methods and results: Weaned piglets received three different diets containing low (50 ppm), medium (150 ppm) and high levels (2500 ppm) of Zn oxide. Half of the animals received a single vaccination with an experimental inactivated PRRSV vaccine in order to test if higher Zn doses could enhance vaccine efficacy. Four weeks after vaccination, all pigs were challenged intranasally with a heterologous PRRSV strain and necropsied at 35 days post-infection (dpi). The clinical course of disease was not significantly influenced by either vaccination or diet. Vaccinated pigs showed faster seroconversion and higher PRRSV-specific IgM/IgG antibody levels early after infection, and this was also seen as a general tendency for animals receiving higher dietary Zn levels. The same was observed for neutralizing antibodies, which were detectable from day 28 post infection. Concerning the cellular immune response, vaccination resulted in increased percentages of blood CD8+, CD4+ cells, γδ T cells and NK cells at early time points after infection, while an influence of diet was not observed. The virological outcome was not reduced by diet or vaccination, and viral loads were even higher in serum and nasal swabs from vaccinated animals compared to their non-vaccinated counterparts.

Conclusions: Taken together, our results suggest that although higher levels of dietary Zn oxide can influence immune responses after PRRSV infection and in response to a prime-only vaccination, this did not result in an improved clinical and virological outcome.

Keywords: PRRSV; inactivated vaccine; Zinc oxide

2.2 Introduction

Porcine reproductive and respiratory syndrome (PRRS) continues to be one of the most significant swine diseases worldwide, which constitutes a persistent challenge to both

vaccinology and control after its first emergence in North America more than 20 years ago [1,2]. PRRS virus (PRRSV) belongs to the *Arteriviridae* family and isolates are divided into two distinct genotypes, the European genotype (EU or type I), represented by the Lelystad virus (LV), and the North American genotype (NA or type II), represented by the ATCC VR-2332 strain. PRRSV mainly infects well-differentiated cells of the monocyte/macrophage lineage, more specifically porcine alveolar macrophages (PAMs), but also interstitial macrophages in other tissues. The virus often causes persistent infections due to the inability of the host's immune system to efficiently eliminate it. PRRSV infection is characterized by reproductive failure in sows and respiratory problems in growing pigs. The virus is a major component of the porcine respiratory disease complex, which is of mixed etiology and occurs at any stage of production causing clinical disease and reduced weight gain [3]. Economic losses in growing pigs caused by PRRSV are estimated to reach as much as $500 million *per annum* [4]. In addition to other eradication strategies, efficient PRRSV vaccines would be invaluable in minimizing the clinical and economic impact of PRRSV infections. Immune protection against a broad spectrum of PRRSV strains is paramount due to the extreme genetic diversity and rapid evolution of PRRSV [2]. Ideally a vaccine would confer sterilizing immunity with high titers of neutralizing antibodies in the blood and at sites of virus entry. In addition it should also stimulate cellular immunity in order to efficiently eliminate infected cells. Currently there are both live-attenuated and inactivated PRRSV vaccines commercially available. Live-attenuated vaccines have the potential to protect animals against viremia, but the level of protection depends on the homology between vaccine virus and circulating virus [5]. Furthermore, there are safety concerns, as vaccine virus may spread and revert to virulence [6]. In contrast, inactivated vaccines are generally safe to use, but do not provide sufficient protection [7].

Zinc (Zn) ion salts have been shown to exhibit a broad-spectrum antiviral activity against a variety of viruses *in vitro*, including the animal viruses, equine arteritis virus and transmissible gastroenteritis virus. These effects include blocking of adsorption and virus entry as well as inhibition of intracellular replication and processing [8,9]. Furthermore, Zn induces the production of antiviral interferon (IFN)-α and IFN-γ and it can potentiate the antiviral action of IFN-α [10]. However, there is a lack of data concerning *in vivo* effects. It is well-known that Zn is indispensable for proper immune function of innate immune cells such as neutrophils and natural killer cells and that Zn deficiency also affects acquired immunity: T cell numbers are reduced and functions like Th1 cytokine production, and B lymphocyte help are compromised. The clearance of viral infections requires cytotoxic T lymphocytes, which are highly dependent on Zn. Similarly, B cell development and antibody production, particularly that of IgG, is

compromised. Important for PRRSV, macrophage functions like intracellular killing, cytokine productions, and phagocytosis, are also adversely affected by Zn deficiency [11]. On the other hand, there is evidence that elevated Zn intake can stimulate immune responses, but it was also shown that prolonged excess Zn intake can result in anemia and immunodepression [12].

Growing pigs require 50 mg Zn per kg diet (50 ppm) according to National Research Council recommendations [13]. Zn oxide (ZnO) is often added as a supplement to the feed to cover these requirements. In swine nutrition, especially in the North American swine industry, high levels of in-feed zinc oxide (2000-3000 ppm) are often added to the diet of weaned pigs since this was shown to reduce non-specific post-weaning diarrhea and improve performance in this critical period of dietary change [14]. Evidence of limited positive effects of these high doses on the immune response in *Salmonella*-infected piglets was also provided [15]. However, because of environmental concerns, the maximum level of Zn allowed in pig diets was set up to 150 ppm in the European Union irrespective of the source or formulation [16].

The objective of this study was to examine, if feed supplementation with different levels of ZnO could influence the success of vaccination with an experimental vaccine and if it could affect the level of protection in vaccinated and/or non-vaccinated pigs challenged with a heterologous PRRSV strain. For the experimental vaccine we followed an approach based on a publication of Vanhee et al. [17] who produced LV-based PRRSV vaccines with different methods of inactivation and various adjuvants. One important result of their study was the discovery that vaccination with a UV-inactivated virus in combination with a suitable adjuvant could strongly prime the virus-neutralizing (VN) response and markedly reduce duration of viremia after infection with LV. In contrast to Vanhee and colleagues, who vaccinated twice, we chose a single-vaccination approach with an UV-inactivated LV adjuvanted with aluminium hydroxide, liquid paraffin and mannid mono-oleate, which are potent enhancers of antibody responses [18] in order test the influence of elevated Zn levels on immunogenicity in response to a suboptimal antigenic stimulus. As another difference to Vanhee et al., who used LV as a challenge virus, we infected the animals with a type I PRRSV field strain in order to evaluate the effects of Zn on heterologous protection.

2.3 Results

2.3.1 Clinical follow-up and pathology

Almost all pigs showed clinical symptoms typical for PRRSV infection like increased rectal temperature for more than 14 days and edema of the eyelids and conjunctivitis for more than 21 days, but there was no significant difference between groups. Other clinical signs such as cough were recorded only for two individuals and lasted just for 1-2 days. Figure 1 shows the development of body temperature and weekly weight gain. Regarding the latter, we only compared the differences in non-vaccinated groups, given that 4 time points were missing in Zn^{high} pigs in vaccinated groups. A significantly lower weight gain was found from 14 to 21 dpi in the control group (Zn^{low} group) relative to the Zn^{med} (P=0.025) and Zn^{high} (P=0.036) groups. Necropsies at 35 dpi revealed, that none of the animals had gross lesions in lungs, and no differences were observed between groups in terms of lymphoid hyperplasia in tonsils, lymph nodes or spleen (data not shown).

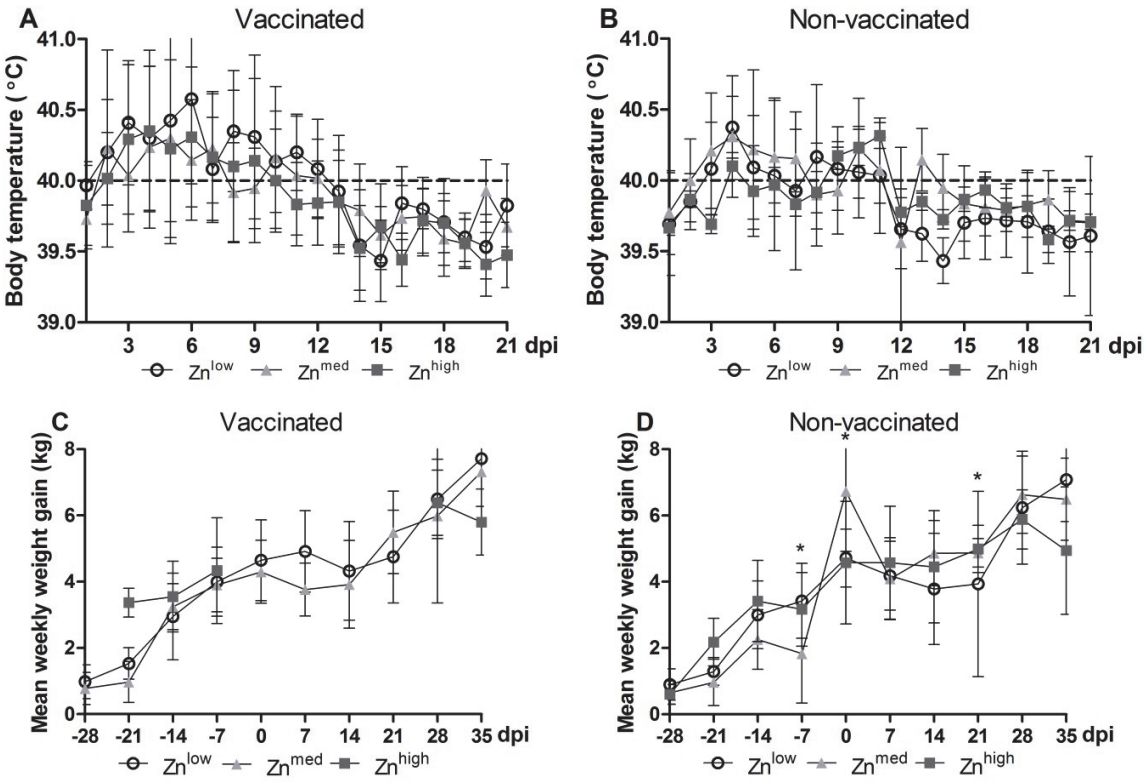

Figure 1 Body temperature and weekly weight gain in pigs infected with PRRSV strain CReSA 3267. Development of body temperatures (Figures 1A and 1B) and weekly weight gains (Figures

1C and 1D). Body temperatures ≥ 40°C were defined as fever (dotted line). Each bar represents the mean value ± standard deviation from 12 pigs. Asterisks indicate differences ($P < 0.05$) between averages at each dpi calculated by Fishers Least Significant Difference (*LSD*) test.

2.3.2 Viral load in serum and tissues of PRRSV infected pigs

After challenge, PRRSV loads were determined by qPCR in serum samples. Results of the development of viremia are shown in Figure 2. Mean PRRSV load in serum peaked at 4 dpi with an average load of 1.3×10^6 genome copies/ml and gradually reduced later on. Vaccination did not influence PRRSV loads at 4, 7, and 14 dpi, and led to greater ($P=0.022$) virus loads at 21 dpi compared to non-vaccinated animals. Higher dietary Zn levels did not significancly influence viral genome copy numbers in serum. Examination of nasal swabs indicated that greater ($P<0.01$) virus shedding was present in vaccinated groups compared to their non-vaccinated counterparts. Considering the Zn effects, lower virus shedding was found in the vaccinated Zn^{med} and Zn^{high} groups only at 4 dpi. At 35 dpi, PRRSV was detectable in the majority of tonsil samples. Comparison between vaccinated and non-vaccinated groups revealed no significant differences. However, higher viral genome copy numbers were determined in Zn^{high} animals when compared to the other two groups regardless of vaccination ($P=0.008$). All lung and tracheobronchial lymph node samples were tested negative for the presence of PRRSV genomes.

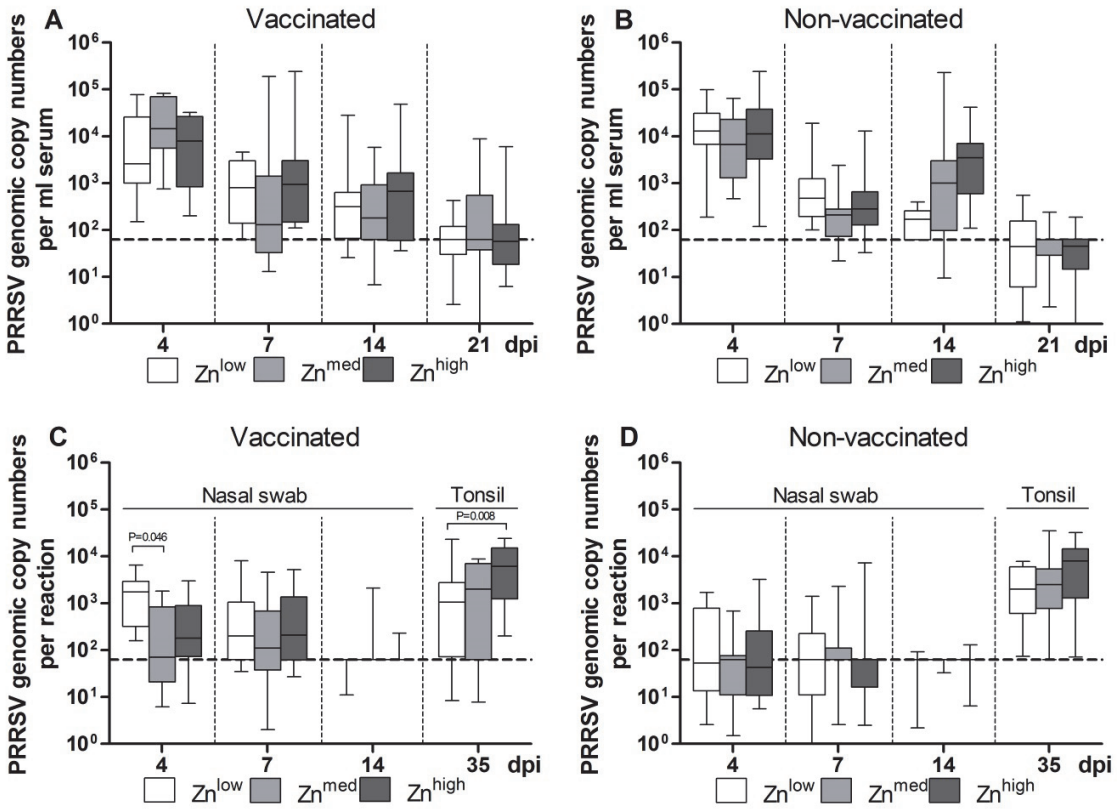

Figure 2 Detection of virus in sera, nasal swabs and tonsils by quantitative RT-PCR after PRRSV infection. Boxes indicate medians (n=12) (horizontal lines) and the lower and upper quartiles (bottoms and tops of boxes). The vertical bars in the box plots indicate the minimal and maximal values recorded. Mean viral loads (copy numbers/ml) of virus load in sera (Figures 2A and 2B), nasal swabs and tonsils (Figures 2C and 2D) are shown. Viral RNA could not be detected if it was less than 6.3×10^{1} (below broken line). Significant differences ($P<0.05$) are marked with an asterisk.

2.3.3 Antibody response against PRRSV infection

Randomly chosen samples (10 vaccinated/13 non-vaccinated) were tested negative for PRRSV antibodies as measured by ELISA on day 7 after vaccination (day 21 before infection). On day 0, only one vaccinated animal tested positive for PRRSV antibodies. Figure 3 shows the development of the humoral response after challenge infection. At 7 dpi, piglets from all groups were seropositive, except for 1 pig each in the Zn^{low}+VAC and Zn^{low} groups. Based on S/P ratios, higher ($P\leq0.01$) antibody levels were detected in vaccinated groups than in non-vaccinated groups at 7 and 21 dpi after PRRSV challenge. Regarding the diets, there was a tendency

towards higher PRRSV antibodies in Zn^{med} and Zn^{high} groups at 7 dpi, but no clear influence was seen at later time points (Figures 3A, B).

Virus neutralizing (VN) antibodies were not detected until 28 dpi in the serum. Higher levels of Zn (Zn^{med}) induced increased (P=0.016) VN antibodies titer at 28 dpi. This tendency continued to 35 dpi in both Zn^{med} and Zn^{high} groups (P=0.072). Furthermore, vaccinated groups developed higher (P=0.045) VN titers than non-vaccinated groups at 35 dpi (Figures 3C, D).

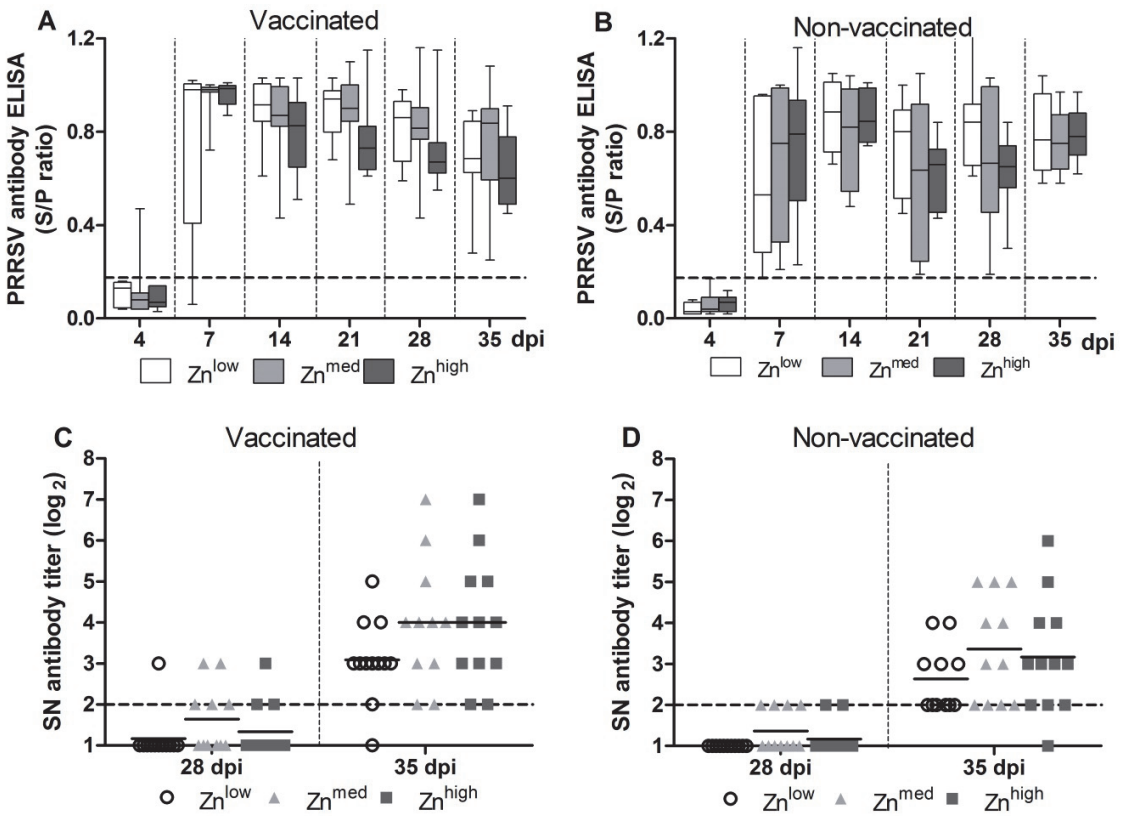

Figure 3 Development of humoral responses against PRRSV. **A and B,** PRRSV-specific antibodies measured by ELISA are shown as average sample to positive ratios (S/P) of optical densities from 4 to 35 dpi. **C and D,** Serum neutralization tests were performed by a standard immunofluorescence assay and the results are shown as titers (log_2). The PRRSV-specific antibody response was considered as negative when S/P < 0.175, and SN antibody was considered as negative when the antibody dilution was lower than 1:4 (below broken line).

2.3.4 Cell-mediated immune response

The IFN-γ ELISpot was performed at 35 dpi using in both PRRSV strain CReSA 3267 and LV in order to examine homologous and heterologous responses. Restimulation with the challenge virus CReSA 3267 revealed no differences in terms of virus-specific IFN-γ secreting cells in all groups (Figures 4A, B). Heterologous stimulation with LV showed a similar tendency as seen in homologous stimulation (Figures 4A, B).

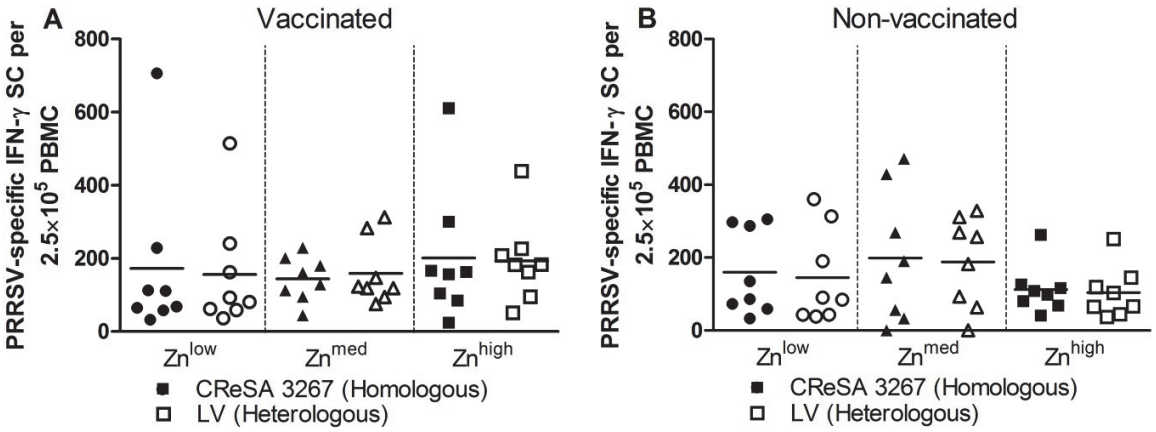

Figure 4 PRRSV-specific cell-mediated immune response was determined by IFN-γ ELISpot at 35 dpi. PBMC were stimulated with either of the PRRSV strains (Lelystad or strain CReSA 3267) used in the study. Results are shown as average frequencies of virus-specific IFN-γ secreting cells per 2.5×10^5 of PBMC as determined by ELISpot. Filled symbols indicate results obtained after *in vitro* stimulation with the same PRRSV used for infections (homologous) while empty symbols show the results of *in vitro* stimulation with PRRSV LV (heterologous).

2.3.5 Modulation in the frequency of immune cells in the blood

Flow cytometry analysis of PBMC phenotypes was performed weekly from day 0 to day 35 post infection. We found a reduction ($P<0.05$) in the frequency of CTLs ($CD4^-CD8\alpha^{high}$) in non-vaccinated groups at 7 dpi, while a slight increase at 7 dpi followed by a drop at 14 dpi was found in vaccinated groups (Figure 5A, B). Dietary effects on CTL frequencies were not observed. A similar tendency was also seen for $CD4^+CD8^+$ T cells (data not shown). Naïve T_H cell freqencies ($CD4^+$ $CD8^-$) dropped at 7 dpi in non-vaccinated animals followed by a gradual recovery until 28 dpi and another drop at 35 dpi. In vaccinated animals the same tendency was observed, but the initial drop was delayed until 14 dpi (Figure 5C, D). The frequency of

activated/regulatory T_H cells ($CD3^+CD4^+CD25^{high}$) was only slightly increased following infection and differences between vaccinated and non-vaccinated groups and diets were not observed (data not shown). Animals in the Zn^{high} groups had significantly increased $CD4^+CD8^+$ T cells population at 28 dpi (P=0.011) compared to those in the Zn^{med} groups, whereas no dietary effect on CTL, naïve T_H cells and activated/regulatory T_H cells were observed.

γδ T cells showed a trend similar to that observed for the CTLs: a delayed reduction of $CD2^+CD8^+$ was found in animals of all vaccinated groups. The frequency of $CD2^+CD8^+$ and $CD2^-CD8^-$ T cells increased in Zn^{med} and Zn^{high} pigs, respectively (Figures 5E, F).

Greater numbers (P<0.01) of antibody producing and/or memory B cells ($CD2^+CD21^-$) were determined in vaccinated groups at 7 dpi (Figure 5G, H), while no significant differences (P=0.181) were found concerning primed and/or activated B cells ($CD2^-CD21^+$). Finally vaccination also significantly increased (P<0.01) the frequency of NK cells at 7 and 14 dpi compared to non-vaccinated groups (Figure 5I, J)

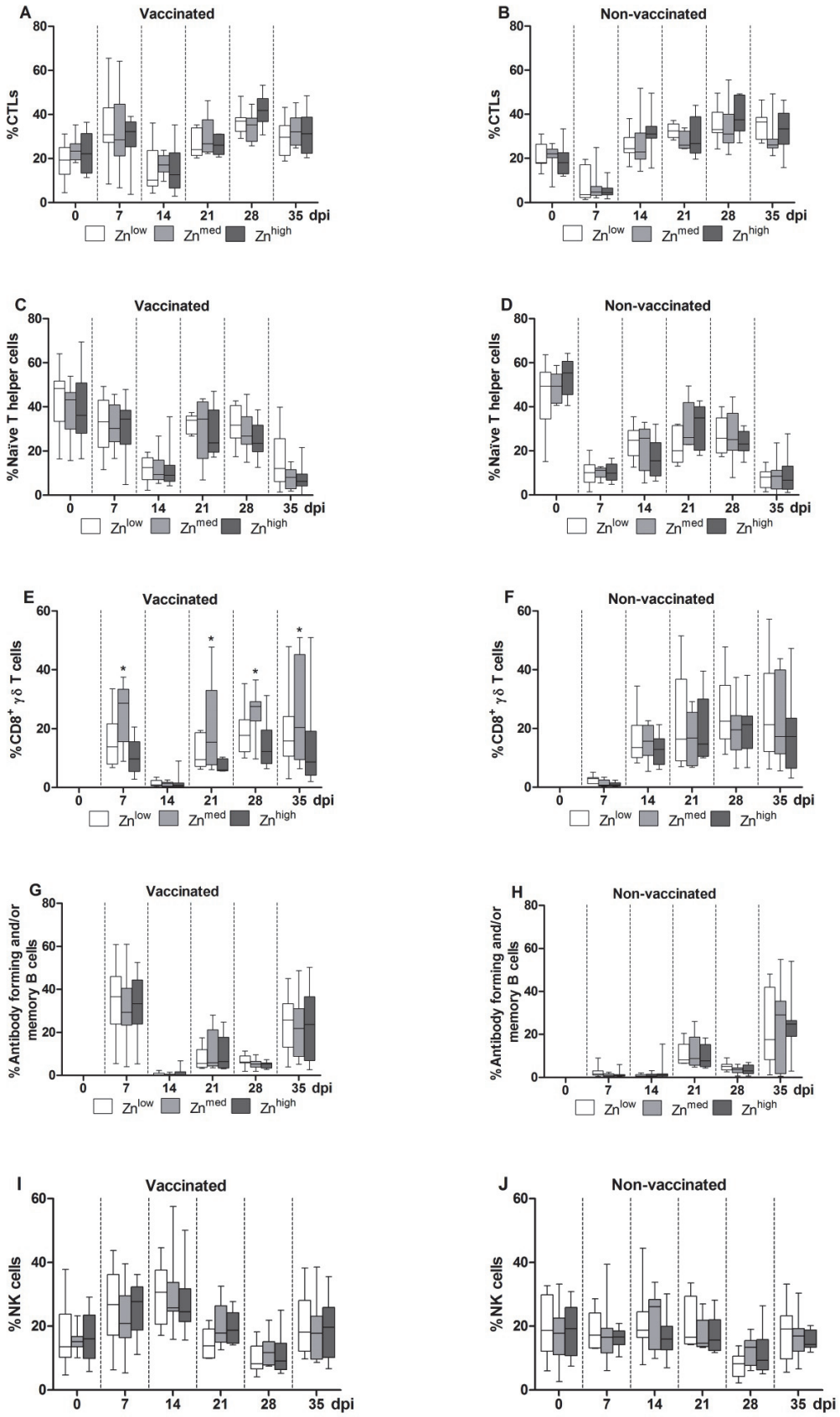
A
Vaccinated
% CTLs
B
Non-vaccinated
% CTLs
C
Vaccinated
% Naïve T helper cells
D
Non-vaccinated
% Naïve T helper cells
E
Vaccinated
% CD8+ γδ T cells
F
Non-vaccinated
% CD8+ γδ T cells
G
Vaccinated
% Antibody forming and/or memory B cells
H
Non-vaccinated
% Antibody forming and/or memory B cells
I
Vaccinated
% NK cells
J
Non-vaccinated
% NK cells
0 7 14 21 28 35 dpi
Zn low Zn med Zn high

Figure 5 Modulation in the frequency of immune cells in pigs with or without vaccination and fed with different concentration of Zn (Zn^{low}, Zn^{med} and Zn^{high}). Boxes indicate the medians (n=12) (horizontal lines) and the lower and upper quartiles (bottoms and tops of boxes). The vertical bars in the box plots indicate the minimal and maximal values recorded. White boxes, control (Zn^{low}); light gray boxes, Zn^{med}; dark gray boxes, Zn^{high}. Blood cells were immunostained to determine the frequency of immune cells: **A and B**, CTLs ($CD3^+CD4^-CD8\ \alpha^{high}$); **C and D**, naïve T_H cells ($CD3^+CD4^+CD8^-$); **E and F**, $CD8^+\ \gamma\delta$ T cells ($CD3^+CD2^+CD8^+$); **G and H**, Antibody forming and/or memory B cells ($CD3^-CD2^+CD21^-$); **I and J**, NK cells, and then subjected to flow cytometry. Asterisks indicate statistically significant differences ($P < 0.05$) between averages at each dpi.

2.4 Discussion

The aim of this study was to determine humoral and cellular responses in pigs after vaccination and subsequent challenge with a European PRRS wild-type virus. Pigs were fed different levels of dietary Zn (Zn^{low}, Zn^{med} and Zn^{high}) and clinical signs and virological as well as immunological parameters were assessed as a function of diet and vaccination. Overall, the study shows that challenge infection with a wild-type PRRSV without additional environmental and social stress results in relatively mild clinical signs. All pigs developed clinical symptoms typical for PRRSV infection and similar to a previous study with the same PRRSV strain [19] irrespective of vaccination and diet. Positive effects of higher dietary Zn levels were only seen between 14 and 21 dpi, when a more prominent increase in weekly body weight gain was found in the non-vaccinated Zn^{med} and Zn^{high} groups. Since fever had ceased in almost all pigs at that period, this would suggest that higher Zn supplementation could positively affect growth in clinically non-affected animals, which is consistent with a study by Sales [20] who showed that pharmacological levels of ZnO corresponding to the Zn^{high} diet used in this study increased weight gain in post-weaning pigs.

Regarding the humoral immune response, it has been shown that PRRSV infection induces antibody production rapidly (5 – 7 dpi) but that antibodies are mainly of the IgM type and do not possess neutralizing capacities. They might even increase infection rates via opsonization of virus, making it a target for macrophage phagocytosis. The development of antibodies with neutralizing capacity is delayed and diminished in comparison to the response to other viruses and appears only after viremia is no longer detectable [21].

In our study, the humoral immune response was measured by an ELISA that detected both virus-specific IgM and IgG antibodies. In vaccinated animals antibodies were not detectable before infection, which was not unexpected, since very few non-live vaccines induce high antibody responses after a single vaccine dose [22]. However, Vanhee et al. observed an IPMA-detectable antibody response already after prime vaccination with a similarly inactivated LV-Virus adjuvanted with incomplete Freund's adjuvant (IFA). Concerning the adjuvans, the vaccine used in our study contained the ingredients that are also part of IFA plus aluminum salt. Apart from a potentially higher sensitivity of the IPMA, one explanation for the discrepancy between our results and those seen by Vanhee et al. might be that we used a cell culture-adapted LV with possibly decreased immunogenicity when compared to purified LV grown on PAMs used in the cited study [17]. After infection, seroconversion was not detectable before 7 dpi in non-vaccinated and only sporadically detectable in vaccinated animals independently of dietary Zn levels. Thus, the heterologous challenge did only slightly boost IgG/IgM antibody production, although a temporary increase of antibody-forming B cell numbers could be observed in vaccinated animals (Figure 5 G) at that time point. Higher dietary Zn levels positively influenced antibody levels only at 7 dpi in both vaccinated and non-vaccinated animals. Since antibody production is affected by Zn deficiency [23,24], this might suggest that the animals in the control group temporarily received Zn at levels too low to allow full development of early antibody responses.

The generation of neutralizing antibodies is delayed in PRRSV infection and usually appears three to four weeks after infection [21]. In accordance, we detected neutralizing antibodies from 28 dpi with a higher incidence of detectable neutralizing antibodies in the vaccinated groups and a tendency towards higher titers in the Zn^{med} and Zn^{high} treatment groups (Figure 3C,D). This might point towards a possible involvement of Zn in the process of affinity maturation of antibodies produced by B cells.

Concerning the cell-mediated immune response, it was shown that the antiviral protection was higher in pigs that developed higher IFN-γ-SC responses [25]. In our study, no differences in the number of IFN-γ-SC in response to vaccination or diet were determined after homologous or heterologous stimulation. The results are in accordance with our flow cytometry data in which percentages of T_H/memory cells and CTLs, which are the main producers of the antiviral cytokine, were also not different between those groups. Since we only determined IFN-γ-SC numbers at 35 dpi, we cannot exclude that Zn treatment and/or vaccination might have had effects on IFN-γ-SC responses at earlier time points.

By analyzing the cellular immune response via flow cytometry, we found a temporary reduction of $CD4^+$, $CD8^+$ and $CD4^+CD8^+$ T cell populations, which are important for viral clearance, in unvaccinated pigs at 7 dpi. Comparable results were observed by Dwivedi et al., suggesting that one mechanism of the PRRSV-mediated delay in initiation of adaptive immunity might be an alteration of function and frequency of those lymphocyte subsets early post-infection [26]. In vaccinated animals, such a decrease was also seen but delayed and less pronounced. A dietary effect on $CD4^+$, $CD8^+$ and $CD4^+CD8^+$ T cell frequencies was not observed.

Regarding the involvement of innate immune cells, we also determined percentages of γδ T cells, particularly the $CD2^+CD8^+$ subset, which has been suggested to have antiviral and anti-inflammatory functions [27] and has been shown to possess antigen-specific cytolytic activity in PRRSV and FMDV infected pigs [28,29]. In non-vaccinated animals we observed a strong increase in blood $CD2^+CD8^+$ γδ T cell at 14 dpi, which persisted throughout the observation period. Similar observations had also been made for a type II PRRSV strain [30]. In vaccinated animals a strong increase of $CD2^+CD8^+$ γδ T cell frequency was already seen at 7 dpi. Regarding dietary effects, we found that in the vaccinated animals the Zn^{med} but not the Zn^{high} diet increased the frequency of γδ T cells compared to the control group, suggesting that positive effects of Zn on immune responses of pigs infected with PRRSV might be seen at optimal but not at excessive Zn levels. The frequencies NK cells, which also play an important role in innate antiviral immunity, were elevated in comparison to the non-vaccinated controls on day 7 and 14 post infection, but dietary Zn levels had no influence

The analysis of virological outcome after challenge revealed that vaccination had no positive effect on viremia and resulted in even higher serum viral loads in the vaccinated groups at 21 dpi. Accordingly, higher virus shedding in nasal swabs in these groups was noticed. Similar phenomenons were reported earlier [31] and since for the vaccinated animals higher antibody titers were obtained by ELISA, a possible explanation could be antibody-dependent enhancement of infection, where virus uptake by macrophages is increased through opsonization by non-neutralizing antibodies.

Considering dietary effects, vaccinated Zn^{med} and Zn^{high} treatment groups had reduced virus shedding in nasal swabs only at 4 dpi. Possibly, higher Zn doses in combination with the vaccine might have elicited unspecific antiviral effects early after infection.

Taken together, higher dietary Zn levels partially influenced humoral and cellular immune parameters in response to vaccination and challenge with PRRSV but were not able to reduce clinical symptoms and viremia.

2.5 Materials and methods

2.5.1 Viruses and cells

Lelystad virus (LV; accession no. M96262) was a kind gift from Prof. H. Nauwynck (Ghent University, Ghent, Belgium) and the European genotype PRRSV strain CReSA 3267 (accession no. JF276435) was kindly provided by Prof. J. Segalés and Prof. E. Mateu (CReSA, Barcelona, Spain). To prepare PRRSV stocks and to perform virus neutralization tests, a stable mycoplasma-free MARC-145 cell line (subclone of MA104 monkey kidney cell line), which is highly permissive for PRRSV was used. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; PAN Biotech) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone), and 1% penicillin/streptomycin (Biochrom) at 37 °C with 5% CO_2. For virus infection, DMEM supplemented with 2% FBS was used.

2.5.2 Vaccine preparation

The vaccine was based on an inactivated LV diluted to a titer of 1 x 10^7 50% tissue culture infective doses ($TCID_{50}$)/ml in DMEM. Inactivation was performed by UV irradiation of the virus suspension with 1000 mJ/cm^2, using a UV crosslinker (HL-2000 hybrilinker UVP). Inactivated virus was mixed with an oil-in-water adjuvant solution prepared similarly to the diluent used in the commercial pseudorabies vaccine Suvaxyn® (Zoetis) containing aluminium hydroxide (Sigma-Aldrich), liquid paraffin, mannid mono-oleate (Gerbu Biotechnik), and Tween 80 (Sigma-Aldrich) before vaccination.

To verify if virus was completely inactivated, 1 ml of inactivated virus suspension was inoculated on MARC-145 cells in a 150 cm^2 tissue culture flask in 50 ml of medium. The cells were cultivated for 1 week at 37°C, followed by transfer of the supernatant to a fresh culture and incubation for another week. Inoculation of MARC-145 cells with 1 ml of non-inactivated virus was included as a positive control. Cells were analyzed for cytopathic effects (CPE) and the presence of viral RNA in the supernatants was further confirmed by qPCR.

2.5.3 Animals and experimental design

The study was approved by the local animal welfare authority (Landesamt für Gesundheit und Soziales, Berlin, Germany) under the registration number G 0116/12. German Landrace piglets (n = 72) of both sexes originating from 13 sows from a PRRSV-free herd (Leibniz-Institut für Nutztierbiologie, Dummerstorf, Germany) were weaned at the age of 28 days and then moved to a biosafety level 2 and environmentally controlled experimental facility (Bundesinstitut für Risikobewertung, Berlin, Germany) where they were randomly allocated to six pens (n = 12 / pen) of 8.5 m^2 each. The concrete floors of the pens were covered with rubber mats. Each pen was equipped with 3 nipple drinkers (water flow of 1000ml/min) and 3 feeding troughs, each with 70 cm length. Rooms were constantly ventilated and kept at ambient temperature of 26 °C at delivery, with a stepwise reduction to 22 °C. For the first 4 weeks, heat lamps provided additional warmth.

Piglets were assigned to three different diets (2 pens per diet) supplemented with 50, 150 or 2,500 ppm of ZnO in the diets (Zn^{low}, Zn^{med} and Zn^{high}). Given that 50 ppm of Zn is the minimally recommended Zn content supplied with the regular diet, Zn^{low} was considered the negative control. Feed was already offered to suckling piglets from 14 days of age and was offered *ad libitum* after weaning. At the age of 63 days, the animals receiving the Zn^{high} diet were switched to the Zn^{med} diet, in order to avoid toxic effects of Zn. One week after commencing the different diets (day 35 of age), one pen per diet was chosen randomly and the animals were vaccinated intramuscularly (i.m.) with a UV-inactivated vaccine based on LV (Vanhee et al., 2009). Four weeks after the first vaccination, at the age of 63 days, all pigs were challenged with the heterologous European genotype PRRSV CReSA 3267 with a titer of 5 x 10^6 $TCID_{50}$/ml by intranasal application of 1 ml of the suspension to each nostril of one pig using a spray nebulizer. All pigs were necropsied on day 35 post infection (dpi), i.e. at age of 98 days (Figure 6).

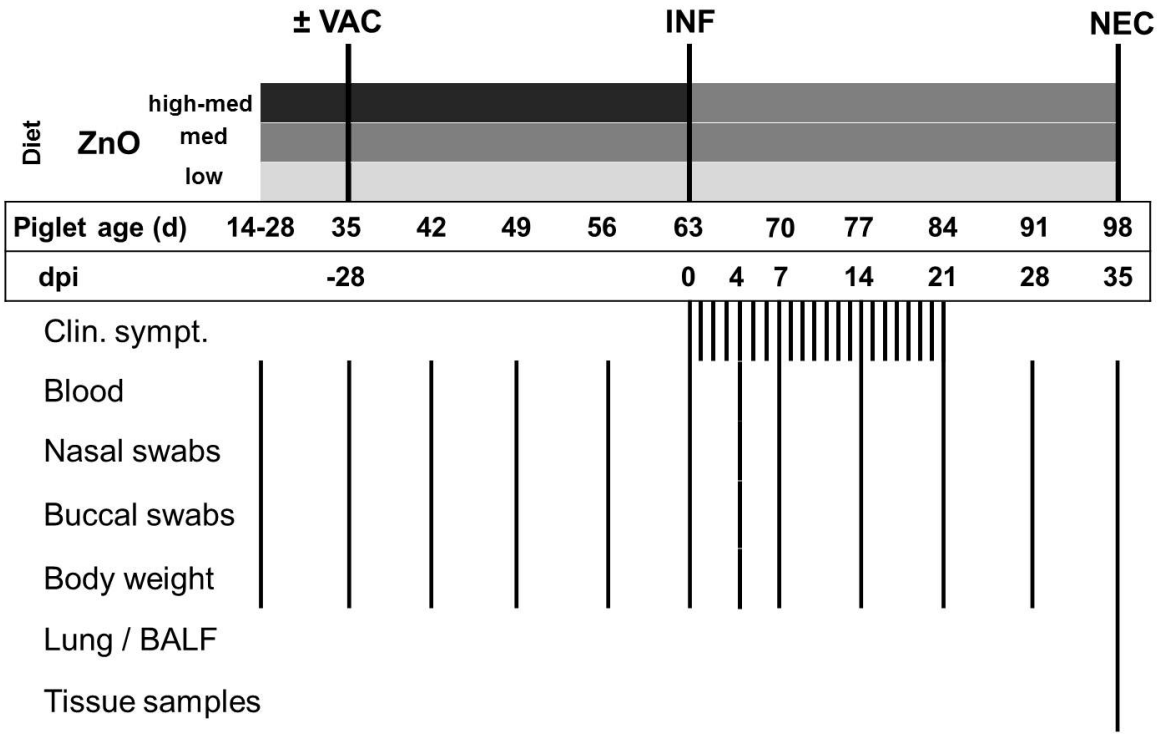

Figure 6 Experimental design showing PRRS vaccination, infection and sampling time points. The Zn^{high} diet was fed for 5 weeks and then reduced to Zn^{med} in order to avoid toxic effects; VAC: vaccination with an UV-inactivated vaccine based on a European genotype PRRSV (Lelystad virus); INF: intranasal infection with 5×10^6 $TCID_{50}$/ml (2 ml) PRRSV strain 3267; NEC: necropsy at 35 dpi.

2.5.4 Clinical follow-up and sampling

Animals were clinically examined on arrival. Blood samples were collected in duplicate (heparinized and siliconized blood collecting tubes) weekly after vaccination and at 4, 7, 14, 21, 28, and 35 dpi sera were collected to determine viremia, PRRSV-specific antibodies and virus neutralizing antibodies (NA). Heparinized blood samples were used to obtain peripheral blood mononuclear cells (PBMC) to measure IFN-γ by ELISpot. Pigs were monitored daily for presence of clinical signs and rectal temperatures were taken. Body weight was recorded once weekly. Nasal and buccal swabs were taken on the same dpi as blood samples for quantification of virus shedding. At necropsy, samples were systematically taken from lungs (portions of all lobes of the right lung plus the accessory lobe), pulmonary, tracheobronchial, mesenteric and inguinal superficial lymph nodes, tonsils, and spleen. Macroscopic lesions of lung and lymphoid tissues were evaluated by visual inspection following the scoring system described in [25]. All

organ samples were immediately frozen in liquid nitrogen and stored at -70 °C. Duplicate lung samples were fixed in 10 % neutral buffered formalin and embedded in paraffin until needed for further analysis.

2.5.5 Virological analysis

Serum samples collected at 4, 7, 14, 21 and 28 dpi were examined by qPCR to determine the presence and amounts of PRRSV. The presence of PRRSV in nasal swabs at 4, 7 and 14 dpi and in tonsils and lungs at 35 dpi was also determined.

RNA extraction was performed using a viral RNA/DNA purification kit (STRATEC) applying 200 µl of serum or 10 mg of tissue each. RNA yields and quality was determined by measuring absorbance at 260 and 280 nm using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies). Reverse transcription (RT) was performed using the DyNAmo cDNA Synthesis Kit (Thermo Scientific). Viral loads were quantified using a TaqMan fluorescent quantitative PCR assay in an iCycler iQ™5 detection system (Bio-Rad). The primers used for PRRSV detection were EU F1 (5'-GCA CCA CCT CAC CCA GAC-3'), EU R1 (5'-CAG TTC CTG CGC CTT GAT-3'), and probe (5'-FAM-CCT CTG CTT GCA ATC GAT CCA GAC-BHQ-3') [33]. PRRSV cDNA quantification was achieved by comparison of the unknown sample with a standard curve derived from known amounts of plasmid DNA.

2.5.6 Determination of humoral responses

Development of PRRSV-specific IgM and IgG antibodies was measured by ELISA (INGEZIM PRRS DR) according to the manufacturer's instructions. The presence of NA against the PRRSV was tested in sera by the viral neutralization test (VNT) as previously described [34,35]. Briefly, after complement inactivation at 56°C for 30 min, serum was diluted in log2 steps and incubated with an equal volume of medium containing 2 x 10^3 $TCID_{50}$ PRRSV (CReSA 3267) per well for 1 h at 37 °C. Then 100 µl of that mixture was transferred into 96-well microtiter plate containing confluent monolayer of MARC-145 cells and incubated for 2 d at 37 °C in a CO_2 incubator. Cytopathic effects were examined following fixation with 4% PFA and addition of PRRSV GP5 specific monoclonal antibody 3H4 (Ingenasa) and Alexa Fluor™ 488 conjugated anti-mouse IgG (H+L) secondary antibody (Invitrogen), and evaluated under a fluorescent microscope.

2.5.7 Evaluation of cell-mediated immune responses

PBMC were isolated from porcine peripheral blood using density centrifugation through a Ficoll gradient (LSM1077, PAA Laboratories). Samples were treated with erythrocyte lysis buffer for 5 min on ice, PBMC were washed with 10 ml of PBS–0.2% BSA and centrifuged for 15 min at 700 x g at 4 °C. In all samples, PBMC were >98% viable as confirmed by trypan blue exclusion.

The cell-mediated PRRSV-specific immune response was measured by using ELISpot for the enumeration of interferon (IFN)-γ secreting cells (IFN-γ-SC) in PBMC using a commercial porcine IFN-γ ELISpot kit (Mabtech). In order to evaluate homologous and heterologous responses, PBMC were stimulated in parallel (2.5 x 10^5 PBMC/well, 3 wells per pig and stimulus) with CReSA 3267 or LV at a multiplicity of infection of 0.1. Unstimulated and PHA-stimulated cells (10 µg/ml) were used as negative and positive controls, respectively. The frequency of PRRSV specific IFN-γ-secreting cells was counted using an ELISpot Reader System (A.EL.VIS GmbH). PRRSV-specific corrected frequencies of IFN-γ-SC were calculated by subtracting counts of spots in unstimulated wells from counts in virus-stimulated wells, and the immune responses were expressed as the number of IFN-γ-secreting cells per 2.5 × 10^5 PBMC.

2.5.8 Flow cytometric study of immune cell populations

Flow cytometry analysis was performed to determine the phenotype and the frequency of different immune cells in whole blood. Table 1 displays the source of antibody and the composition of antibody mixes used in the study. Briefly, 50 µl of heparinized blood was mixed with 100 µl of antibody mix 1. After incubation for 15 min in the dark at room temperature, cells were washed with fluorescence-activated cell sorting (FACS) buffer (0.1% BSA, 0.035% sodium bicarbonate and 0.02% sodium azide in HBSS) and centrifuged for 5 min at 700 x g. Then antibody mix 2, mix 3 and mix 4 were added and cells were washed and centrifuged accordingly. After the last wash step, contaminating erythrocytes were lysed by adding 1 ml erythrolysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4) for 15 min at room temperature. Finally, the cells were washed and resuspended in 0.5 ml of FACS buffer and set aside for flow cytometry analysis. Different T lymphocyte subsets were determined by first gating for CD3 cells and subsequently, for expression of the γδTCR. Based on the expression of further cell surface markers, the frequencies of the following phenotypes were determined: naïve T-helper (T_H) cells ($CD3^+CD4^+CD8^-$); cytotoxic T cells (CTLs) ($CD3^+CD4^-CD8\alpha^{high}$); T_H/memory cells ($CD3^+CD4^+CD8^+$); NK cells ($CD3^-CD4^-CD8^+$); $CD8^-$ γδ T cells ($CD3^+CD2^-CD8^-$); $CD8^+$ γδ T cells

($CD3^+CD2^+CD8^+$), antibody-forming and/or memory B cells ($CD3^-CD2^+CD21^-$); primed and/or activated B cells ($CD3^-CD2^-CD21^+$). Flow cytometry was performed using a BD FACSCanto™ flow cytometer (BD Biosciences). Data were analyzed with FlowJo™ software (TreeStar).

Table 1 Primary and secondary antibodies used for flow cytometry staining

Mix	Antibody	Isotype	Labeling	Clone	Source
1	Mouse anti-pig CD2	IgG2a	None	MSA 4	Hybridoma-supernatant
	Mouse anti-pig CD25	IgG1	None	K231.3B2	AbD Serotec
	Mouse anti-pig TCRγδ	IgG2b	None	PPT 16	Hybridoma-supernatant
2	Goat anti-mouse IgG1	IgG1	Dylight 405	polyclonal	Dianova
	Goat anti-mouse IgG2a	IgG2a	APC –Cy7		Southern Biotech
	Goat anti-mouse IgG2b	IgG2b	PE	polyclonal	Dianova
3	Mouse anti-pig CD3	IgG2a	Alexa 648	BB23-8E6-8C8	BD Pharmingen
	Mouse anti-pig CD4	IgG2b	PerCP-Cy™5.5	74-12-4	BD Pharmingen
	Mouse anti-pig CD8α	IgG2a	FITC	76-2-11	Southern Biotech
	Mouse anti-pig CD21	IgG1	BIOT	BB6-11C9.6	Southern Biotech
4	Streptavidin		PE–Cy7		Southern Biotech

2.5.9 Statistical analysis

All data are given as the mean ± SD. Calculations were performed with SPSS® Version 21 (IBM) and Prism 5 (GraphPad Software). Two-factorial models (diet, vaccination, diet*vaccination) were applied to calculate residuals for all variables. Normality of residuals was validated using the Kolmogorov-Smirnov test. In case of normally distributed residuals, further tests were applied on raw data. In case of not normally distributed residuals, further tests were applied on rank-transformed data. Flow cytometry data were analyzed by a mixed model with fixed effects (time, diet, vaccination, diet*vaccination, diet*time, time*vaccination) and a random effect (animal). Post hoc tests (LSD) were applied in case of significant diet or interactions effects. Significance was set at $P < 0.05$, and tendency at $0.05 \leq P \leq 0.1$.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MB, WC and NO conceived and designed experiments. WC and ZW performed the experiments, UB carried out flow cytometry analyses. PJ supervised and coordinated the animal experiments and provided clinical and performance parameter data. WC, MB and ST performed statistical analyses of experimental data. WC, MB and NO prepared the draft of the manuscript. All authors critically revised the manuscript and approved of the final version.

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Chapter 3: High-dose dietary zinc oxide mitigates infection with transmissible gastroenteritis virus in piglets

3.1 Abstract

Background: Zinc (Zn) supplementation has been shown to reduce the incidence of diarrhea and to protect animals from intestinal diseases, but the mechanisms of this protective effect against virus infection *in vivo* have not yet been elucidated. Transmissible gastroenteritis virus (TGEV) causes diarrhea in piglets with an age-dependent decrease of severity.

Results: We used 60 weaned piglets that were divided into three groups to evaluate the effect different Zn levels added to a conventional diet (50 mg Zn/kg diet, Zn^{low}, control group). The other groups received the diet supplemented with ZnO at final concentrations of 150 mg Zn/kg diet (Zn^{med}), or 2,500 mg/kg diet (Zn^{high}). Oral challenge infection with TGEV was performed when the pigs had been fed for 1 week with the respective diet. Half of the piglets of each group were sacrificed at day 1 and 18 after challenge infection. Fecal consistency was improved and body weights increased in the Zn^{high} group when compared to the other groups, but no direct effect of Zn concentrations in the diet on fecal TGEV shedding and mucosal immune responses was detectable. However, in the Zn^{high} group, we found a prevention of villus atrophy and decreased caspase-3-mediated apoptosis of jejunal epithelium. Furthermore, pigs receiving high Zn diet showed a down-regulation of interferon (IFN)-α, oligoadenylate synthetase (OAS) expression, Zn transporter *SLC39A4* (ZIP4), but up-regulation of metallothionein-1 (MT), Zn transporters *SLC30A1* (ZnT1) and *SLC30A5* (ZnT5). In addition, forskolin-induced chloride secretion and epithelial resistance were controlled at a physiological level in the Zn^{high} but not the other groups. Finally, in the Zn^{high} group, we documented an earlier and higher systemic TGEV-specific serum antibody response.

Conclusions: These results suggest that high dietary Zn could provide enhanced protection in the intestinal tract and stimulate the systemic humoral immune response against TGEV infection.

3.2 Background

Several *in vitro* studies have shown that zinc (Zn) has broad-spectrum antiviral activity against a variety of viruses, such as human immunodeficiency virus, transmissible gastroenteritis virus

(TGEV), equine arteritis virus, and severe acute respiratory syndrome coronavirus [1-6]. Many potential mechanisms have been suggested to explain the potential beneficial effect of Zn against virus infections. For example, Zn mediates antiviral effects through the inhibition of nidovirus RNA-dependent RNA polymerases or other proteins essential for the different phases of the viral life cycle [5, 6]. In addition, Zn participates in initiating and maintaining robust immune responses, in particular cytokine production and modulation of the activity of immune cells [7]. Zn induces the production of innate interferon (IFN)-α and also immune IFN-γ, and can potentiate the antiviral action of IFN-α, but not of IFN-γ [8]. Clearance of viral infections requires cytotoxic T lymphocytes, which are also highly dependent on the presence of Zn [7]. Antibody production during both the first and an immunological memory response is disturbed by Zn deficiency [9, 10], indicating that Zn is necessary for optimal results following vaccination.

In swine nutrition, especially in the North American swine industry, high levels of Zn oxide (ZnO, 2,000-3,000 ppm) are often added to the diet of weaned pigs, since such addition was shown to reduce non-specific post-weaning diarrhea and improve performance in this critical period of dietary change [11-13]. Diarrhea is caused by impaired intestinal epithelial barrier function, which most likely leads to malnutrition and decreased uptake of micronutrients, including Zn. It was shown that oral Zn supplementation with high doses was able to counteract this loss, improve intestinal mucosal integrity as well as absorption of water and electrolytes [12,14]. Furthermore, it leads to a faster regeneration of the gut epithelium [15]. However, because of environmental concerns, the maximum level of Zn allowed in pig diets was set up to 150 ppm in the European Union, irrespective of the Zn formulation [16].

Zn homeostasis is maintained in the body through a variety of transporters and Zn binding proteins [17]. High levels of dietary Zn provided as ZnO have been recently shown to outbalance Zn homeostasis with increased accumulation of Zn in various organs including the small intestine of piglets [18, 19]. Since intestinal Zn uptake can also take place through passive diffusion, it is likely that very high dietary Zn levels would indirectly increase the intestinal barrier function as a protection mechanism of the epithelium. In addition, metallothionein that is induced by Zn accumulation in intestinal tissue may also protect the tissue from oxidative damage.

Due to suboptimal immune functions, newborn as well as weaned piglets are particularly susceptible to infection by various pathogens, among them TGEV, which causes severe to mild gastroenteritis in piglets, depending on the age [20, 21]. Our previous study [5] showed that high Zn levels markedly reduced TGEV titers as well as viral RNA and protein synthesis *in vitro*, but there is no report about the antiviral effect of Zn in pigs. The aim of this study was to close the

knowledge gap and evaluate the antiviral potential and possible protection mechanisms of increased dietary Zn supplementation against TGEV infection in weaned piglets.

3.3 Results

3.3.1 High-dose Zn prevents diarrhea in piglets but does not affect other trace elements and virus shedding

TGEV infection caused only mild symptoms and there was no difference in dehydration, anorexia, lethargy and body temperature when the different Zn treatment groups were compared. However, the body weight in Zn^{high} was higher at 7, 14 and 18 dpi in comparison to other groups (Figure 1A). Furthermore, the fecal score from 2 to 7 dpi was also higher in Zn^{high} compared to the Zn^{low} group (Figure 1B).

Serum and liver Zn concentration was higher in Zn^{high} group as compared to the Zn^{med} and Zn^{low} groups, but other trace elements were not affected. In addition, there was an increased Zn, manganese and iron concentration, but decreased copper concentration, in both liver and serum from 1 to 18 dpi (Additional file 1: Table S1).

Low amounts of shedding TGEV could be detected by qPCR from 1 to 6 dpi, irrespective of Zn treatment group. The highest incidence was at 4 dpi with 4 out of 10 positive TGEV shedding piglets in each group.

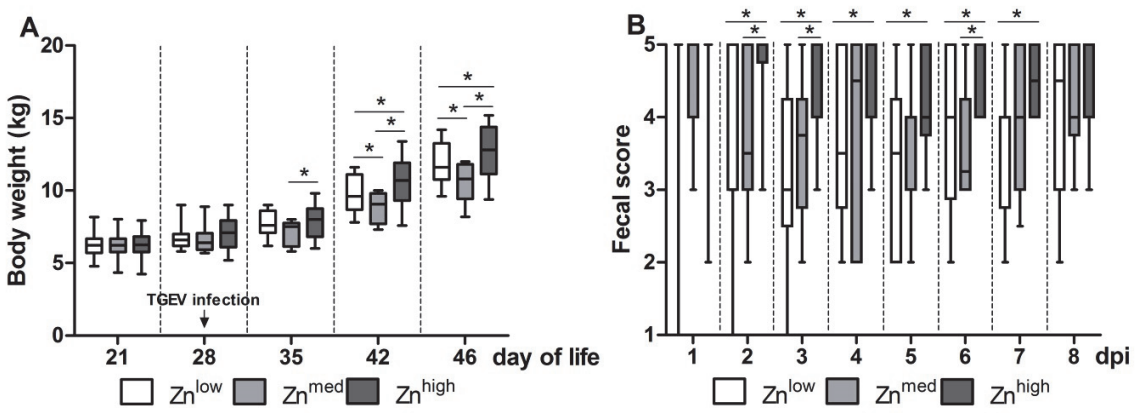

Figure 1 Body weight and fecal score of TGEV-infected piglets. Piglets fed with Zn^{low}, Zn^{med} and Zn^{high} were orally infected with TGEV. **(A)** Body weight was recorded at given time points and **(B)** fecal scores (from 1 to 5, where 1 means watery and 5 hard and dry stool) were

recorded daily after infection. Boxes indicate medians (n = 10) (horizontal lines) and the lower and upper quartiles (bottoms and tops of the boxes). The vertical bars in the box plots indicate the minimal and maximal values recorded. Asterisk indicates statistically significant difference (p≤0.05) between the groups.

3.3.2 Systemic and mucosal immune responses

Figure 2 shows the systemic and mucosal immune responses of piglets as measured by ELISA. At the time of challenge, all piglets fed with different concentrations of Zn were negative for TGEV-specific antibodies. The serum antibody response after infection occurred earlier in Zn^{high} piglets and was measurable already at 7 dpi, when all piglets from this group tended to develop higher (*P* = 0.06) TGEV-specific serum antibody titers than piglets in the Zn^{low} group. At 11 dpi, seroconversion was clearly detectable in almost all animals. Greater antibody levels were detected in Zn^{med} (*P* = 0.009) and Zn^{high} (*P* = 0.03) groups compared to Zn^{low} group at 14 dpi (Figure 2A).

The levels of sIgA antibodies in intestinal wash fluids were increased 6 to 7-fold from 1 to 18 dpi, showing that mucosal adaptive humoral immune response had also been induced after infection but the differences between the dietary groups were only marginal (Figure 2B).

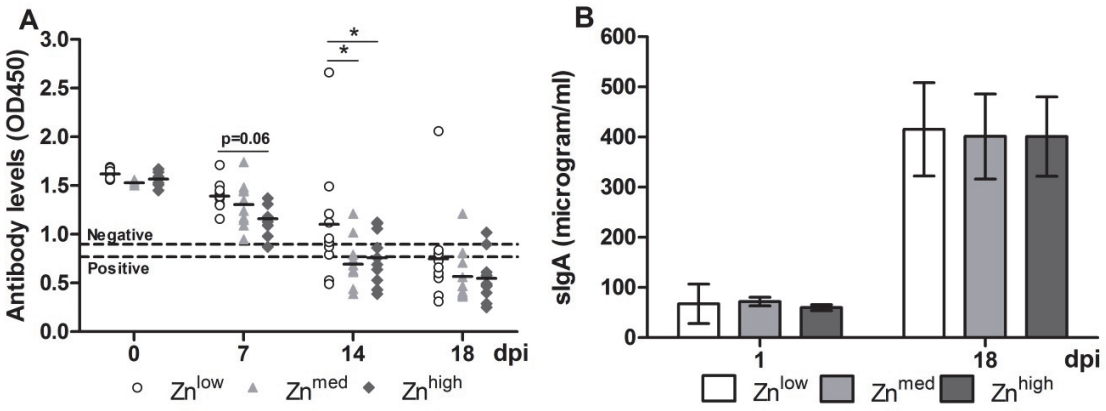

Figure 2 Development of systemic and mucosal immune responses after TGEV infection. **(A)** TGEV-specific antibodies measured by competitive ELISA are shown as optical densities (OD), measured at 450 nm, from 0 to 18 dpi. An OD_{450} value higher than 0.9 was considered negative (upper dashed line), an OD_{450} value lower than 0.77 was considered positive (lower dashed line), and an OD_{450} value between 0.9 and 0.77 was considered questionable. **(B)** The concentration of sIgA in intestinal wash fluid was measured by a direct ELISA. Results are

mitigates infection with transmissible

shown as the mean value ± standard deviation. Asterisk indicates statistically significant difference ($P \leq 0.05$) between the groups.

Additional file 1: Table S1 Mean concentration of trace elements (mg/kg fresh matter) in liver and serum at 1 and 18 dpi.

	1 dpi			18 dpi			Significance		
	Zn^{low}	Zn^{med}	Zn^{high}	Zn^{low}	Zn^{med}	Zn^{high}	Diet	Time	Diet x Time
Liver									
Zinc	183,8 ± 24,5	312,9 ± 141,8	595,6 ± 33,4	88,4 ± 2,9	116,1 ± 7,1	708,3 ± 106,9	**	ns	ns
Copper	108,7 ± 16,1	192,6 ± 62,1	105,4 ± 11,1	34,7 ± 5,9	17,6 ± 3,7	27,4 ± 4,8	ns	**	ns
Manganese	6,1 ± 0,4	5,7 ± 0,3	4,9 ± 0,3	7,7 ± 0,6	6,6 ± 0,3	6,3 ± 0,2	ns	**	ns
Iron	788,6 ± 100,1	686,4 ± 111,2	684,7 ± 47,3	249,4 ± 33,0	330,8 ± 61,6	196,4 ± 24,4	ns	**	ns
Serum									
Zinc	0,63 ± 0,13	0,56 ± 0,03	1,00 ± 0,12	0,91 ± 0,22	0,93 ± 0,69	1,65 ± 0,09	**	**	ns
Copper	1,68 ± 0,09	1,60 ± 0,08	1,88 ± 0,29	1,12 ± 0,11	1,07 ± 0,07	1,12 ± 0,11	ns	***	ns
Manganese	0,03 ± 0,00	0,04 ± 0,01	0,04 ± 0,01	0,12 ± 0,01	0,10 ± 0,02	0,1 ± 0,01	ns	***	ns
Iron	0,85 ± 0,11	0,92 ± 0,08	0,92 ± 0,09	1,41 ± 0,16	0,99 ± 0,07	0,92 ± 0,13	ns	*	*

Data are means ± SEs, n = 10/group. Significances are depicted as: **, $P < 0.01$; ***, $P < 0.001$.

3.3.3 Gene expression profiles

To further elucidate the potential effect of Zn supplementation on the immune response and on Zn transport, we examined the expression of genes for cytokines and metal binding/transport proteins in intestinal tissues in the three Zn treatment groups. There was a statistically significant increase of IFN-α expression in the Zn^{low} group compared to Zn^{high} group (P = 0.009). 2´, 5´-oligoadenylate synthetase (OAS) is one of IFN-stimulated gene products (ISGs), and there was a decreased expression of this enzyme in the Zn^{low} group (P = 0.01) (Figure 3). Expression of *ZIP4* was higher and *ZnT1*, *ZnT5* and *MT1* were lower in Zn^{high} group compared with two other groups (Table 1). Expression of IL-6, TNF-α and *ZnT2* did not differ between treatments.

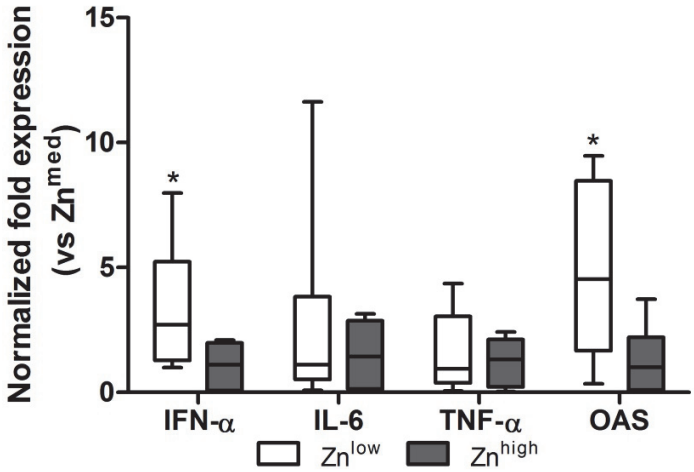

Figure 3 Cytokine expression in intestinal tissues of Zn-treated piglets at 1 dpi. The expression of selected cytokines was assessed by quantitative RT-PCR. The expression of IFN-α and OAS was significantly increased in the Zn^{low} compared to the Zn^{high} group. Boxes indicate medians (n = 10) (horizontal lines) and the lower and upper quartiles (bottoms and tops of boxes). The vertical bars in the box plots indicate the minimal and maximal values recorded. Asterisk indicates statistically significant differences ($P \leq 0.05$) between Zn treatment groups.

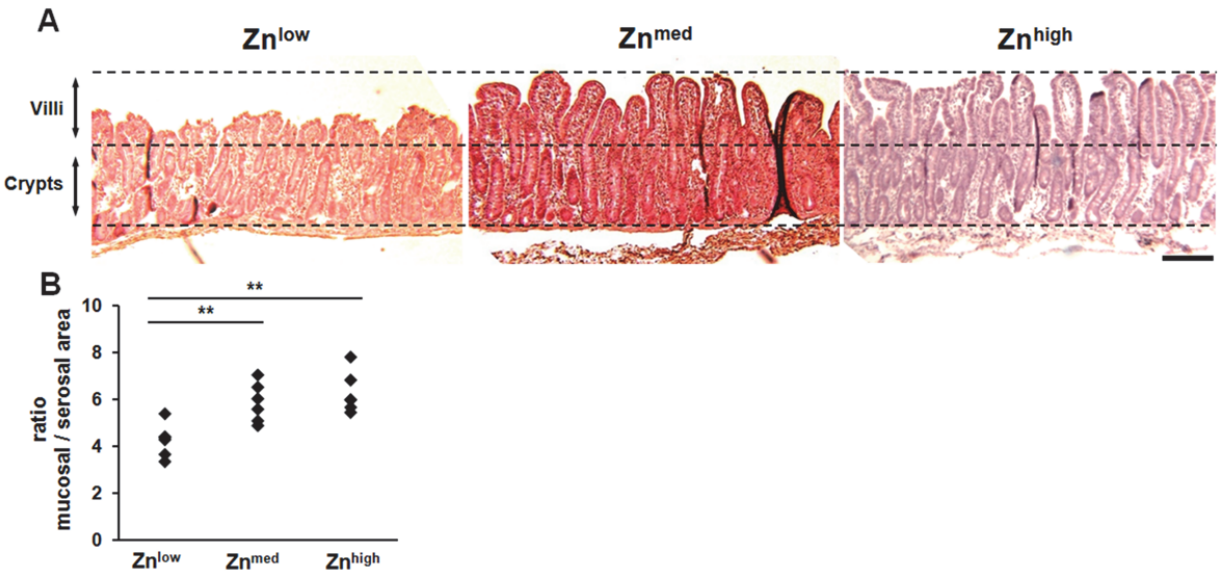

Figure 4 Morphometrical analysis of jejunal tissue at 1 dpi. (**A**) Jejunal tissue from piglets fed with Zn^{low}, Zn^{med}, and Zn^{high} diets, respectively, were sliced and H&E stained. The bottom and upper dashed lines indicate the basal and apical borders of the epithelium. The middle dashed line represents the transition zone from where crypts go down and villi go up. TGEV infection in Zn^{low} piglets resulted in villus atrophy, which could be prevented by Zn^{med} and Zn^{high} diets. Scale bar = 200 µm. (**B**) H&E-stained jejunal specimens were morphometrically analyzed by measuring the lengths of apical epithelial and the mucosa's muscular linings. The ratio of mucosal-to-serosal surface area represents a measurement for the effective epithelial area, which was significantly decreased under Zn^{low} ($P \leq 0.01$, n =6) compared to Zn^{med} and Zn^{high} (n = 6 each). The latter conditions were not significantly different.

3.3.4 Histology and immunohistochemistry

To further investigate the effect of Zn supplementation on TGEV infection in piglets, histological changes in intestinal tissues of piglets were examined. Piglets from the Zn^{low} group showed a destruction of the architecture of intestinal tissue with villous atrophied, which resulted in a significant reduction of the intestinal surface area by a factor of 1.38 (n = 6, $P = 0.009$) compared to the Zn^{med} group where no villus atrophy was observed (n = 6) (Figure 4A and B). No further changes in villus morphology in the Zn^{high} group (n = 6) could be detected (Figure 4A and B). In piglets from the Zn^{low} group, the majority of jejunal enterocytes was caspase-3-positive while being morphologically altered, whereas in Zn^{med} jejunal tissue the number of caspase-3-positive cells was drastically reduced and there was a further reduction of apoptotic

cells in Zn^{high} animals (Figure 5A and B). The epithelial architecture of Zn^{med} and Zn^{high} jejunum was apparently not impaired. The crypt epithelium was not affected by TGEV infection at any Zn concentration.

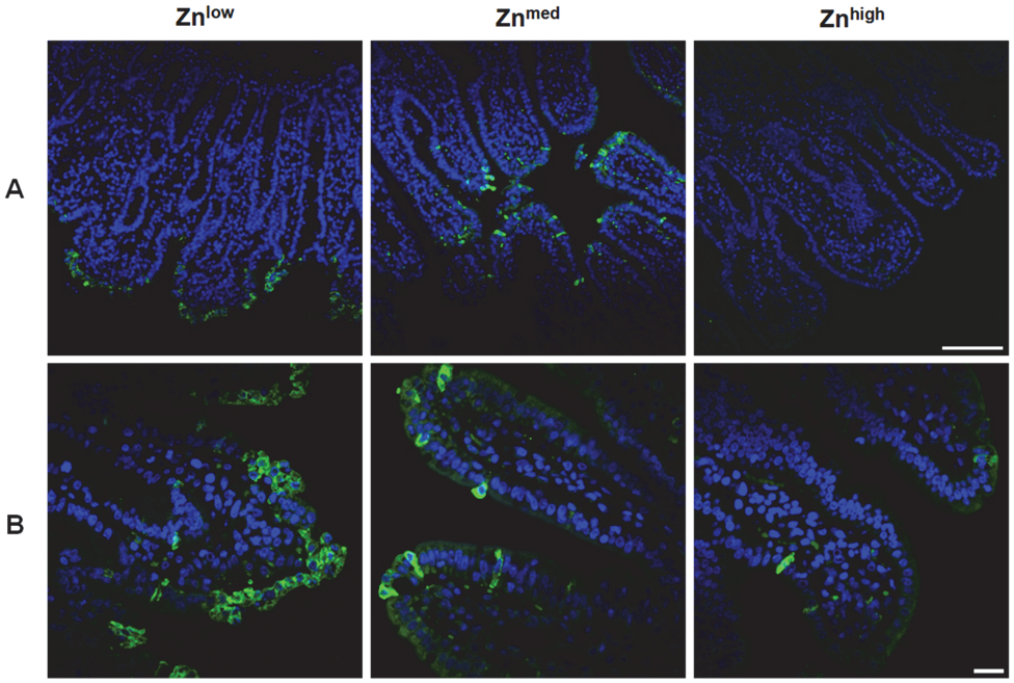

Figure 5 Immunofluorescence staining of caspase-3 in jejunal epithelium at 1 dpi. Apoptotic cells are presented by cells being positive for cleaved caspase-3 (as depicted in green). Nuclei (DAPI staining) are presented in blue. (**A**) When the jejunal epithelium was affected by TGEV infection, only the villus lining was apoptotic, while the crypt epithelium stayed intact. Scale bar = 100 µm. (**B**) Under Zn^{low} diet, most of jejunal enterocytes were cleaved caspase-3-positive and their shape was distorted, whereas under Zn^{med} treatment, the amount of cleaved caspase-3 signals was reduced and the cell shape was not affected. Under Zn^{high}, apoptotic cells were extremely rare and cell morphology was not impaired. Scale bar = 20 µm.

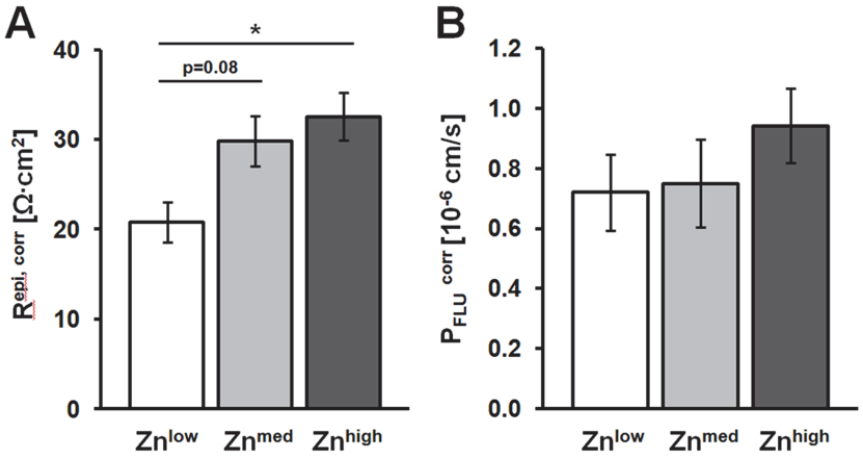

Figure 6 Epithelial resistance and paracellular permeability at 1 dpi. All values were corrected for the epithelial surface area (^{corr}). (**A**) Epithelia from the Zn^{low} group exhibited significantly decreased $R^{epi,\ corr}$ values (P = 0.01, n = 8) compared to the Zn^{high} group (n = 10) and a trend to values lower than those of the Zn^{med} group (P = 0.08, n = 10). (**B**) Fluorescein permeability ($P_{FLU}^{\ corr}$) did not significantly differ between Zn groups.

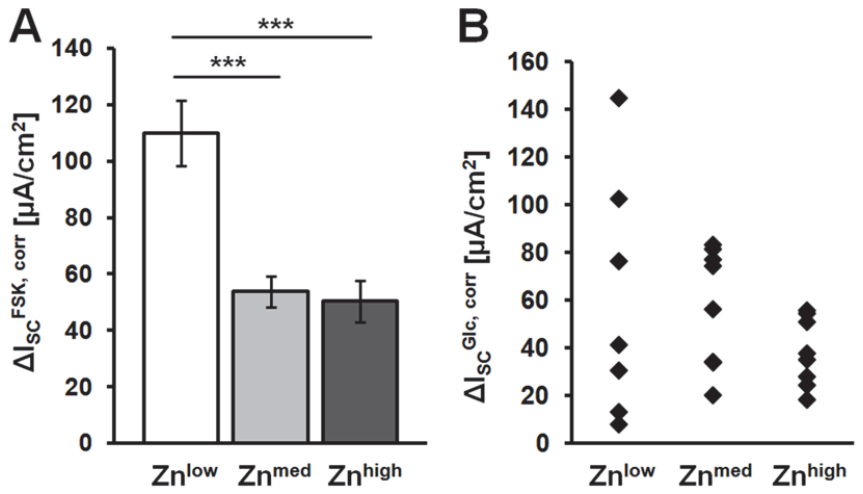

Figure 7 Active transport of jejunal epithelium at 1 dpi. All values were corrected for the epithelial surface area (^{corr}). (**A**) Stimulation by forskolin (FSK) resulted in a significantly increased chloride secretory response ($\Delta I_{SC}^{FSK,\ corr}$) in the Zn^{low} group ($P \leq 0.001$, n = 8) when compared to the Zn^{med} (n = 9) and Zn^{high} groups (n = 10). (**B**) Glucose-induced short-circuit currents ($\Delta I_{SC}^{Glc,\ corr}$) of Zn^{low} (n=7), Zn^{med} (n = 8), and Zn^{high} (n = 8) were not significantly different. Note the big spread of individual values in the Zn^{low} group.

3.3.5 Intestinal epithelial resistance, paracellular permeability, and active transport

Taking into account the changes in the surface area (^{corr}), $R^{epi,\ corr}$, as a measure of epithelial integrity, was lower in tissues from the Zn^{low} group (20.8 ± 2.2 Ω•cm^2, n = 8), compared to Zn^{med} (29.8 ± 2.8 Ω•cm^2, n = 10; Zn^{low} vs. Zn^{med}, *P* = 0.08) and Zn^{high} (31.4 ± 2.7 Ω•cm^2, n=10; Zn^{low} vs. Zn^{high}, *P* = 0.01) (Figure 6A). Surprisingly, permeability to the paracellular marker fluorescein did not significantly differ in TGEV-challenged jejunal epithelia in any of the animals (Figure 6B). As an *ex vivo* measure for *in vivo* diarrhea susceptibility, forskolin (FSK)-induced chloride secretion was quantified as increase in short-circuit current, $\Delta I_{SC}^{FSK,corr}$. It was found to be increased in jejunal tissue from TGEV-infected Zn^{low} piglets ($\Delta I_{SC}^{FSK,\ corr}$, 110 ± 11 µA/cm^2, n = 8) compared to Zn^{med} (54 ± 5 µA/cm^2, Zn^{low} vs. Zn^{med}, *P* = 0.0002, n = 9) and Zn^{high} (50 ± 7 µA/cm^2, Zn^{low} vs. Zn^{high}, *P* < 0.0001, n = 10) (Figure 7A), whereas $\Delta I_{SC}^{FSK,\ corr}$ of Zn^{med} and Zn^{high} did not significantly differ. In contrast, average glucose-induced short-circuit currents ($\Delta I_{SC}^{Glc,\ corr}$) were not different in jejunal tissues from piglets fed the tested Zn concentrations, however, against expectations, the range of $\Delta I_{SC}^{Glc,\ corr}$ values was greatly increased in Zn^{low} compared to the other two conditions (Figure 7B).

3.4 Discussion

High doses of ZnO (2000 – 3000 mg ZnO/kg diet) added to the diets of newly-weaned piglets were shown to improve performance and to reduce the occurrence of unspecific diarrhea [13]. TGEV infection causes villus atrophy with severe and frequently fatal diarrhea in newborn pigs, while the clinical signs in older piglets or in adult pigs are milder or inapparent because of a higher replacement rate of enterocytes compared to newborn piglet [22-24]. However, asymptomatic older piglets may serve as carriers, and the high mutation rates of coronavirus genomes may lead to the generation of more virulent genotypes. For this study we infected weaned piglets with a cell-culture adapted TGEV strain, for which direct antiviral effects of Zn were proven *in vitro* [5], and tested if direct antiviral and/or systemic effects of different Zn levels could be observed *in vivo*. Critical factors for the reduction in the mortality and morbidity of piglets from TGE include a reduction in the infectious agent, and oral rehydration therapy for the treatment of dehydration and metabolic acidosis associated with acute diarrhea [12].

In this study, TGEV infection caused only mild clinical symptoms, which can be explained by the piglets' age and by the use of a tissue-culture adapted virus strain, which was chosen on purpose in order to directly compare results from this study with previous *in vitro* results [25].

Furthermore, in our study the piglets were provided with a relatively comfortable environment to minimize stress other than that induced by infection. It should be stressed that our experimental conditions vary substantially from those in commercial farming conditions. This could also contribute to the absence of severe clinical signs after infection. However, this study demonstrated that feeding the Zn^{high} diet improved the fecal score and led to higher body weights after infection in comparison to the Zn^{med} and Zn^{low} groups. Several studies demonstrated that feeding high levels of Zn reduces the incidence and severity of diarrhea and improved fecal consistency [25, 26]. Our results are consistent with these findings showing that the Zn^{high} group had higher fecal scores compared to the Zn^{med} and Zn^{low} groups after infection. There was also a direct correlation between Zn levels and histological changes. TGEV infection in the Zn^{low} group led to destruction of the enterocytes of jejunal villi as reflected by marked villus atrophy. It has been reported that Zn plays a fundamental role in maintaining epithelial barrier integrity and function. For example, supplementation of Zn reduced methotrexate-induced intestinal damage and resulted in faster recovery [28], while it reduced intestinal permeability in Bangladeshi children with acute diarrhea and persistent diarrhea syndrome [29]. Furthermore, feeding supplemental Zn to rats with experimental colitis improved mucosal repair by regulating tight junction permeability [30]. In agreement with these data, higher $R^{epi, corr}$ values in the Zn^{med} and Zn^{high} group indicate that Zn may prevent epithelial barrier loss induced by TGEV. The dramatic increase in $\Delta I_{SC}^{FSK, corr}$ observed in the Zn^{low} group despite the reduced surface area may be interpreted as a protective mechanism. Chloride secretion is the basis of secretory diarrhea and might be regarded as a mechanism to rapidly extrude pathogens. The increase in $\Delta I_{SC}^{Glc, corr}$ observed in some of the animals from the Zn^{low} group may be a compensatory effect and is in agreement with the observation that, even in the Zn^{low} group, animals gained weight at a normal rate. This considerably larger variance in the Zn^{low} group indicates that some animals were able to compensate reduced glucose uptake due to the loss of villi by increasing glucose transport capacity.

Zn is also important for the production of antibodies against intestinal pathogens [31]. In agreement with such finding, TGEV-specific serum antibody titers were detected earlier and at higher levels in Zn^{high} when compared to Zn^{low} piglets. This finding may be the result of multiple effects of Zn on antigen-presenting cells, T-cells and antibody-producing B-lymphocytes. IgA is the primary immunoglobulin isotype induced at the mucosal surface. Secretory IgA (sIgA) in mucosal secretions provides protection against bacterial and viral pathogens and neutralizes microbial toxins [32]. Zn can influence sIgA levels by altering the cytokine profile of stimulated immune cells residing in the gut-associated lymphatic tissue (GALT) [33]. Furthermore, sIgA

responses are mediated through activated Th2 cells producing, among other, abundant amount of IL-6 [34]. In this study, there was an increased sIgA levels from 1 to 18 dpi, indicating that adaptive mucosal immune responses were induced by TGEV infection but not influenced by the diet. This finding is in accordance with results from Broom and colleagues [33], who also showed only slight differences of intestinal IgA concentration between animals either receiving low or high levels of dietary ZnO. In accordance with these fingdings, the expression of IL-6 in the intestinal tissues also showed no difference between the dietary groups. Therefore, it is questionable if Zn plays a role in enhancing intestinal IgA concentration.

TGEV infection in piglets is characterized by a robust and early IFN-α production in intestinal secretions and in other organs [35, 36]. At 1 dpi, higher IFN-α gene expression levels in the Zn^{low} group may be consistent with a more severe TGEV infection or higher virus loads in this group compared to the Zn^{high} group. The activation of OAS, one of the IFN-stimulated gene products (ISGs), can lead to apoptosis [37], potentially by indirectly triggering cleavage of caspase-3 [38]. The increased OAS level in the Zn^{low} group may reflect TGEV-induced apoptosis in intestinal epithelial, as it was shown, that the amount of caspase-3-positive cells was markedly increased in the Zn^{low} compared with the Zn^{med} and Zn^{high} group. This finding is in agreement with the histological observations: the high-grade villus atrophy in the Zn^{low} and normal jejunal mucosal morphology in the Zn^{high} group.

Metallothionein is known to be induced by exposure to heavy metal cations [39], and in line with former study [18], the expression level of *MT1* was higher with concomitant high level of Zn in piglets (Additional file 2: Table S2). As reported previously, this indicates an outbalanced Zn homeostasis although the expression of Zn transporters in the jejunum was regulated to reduce Zn uptake (*ZIP4*) from the gut lumen and increase the export of Zn from epithelial cells (*ZnT1*, *ZnT5*) in the Zn^{high} group. However, an increased level of Zn in intestinal tissue and the induction of metallothionein may explain our observations of improved intestinal mucosal integrity and histological changes, which are in line with other studies [12, 14]. Thus, changes in absorption of water and electrolytes may counteract TGEV caused impaired intestinal epithelial barrier function and decreased uptake of micronutrients.

The paracellular epithelial barrier is highly regulated both under normal conditions and in disease. During infection, TNF-α is a mediator between the immune system and the intestinal epithelial barrier by altering tight junction proteins and their cellular localization. More specifically, the cytokine up-regulates the pore-forming protein claudin-2 [40] and redistributes the tightening protein claudin-1 [41]. This leads to an impaired barrier function which is associated with

increased paracellular permeability. However, our *in vitro* work [25] and the *in vivo* study presented here did not find any up- or down-regulation of TNF-α after TGEV infection in any of the experimental groups. The outcome is, however, in accordance with the data set of permeability to fluorescein (P_{FLU}^{corr}), as paracellular permeability was not affected. The data could, therefore, be interpreted as a result of a different pathological mechanism, possibly mediated by IFN-α, rather than by TNF-α, as up-regulation of the former in Zn^{low} piglets was clearly evident.

3.5 Conclusions

This study provides data that supplementation of the post-weaning diet with high levels of ZnO resulted in earlier and higher TGEV-specific antibody response, modulation of cytokine expression, and prevention of disruption of the intestinal barrier integrity. Our findings might also be interesting for infections with other coronaviruses like SARS-CoV, which could cause gastrointestinal symptoms and diarrhea as well.

3.6 Methods

3.6.1 Virus and cells

The TGEV strain Purdue 46-MAD (kindly provided by Dr. C. Schwegmann-Wessels, Institut für Virologie, Tierärztliche Hochschule Hannover) was used in this study. To prepare TGEV stocks, a stable mycoplasma-free swine testicle (ST) cell line supporting the growth of TGEV was used. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; PAN Biotech) supplemented with 10% heat-inactivated fetal calf serum (Hyclone), and 1% penicillin/streptomycin (Biochrom) at 37°C with 5% CO_2. Stock virus was propagated in ST cells to a titer of 1.00E+07 plaque-forming unit (PFU)/ml.

3.6.2 Animals and experimental design

German landrace piglets (n = 60) of both sexes from a TGEV-free herd (Leibniz Institut für Nutztierbiologie, Dummerstorf, Germany) were weaned at the age of 21 days and were randomly assigned to three different dietary groups (20 animals each). Diets either contained no additional ZnO (Zn^{low}: 50 mg Zn/kg diet), or were supplemented with analytical grade ZnO (>98% purity) to contain 150 mg Zn/kg diet (Zn^{med}) or 2,500 mg Zn/kg diet (Zn^{high}). The Zn^{low} diet represents the regular feed of the animals and the Zn^{low} group, therefore, represented the

control group for this experiment. At 26 days of age, animals were moved to a containment facility (Bundesinstitut für Risikobewertung, Berlin, Germany) where they were randomly allocated to 6 pens per group. The piglets were fed the respective diet *ad libitum* in a pelleted form, water was also provided *ad libitum* by nipple drinkers. The pens were of equal size (2.8 m^2), equipped with a feeding automate with 5 feeding places and a nipple drinker. The floors were covered with rubber mate and a red light lamp was placed above to provide additional heat. Room temperature was kept at 25 ± 1°C with humidity of 50 - 60% and constant air volume exchange. The pens were thoroughly cleaned by brushing the floors and walls with consecutive flushing with lukewarm water in the mornings. Superficial flushing of the floors was additionally performed in the late afternoon. At day 28 of age, all piglets were challenged orally with 2 ml TGEV with a titer of 1.0E+07 PFU/ml. In each group, half of the piglets (n=10 per group) were sacrificed 1 day post infection (dpi) in order to examine acute infection, since piglets reportedly display strong symptoms of gastroenteritis within 20 h post-infection [42], and the other half (n=10 per group) were sacrificed 18 dpi to see the effect of Zn on adaptive immune response. Pawel Janczyk from Bundesinstitut für Risikobewertung supervised and coordinated the animal experiments and provided clinical and performance parameter data.

3.6.3 Clinical follow-up and sampling

The study was approved by the local animal welfare authority (Landesamt für Gesundheit und Soziales, Berlin, Germany) under the registration number G 0116/12. Animals were clinically examined on arrival. Blood samples were collected at 0, 4, 7, 11, 14 and 18 dpi. Sera were used to determine TGEV-specific antibodies. Piglets were monitored daily for rectal temperature and body weight was recorded once weekly. Fecal scores (from 1 to 5, where 1 means watery and 5 dry and hard stool) were also recorded daily up to 12 dpi. Fecal swabs were taken before and then daily after infection for the detection of virus shedding. At necropsy (both 1 and 18 dpi), 10 – 15 cm long samples from the descending duodenum, jejunum (approximately 100 cm distal to the duodeno-jejunal flexure), ileum (distal 15 cm), spleen and jejunal mesenteric lymph nodes were taken to determine gene expression profiles. Furthermore, defined pieces of the jejunum of the same length were washed with 25 ml PBS and intestinal fluid was collected to detect adaptive immune response (sIgA). Additionally, 15 cm of mid jejunum (1 dpi) were removed, cut open, and rinsed with and transported in cooled saline solution (0.9% NaCl, 1 mM $CaCl_2$). Jejunal tissue was stripped off the muscle layer and explants were mounted in Ussing chambers for electrophysiological analysis as described in detail below. Part of each intestinal tissue sample was additionally fixed in buffered 4% formalin.

3.6.4 Confirmation of Zn status

Trace element status of the pigs was determined in serum and liver tissue as described previously [18]. Briefly, organs were freeze dried, incinerated and hydrolyzed in concentrated hydrochloric acid. Serum samples were hydrolyzed directly. Trace element concentration was determined by atomic absorption spectrometry in an AAS vario 6 spectrometer (Analytik Jena, Jena, Germany). Experimental procedures were performed by Robert Pieper, Freie Universität Berlin.

3.6.5 Real-time quantitative RT-PCR (qRT-PCR) analysis

Total RNA was extracted from 20 mg of jejunum sample or 200 µL of serum using a Nucleo-Spin® RNA II Kit for Tissue (Macherey & Nagel, Düren, Germany) following the manufacturer's instructions. Reverse transcription (RT) was performed using the RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. PCR reactions were performed in a total volume of 25 µL in an iCycler iQ5® detection system (Bio-Rad Laboratories, München, Germany). Melt curve analysis and agarose gel electrophoresis were performed after completion of each assay to confirm specificity of the amplification. For the following target genes: zinc transporters *SLC30A1 (ZnT1)*, *SLC30A2 (ZnT2)*, *SLC30A5 (ZnT5)*, *SLC39A4 (ZIP4)*, and metallothionein *(MT1)*, quantitative real-time RT-PCR was performed using the one-step QRT-PCR master mix kit (Brilliant®II SYBR®Green, Agilent Technologies, Santa Clara, USA) as described previously [18], and performed by Robert Pieper, Freie Universität Berlin.

TGEV genome copy numbers were quantified using a TaqMan fluorescent quantitative (q) PCR assay as previously shown [25]. Expression levels of IFN-α, IL-6, OAS, and TNF-α were calculated using the Delta-Delta-Ct-Method calculation [43]. Four commonly used reference genes (β-2 microglobulin, RPL13, RPL19 and SDHA) were selected for normalization of gene expression. A mean expression value (normalization factor) for the four reference genes was calculated to enable normalization of gene expression data for all the genes of interest. Samples from Zn^{med} group were used the references for the calculations. For the expression of Zn transporters *ZnT1*, *ZnT2*, *ZnT5*, *ZIP4* and *MT1*, standard curves were generated using serial dilutions of pooled RNA (within a range from 5-200 ng/µL) from 20 samples to convert Ct values into arbitrary values. These values were then normalized using the mean values of the house-keeping genes and then used for statistical comparisons. The names of genes, primer sequences, annealing temperature, and references are listed in Additional file 2: Table S2.

Additional file 2: Table S2 Detailed qRT-PCR primers and conditions used in this study

Target[1]	Primer pairs (5'- 3')	Annealing temp. (°C)	Reference
β-2 microglubin	CCCCGAAGGTTCAGGTTTAC	60	(46)
β-2 microglubin	CGGCAGCTATACTGATCCAC		
IFN-α	GCTCCTGGCACAAATG	60	(21)
IFN-α	GCTGCTGATCCAGTCC		
IL-6	AACGCCTGGAAGAAGA	53	(21)
IL-6	AACCCAGATTGGAAGC		
MT1	GTGAATCCGCGTTGCTCTCTGCT	60	(17)
MT1	CTGTGGGGCAGGAGCAGTTGG		
OAS	GAGCTGCAGCGAGACTTCCT	62	(27)
OAS	TGCTTGACAAGGCGGATGA		
RPL13	CCGTCTCAAGGTGTTCGATG	60	(46)
RPL13	GGATCTTGGCCTTCTCCTTC		
RPL19	GCTTGCCTCCAGTGTCCTC	60	(46)
RPL19	GCGTTGGCGATTTCATTAG		
SDHA	CAAACTCGCTCCTGGACCTC	60	(46)
SDHA	CCGGAGGATCTTCTCACAGC		
TGEV	GTATTGGGATTATGCT	55	(5)
TGEV	GGTGGTGGTAGTAGGT		
TNF-α	ACGCTCTTCTGCCTACTGC	58	(21)
TNF-α	TGGGCGACGGGCTTATC		
ZIP4	TGCTGAACTTGGCATCTGGG	60	(20)
ZIP4	CGCCACGTAGAGAAAGAGGC		
ZnT1	CCAGGGGAGCAGGGAACCGA	60	(20)
ZnT1	TCAGCCCGTTGGAGTTGCTGC		
ZnT2	GACAGCGCCAGCCAGCATCA	60	(20)
ZnT2	GGCAGCCACCAAAACGCCCA		
ZnT5	ACACCAGTCTCAGTTGGAGGGCT	60	(20)
ZnT5	GGTATGGGTGTGGGCATGGCT		

3.6.6 Enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected on 0, 4, 7, 11, 14 and 18 dpi, and TGEV-specific IgG antibodies were determined using a commercial ELISA (INGEZIM TGEV 2.0, Ingenasa). Intestinal wash fluid was collected on 1 and 18 dpi and mucosal sIgA antibodies in the supernatants were also measured using a commercial ELISA (Pig IgA ELISA Kit, Biomol) following the manufacturer's instructions.

3.6.7 Electrophysiology

Jejunal specimens were mounted in modified Ussing chambers to carry out impedance measurements as described previously [44]. In brief, one-path impedance spectroscopy was performed to determine epithelial (R^{epi}) and subepithelial (R^{sub}) contributions to the transepithelial resistance (TER). Resistances of bath solution without tissue as well as electrode offsets were recorded prior to each experiment and subtracted from experimental data. Preparations were allowed to equilibrate for 45 min and R^{epi} was measured as previously described [45].

As pathogen-induced secretory diarrhea is caused by excessive chloride secretion with accompanied osmotically driven water flux, forskolin (Calbiochem®, Merck, final concentration 10 µM), a secretagogue agent, was added basolaterally in supplemented Ringer's solution (113.6 mM NaCl, 5.4 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 21 mM $NaHCO_3$, 0.6 mM NaH_2PO_4, 2.4 mM Na_2HPO_4, 10 mM D(+)-glucose, 2.5 mM glutamine, 10 mM D(+)-mannose, 0.5 mM β-OH-butyrate, 50 mg/l piperacillin, 4 mg/l imipenem; pH 7.4 when equilibrated with carbogen) in order to trigger a chloride secretory response. For testing the sodium-coupled and therefore electrogenic glucose absorption via SGLT1, glucose (Roth, final concentration 10 mM) was added apically in glucose-free Ringer's solution (113.6 mM NaCl, 5.4 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 21 mM $NaHCO_3$, 0.6 mM NaH_2PO_4, 2.4 mM Na_2HPO_4; pH 7.4 when equilibrated with carbogen). Post administration of effectors, changes in short-circuit current (ΔI_{SC}) were recorded.

Permeability to fluorescein (332 Da) represents a measure for the paracellular leakiness/tightness of the epithelial layer and was ascertained in Ussing chamber experiments under voltage clamp conditions. After equilibrating jejunal specimens in supplemented Ringer's solution, fluorescein (Sigma) was added apically (final concentration, 50 µM). At 30 and 90 min post administration, basolateral samples were replaced with Ringer's solution. Fluorescein concentrations were determined with a fluorometer at 525 nm (Infinite M200, Tecan) and permeabilities were calculated. Experimental procedures were performed by Silke S. Zakrzewski from Charité - Universitätsmedizin Berlin.

3.6.8 Morphometry

Formalin-fixed tissue sections were stained with hematoxylin and eosin (H&E) using standard staining protocols and analyzed using the freehand line selection tool of Image J (Rasband, ImageJ, NIH, Bethesda, Maryland; http://rsb.info.nih.gov/ij/).

The surface area of the jejunal mucosa was assessed as the ratio of mucosal-to-serosal surface area from the lengths of apical epithelial as well as muscular mucosa linings in equivalent fields of view of adjacent sections. Crypt/villus height and density were determined from five sections per piglet, each ~750 µm in width. Experimental procedures were performed by Silke S. Zakrzewski from Charité - Universitätsmedizin Berlin.

3.6.9 Immunofluorescence staining

In order to highlight caspase-3-mediated apoptotic events in jejunal tissue, formalin-fixed tissue slices were rehydrated (xylene, increasing ethanol series), heated at 95°C in citrate buffer (10 mM citric acid, pH 6.0) for 15 min, and then washed in phosphate-buffered saline (PBS). Tissue slices were then incubated in blocking solution (6% goat serum + 1% BSA in PBS) for 30 min at room temperature (RT) before incubation with rabbit anti-cleaved caspase-3 (Cell Signaling Technology, Cambridge, UK) at a 1:250 dilution in blocking solution for 60 min at RT. After several washings, tissue slices were incubated with goat anti-rabbit $F(ab')_2$ conjugated with DyLight™488 (Jackson ImmunoResearch, Newmarket, UK) at a 1:500 dilution and 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI, 1 µg/ml; Roche, Mannheim, Germany) for 60 min at RT in the dark. After washing, sections were embedded using ProTaqs Mount Fluor (Biocyc, Luckenwalde, Germany). Experimental procedures were performed by Silke S. Zakrzewski from Charité - Universitätsmedizin Berlin.

Images were taken with an inverted Zeiss LSM 510 META confocal laser-scanning microscope (Carl Zeiss, Jena, Germany). Digital images were processed using Fiji imaging software [46] and Zeiss LSM 510 META software.

3.6.10 Statistical analyses

Calculations were performed with SPSS® Version 21 (IBM, Armonk, NY, USA) and GraphPad Prism 5 (GraphPad Software Inc, La Jolla, CA, USA). Two-factorial mixed models were applied to calculate residuals for all variables (fixed factor: diet, time, diet*time) and a random effect (animal). Data from gene expression were used for the one-factorial model (fixed factor: diet). F-

Test was applied for fixed effects and interaction effects with subsequent LSD post hoc test. Electrophysiology and morphometric data are expressed as means ± standard error of the mean (SEM), and statistical analyses were carried out using a one-way ANOVA with Tukey HSD post hoc test. Significances are depicted as: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; tendencies are given as $0.05 \leq P \leq 0.1$.

Abbreviations

dpi, Day post infection; H&E, Hematoxylin and eosin; *IFN-α*, Interferon alpha; *MT1*, Metallothionein-1; *IL-6*, Interleukin-6; *OAS*, Oligoadenylate synthetase; *RPL13*, 60S ribosomal protein L13; SDHA, Succinate dehydrogenase subunit A; ST cells, Swine testicle cells; TER, Transepithelial resistance; TGEV, Transmissible gastroenteritis virus; *TNF-α*, Tumor necrosis factor-alpha; *ZIP4*, Zn transporter SLC39A4; *ZnT1*, Zn transporter SLC30A1; *ZnT2*, Zn transporter SLC30A2; ZnT5, Zn transporter SLC30A5

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

M.B., W.C. and N.O. conceived and designed experiments. W.C., S.S.Z., D. G., R.P., P.J. and Z.W. performed the experiments; W.C., M.B. S.S.Z., R.P., and S.T. performed statistical analyses of experimental data. W.C., M.B. and N.O. prepared the draft of the manuscript; and M.B., and N.O. had primary responsibility for the final content. All authors critically revised the manuscript and approved the final version.

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Chapter 4: General discussion

Zn is involved in numerous cellular functions, including replication, transcription, and signal transduction. In addition, it is essential in the maintenance of membrane barrier integrity, protection against pathogens, and regulation of immune responses. The therapeutic application of Zn to treat or prevent various diseases has been studied in different experimental settings, but there is no report on the proposed effects of Zn treatment on virus infection in pigs. Here we filled in the gap by investigating the potential benefits of ZnO in protecting pigs from infections with one important porcine respiratory virus (PRRSV) and one important porcine enteric virus (TGEV).

4.1 Supplementation of diet with high levels of ZnO improved clinical performance after virus infection

ZnO is frequently added at high dose of 2,000-3,000 mg/kg diet of the weaned pigs since this was shown to reduce diarrhea and improve the performance of piglets (Hu et al., 2012; Janczyk et al., 2013; Martin et al., 2013; Molist et al., 2011; Poulsen, 1995; Sales, 2013). In agreement with these reports, in the present study, Zn^{high} corresponding to pharmacological levels of ZnO caused increased body weight gain of piglets after both TGEV and PRRSV infections. However, in contrast to another study (Martin et al., 2013), which showed the growth promoting effect of pharmacological dietary ZnO concentration only during first two weeks after the weaning, the current studies observed this beneficial effect of Zn^{high} during the third and fourth week period after the weaning instead of the first two weeks. The possible reasons for this discrepancy could contribute to the virus infection in TGEV challenge trial and vaccination in PRRSV challenge trial, or to the different experimental and sanitary conditions, which differ between experiments and affect the effects of ZnO supplements (Janczyk et al., 2013).

The mechanisms and modes of action of the growth-promoting effect of high dose of Zn are still not fully elucidated. It was reported that ZnO could stimulate the secretion of ghrelin, a gastrointestinal hormone, which acts on the small intestine and brain to promote feed intake (Yin et al., 2009). Antimicrobial properties of ZnO were illustrated by changes in the gastrointestinal ecosystem of the piglet (Hojberg et al., 2005; Pieper et al., 2012; Vahjen et al., 2011), leading to the assumption that high levels of dietary ZnO enhanced the growth of weaned pigs by controlling pathogenic bacterial scours. While Carlson et al. (Carlson et al., 1999) suggested a systemic effect via the blood rather than a direct influence on the gastrointestinal tract. So far it

is not known which of these explanations are relevant for the growth promoting effect of Zn treatment *in vivo*.

Zn plays a fundamental role in maintaining membrane function and stability. Some authors suggest that the mechanism of this activity involves the stabilization of the membrane structure (O'Dell, 2000) or the displacement of redox-active metals to prevent free-radical oxidative damage (Canali et al., 2000; Powell, 2000). The integrity of the intestinal barrier is fundamental to the proper functioning of the epithelial cells and to preventing the entry of pathogens that cause inflammation (Lu and Walker, 2001). TGEV causes localized infection of the intestinal tract that induce villus atrophy and, consequently, malabsorptive diarrhea in newborn piglets (Lee et al., 2011). We infected weaned piglets with TGEV in order to determine possible effects of different dietary Zn levels on clinical disease. Our results showed that the Zn^{high} group had higher fecal scores compared to the Zn^{med} and Zn^{low} groups after infection, which is consistent with the findings that feeding pharmacological levels of Zn reduces the incidence and severity of diarrhea and improved fecal consistency (Mavromichalis et al., 2001; Zhang and Guo, 2009). This beneficial effect of Zn^{high} could results from its role in maintaining epithelial barrier integrity and function (higher $R^{epi,\ corr}$ values in the Zn^{high} group) and reducing intestinal permeability, since we observed destruction of the enterocytes of jejunal villi as reflected by severe villus atrophy induced by TGEV infection in Zn^{med} and Zn^{low} groups. Furthermore, Zn suppressed caspase activation and in consequence apoptosis in intestinal epithelial cells. The activation of 2′-5′ OAS, one of the IFN-stimulated gene products (ISGs), can lead to apoptosis, potentially by indirectly triggering cleavage of caspase-3 (Rusch et al., 2000). The increased OAS level in the Zn^{low} group may reflect TGEV-induced apoptosis in intestinal epithelial cells, as it was shown, that the amount of caspase-3-positive cells was greatly increased in the Zn^{low} compared with the Zn^{high} group. This finding is in agreement with the histological observations: the high-grade villus atrophy in the Zn^{low} group and normal jejunal mucosal morphology in the Zn^{high} group.

4.2 Effect of Zn on virus replication *in vivo*

It has long been recognized that Zn^{2+} is an important cofactor not only of cellular proteins but of many viral proteins as well. One of the arguments used to support the use of Zn salts was that Zn *per se* has antiviral activity in *in vitro* experiments. Studies with purified rhinovirus and poliovirus 3C proteases revealed that protease activity was inhibited by Zn^{2+} (Baum et al., 1991; Cordingley et al., 1989). Haraguchi et al. indicated that Zn^{2+} could more specifically inhibit HIV-1RNA transcription than cellular RNA transcription (Haraguchi et al., 1999). The inhibition of

polyprotein processing by Zn^{2+} was observed in cells infected with human rhinovirus and coxsackievirus B3 (Hulisz, 2004; Krenn et al., 2009). The inhibition effect of Zn^{2+} on coronavirus and arterivirus RNA polymerase activity was also reported *in vitro* (te Velthuis et al., 2010). However, the direct antiviral action of Zn was questioned by other studies. Bourne et al. suggest that the observed protection effect of Zn in the efficacy studies was due, at least in part, not to direct antiviral activity but to infected epithelial cells sloughing off before the virus could enter peripheral neurons (Bourne et al., 2005). Zn inhibits RSV by altering the ability of cells to support RSV replication rather than by a direct effect on the virus (Suara and Crowe, 2004).

The results in this study showed that ZnO used in PRRSV challenge trial did not reduce viral load and/or viral shedding. These results are in line with another challenge trial performed by our group with swine influenza virus (SIV), which also indicated that ZnO has no effect on viral shedding (Wang et al., 2014), and also by several other studies (Bobat et al., 2005; Green et al., 2005). The reasons for the absence of antiviral activity of Zn^{high} *in vivo* are not clear, but it could due to the relatively low amount of Zn in serum or tissues. There were around 1 µg/ml (~15 µM) Zn ions in serum samples in Zn^{high} group in our *in vivo* studies. While the *in vitro* studies, which showed that Zn inhibit viral replication applied concentrations of 100 µM Zn ions to the cell culture medium (Haraguchi et al., 1999; Kumel et al., 1990; te Velthuis et al., 2010; Yuasa et al., 2006). It was even higher for HIV with a concentration of 1.5 mM. All these amounts seem to be relatively higher when comparing to the real Zn level *in vivo*. Therefore, the relevance of a direct inhibition of viral replication as a mechanism for antiviral actions of Zn *in vivo* could be questionable.

The evident of direct antiviral activity from Zn was very limited in this study, although we could not rule out the possibility that Zn, via some unknown mechanisms, could have indirect antiviral activity by stimulation and/or improvement of other systems, such as the immune system.

4.3 Influences of high levels of Zn on immune responses

The effects of Zn deficiency on immunity are hypoplasia of lymphoid tissues, and reductions in T-helper cell numbers, NK cell activity, antibody production, cell mediated immunity, and phagocytosis (Beisel, 1982; Haase and Rink, 2009), indicating the important role of Zn in immune responses. In both TGEV and PRRSV challenge trials, we found higher dietary Zn levels had a tendency to induce earlier and higher viruses-specific antibody levels after virus infection. Since not only B cell lymphopoiesis, but also antibody-mediated immune defense are affected by Zn deficiency (Fraker and King, 2004), this might suggest that the pigs in the Zn^{low}

group received not enough Zn to allow full development of early antibody responses and that under the circumstances of an immune challenge there is a need for a higher Zn supply. In the PRRSV challenge trial, we also analyzed the levels of neutralizing antibodies. It is well-known that the generation of neutralizing antibodies is delayed in PRRSV infection and usually appears three to four weeks after infection (Diaz et al., 2005). Accordingly, we detected neutralizing antibodies from 28 dpi rising until 35 dpi and with a tendency towards higher titers in the Zn^{med} and Zn^{high} treatment groups. The obvious booster effect on antibody responses in pigs receiving dietary Zn at higher levels could be attributed to multiple effects of Zn on antigen presenting cells, T- and antibody-producing B-lymphocytes (Haase and Rink, 2009).

Zn supplementation can modulate T cell dependent immune reactions. It was reported that the protective immunity depends on the capacity of PRRSV to induce a strong immune response (CMI) and correlated with higher IFN-γ-SC responses (Diaz et al., 2006; Zuckermann et al., 2007). In the PRRSV challenge trial, no differences in the number of IFN-γ-SC were determined after homologous or heterologous stimulation at 35 dpi, irrespective of Zn treatments. This result was in accordance with our flow cytometry results showing that the percentages of Th/memory cells and CTLs, which are the main producers of the antiviral cytokine IFN-γ, were unaffected by Zn treatments either at that time point. However, we cannot rule out the possibility that high levels of Zn could have influence on CMI at earlier or later time point. This result would have been stronger if another two additional time points (one earlier and one later) had been included. Although we found no effect of Zn treatments regarding modulation in the frequency of immune cells in pigs, there was a tendency that Zn^{high} could induce higher number of CTLs and $CD8^-$ γδ T cell. In contrast, $CD8^+$ γδ T cell seems to be inhibited by Zn^{high} group. The reason for this discrepancy is not clear in this work, but it can be explained from other authors' conclusion that Zn supplementation leads to T cell activation, an indirect effect that is mediated by cytokine production by other immune cells, but higher concentrations of Zn can also directly suppress T cell function (Haase and Rink, 2007).

Zn has been characterized as a regulator of pro-inflammatory cytokines. In the TGEV challenge trial, we found that Zn^{high} significantly down-regulated IFN-α expression level in intestinal tissue, but the expression of IL-6 and TNF-α did not differ between treatments. Some reports describe that Zn supplementation to human PBMCs leads to an increased mRNA production and release of the monokines IL-6, IL-1β, and TNF-α, (Wellinghausen et al., 1997). On the other hand, several reports indicate that Zn treatment suppresses the formation of pro-inflammatory

cytokines (Bao et al., 2003; Zhou et al., 2004). The difference could due to the concentration dependent effect of Zn (Haase and Rink, 2007), and the different experimental drawn part.

IgA is the primary immunoglobulin isotype induced at the mucosal surface. Secretory IgA (sIgA) in mucosal secretions provides protection against bacterial and viral pathogens and neutralizes microbial toxins (Zhang et al., 2002). By altering the cytokine profile of stimulated immune cells residing in the gut-associated lymphatic tissue (GALT), it has been proposed that ZnO supplementation may influence intestinal IgA concentrations (Broom et al., 2006). For example, sIgA responses are mediated through activated Th2 cells producing cytokine such as IL-4, IL-6 and transforming growth factor (Simecka, 1998). The mucosal immune response in this study was induced by TGEV infection since an increased sIgA level from 1 to 18 dpi was observed. However, Zn treatments had no effect on the intestinal sIgA level at both 1 and 18 dpi. It cannot be ruled out that, at other time points in between, there might have been a difference, since 1 dpi was early for the development of mucosal immune response and 18 dpi was already late. But in accordance with another study (Broom et al., 2006), which also showed only slight differences of intestinal IgA concentration in animals that were treated with low or pharmacological levels of ZnO, it seems that our results are fairly convincing. Furthermore, IL-6, which mediated sIgA responses, expression in the intestinal tissues showed a tendency similar to that of sIgA production at 1 dpi, with no difference between the dietary groups. Therefore, given that no effects of Zn^{high} treatment were found on sIgA level in intestinal washing and on IL-6 expression in intestinal tissue, the possible role of ZnO in enhancing mucosal immune response needs further investigation.

4.4 Supplementation with high levels of ZnO had no adjuvant effect of vaccination

Antibody production during both the first and an immunological memory response is disturbed by Zn deficiency, suggesting that Zn supplementation could improve vaccination results (Overbeck et al., 2008). However, various vaccination studies were done with additional Zn supplementation, but in most cases, there was no increase of the antibody titer against the vaccine (Table 1). For example, Spears et al. reported that humoral and cellular immune responses following vaccination with bovine rhinotracheitis were not affected by Zn treatment (Spears and Kegley, 2002). No effect of Zn supplementation on influenza vaccination was found either (Provinciali et al., 1998; Turk et al., 1998).

In this study, a UV-inactivated LV-based PRRSV vaccine which had been shown to reduce viremia and to induce an earlier and elevated VN antibody response was used (Vanhee et al.,

2009). In contrast to Vanhee and colleagues, we applied a single-vaccination approach, in order to test the influence of different Zn levels on immunogenicity in response to a suboptimal antigenic stimulus. After a single-vaccination with a heterologous PRRSV inactivated vaccine, the antibodies responses were greater in vaccinated groups, which are in line with Vanhee's results. However, Zn supplementation did not result in a significantly enhanced PRRSV-specific as well as neutralizing antibodies responses after vaccination when compared to the non-vaccinated groups.

In the studies that find increased antibody titers after vaccination, Zn administration was done for 2 – 4 weeks, but stopped before vaccination (Duchateau et al., 1981; Tipu et al., 2012). The major problem concerning ours and others most of the data contradicting the hypothesis that Zn supplementation can increase antibody titer after vaccination could be due to the fact that the Zn uptake after vaccination was not controlled and sometimes definitely too high. Because high dosage of Zn has a direct inhibitory effect on immune functions (Haase and Rink, 2007; Reinhold et al., 1999) and the formation of IgA and IgG requires T cell help, Zn supplementation after the vaccination may have suppressed T cells and hindered efficient vaccination response. This could be the reason for the studies without an effect of Zn, which had continued the supplementation regime after the immunization.

In conclusion, by assessing the clinical performance, viral replication and distribution, humoral and cellular immune response, vaccination, as well as morphometric measurements of and electrophysiology of intestinal tissue, we found that supplementation of diet with high levels of ZnO could improve clinical performance after virus challenge infection, be beneficial to humoral response in both challenge trials, and protect intestinal epithelial cells and prevent the disruption of the intestinal barrier integrity after infection. However, it had no effect on viral load and shedding, on mucosal immune response, and on vaccination.

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Publications

1. **Chai, W.**, Burwinkel, M., Wang, Z., Palissa, C., Esch, B., Twardziok, S., Rieger, J., Wrede, P., Schmidt, M.F., 2013. Antiviral effects of a probiotic *Enterococcus faecium* strain against transmissible gastroenteritis coronavirus. Archives of virology 158, 799-807.
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3. **Weidong Chai**, Michael Burwinkel, Zhenya Wang, Pawel Janczyk, Sven Twardziok Ulrike Blohm, Nikolaus Osterrieder. Effect of dietary zinc oxide on porcine reproductive and respiratory syndrome virus (PPRSV) infection in pigs. (Submitted).
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Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe.
Ich versichere dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, 19.12.2013 Weidong Chai