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Using piglets as an animal model: Dose-response study on the impact of short-term marginal zinc supply on oxidative stress dependent and cell fate associated gene expression in the heart muscle

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Running title: Zinc deficiency induced oxidative stress in piglet heart muscles

Abstract

During the last decade, zinc was shown to be involved in oxidative stress reactions demonstrating the pathological significance of zinc deficiency. However, those investigations mainly focused on situations of massive zinc depletion compared to sufficiently fed and oversupplied animals [1]. Though, the more common zinc deficiency phenotype in men and animals is marginal alimentary undersupply, due to plant based diets rich in anti-nutritive factors like phytate and fibre [2]. In order to mimic this situation, a dose-response study of gradually increasing zinc addition to a zinc deficient diet (corn soybean based diet; 28 mg native zinc content/kg diet; +0, +5, +10, +15, +20, +30, +40, +60 mg zinc/kg as ZnSO₄) with 48 weaning

piglets (n = 6 animals per group) was conducted. The diet with +60 mg zinc/kg served as baseline and was fed to all animals for two weeks prior to the study. Treatment lasted for eight days in order to stay within margin of physiological capacity of zinc mobilization and to avoid clinical symptoms of zinc deficiency. Analysis included blood plasma zinc levels and mRNA expression patterns of genes associated with antioxidative defense and cell fate in the heart muscle. The plasma zinc content of the groups increased linearly ($R^2 = 0.93$) from 0.21 to 0.63 mg zinc/L in group +0 mg zinc/kg to +60 mg zinc/kg respectively, proving the efficacy of the model to produce a fine-graded undersupply in dietary zinc. A statistically significant up-regulation of proapoptotic factors was monitored in the +0 mg zinc/kg group compared to all other groups (Bax, Fas, Casp9), in concordance to an up-regulation of the transcription factor p53. This might be due to increasing abundance of reactive oxygen species as zinc supply declines marked by an increased transcription of glutathione reductase and catalase. Simultaneously, p21 and Gadd45a (regulating cell cycle arrest in a p53 dependent manner) were lower expressed with reduced dietary zinc supply. In summary, undersupply of zinc proved to increase the transcription of genes associated with oxidative stress and apoptosis in the heart muscle, even under condition of short term exposure.

Introduction

During the last decades increasing evidence suggested a strong relationship between insufficient zinc intake and the occurrence of oxidative stress. Several hypotheses were formulated to explain this examinations, from decreased activity of key antioxidant enzymes (e.g. Cu/Zn-superoxide dismutase (SOD)) over lower metallothionein abundance and competition of zinc with redox active metal ions for binding sites (e.g. Fe, Cu) to loss of zinc binding to free protein sulfhydryl groups [1].

Under basal conditions, the abundance of reactive oxygen species (ROS) is balanced by a variety of enzymes, thiols, thioneins and vitamins which detoxify ROS metabolites through chemical reduction. However, under pathological conditions like nutrient deprivation, the compensation capacity of the antioxidative defense machinery may be exceeded, leading

to a markedly increased abundance of ROS. High ROS contents promote peroxidation of lipids and proteins as well as DNA strand breaks [3].

Cardiac function is inevitable for wellbeing and longevity due to its important role in delivering oxygen and nutrients to every part of the body. Increased oxidative stress in the heart muscle has been associated with the development of heart failure in adult individuals [4]. The consequences for a developing heart muscle can be assumed to be much more dramatic and are possibly involved in the growth retardation observed in growing, zinc deficient individuals [5].

Tumor suppressor p53 is a stress responsive transcription factor which has been shown to be involved in the apoptosis of cardiomyocytes resulting in the development of heart failure [6-8]. At basal intracellular ROS levels, p53 is short-lived and induces several antioxidative factors like glutathion-peroxidase 1 (Gpx1) and manganese-SOD (SOD2) as well as cell cycle regulators like cyclin-dependent kinase inhibitor 1a (p21) in order to maintain ROS homeostasis. At increasing stress levels, the activity of p53 changes towards the activation of prooxidant genes, leading to apoptosis [9]. This stress level dependent reactivity shows clearly that the p53 signaling pathway might be not only an indicator of cell fate, but also of the extent of intracellular stress.

Most in vivo studies considering zinc supply and oxidative stress are using experimental designs to compare situations of massive, long-term dietary zinc deficiency to sufficiently fed and/or oversupplied animals [1]. Though, the more common zinc deficiency phenotype in men and animals is a marginal, alimentary undersupply, due to plant based diets rich of anti-nutritive factors like phytate and fibre [2]. To mimic this situation, an experiment was conducted in which eight groups of weaned piglets were exposed to a fine-graded zinc supply for a short period of time, in order to avoid metabolic imbalance due to a clinical manifestation of zinc deficiency. The aim of the study was to investigate the influence of a short term, marginal zinc supply on the transcription of several factors associated with antioxidative defense and the p53 pathway.

Material and Methods

Experimental Design

48 weaned piglets (50% female, 50% male-castrated) were randomly assigned to eight treatment groups (n = 6) in a complete randomized block design.

All animals received a corn-soybean-meal based diet (13.0 MJ ME/kg, 24% CP), supplemented with 60 mg zinc/kg as $\text{ZnSO}_4 \times 7 \text{ H}_2\text{O}$, for two weeks prior to the study.

The animals showed an average life weight of 13.4 kg at the first day of the experiment. During the experimental phase (8d) they received the same corn-soybean-meal based diet as during the 14d acclimatization phase, but with varying zinc doses (Table 1). The experimental diets were pelletized twice in order to markedly reduce the activity of native phytase.

Table 1: Zinc supplementation levels of the experimental diets [mg/kg].

Treatment group	1	2	3	4	5	6	7	8
Zn dosage	0	5	10	15	20	30	40	60

Sample collection and storage

At the end of the experiment, blood samples were taken in Li-Heparin-Tubes and the blood plasma was recovered by centrifugation. The blood plasma samples were stored at -20°C. All animals were euthanized and tissue samples were taken from the tip of the heart muscle, placed in RNeasy (Qiagen, Hilden, Germany), incubated at 5°C overnight and stored at -80°C.

Zinc content analysis

The Zinc contents of the experimental diets and blood plasma were measured using atom-absorption-spectrophotometry (AAS) (novAA 350, Analytik Jena AG, Jena, Germany) directly (blood plasma) or after microwave extraction (Ethos 1, MLS GmbH, Leutkirch, Germany) (experimental diets).

Total RNA extraction and quality control

RNA was extracted from 50 mg heart muscle tissue using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer instructions. The tissue was homogenized with the MagnaLyzer System (MP Biomedicals, Illkirch, France). The total RNA extracts were diluted in RNase free water. The total RNA yield of every sample was determined with the Biophotometer (Eppendorf, Hamburg, Germany). $OD_{260/280}$ and $OD_{260/230}$ ratios of >2.0 were considered as indicators of high purity total RNA extracts.

The total RNA integrity was evaluated using the Experion System (Bio-Rad, Munich, Germany). A RNA quality index (RQI) of >5.0 was considered as threshold for satisfactory sample integrity.

cDNA synthesis

500 ng total RNA/sample were used for cDNA synthesis with the iScript Reverse Transcription Kit (Bio-Rad, Munich, Germany) in a total reaction volume of 20 μ l, according to manufacturer instructions. This kit uses a mixture of random hexamer and oligo-dT Primers. The reverse transcription (RT) was performed in duplicate per sample on the Mastercycler[®] gradient (Eppendorf, Hamburg, Germany). One sample duplicate was additionally used with the noRT mix of the kit to serve as a negative control. DEPC water was loaded on the plates in duplicate as an additional negative control. To reduce interplate variation an interplate calibrator (IPC) was introduced. The IPC consisted of a 1:1 mixture of five samples.

The RT was performed with the Mastercycler® gradient (Eppendorf, Hamburg, Germany): priming (25°C, 5 min), RT step (42°C, 30 min), RT inactivation (85°C, 5 min), hold (4°C).

After the reverse transcription, all samples were diluted 1:5 with 80 µl DEPC water.

The cDNA plates were stored at -20°C until further usage.

Primer design and optimization

Oligonucleotides for RT-qPCR applications were designed with Primer Blast [10] and ordered at Eurofins MWG Operon (Ebersberg, Germany). Primer pair specifications are summarized in table 2. If information on the exon-intron-positions of the template sequences (NCBI database, Ensemble database) was available, primer pairs were designed to span at least one exon-exon-junction. Primer sequences were blasted against the *Sus scrofa* and *Homo sapiens* reference sequence databases (RefSeq mRNA) [11] to check for their target specificity.

The primer pairs were used in a gradient PCR (54°C - 64°C) to evaluate the optimal annealing temperature and their specificity by melting curve analysis.

Table 2: Primer pair specifications.

Gene name	Accession number	Forward sequence	Backward sequence	Annealing Temperature [°C]
18S	NM_213940.1	tgtgggtgtgaggaaagcag	tcccatccttcacatccttc	58.0
GSR	XM_003483638.1	gtcgggatgtgtacccaaaa	gtgttcagtcggctcacgta	58.0
Sod1	NM_001190422.1	aggctgtaccagtgcaggtc	ccaatgatggaatgtctccc	61.0
Sod2	NM_214127.2	attgctggaagccatcaaac	ggtagaacaacgggcaatc	58.0
Gpx1	NM_214201.1	caagaatggggagatcctga	gataaacttggggtcgggtca	61.0
Cat	NM_214301.2	gcacgttggaaaggagacac	ggctgtggataaaggatgga	58.0
Prdx1	ENSSSCT00000004329	tgcttcgcgtgtctgcttc	gaccatctggcataacagcag	58.6
Prdx3	NM_001244531.1	gtgaaggcgttcagtttgt	tcccaactgtggctctcttc	58.0
Prdx4	XM_001927369.1	attccaagccagcacctta	gcgataatttcagttggacag	58.0
p53	NM_213824.3	cccttttgaagtcctggca	gccccagccaagcatatag	60.0
Bax	XM_003127290.2	ccgagaagtcttttccgagt	cgatctcgaaggaaagtcag	58.0
Fas	NM_213839.1	agtgactgaccccgattctg	atgtttccgtttgccaggag	61.3
PIG8	XM_003130045.1	tgtggtctctgtctccactg	ggcacttgcctcaaacctggt	61.0
Scotin	NM_016479.3	atgggggttggaaacgactgt	gtgggtgtagtcacgactgg	58.0
IGF-BP3	NM_001005156.1	gggtgcctgactccaaactc	gaggagaagttctgggtgtcc	60.0
Casp3	NM_214131.1	gccatggtgaagaaggaaaa	gtcgcgtctcaatcccacagt	61.0
Casp8	NM_001031779.2	gcctggactacatcccacat	tcctcctcattggtttccag	58.0
Casp9	XM_003127619.1	gaccccttaccctgccttac	ctcttttccatcgtcggtc	61.0
p21	XM_001929558.1	gggttccccagttctacctc	cctcctggaaatgtctgctc	61.0
SFN	NM_001044564.1	ctgaactgtgtggcagagact	tttccctctcatcctcggtct	64.0
Gadd45a	NM_001044599.1	atcttcctgaacgggatgg	catctatcttcgggctcctg	61.0

QPCR

QPCR experiments were performed with the SensiFAST™ SYBR No-ROX Kit (Bioline, Luckenwalde, Germany).

The Mastercycler® gradient was programmed with one initial 95°C step for 2 min in order to activate the polymerase. The polymerase chain reaction was performed for 40 cycles, with 5 sec denaturation at 95°C, 10 sec annealing at the specific temperatures shown in table 2 and 8 sec elongation at 72°C. A dissociation step was programmed to determine the melting curve of the amplicons after the PCR.

An internal standard was loaded in duplicate on each plate, to create a standard curve in order to calculate the amplification efficiencies for subsequent data normalization. The internal standard for one primer pair consisted of its purified amplicons from the gradient PCR (MinElute Purification Kit from Qiagen) in different dilutions: 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10^1 , 10^0 copies/μl. To evaluate the copy number in an amplicon solution, its dsDNA content was measured with the Biophotometer (Eppendorf). The dsDNA content of the solution in combination with the specific amplicon length was used to calculate the copy number (<http://www.uri.edu/research/gsc/resources/cndna.html>).

The qPCR runs were performed in a 96 well format. The plates were prepared with the EpMotion System (Eppendorf) and heat sealed (Thermal sealer, 4titude®, Wotton, Surrey, UK).

Normalization of gene expression data

The raw mean cq datasets of the second reaction plate of each plate duplicate was corrected for interplate variation using the mean Cq's of the IPC on each reaction plate in the following formula:

$$\text{Corrected mean Cq} = \text{Mean Cq}_{\text{plate2}} - (\text{Mean_IPC}_{\text{plate1}} - \text{Mean_IPC}_{\text{plate2}})$$

To search for useful reference genes, the complete mean Cq dataset of the RT-qPCR assay was screened with Genorm and Normfinder algorithms, using the GenEx software (Multi D Analysis, Gothenborg, Sweden). The mean Cq's of the reference genes in each sample were used to calculate the geometric mean to serve as reference gene index (RGI).

The efficiency corrected normalization model [12] was used for evaluation of gene expression data:

$$ratio = \frac{(E_{target})^{\Delta Cq_{target}(control-sample)}}{(E_{ref})^{\Delta Cq_{ref}(control-sample)}}$$

Amplification efficiencies of target and reference gene reactions were calculated according to [13]:

$$E_n = 10^{[-1/slope]}$$

The +60 mg/kg group served as control group.

The normalized dataset was purified from extreme values by removing all data which scattered more than three times the 25% quantile around the median.

Statistical analysis

A two factorial ANOVA (treatment, block) was performed, using the procedure GLM (SAS 9.3; SAS Institute Inc., Cary, United States of America). Significantly different treatment means regarding the blood plasma zinc content were identified with the Tukey test. Differences between treatment groups regarding the normalized gene expression results were compared in a linear contrast model using the CONTRAST function within the GLM procedure.

Results

Zinc contents in feed and blood plasma

The zinc content of the experimental diets increased linearly with increasing zinc supplementation ($R^2 = 0.99$; $p < 0.0001$; Table 3), ranging from 28.1 mg/kg in the +0 mg/kg group to 88.0 mg/kg in the +60 mg/kg group (Table 3).

Table 3: Zinc supplementation levels and analyzed zinc contents of the experimental diets [mg/kg].

Treatment Group	1	2	3	4	5	6	7	8
Zinc supplementation level	0	5	10	15	20	30	40	60
Analyzed diet zinc content	28.1	33.6	38.8	42.7	47.5	58.2	67.8	88.0

The zinc content in the blood plasma was also directly correlated with the dietary zinc supply ($R^2 = 0.93$; $p < 0.0001$; Figure 1), ranging from 0.21 mg/L in the +0 mg/kg group to 0.63 mg/L in the +60 mg/kg group (Figure 1).

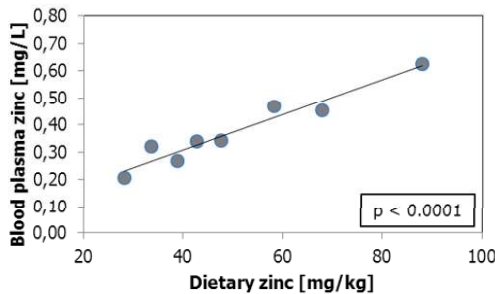


Figure 1: Effect of increasing dietary concentrations of zinc supply on the blood plasma zinc content.

Total RNA Quality and evaluation of reference genes

The total RNA extracts showed sufficient yield ($909 \pm 232 \mu\text{g/mL}$), purity ($\text{OD}_{260/280}$: 2.1 ± 0.1 ; $\text{OD}_{260/230}$: 2.2 ± 0.2) and integrity ($\text{RQI} = 6.6 \pm 1.1$) for RT-qPCR-experiments.

Screening the raw Cq dataset with Genorm and Normfinder algorithms revealed 18S, PIG8 and SOD2 as useful reference genes.

Gene expression data

The expression of catalase (CAT) and glutathione reductase (GSR) was significantly increased ($p = 0.04$; $p < 0.0001$) in the +0 mg/kg group (Figure 2 A + B). The remaining antioxidative factors examined, showed no differences in expression ratios, compared between treatment groups (data not shown).

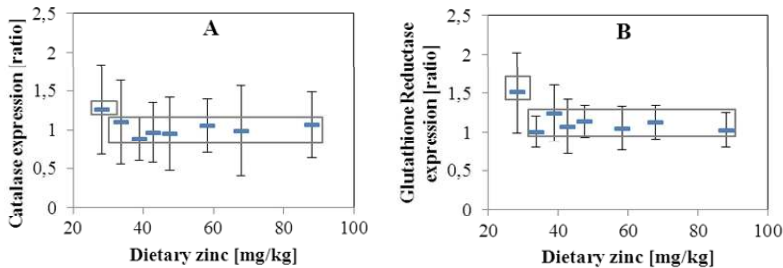


Figure 2: Effect of varying dietary zinc supply on the expression of (A) catalase and (B) glutathione reductase. Frames mark the treatment blocks with significant differences between each other, evaluated by linear contrast ($p = 0.04$ and < 0.0001 , respectively).

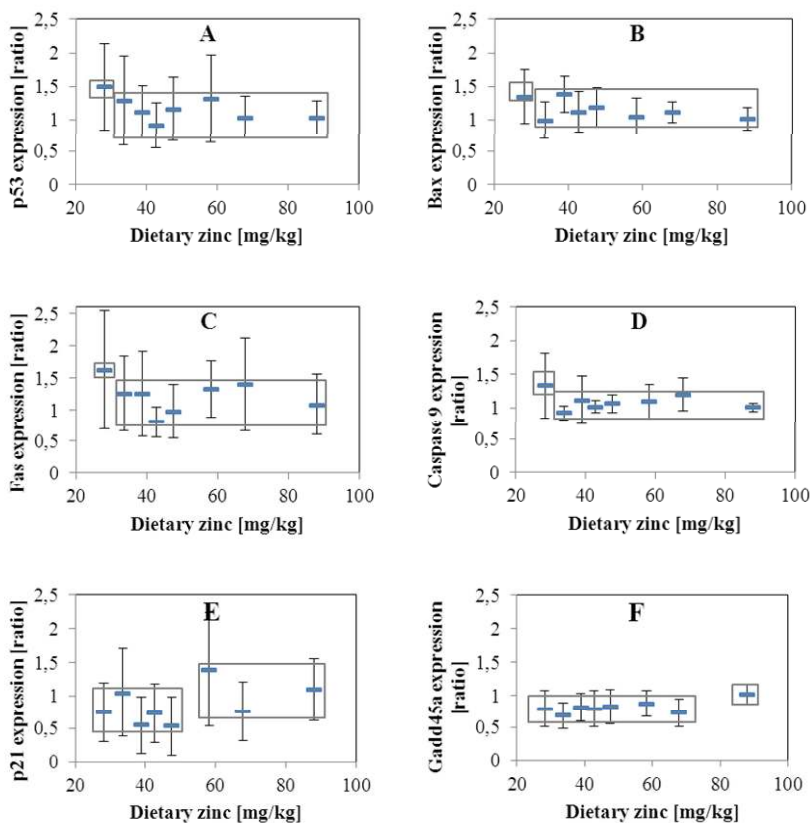


Figure 3: Effect of varying dietary zinc supply on the expression of (A) p53, (B) Bax, (C) Fas, (D) Casp9, (E) p21 and (F) Gadd45a. Frames mark the treatment blocks with significant differences between each other, evaluated by linear contrast ($p = 0.03$; 0.05 ; 0.02 ; 0.02 ; 0.01 and 0.02 , respectively).

p53 as well as its target genes Bcl2-like x-protein (Bax) and Fas-receptor (Fas) were up-regulated in the +0 mg/kg group compared to all other treatment groups (Figure 3 A, B and C; $p = 0.03$, $p = 0.05$, $p = 0.02$). The expression of caspase 9 (Casp9) showed the same expression pattern (Figure 3 D; $p = 0.02$). The remaining proapoptotic p53 target genes as well

as Casp3 and 8 were not differently regulated between treatment groups (data not shown). The p53 target genes p21 and growth arrest and damage inducible alpha (Gadd45a) showed an expression pattern which was contrary to the differential regulated proapoptotic p53 targets. p21 was down-regulated under conditions of insufficient zinc supply (< 60 mg/kg; [14]), compared to sufficient and mild oversupply (Figure 3 E; $p = 0.01$). Gadd45a was lower expressed in all groups, compared to control (+60 mg/kg) (Fig. 3 F; $p = 0.02$).

Discussion

The observed increase of the plasma zinc content with increasing dietary zinc supply is in concordance with earlier published data [15, 16]. Though, in the actual investigation no plateau was reached at the estimated level of sufficient zinc supply for piglets (60 mg/kg; NRC [14]). This might be due to ongoing homeostatic adaptations because of the change in alimentary supply compared to the supply level during the acclimatization phase. Nevertheless it proves the treatment groups were in different states of zinc supply, ranging from deficient states to potential mild oversupply.

Zinc deficiency was discussed as a cause of oxidative stress [1]. This could be supported in the actual project in which we investigated several anti-oxidative factors on the transcriptional level. Two of them, GSR and Cat were up-regulated in the group with the lowest zinc supply level, which points towards an increased abundance of ROS in the marginal zinc supplied heart muscle.

p53 is a stress responsive transcription factor. Under the terms of physiological stress, it is expressed on a basal level to induce antioxidative and cell cycle regulators like Gpx1 and p21, while at high stress levels transcription is up-regulated leading to the initiation of proapoptotic and prooxidative target genes like Fas and Bax [9]. Gpx1 and SOD2 (anti-oxidative p53 targets) showed a very consistent expression pattern over all treatment groups, while p21 and Gadd45a (p53 dependent cell cycle regulators) were down-regulated with decreasing dietary zinc supply. On the contrary, Bax and Fas were up-regulated in the +0 mg/kg group. This is

in concordance with a higher expression of p53 under this treatment condition, which has been already shown in human lung fibroblasts [17]. The results indicate high intracellular stress levels, which is in context to the significantly increased GSR and CAT expression.

The increase in Bax expression is in good context to the up-regulation of Casp9 in the same group. This factor is activated in the presence of cytochrome-c after its release due to a higher permeabilisation of the outer mitochondrial membrane by Bax, leading to the activation of Casp3 and endonucleases [18]. Though, no up-regulation of Casp3 could be detected (data not shown). This also applies for Casp8 (data not shown) which has been proven to be activated after the binding of death factors like the Fas-ligand to their membrane-bound receptors, resulting in subsequent activation of Casp3 and DNA degradation [19, 20]. The fact that Casp3 and 8 showed no significant differences between treatment groups suggests their regulation occurs primarily on the proteomic level.

The initiation of prooxidative factors like Bax might increase the amount of reactive oxygen species making it difficult to determine, if high oxidative stress in a tissue is the inducer of apoptosis or its catalyzing by product. One of the hypotheses regarding the promotion of oxidative stress by zinc deficiency is the lower abundance of metallothioneins [1], which has been shown for metallothionein 1 and 2 in zinc deficiency [21]. This was confirmed in own investigations regarding the expression of Mt1a and Mt2b proving differential zinc supply of the heart muscles (data not shown). Metallothioneins are not only the regulators of intracellular zinc, but potent antioxidative factors [22-24]. The significantly reduced expression of Mt1a and Mt2b potentially decreased the overall antioxidative capacity of cardiomyocytes, leading to increased oxidative stress and apoptosis.

Conclusions

A varying dietary zinc supply (28 mg/kg to 88 mg/kg) for eight days induced differential states of zinc homeostatic regulation in the piglets, as indicated by a linear increase in the blood plasma zinc content (0.21 mg/L to

0.63 mg/L). Despite the short experimental period, the lowest supplied treatment group showed an up-regulation of GSR and CAT, suggesting an increased abundance of ROS. In concordance with this observation, the stress responsive factor p53, its proapoptotic and prooxidative target genes Bax and Fas as well as Casp9 were up-regulated. On the contrary, the expression of the p53-dependent cell cycle regulators p21 and Gadd45a was reduced in the groups with a dietary zinc content of < 60 mg/kg and < 88 mg/kg, respectively. In summary, our results indicate dramatically increased stress resulting in proapoptotic stimuli in the heart muscle of piglets after just eight days of marginal supply with zinc. This highlights the necessity to avoid even short periods of zinc deprivation.

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