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Accumulation of copper in the kidney of pigs fed high dietary zinc is due to metallothionein expression with minor effects on genes involved in copper metabolism

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ABSTRACT

A study was conducted to determine the effect of high dietary zinc (Zn) oxide on trace element accumulation in various organs with special emphasis on the kidney. A total of 40 weaned piglets were allocated into two groups with 16 and 24 piglets each receiving a diet containing normal (NZn; 100 mg Zn/kg) or high (HZn; 2,100 mg Zn/kg) Zn concentration, respectively. After two weeks, eight piglets from each treatment were killed and organ samples were taken. Eight piglets from the remaining 16 pigs fed HZn diets were changed to NZn diets (CZn). All remaining piglets were killed after another two weeks for organ sampling. Trace element concentration was determined in the jejunum, liver, kidney, pancreas, bone (metacarpal IV), spleen, lung, thymus, tonsils and lymph nodes of jejunum, ileum and colon. Kidney mRNA expression of Zn transporter *ZnT1* and *ZIP4*, genes involved in Cu metabolism (*Ctr1*, *Atox1*, *SOD1*, *ATP7A*, *CCS*, *CP*) and divalent metal ion transport (*DMT1*) and binding (*MT-1a, MT-2b, MT-3*) were determined. The Zn concentration in jejunum, liver, pancreas tissue and metacarpal IV was higher ($P < 0.05$) in HZn group compared with NZn and CZn groups. Trace element concentration in organs of CZn pigs was similar to those fed NZn diets. Zn concentration in muscle, lung and lymphatic organs as thymus, tonsils, spleen and lymph nodes of jejunum, ileum and colon did not differ between the groups. Zn and Cu were positively correlated ($R = 0.67$; $P < 0.05$) in the kidney. No significant differences for Cu chaperones, Cu transporters and Cu-dependent factors were determined despite decreased expression of *Atox1* after two weeks and increased *Ctr1* expression over time in the HZn group. Expression of *MT-1a, MT-2b* and *MT-3* were significantly higher in HZn fed pigs with most pronounced effects for *MT-1a* > *MT-2b* > *MT-3*. Gene expression of MTs in pigs fed CZn diets did not differ from pigs fed NZn diets. The data suggest that high dietary Zn feeding in pigs leads to Cu co-accumulation in the kidney of pigs with minor effect on genes relevant for Cu metabolism. In addition, the organ Zn and Cu accumulation is reversible after two weeks of withdrawal of high dietary Zn.

1. Introduction

The time after weaning in pigs is often accompanied with an increased risk for gastrointestinal disorders and reduced growth performance. High levels of dietary zinc oxide (ZnO) (1500–3000 mg Zn/kg diet), exceeding the dietary recommendations and maximum allowances in the EU by 10- to 20-fold, have been frequently shown to reduce diarrhea and improve the performance of weaned piglets [1]. However, these high Zn concentrations in the diet have been shown to outbalance Zn homeostasis in the body with subsequent Zn accumulation and change of metabolic reactions in different organs including the small intestine, liver and pancreas [2–5]. The accumulation of Zn goes along with increased abundance of metallothioneins (MTs), which are essential for the regulation of intracellular heavy metal homeostasis and detoxification [6,7]. Dietary Zn increases the MT expression in a dose-depended manner, and the expression and metal-binding affinity differs between the different MT isoforms [6,8]. Thus, it is likely that feeding high dietary Zn levels with subsequent Zn

Abbreverations: ADG, average daily gain; Atox1, antioxidant 1 copper chaperon; ATP7A, copper-transorting *P*-type ATPase; BW, body weight; CZn, changed dietary Zn group; CCS, copper chaperone for superoxide dismutase; CP, ceruloplasmin; Ctr1, copper transporter 1; DM, dry matter; HZn, high dietary zinc group; DMT1, divalent metal ion transporter 1; MT, metallothionein; NZn, normal dietary zinc group; SDHA, succinate dehydrogenase subunit A; SOD1, super oxide dismutase 1; ZIP, Zrt- and Irt- like protein; ZnT, zinc transporter.

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Table 1
Ingredients and chemical composition of the diets used in the study.

Ingredients	g/kg diet	Chemical composition	NZn[b]	HZn[c]
Wheat	300	g/kg		
Barley	200	Dry matter	892	891
Corn	230	Crude protein	188	192
Soybean meal	200	Crude fiber	30	32
Monocalcium phosphate	13	Ether extract	29	30
Limestone	14	Starch	424	445
Vitamine–mineral pre-mix[a]	15	Ash	51	52
Soy oil	10			
Lysine–HCl	3.5	mg/kg		
Tryptophan	1	Iron	183	156
Methionine	1	Manganese	96	113
Salt	2.5	Copper	18	19
Zinc oxide/corn starch	10	Zinc	72	2103

[a] Containing per kg: 600,000 IU vitamin, 120,000 IU vitamin D3, 8,000 mg vitamin E, 300 mg vitamin K3, 250 mg vitamin B2, 400 mg vitamin B6, 2,000 μg vitamin B12, 2,500 mg nicotine acid, 100 mg folic acid, 1,000 mg pantothenic acid, 80,000 mg choline chloride, 30 mg cobalt, 45 mg iodine, 35 mg selenium, 6,000 mg manganese, 1,000 mg copper, 5,000 mg Iron.
[b] Normal dietary zinc group.
[c] High dietary zinc group.

accumulation and MT induction in different organs would also affect other trace elements such as copper (Cu).

Usually, the Cu concentration in extrahepatic tissues is low and maintenance is regulated through Cu storage in the liver and biliary excretion. Genetic defects such as Wilson disease are associated with toxic Cu accumulation in the liver, brain, kidney and cornea in humans [9] and livestock [10]. Interestingly, the administration of Zn to patients suffering from Wilson disease could reduce the toxic effects of Cu overload in the liver due to the induction of MT [9]. In addition, a chronic intake of Zn can lead to severe Cu deficiency in humans, likely due to MT induction and Cu fixation in intestinal epithelial cells [11]. Compared to humans or ruminants, pigs are relatively tolerant against high dietary levels of Zn and reports about secondary Cu deficiency are scarce [12]. Interestingly, previous studies with piglets fed high dietary Zn showed an increased Cu accumulation in the kidney but no other organ [13,14]. The reasons or consequences for Cu metabolism in the kidney are yet not known.

The present study was conducted to determine the influence of feeding high dietary levels of ZnO to weaned piglets on the accumulation Zn and Cu in various organs with special emphasis on the kidney and the influence on Zn- and Cu-specific transporters and binding protein in this organ. Furthermore, the change in trace element metabolism and accumulation after the switch from very high to normal dietary Zn was studied.

2. Material and methods

The study followed the institutional and national guidelines for the care and use of animals and the study was approved by the State Office of Health and Social Affairs 'Landesamt für Gesundheit und Soziales Berlin'(LaGeSo Reg. Nr 0296/13).

2.1. Animals, diets and sampling

A total of 40 piglets (7.6 ± 0.7 kg) were weaned at the age of 26 ± 2 days and randomly assigned into two groups. One group received normal dietary Zn concentration (NZn, $n = 16$) the other group received a very high level of dietary Zn (HZn, $n = 24$). The experimental diets are given in Table 1. To achieve the respective Zn concentrations in the diets, corn starch was partially replaced by analytical grade ZnO. Piglets were kept in flatdeck pens ($n = 2$ per pen) and had ad libitum access to feed and fresh water. Body weight and feed intake per pen were recorded throughout the experimental period. None of the piglets received medication before or during the experiment. After 14 days, eight piglets from each group were killed and samples from jejunum, liver, kidney, pancreas, bone, spleen, lung, thymus, tonsils and lymph nodes of jejunum, ileum and colon were taken, immediately snap-frozen in liquid nitrogen and stored at −80 °C until further analyses. In addition, a 1.5 cm × 1.5 cm part of left kidney was immediately placed in RNA*later*® (Sigma–Aldrich) for 30 min and subsequently stored at −80 °C. The remaining eight piglets in the NZn group and eight piglets of the HZn group were fed their respective diets another 14 days, whereas eight piglets from the HZn group were subjected to NZn for the next 14 days (CZn group). At the end of this period, all remaining piglets were killed and organ samples takes as described above.

2.3. Chemical analyses

Proximate nutrients in the diets were analysed by standard Weende procedures [15]. After hydrolysing ash in hydrochloric acid, concentration of Zn, Cu, Mn and Fe in diets and organs were analysed by atomic absorption spectrometry in an AAS vario 6 spectrometer (Analytik Jena, Jena, Germany) as described previously [5].

2.4. Gene expression

Total RNA from kidney tissue was extracted using the Nucleo Spin® kit (Macherey Nagel) and mRNA quality and quantity were determined on an Agilent Bioanalyzer 2100 (Agilent). Equimolar mRNA (104 ng RNA) was transcribed into complementary DNA (cDNA) by Superscript® reverse transcriptase kit (Life Technologies). Subsequently, real-time qPCR was performed using Brilliant II SYBR® Green QPCR Master Mix low Rox (Agilent Technologies) on a Stratagene Mx3005P (Agilent Technologies) with cycling conditions as described previously [16,17]. Primers (Supplemental Table 1) were designed targeting zinc transporter (*ZnT1, ZIP4*), copper transport 1 (*Ctr1*), antioxidant 1 copper chaperon (*Atox1*), superoxide dismutase 1 (*SOD1*), copper-transporting *P*-type ATPase (*ATP7A*), copper chaperone for superoxide dismutase (*CCS*), ceruloplasmin (*CP*), divalent metal ion transporter 1 (*DMT1*) and three metallothionein isoforms (*MT-1a, MT-2b, MT-3*) using the NCBI online primer designer tool. Relative gene expression was calculated based on PCR efficiency and Ct values of target genes normalized using *Succinate Dehydrogenase subunit A, β-2-microglobulin* and *β-actin* [18].

2.5. Statistical analyses

For statistical analyses, normally distributed data were analyzed by student's *t*-test to compare means within a group at the different time points and group differences after 2 weeks of feeding several dietary Zn concentrations. Furthermore, ANOVA followed Bonferroni post hoc test for group differences after four feeding weeks using SPSS (version 22.0, Chicago, USA). Pearson correlation coefficients were determined for trace elements within organs and for gene expression data with trace elements in the kidney. Unless otherwise stated, results are presented as mean ± SD. Differences at $P < 0.05$ were considered as significant.

3. Results

3.1. Performance

After the first two weeks, piglets in the HZn group had higher weight gain ($P < 0.05$) compared to NZn group (10.6 ± 1.00 kg vs.