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Nutrition

Influence of zinc supplementation on immune parameters in weaned pigs

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ABSTRACT

Zinc is an essential trace element, highly important for a well functioning immune system. In case of zinc deficiency, proper immune functions are not ensured thus leading to various diseases. Weaning of pigs from the sow causes stress, increasing susceptibility to infections. Moreover, low feed intake during the first two weeks post-weaning, accompanied by low zinc intake, results in temporary zinc deficiency. Therefore, supporting the immune system by zinc supplementation might improve its function and thereby the pigs' health and well-being. In this study, the immune status of weaned pigs was analyzed under different conditions of zinc supplementation. More precisely, the daily porcine diet was either left unsupplemented (0 ppm), or was supplemented with low (100 ppm), or high (2500 ppm) amounts of additional zinc in the form of zinc oxide (ZnO) (Zn0, Zn100, and Zn2500, respectively). Porcine innate and adaptive immune cells of the different dietary groups were analyzed. Results revealed an improved innate immune capacity, represented by increased phagocytosis and slightly increased oxidative burst in cells from the Zn2500 pigs and Zn100 pigs, respectively. Apart from that, zinc supplementation improved adaptive immunity, as seen by increased numbers of $CD3^+$ T cells as well as increased numbers of $CD3^+CD4^+Foxp3^+$ regulatory T cells, elevated interleukin (IL)-2 production and decreased IL-10 production. Although not significant, supplementing 2500 ppm zinc slightly decreased killing activity of natural killer (NK) cells. Thus, the optimal concentration for zinc supplementation of weaned pigs two weeks post-weaning needs to be further studied, presumably establishing an optimal concentration between 100 ppm and 2500 ppm zinc. Genome comparisons indicate that the porcine genome is more closely related to the human genome than the murine genome is related to the human genome. Therefore, the pig seems to be a suitable organism to study human immunity and diseases. Results obtained in the current study might therefore be transferable to the human immune system.

1. Introduction

Zinc is involved in various biological processes, especially in cell development and metabolism. Since the immune system presents the highest proliferation rate, zinc plays an important role in its development and function. Thus, it is not surprising that zinc deficiency affects cell differentiation and proliferation, resulting in impaired immune functions [1,2]. Weaning seems to be one of the most stressful events in a pig's life, contributing to dysfunctions of their immune system and diarrhea [3]. To reduce the incidence of post-weaning diarrhea, high dietary zinc levels (2500 ppm) were used in pigs since the 1990s [4]. The underlying antidiarrheal mechanisms of high zinc supplementation are not completely understood, but an improved zinc status seems to be beneficial [5,6]. Supplementing Zn2500 was especially effective during the first two weeks post-weaning, probably as zinc intake was low due to overall low feed intake during this period [4,5,7,8]. Moreover, at the age of weaning passive immunity declines, but active immunity is yet not fully developed, so that immune support is warranted [9].

Comparison of the porcine and human genomes with special respect to immunologically associated genes revealed 80% similarities, which is higher than comparing human and mouse genomes [10]. Thus, using a pig model rather than a rodent model might represent an improved approach to address immunological questions. Furthermore, analyses of porcine compared to human innate immune cells revealed similar effector functions and the comparable release of cytokines [11]. Additionally, adaptive immunity shows significant similarities between

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humans and pigs [12].

The aim of this study was to assess the immune status of zinc-supplemented pigs 1–2 weeks post-weaning. Due to the high value of the pig model, results from the current study may be relevant for research concerning the influence of zinc on human immune competence.

2. Materials and methods

2.1. Animals and diets

The animal experiment was conducted according to license obtained from the Danish Animal Experiments Inspectorate, Ministry of Food, Agriculture and Fisheries, Danish Veterinary and Food Administration. This study was in compliance with the guidelines concerning animal experiments and care of animals under study according to the Danish Ministry of Justice, Act. 726 of September 9, 1993, and as amended in Act. 1306 of November 23, 2007, which complies with the EU Directive 2010/63/EU for animal experiments. This study comprised 18 pigs (3 litters of 6 pigs each) with an average initial body weight of 6.8 kg ± 0.7 kg. The pigs were cross-bred (Duroc x Danish Landrace x Yorkshire) females and castrate males. They were allowed creep feed, containing 100 ppm zinc as zinc oxide (ZnO) from 7 days of age and were weaned at day 28 of age (day 0). One lot of basal diet was prepared according to the Danish recommendations of all nutrients for pigs between 6 and 9 kg except for zinc [13] (Table 1). No inorganic zinc was supplemented. The basal diet was divided into three portions which were supplemented with 0, 100 or 2500 ppm zinc in the form of ZnO (treatment: Zn0, Zn100 and Zn2500, respectively). However, the diet contained in total 59 ppm (Zn0), 198 ppm (Zn100) and 3515 ppm (Zn2500) zinc after the respective zinc supplementation. The total zinc amount of the respective porcine diet was measured in dry weight. Supplementation of zinc was carried out on wet weight. In the following article we will only talk about the supplemented zinc amounts to distinguish between the different supplemented pig groups. Two of the six littermates were fed one of the three diets either from day 0 to day 6–7 post-weaning or from day 0 to day 13–14 post-weaning. The pigs were randomly allocated to the dietary treatments balancing for body weight and were housed individually in pens with *ad libitum* access to feed and water until the time of euthanasia. At the day of slaughtering, blood was drawn from the pigs and used to assess the immune status of the pigs. The complete set of experiments was performed for each pig, except for one pig fed a 2500 ppm zinc-supplemented diet, which suffered an infection in the abdominal cavity, and was therefore excluded from the study. Moreover, one pig of the unsupplemented (Zn0) group showed abnormally high intracellular zinc levels, probably due to a very high feed intake. Therefore, the Nalimov outlier test was performed, leading to subsequent exclusion of the pig from this study. Other outliers throughout this study were as well determined using the Nalimov outlier test.

Table 1
Dietary ingredients (g/kg diet) of the basal diet and analyzed dry matter and zinc and copper content of the three experimental diets (Zn0, Zn100, Zn2500).

Ingredients	Basal
Barley	211.8
Wheat	423.5
Soybean meal	207.6
Fish meal	80.0
Animal fat	50.0
Mono calcium phosphate	9.9
Calcium phosphate	8.5
L-Lysine·HCl, (78.8%)	2.9
DL-Met (99.0%)	0.7
L-Thr (98.5%)	1.1
L-Trp (100%)	0.4
Sodium chloride	1.6
Vitamin and mineral mixture[a]	2.0

Analyzed	Zn_0	Zn_{100}	Zn_{2500}
Dry matter (%)	90	90	90
Zinc (ppm, dry matter basis)	59	198	3515
Copper (ppm, dry matter basis)	34	36	33

[a] Zinc-free vitamin-mineral mixture provided the following quantities of vitamins and minerals per kg of complete diet: 5000 IU vitamin A, 500 IU Vitamin D_3, 140 mg Vitamin E, 140 mg α-tocopherol, 2 mg Vitamin K_3, 2 mg Vitamin B_1, 4 mg Vitamin B_2, 3 mg Vitamin B_6, 0.02 mg Vitamin B_{12}, 20 mg niacin, 0.2 mg biotin, 10 mg D-panthotenic acid, 50 mg Fe ($FeSO_4$), 20 mg Cu ($CuSO_4 \cdot 5H_2O$), 40 mg Mn (MnO), 0.2 mg I ($Ca(IO_3)_2$), 0.3 mg Se (Na_2SeO_3).

2.2. Cell culture and isolation of peripheral blood mononuclear cells (PBMC)

K562 cells were cultivated at 37 °C in a humidified 5% CO_2 atmosphere and seeded in RPMI 1640 (Sigma-Aldrich, Germany) containing 10% heat-inactivated fetal calf serum (FCS) (PAA, Germany), 2 mM L-glutamine, 100 U/mL potassium penicillin and 100 U/mL streptomycin sulfate (all from Sigma-Aldrich, Germany).

Porcine PBMC were isolated from sodium-heparinized whole blood samples (BD Vacutainer, Denmark). Briefly, blood was diluted with PBS (Sigma-Aldrich, Germany) in a ratio of 1:3. After density centrifugation over Biocoll separating solution with a density of 1.077 g/mL (Biochrom, Germany), cells were collected from the interface and washed with PBS. In a next step, erythrocytes were lysed with water and non-lysed PBMC were washed again with PBS. Cells were resuspended in RPMI 1640 medium supplemented with the above mentioned supplements. Cells were counted with trypan blue staining (Sigma, Germany) and adjusted to the concentration needed for the specific assays.

2.3. Inductively coupled plasma-mass spectrometry (ICP-MS)

The destruction of feed samples for zinc and copper analyses was conducted using a microwave (UltraWave, single reaction chamber microwave digestion system from Milestone SRL, Italy) with pressure of 85–115 bar, depending on the sample type, temperature of 230 °C for 35 min. Zinc and copper concentrations were analyzed by ICP-MS on a X series II ICP-MS equipped with a conventional Meinhard nebulizer and a Peltier cooled quartz impact bead spray chamber operated at 3 °C (Thermo Electron Corporation, Germany) set with a CETAC autosampler model ASX-520 (Thermo Fisher Scientific, USA). Data were collected using the PlasmaLab version 2.5.9.30 (Thermo Electron Corporation, Germany). The instrument settings comprised 1400 W forward power, 13 L/min plasma gas (Ar), 0.9 L/min nebulizer gas (Ar) and 0.7 L/min auxiliary gas. The sample uptake comprised approx. 0.4 mL/min and ^{45}Sc, ^{71}Ga and ^{103}Rh were used as internal standards with interpolation. Detection of serum zinc levels within blood collected from the jugular vein of the pigs (BD Vacutainer, Denmark) was as well performed using ICP-MS.

2.4. Flame atomic absorption spectrophotometry (AAS)

Liver tissue was collected (approx. 50 mg) and digested in 2 mL HNO_3 for 3 h at 90 °C. Liver tissue zinc concentrations were measured by AAS (Analyst 800, Perkin Elmer, Germany). Absorbance values for tissue zinc were normalized to the wet tissue weight. This method allowed determination of µg zinc per gram wet tissue.

2.5. Whole blood analysis

Porcine blood was analyzed using a ProCyte Dx Hematology Analyzer and a corresponding IDEXX ProCyte Dx* reagent kit (IDEXX Europe, The Netherlands), according to the manufacturer's instructions. Parameters analyzed can be found in Table 2.