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Research paper

Effects of dietary sodium selenite and organic selenium sources on immune and inflammatory responses and selenium deposition in growing pigs

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ABSTRACT

The study was conducted to compare effects of different dietary Se sources (sodium selenite [NaSe], Se-enriched yeast [Se yeast] or L-selenomethionine [SeMet]) and one Se-deficient control diet on the expression of selected genes, hematological and clinical biochemical parameters, and muscle morphology in two parallel trials with finisher pigs. Se concentrations in blood plasma and tissues were also monitored. From the pigs in one of the parallel groups, muscle samples obtained from *Musculus longissimus dorsi* (LD) before and during the trial were examined. The pigs in the other parallel group were challenged once with lipopolysaccharide (LPS) intravenously.

Transcriptional analyses of LD showed that selenogenes *SelenoW* and *H* were higher expressed in pigs fed Se-supplemented diets compared with control. Furthermore, the expression of interferon gamma and cyclooxygenase 2 was lower in the Se-supplemented pigs versus control. In whole blood samples prior to LPS, *SelenoN, SelenoS* and *thioredoxin reductase 1* were higher expressed in pigs fed NaSe supplemented feed compared with the other groups, possibly indicating a higher level of oxidative stress. After LPS exposure *glutathione peroxidase 1* and *SelenoN* were more reduced in pigs fed NaSe compared with pigs fed organic Se. Products of most above-mentioned genes are intertwined with the oxidant-antioxidant system. No significant effects of Se-source were found on hematologic parameters or microscopic anatomy. The Se-concentrations in various skeletal muscles and heart muscle were significantly different between the groups, with highest concentrations in pigs fed SeMet, followed by those fed Se yeast, NaSe, and control diet.

Consistent with previous reports our results indicate that dietary Se at adequate levels can support the body's antioxidant system. Our results indicate that muscle fibers of pigs fed organic Se are less vulnerable to oxidative stress compared with the other groups.

1. Introduction

Mulberry Heart Disease (MHD) and nutritional myopathy (skeletal muscle degeneration, NMD) are challenges in Norwegian pig production [1]. They are linked to selenium (Se)/vitamin E (vitE) deficiency [2]. Commercial feed mills fortify diets with the maximum allowable quantity of Se combined with high dietary vitE levels, but MHD and NMD still occur [1]. In intensive pig production daily weight gain and feed efficiency are high and infectious disease incidence is low [3–5]. However, high growth rate is associated with enhanced levels of oxidative stress [6,7] and is possibly linked to higher prevalence of MHD [8]. Se-/vitE-levels previously considered satisfactory are reported in pigs with pathognomonic MHD changes [8–11]. This raises the question whether existing feeding strategies meet Se requirements in high-yielding pigs [12,13]. Tissue Se concentrations and some selenoproteins mirror the Se status [14–16]. Biomarkers for detection of subclinical Se deficiency could possibly contribute to reveal MHD and NMD cases at an early stage.

Borella et al. [17] suggested that biological effects of Se in mammals are strongly influenced by the chemical Se form absorbed. Se source

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and status may also influence other body mineral elements. Molybdenum (Mo) is involved in the Se metabolism as an enzymatic cofactor [18,19], whereas magnesium (Mg) shows antioxidant properties and possibly contributes to the protection against MHD via its involvement in calcium homeostasis and glutathione synthesis [20–22].

The objectives of this study were to compare effects of three Se supplemented diets with a low Se control diet on: 1) immune and inflammatory responses in blood and muscle, 2) hematology and clinical biochemistry, 3) plasma mineral concentrations, 4) muscle histopathology and 5) tissue Se deposition. In addition, we wanted to identify biomarkers reflecting porcine Se status.

2. Material and methods

2.1. Experimental design and sampling

The trial was approved by the Norwegian Food Safety Authority. It complies with the current European and Norwegian Animal Welfare Act (LOV-2009-06-19-97) and the Norwegian regulations on swine husbandry (FOR-2003-02-18-175).

Twenty-four female finisher pigs, Landrace × Yorkshire × Duroc (LYDD) hybrids, with an average body weight of 25.6 (SD 4.4) kg were included in this study. The pigs were randomly allocated to four groups with two replicates in two rooms (room A and B), where they were kept in groups of three animals/pen. The pen size was 3.5 m^2 in room A and 2.2 m^2 in room B. Pigs in room A were kept until reaching slaughter weight (day 64 of trial period), thus requiring more space. Those in room B were killed when reaching 70 (SD 9.34) kg (see *LPS treatment*). The room temperature was initially set to 22 °C and successively reduced to 19 °C over the trial period. Dried compound feed was provided through a feed automate (Domino, type FR-H3 45L, Felleskjøpet, Norway). Feed and water were given *ad libitum*. Before entering the study, the pigs were fed standard compound starter feed containing 18% crude protein, 1.2% lysine, 5.3% crude fat, 8000 IU vitamin A, 1500 IU vitamin D, 150 mg vitE/kg, 12 mg copper/kg (from copper sulphate), 0.4 mg Se/kg (from NaSe) and 141 mg zinc/kg. After an acclimatization period of one week, the pigs were introduced to the trial diets. The composition of the trial feed is listed in Table 1. The trial diets were either the Se deficient, non-supplemented basal diet containing 0.05 mg Se/kg (control) or the same diet supplemented with NaSe (Mikromin Selen 300 FK,Vilomix, Norway), selenized yeast (Se yeast, Sel-Plex®, Alltech, USA) or L-selenomethionine (SeMet, Excential Selenium 4000 ®, Orffa, Netherlands). The NaSe-, Se yeast- and SeMet-enriched diets contained 0.33, 0.32 and 0.32 mg Se/kg, respectively. All diets were added 100 mg vitE/kg feed. The pigs in room A were fed with the trial feed to for 64 days, and were then slaughtered by standard procedures at the abattoir (CO_2). The pigs in room B received the trial feed for 47–48 days before they were euthanized by captive bolt stunning and exsanguination. These pigs were necropsied.

2.1.1. Muscle biopsies

Two muscle biopsies were obtained from the pigs in room A, the first one before starting the trial and the second biopsy on day 38. The 12 pigs were sedated using 2 mg azaperone/kg BW and locally anesthetized with lidocaine-adrenaline (20 mg/mL + 0.036 mg/mL). Biopsies were obtained by using a biopsy gun (Biopty-Cut®, BARD, USA) and disposable needles (14 g × 10 cm length, Biopty-Cut®, BARD, USA). Biopsy samples of approximately 25 mg and 75 mg were fixed in RNAlater for subsequent RNA isolation or frozen at −20 °C for total Se measurements by inductively coupled plasma mass spectrometry (ICP-MS), respectively. After the biopsy procedure, pigs received an i.m. injection of 0.4 mg meloxicam/kg BW.

2.1.2. Post mortem collection of tissue samples

Samples of selected skeletal muscles (LD, *M. semitendinosus*, *M. semimembranosus*, *M. biceps brachii*, *M. psoas major*, *M. quadriceps femoris*, diaphragm), heart muscle (left and right ventricle, septum), liver and kidney (cortex) were collected *post mortem.* Tissue samples of approximately 15 × 10 × 5 mm were fixed in 4% neutral buffered formaldehyde and processed routinely for light microscopic examination. Sections were cut at 5 µm and stained with hematoxylin and eosin (HE). For ICP-MS- analysis tissue samples were stored at −20 °C until analyses. For subsequent gene expression studies samples from LD were fixed in RNA-later for 24 h at 4 °C and subsequently stored at -20 °C until analysis.

Table 1
Composition of the trial diets.

Raw materials	%
Barley	59.2
Extracted soy	13.4
Wheat	10.0
Rapeseed cake Mestilla	5.00
Oats	5.00
Soybean oil (raw)	3.24
Limestone, Visnes	1.32
Mono Calcium Phosphate	0.45
NaCl	0.58
Mikromin Pig[a]	0.16
Vitamine A	0.05
VitE (V5)	0.06
Vitamin ADKB[b]	0.06
L-lysine	0.37
DL-metionine	0.11
L-treonine	0.17
L-tryptophan	0.01
Formic acid 85%	0.75
Physyme XP 5000 TPT	0.01
Total Selenium in mg Se/kg diet[c]	
None (control)	0.05
Sodium selenite (NaSe)	0.33
Se yeast (Se yeast)	0.32
L-SeMet	0.32

[a,b]Supplied the following per kilogram of diet.
[a] Fe 96 mg; Cu 20.8 mg; Mn 48 mg; Zn 96 mg; I 0.48 mg.
[b] Vitamin A 5700 IU; Vitamin D 1200 IU; Vitamin E 100 mg; Vitamin K 3.72 mg; Vitamin B1 2.4 mg; Vitamin B2 4.5 mg; Vitamin B5 12.0 mg; Vitamin B6 7.2 mg; Vitamin B12 0.012 mg; Folic acid 1.8 mg; Biotin 0.24 mg.
[c] as measured with ICP-MS.

2.1.3. Blood samples

Nine mL PAXgene®-tubes, Vacuette® Z serum clot activator and six mL Vacuette® Lithium Heparin tubes (Greiner Bio-One, Austria) along with Venoject needles (20G × 1½"UTW, USA) were used for blood sampling from the *Vena jugularis externa*. PAXgene®-tubes were frozen at -20 °C after two hours at room temperature and stored at -70 °C until RNA isolation. Blood samples for serum and plasma isolation were centrifuged at 3500 × *g* for 15 min (Megafuge 1.0 R, Heraeus SEPATECH, USA). Initial blood samples were obtained from pigs in both rooms after the acclimatization period. Subsequent blood samples were drawn from the pigs in room B prior to LPS-application (initiation LPS), one hour and 24 h after LPS treatment.

2.1.4. LPS treatment

Forty-five to forty-seven days into the trial, the twelve pigs in room B, with an average BW of 70 (SD 9.34) kg, received an injection of 2 µg LPS/kg BW (*E. coli* O111:B4, L4391, lot: 014M4019 V, G-irradiated, Sigma Aldrich, USA). Clinical examinations including rectal temperature, respiratory and heart rate were conducted before each blood sampling and at 90 min, 3 and 5 and 8 h after LPS application.

2.2. RNA isolation and quantitative Real Time PCR (qPCR) analysis

Total RNA was extracted in a randomized order. Blood RNA was isolated using the PAXgene®-products and the appendant protocol (PreAnalytiX®, UK). Muscle RNA was isolated using RNeasy Fibrous Tissue Mini Kit (Qiagen) according to the manufacturer's protocol. RNA integrity was verified by the 2100 Bioanalyzer in combination with an RNA Nano Chip (Agilent Technologies), and RNA purity and concentrations were measured using the NanoDrop ND-1000 (NanoDrop Technologies). Samples with RNA integrity number (RIN) >7.5 were accepted for gene expression analysis. Total RNA was stored at −80 °C until use.

The qPCR assays were performed according to MIQE standards [23] on three animals from each of the four diet groups (Table 1) and at three time points. First-strand cDNA was synthesized from 0.8 µg total RNA from all samples using SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen™) following manufacturer's protocol. Negative controls were performed in parallel by omitting RNA or enzyme. Obtained cDNA was diluted 1:10 in molecular grade H_2O and stored at − 20 °C. The qPCR primers were obtained from the literature or designed using Primer3 (http://bioinfo.ut.ee/primer3/). Primer details are shown in Table A1 (supplementary material). All primer pairs gave a single band pattern for the expected amplicon of interest in all reactions. PCR reaction efficiency for each gene assay was determined using 2-fold serial dilutions of randomly pooled cDNA. The qPCR assays were performed using a LightCycler® 96 Real-Time PCR System (Roche Diagnostics). Each 10 µL DNA amplification reaction contained 2 µL PCR-grade water, 2 µL of 1:10 diluted cDNA template, 5 µL of Lightcycler 480 SYBR Green I Master (Roche Diagnostics) and 0.5 µL (final concentration 500 nM) of each forward and reverse primer. Each sample was assayed in duplicate, including a no template control (NTC). The three-step qPCR program included an enzyme activation step at 95 °C (5 min) and a minimum of 40 cycles of 95 °C (10 s), primer specific annealing temperature (10 s) and 72 °C (15 s). To confirm amplification specificity the PCR products from each primer pair were subjected to melting curve analysis and visual inspection of PCR products after each run by agarose gel electrophoresis. For target gene normalization, *Rpl4, Hprt1, B2m, Actb* and *Gapdh* were evaluated for use as reference genes by ranking relative gene expression according to their overall coefficient of variation (CV) and their interspecific variance, as described previously [24]. For muscle samples, *Hprt1* was used as a normalization factor, whereas the geometric average of *Hprt1*, *Rpl4* and *Gapdh* was used for blood samples. Mean normalized levels (MNE) of target genes were calculated from raw quantification cycle (Cq) values [25]. The panel of genes (font: *italic*) included in the study encode for different selenoproteins (font: regular), immune and inflammation related proteins (font: regular): a.) glutathione peroxidase 1 and 3 (*Gpx1, Gpx3*), iodothyronine deiodinases 1 and 3 (*Dio1*, *Dio3*) selenoproteins (Sel) H, K, S, W1, P1 and N1 (*SelenoH, SelenoK, SelenoS, SelenoW, SelenoP, SelenoN*) and thioredoxin reductase 1 (*Txnrd1*), b.) interleukin 1 β, 6 and 10 (*Il1β, Il6, Il10*), tumor necrosis factor alpha (*Tnfα*) and interferon gamma (*Ifnγ*), c.) superoxide dismutase 1 (*Sod1*), catalase (*Cat*), inducible nitric oxide synthase (*iNos*), cluster of differentiation 4 (*CD4*) and cyclooxygenase-2 (*Cox2*, syn. inducible prostaglandin endoperoxide H synthase) were measured in blood prior to and after LPS-challenge as well as in loin muscle biopsies (LD) collected repeatedly. Additionally, the gene expression of F-Box protein 32 (*Fbxo32*) and caspase 3 (*Casp3*) was analyzed in LD samples.

2.3. ICP-MS analysis

The concentrations of selected elements in tissue (dry matter, DM) and plasma samples were measured by ICP-MS (Agilent 8800) [26]. The mineral panel included Se for all samples and additionally, concentrations of Mg, and Mo were measured in plasma. Plasma samples were thawed and sonicated for 10 min at room temperature (Ultrasonic cleaner, Biltema, Norway). Then, 0.5 mL were transferred to new plastic vials, weighed (Analytical Balance LC 620 P, Sartorius, Germany) and mixed with 4.4 mL of an alkaline diluent [10]. A tellurium (Te) internal standard was added to the samples to a final sample dilution of 1:9 [27], a modification of the method described by Gajek et al. [28].

2.4. Hematology and biochemistry

Blood samples were subjected to a complete multi parametric hematological analysis (ADVIA 2120 Hematology System, Siemens Healthcare GmbH). The hematological parameters analyzed were red blood cell count (RBC), haematocrit (Hct), haemoglobin (Hgb), white blood cell count (WBC), thrombocyte count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). The clinical biochemical parameters C-reactive protein (CRP, Randox, UK), γ-glutamyl transferase (GGT, Siemens Medical Solutions Diagnostics), glutamate dehydrogenase (GLDH, Randox, UK), creatine kinase (CK, Siemens Healthcare GmbH), and aspartate transaminase (AST, Siemens Healthcare GmbH) were analyzed on ADVIA 1800 (Siemens Healthcare GmbH)

2.5. Histopathological analysis

Samples from liver, myocardium (right and left ventricle, septum) and seven skeletal muscles, incl. diaphragm, were analyzed.

The histopathological findings in skeletal muscles were graded blindly on a semi-quantitative scale of severity (0–normal, 1–mild changes, 2–moderate changes, 3–severe changes). Mild changes were defined as scattered, swollen myocytes with loss of striation or a very low number of degenerating myocytes, sometimes with mild infiltration of macrophages. Moderate changes were defined as multifocal degenerating or necrotic myocytes with or without macrophage infiltration, and severe changes were defined as multifocal, relatively widespread degenerating or necrotic myocytes with or without macrophage infiltration. Findings in liver and heart were not graded.

2.6. Statistics

Levels of mRNA in samples from the skeletal muscle *M. longissimus dorsi* (LD) were measured at day 0, 38 and 64 days into the trial. Linear regression both with and without random effect was used to analyze the q-PCR values using the functions lm and lmer (package *lme4*) in RStudio [29]. *Diet* and *Time* were tested as factors describing the fixed effect and pig ID was tested as random effect. The effect of including/excluding variables and random effect was compared using Akaike's 'Information Criterion' (AIC). Prediction from the model selected was plotted to show the differences between diets over time, for each time point the differences between diets were marked with different letters.

The measured Se concentrations in LD were plotted using Excel 2013.

For qPCR-results from the LPS-study, we reported mean normalized values (MNE) as mean ± SEM (time point 0 h) and the percentaged change compared with prior to LPS for 1 h and 24 h (Table 2). MNE were obtained by determining the geometric mean of three of the reference genes used and normalizing the target genes to this geometric mean. The percentaged difference between prior to LPS and 1 and 24 h after LPS application, respectively, was calculated for all blood parameters, plasma mineral concentrations and qPCR-results as value after LPS minus value prior to LPS-application divided by value prior to LPS-application. Linear regression (lm in R) was used to compare initial values as well as the percentaged differences for each diet, to obtain eventual significant differences between the diets. The regressions were repeated such that all diets were used as control. Significant differences between diets are reported with different letters in Table 2.

Table 2
Gene expression in whole blood (MNE) and mineral concentrations in plasma (mg/kg) prior to LPS application and change after LPS-application compared with values at 0 h (%).

		Change over time after LPS (Mean ± SEM; %)	
	Initial MNE- levels (Mean ± SEM)	1 h	24 h
GENE EXPRESSION			
SelenoW			
Control	0.575 ± 0.049	−20 ± 14	−6 ± 11
NaSe	0.648 ± 0.096	−17 ± 9	−10 ± 6
Se yeast	0.810 ± 0.097	−19 ± 12	−19 ± 7
SeMet	0.780 ± 0.061	−27 ± 5	−10 ± 5
SelenoH			
Control	0.150 ± 0.016	−32 ± 7	−15 ± 3
NaSe	0.143 ± 0.008	−24 ± 5	−10 ± 6
Se yeast	0.150 ± 0.011	−13 ± 17	−8 ± 12
SeMet	0.150 ± 0.003	−28 ± 3	−1 ± 2
Gpx1			
Control	14.8 ± 1.35	111 ± 46	-22 ± 11^{ab}
NaSe	33.3 ± 11.8	44 ± 43	-45 ± 11^{a}
Se yeast	40.4 ± 5.60	51 ± 32	-4 ± 1^{b}
SeMet	29.4 ± 4.75	74 ± 27	3 ± 11^{b}
Gpx3			
Control	0.067 ± 0.062	313 ± 261	−2 ± 34
NaSe	0.528 ± 0.154	103 ± 76	−61 ± 11
Se yeast	0.296 ± 0.061	131 ± 72	−6 ± 6
SeMet	0.392 ± 0.267	206 ± 61	−13 ± 33
SelenoK			
Control	0.738 ± 0.060	79 ± 9	−24 ± 6
NaSe	0.751 ± 0.107	43 ± 15	−5 ± 23
Se yeast	0.666 ± 0.011	54 ± 23	−17 ± 9
SeMet	0.636 ± 0.092	90 ± 46	10 ± 12
SelenoN			
Control	0.014 ± 0.001^{a}	−75 ± 2	-26 ± 5^{ab}
NaSe	0.030 ± 0.001^{b}	−75 ± 3	-49 ± 4^{a}
Se yeast	0.021 ± 0.001^{c}	−68 ± 8	-25 ± 8^{ab}
SeMet	0.015 ± 0.001^{a}	−72 ± 1	-22 ± 6^{b}
SelenoP			
Control	0.098 ± 0.006	−18 ± 8	−20 ± 7
NaSe	0.099 ± 0.013	2 ± 24	−22 ± 17
Se yeast	0.113 ± 0.009	−15 ± 11	−33 ± 6
SeMet	0.112 ± 0.034	14 ± 32	−20 ± 20
SelenoS			
Control	0.173 ± 0.015^{a}	−44 ± 6	−16 ± 5
NaSe	0.230 ± 0.010^{b}	−45 ± 5	−28 ± 8
Se yeast	0.185 ± 0.007^{a}	−34 ± 12	−17 ± 10
SeMet	0.173 ± 0.008^{a}	−44 ± 6	−12 ± 4
Txnrd1			
Control	0.293 ± 0.024^{a}	-24 ± 4^{a}	16 ± 11
NaSe	0.355 ± 0.014^{b}	-1 ± 7^{b}	5 ± 12
Se yeast	0.296 ± 0.014^{a}	-1 ± 5^{b}	< 1 ± 3
SeMet	0.269 ± 0.013^{a}	-21 ± 3^{a}	14 ± 14
Cat			
Control	0.663 ± 0.039^{a}	12 ± 47	−9 ± 8
NaSe	1.295 ± 0.251^{b}	84 ± 29	−9 ± 23
Se yeast	0.566 ± 0.032^{a}	41 ± 21	5 ± 7
SeMet	0.617 ± 0.123^{a}	54 ± 13	12 ± 15
Il10			
Control	0.015 ± 0.002	48 ± 23	-18 ± 4^{a}
NaSe	0.021 ± 0.002	22 ± 36	-15 ± 7^{a}
Se yeast	0.017 ± 0.001	−17 ± 13	-31 ± 9^{a}
SeMet	0.008 ± 0.004	62 ± 22	46 ± 24^{b}
PLASMA-MINERAL CONCENTRATIONS			
Se			
Control	0.06 ± 0.01^{a}	−6 ± 3	−9 ± 2
NaSe	$0.14 \pm 0.00^{b*}$	−13 ± 3	−17 ± 5
Se yeast	$0.16 \pm 0.00^{b*}$	−8 ± 2	−10 ± 5
SeMet	$0.15 \pm 0.00^{b*}$	−9 ± 2	−13 ± 4
Mg			
Control	0.021 ± 0.00^{a}	19 ± 5	5 ± 1^{a}
NaSe	0.022 ± 0.00^{ab}	11 ± 2	15 ± 5^{b}
Se yeast	0.025 ± 0.00^{bc}	16 ± 7	0 ± 5^{c}
SeMet	0.026 ± 0.00^{c}	19 ± 3	0 ± 4^{c}

(*continued on next page*)

Table 2 (*continued*)

	Initial MNE- levels (Mean ± SEM)	Change over time after LPS (Mean ± SEM; %)	
		1 h	24 h
Mo			
Control	0.006 ± 0.00[a]	−1 ± 3[a]	−27 ± 6[ab]
NaSe	0.010 ± 0.00[b]	8 ± 1[ab]	−51 ± 7[b]
Se yeast	0.008 ± 0.00[ab]	11 ± 1[ab]	−35 ± 6[ab]
SeMet	0.007 ± 0.00[a]	12 ± 2[b]	−27 ± 2[a]

Genes are ordered in accordance to their sequence in Fig. 2. Data in column 1 beneath heading "Gene expression" are mean normalized expression (MNE) levels of n = 3 animals per diet in whole blood isolated prior to LPS challenge. Data shown in column 1 beneath heading "Plasma mineral concentrations" are plasma concentrations of selenium (Se), molybdenum (Mo) and magnesium (Mg) as measured prior to application of LPS (mg/kg). Data presented in columns 2 and 3 are percentaged changes over time after application of LPS compared with values obtained prior to LPS. The regressions were repeated such that all diets were used as control. Values in a column not connected by the same letter are significantly different ($P < 0.05$). *$P < 0.001$.
Control: 0.05 mg Se/kg diet; NaSe: sodium selenite 0.33 mg Se/kg diet; Se yeast: selenium yeast 0.32 mg Se/kg diet; SeMet: L-selenomethionine 0.32 mg Se/kg diet.

The significance level was set to $P < 0.05$. Deviant significance levels were indicated in the text.

3. Results

Prior to initiation of the feeding trial, the Se-concentrations (mg/kg) measured in LD (shown in Fig. 1) and in plasma (control 0.13 (0.02), NaSe 0.12 (0.01), Se yeast 0.13 (0.01), SeMet 0.11 (0.01)) were similar in the pigs in all four groups.

During the feeding trial, no clinical signs of Se deficiency were observed. Furthermore, no significant effects on complete blood count or clinical biochemical parameters related to diet were revealed (data not shown).

The i.v. application of LPS provoked hyperthermia, which peaked similarly in all four groups after about 1 ½ hours at a mean rectal temperature of 41.2 (0.3) °C. Additionally, the pigs showed typical clinical signs associated with pyrexia including anorexia, lethargy and somnolence for up to 5 h after LPS application (data not shown) [30].

3.1. Gene expression patterns in M. longissimus dorsi

Fig. 2 shows expression levels of selected genes in LD samples. The selenogenes *SelenoW* and *SelenoH* showed similar expression patterns on days 38 and 64. These genes were higher expressed in all groups fed Se supplemented feed compared with the control group, but *SelenoH* in pigs fed SeMet was not statistically significantly different from control at day 38.

The expression of *Gpx3* was lower in pigs receiving Se supplemented feed (trend Se yeast: P = 0.07) at day 38 compared with control. However, at day 64 *Gpx3* expression was equal or significantly increased (SeMet) compared with control. The different Se treatments did not influence the expression of the selenogenes *SelenoK*, *SelenoN*, *SelenoP*, *SelenoS, Txnrd1* and *Dio1* and *3*. Data regarding *Dio1* and *3* are not shown. The expression levels of *Cox2*, the gene encoding the key inflammatory enzyme Cox2, were lower in pigs fed organic Se (Se yeast only at 38 days) compared with those fed NaSe and the control diet. In addition, the gene encoding the key apoptotic protease Casp3 was significantly lower expressed in pigs fed organic Se compared with pigs fed NaSe and the control diet at day 38 but not at day 64.

The expression of the gene *Sod1*, encoding the copper and zinc-dependent key antioxidant enzyme Sod1, was lower in pigs fed the Se supplemented diets compared with control at day 38. On day 64, there was no difference between groups. For the gene encoding the cytokine Ifnγ, no difference between feed groups were found at day 38, but lower expression in the Se-supplemented pigs, particularly in those fed organic Se, was found at day 64.

The expression of the non-selenogenes *Cat*, *CD4, Fbxo32, Il1β*, *Il6*, *Il10* and *iNos* was not influenced by Se treatment (only *Cat*-related data shown in Fig. 2).

3.2. Gene expression patterns and mineral-concentrations in blood prior to LPS injection

SelenoN, *SelenoS* and *Txnrd1*, as well as the gene *Cat*, encoding a key antioxidant enzyme, were highest expressed in the pigs fed NaSe compared with the other groups (Table 2). However, for the selenogenes *SelenoW, SelenoH*, *Gpx1* and *3*, *SelenoK*, *SelenoP, Dio1* and *3* as well as for the non-selenogenes *Cox2*, *Sod1*, *Ifnγ*, *Il1β*, *Il6*, *Il10*, *CD4* and *iNos* no differences between groups were found. Data for *Dio1* and *3*, *CD4*, *Cox2*, *Sod1*, *Il1β, Il6*, *Ifnγ*, and *iNos* are not shown.

Pigs receiving Se-supplemented diets showed significant higher plasma-Se compared with the control ($P < 0.001$), but no difference in plasma-Se was found between pigs fed the various Se supplemented diets (Table 2).

The plasma concentration of Mg was higher in the pigs fed organic Se (Se yeast and SeMet) compared with the control and pigs fed NaSe (Table 2). The plasma concentration of Mo was higher in the pigs fed the NaSe supplemented diet compared with the control and pigs fed SeMet (Table 2).

3.3. Gene expression profiles and mineral concentrations in blood after LPS challenge

Without significant differences between groups, the selenogenes *Gpx1*, *Gpx3* and *SelenoK* as well as the non-selenogene *Il1β* were up-regulated within 1 h after LPS challenge, whereas *SelenoN*, *SelenoS*, *SelenoH* and *SelenoW* were down-regulated. Most of these genes had returned to the baseline levels after 24 h. For *SelenoP* and *SelenoS* lower levels than initial expression levels were found after 24 h.

While unchanged in pigs supplemented with NaSe and Se yeast, the expression of *Txnrd1* was down-regulated from the initiation to 1 h after LPS-injection in control and SeMet fed pigs (Table 2).

Twenty-four hours after LPS-injection (Table 2), in pigs fed NaSe, *Gpx1* was more reduced compared with pigs fed SeMet, and *SelenoN* compared with SeMet and Se yeast. The *Il10*-gene expression was down-regulated in the control, NaSe and Se yeast compared with SeMet which was up-regulated. Diet nor LPS did not influence the expression of *Dio1* and *3*, *CD4*, *Il6* and *iNos* (data not shown).

The plasma-Mo-concentrations reached higher concentrations in the pigs supplemented with SeMet compared with the control group at 1 h after LPS. A following decrease in plasma-Mo was strongest in NaSe supplemented pigs compared with SeMet. The plasma-Mg-levels

increased more in control and NaSe-group compared with the groups supplemented with Se yeast and SeMet at 24 h.

3.4. Selenium concentrations in tissue samples

Se concentrations in LD biopsies showed a pronounced increase during the trial period in pigs fed organic Se (SeMet or Se yeast) supplemented feed compared with pigs fed dietary NaSe and control ($P < 0.001$, Fig. 1). Furthermore, at the end of the trial, all skeletal muscle and heart muscle samples from pigs fed organic dietary Se sources showed increased Se concentrations compared with those fed NaSe or no Se supplement (Table 3); SeMet > Se yeast > NaSe > unsupplemented diets. Pigs fed Se supplemented feed also showed higher Se concentrations in liver samples compared with control. However, Se-concentrations in liver and kidney of all Se supplemented pigs were more similar than those in muscle samples.

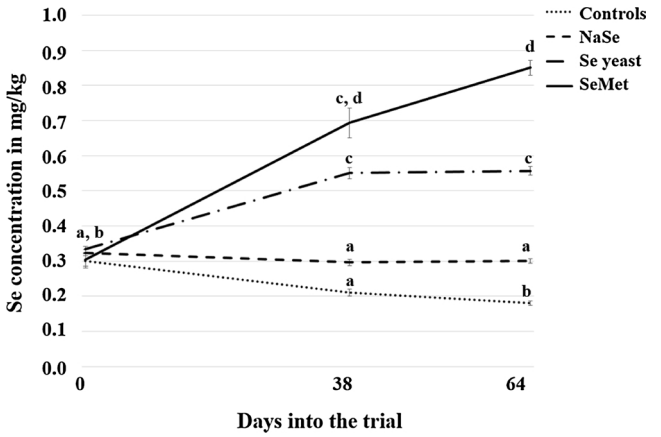

Fig. 1. Effect of time and diet on Se-concentration in *M. longissimus dorsi* (LD, mg/kg DM, Mean ± SEM).
Se-concentrations in LD during the trial period of 64 days (mean ± SEM). Different letters denote significant differences in Se concentration in LD between groups (control: 0.05 mg Se/kg diet; NaSe: sodium selenite 0.33 mg Se/kg diet; Se yeast: selenium yeast 0.32 mg Se/kg diet; SeMet: L-selenomethionine 0.32 mg Se/kg diet) and time point ($P < 0.05$ for the linear model (R lm)).

3.5. Histopathology

The two muscles most commonly showing degenerative changes were *M. semitendinosus* and LD. In all groups, these two muscles showed mild to moderate degenerative changes in most individuals. In the myocardium (heart muscle), scattered myocytes with vacuolization of the cytoplasm were observed in several pigs without significant differences between groups. Within all four groups, multifocal hepatic hemorrhages were more often observed in LPS-treated animals than in slaughtered animals.

Unfortunately, the low number of animals per group in this study potentially increased the influence of inter-individual variance. This might have reduced the possibilities to find significant differences in clinical observations, haematology, biochemistry, and histopathology as well as for the expression of some seleno- and non-selenogenes and presented mineral concentrations.

4. Discussion

Diet significantly influenced gene expression of some seleno- and non-selenogenes in both LD and whole blood obtained prior to and after application of LPS. Furthermore, we observed effects of diet and LPS on plasma-Mo and Mg in addition to the influence on plasma- and tissue Se concentrations.

4.1. Gene expression in M. longissimus dorsi

The expression of *SelenoW* and *SelenoH* in LD samples was found to depend on dietary Se, which is in line with other studies [31–35]. The marked reduction of the expression of *SelenoW* in the control pigs might have been a result of biological readjustment to stabilize the Se levels in brain and endocrine organs at the expense of tissues like skeletal muscle in cases of limited Se supply [36]. Resistance to oxidative stress conferred by SelW depended on GSH [37]. SelH was shown to regulate redox homeostasis and to suppress DNA damage [38]. In addition, SelH regulated expression levels of genes involved in *de novo* GSH synthesis [38]. Gene expression of *SelenoW* and *SelenoH* might be coupled via the GSH metabolism. Other authors found a positive correlation between *SelenoW* mRNA levels and intracellular GSH [39–41], which could also explain the above mentioned downregulation of *SelenoW* in the control group. The stronger upregulation of *SelenoH* in NaSe fed pigs compared with those fed Se yeast at day 64 might relate to the reactive oxygen species (ROS) generation during the NaSe metabolism [42–47]. The reason for the concurrent different expression of *SelenoH* in Se yeast and SeMet fed pigs remained unknown to the authors.

In LD biopsies from day 38, both *Gpx3* and *Sod1* were higher expressed in control compared with pigs fed Se-supplemented diets. This suggests increased O_2^- dismutation (Sod1) followed by upregulated decomposition of a possible product, hydrogen peroxide (H_2O_2), catalyzed by Gpx3. It is possible that higher levels of ROS upregulated both *Cox2* and *Casp3* expression in pigs fed NaSe and in control pigs, compared with those fed SeMet supplemented feed. Low Se status has been shown to aggravate the Casp3-dependent apoptotic response, probably due to impaired capacity of Gpx1 to degrade H_2O_2 [48]. On the other hand, NaSe induced ROS production in combination with growth-dependent ROS production [6,7], might have exhausted this capacity of Gpx1. Higher *Gpx3*-expression levels in LD from SeMet fed pigs at day 64, compared with control, potentially led to stronger cellular protection against oxidative stress as described by Stiegler et al. [49]. In contrast to the biopsies, the last LD samples were isolated after commercial slaughter. Ex-sanguination increases the level of oxidative stress and could explain the observed upregulation of the genes encoding the antioxidative selenoproteins Gpx3 and SelH in the Se-supplemented groups. The missing upregulation of *Gpx3* and *SelenoH* in the control pigs might result from the hierarchy of selenoproteins, since the two corresponding proteins are not prioritized in case of low Se supply [50,51]. The reason for the different expression of *Gpx1* (day 38) in the LD of pigs fed Se yeast versus those fed SeMet remained unknown to the authors but might relate to the antioxidative effects of other Se compounds found in Se yeast [41]. Looking at the gene expression in samples isolated prior to the feeding trial, the same patterns for *SelenoH* and *GPx1* could be seen. Thus, something extraneous to our trial might have had influence.

The lower expression of *Ifn*γ in the Se-supplemented pigs compared with control might also relate to ROS, which have been shown to enhance the *Ifn*γ expression [52]. Dependent on time and diet, the expression of *Cox2* in LD samples increased throughout the study period, but more in control pigs and those fed the NaSe supplemented diet. Low Se status and elevated levels of ROS have been shown to upregulate the expression of *Cox2* [53–55]. In summary, our observations might mirror a higher antioxidant capacity in skeletal muscle from pigs supplemented with organic Se.

The expression of several selenogenes in the control pigs was down-

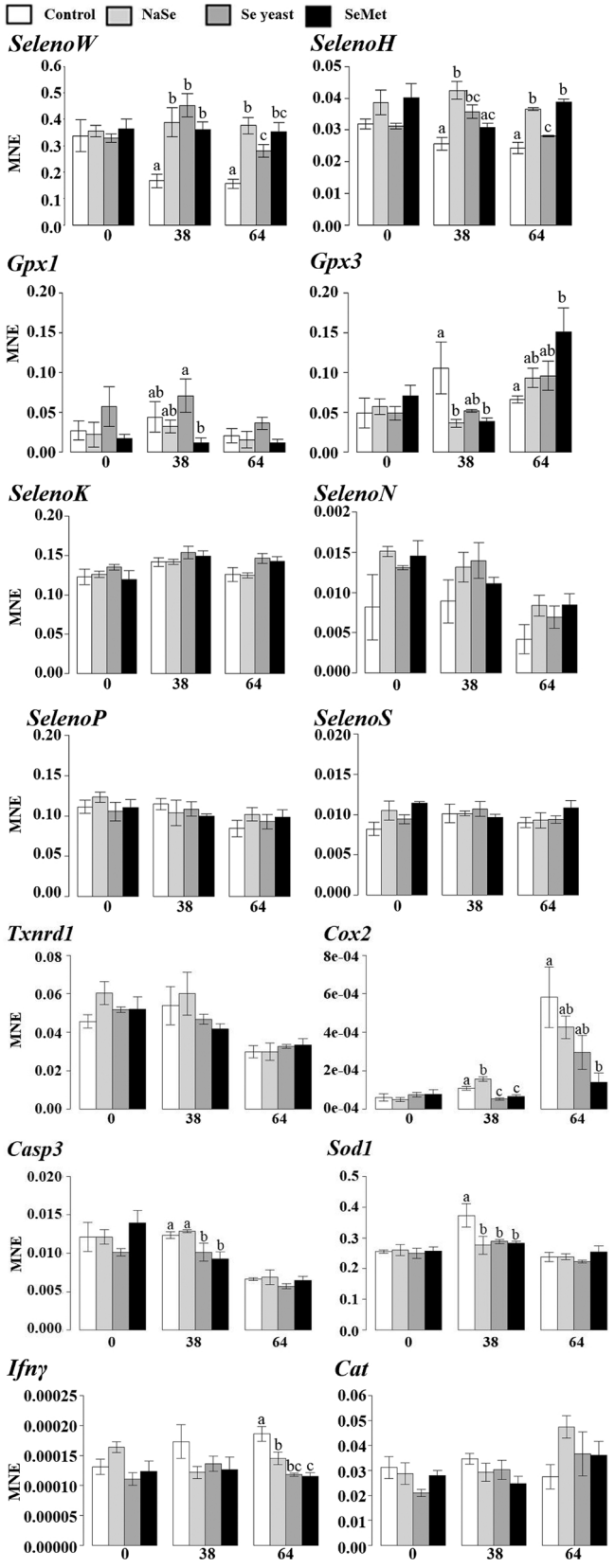

Fig. 2. Gene expression in *M. longissimus dorsi* of selected genes included in the study (MNE, Mean ± SEM).
Data are mean normalized levels of expression (MNE) of n = 3 animals per diet group and sampling time point in *M. longissimus dorsi*. Mean levels are shown in barplots with standard error of the mean (SEM). Different letters denote significant differences in expression levels between groups (control: 0.05 mg Se/kg diet; NaSe: sodium selenite 0.33 mg Se/kg diet; Se yeast: selenium yeast 0.32 mg Se/kg diet; SeMet: L-selenomethionine 0.32 mg Se/kg diet) at each time point. Different letters indicate $P < 0.05$ for the linear model (R lm).

regulated. The parallel-observed higher expression of some genes encoding important Se independent antioxidants might indicate compensatory up-regulation in the control pigs.

4.2. Gene expression in whole blood before and after LPS injection

Prior to LPS injection, *SelenoN*, *SelenoS* and *Txnrd1* as well as *Cat* were higher expressed in the blood of pigs fed NaSe. NaSe increases the Txnrd1 activity [56] as this selenoenzyme is implicated in the reductive assimilation of NaSe by generating selenide for selenoprotein synthesis [57]. Metabolizing the rather strong oxidizing NaSe can trigger endoplasmic reticulum (ER) stress due to generation of ROS [42–47]. Both SelS, an ER stress response protein [58], and SelN1 protect the ER from ROS [59,60]. *Cat* was higher expressed in whole blood of pigs fed NaSe. The antioxidant enzyme Cat, found in all aerobic organisms, catalyzes the decomposition of the ROS H_2O_2 to oxygen and water [61]. As mentioned above, NaSe metabolism can increase the ROS production, which in turn may have increased the requirement for the enzyme Cat to prevent oxidative damage.

The LPS dose of 2 µg/kg BW has previously been shown to induce the aforementioned clinical signs in pigs [62]. In accordance with others, our results clearly showed significant influence of LPS on gene expression [63]. LPS triggered oxidative stress may have caused the upregulation of *Gpx1* and *Gpx3* during the acute phase [64,65]. The increased *SelenoK* mRNA-levels 1 h after LPS challenge coincided with a drop in WBC-counts (data not shown) which can be explained by transendothelial migration of leucocytes. SelK is important for Ca^{2+}-dependent functions in leucocytes, which include transendothelial migration [66–68] explaining its early upregulation. The parallel downregulation of *SelenoN* could be linked to the functional relationship of SelN1 with the ER Ca^{2+} import SERCA2 pump [69]. SelN1 enhances SERCA2 activity [69], which would counteract the aforementioned function of SelK. A distinct SelN1 to SelK ratio might be the prerequisite for transendothelial migration of leucocytes. The enhanced downregulation of *SelenoN* in NaSe fed pigs might be related to its function in regulation of the Ca^{2+} homeostasis in the cell [69] and possibly disturbs the SelN1 to SelK ratio. SelH protects intracellular GSH and antioxidant levels and increases the expression of key enzymes in GSH biosynthesis [38]. In mice, LPS mediated the inhibition of GSH synthesis [70] possibly explaining the observed downregulation of *SelenoH*-expression in blood samples from the pigs 1 h after LPS injection. Fast growing animals experience a higher level of oxidative stress as ROS-production is proportional to the respiratory activity of the cell [71,72] which may be added to NaSe- and LPS-related ROS in these pigs. The stronger downregulation of *Gpx1* in NaSe-fed pigs 24 h after LPS challenge compared with those fed organic Se could be due to re-direction of Se to more important selenoproteins [50,51,73].

In summary, these observations might reflect increased oxidative stress, in case of NaSe supply.

Table 3
Selenium concentration (mg/kg DM) in tissues collected post mortem after 64 days of trial duration.

Tissue	n	Control diet (0.05 mg Se/kg)	n	NaSe (0.33 mg Se/kg)	n	Se yeast (0.32 mg Se/kg)	n	SeMet (0.32 mg Se/kg)
Kidney	2	5.70 (0.28)	3	7.67 (0.84)	3	7.80 (0.95)	2	8.80 (1.56)
Liver	2	0.71 (0.01)^{a}	3	1.57 (0.06)^{b}	3	1.83 (0.12)^{c}	2	1.75 (0.07)^{c}
RV	3	0.34 (0.12)^{a}	3	0.78 (0.12)^{b}	3	1.05 (0.09)^{c}	3	1.20 (0.17)^{c}
LV	3	0.47 (0.01)^{a}	3	0.90 (0.05)^{b}	3	1.20 (0.00)^{c}	3	1.37 (0.06)^{d}
Septum	3	0.49 (0.04)^{a}	3	0.90 (0.01)^{b}	3	1.13 (0.06)^{c}	3	1.47 (0.06)^{d}
LD	3	0.18 (0.01)^{a}	3	0.30 (0.01)^{b}	3	0.56 (0.02)^{c}	3	0.85 (0.04)^{d}
MBB	3	0.21 (0.01)^{a}	3	0.40 (0.01)^{b}	3	0.63 (0.02)^{c}	3	0.96 (0.03)^{d}
MST	3	0.18 (0.03)^{a}	3	0.36 (0.02)^{b}	3	0.61 (0.02)^{c}	3	0.92 (0.01)^{d}
MQF	3	0.19 (0.01)^{a}	3	0.37 (0.01)^{b}	3	0.62 (0.03)^{c}	3	0.96 (0.01)^{d}
MPM	3	0.19 (0.01)^{a}	3	0.35 (0.02)^{b}	3	0.63 (0.02)^{c}	3	0.92 (0.03)^{d}
MSM	3	0.16 (0.02)^{a}	3	0.32 (0.01)^{b}	3	0.49 (0.04)^{c}	3	0.75 (0.10)^{d}
Diaphragm	2	0.21 (0.01)^{a}	3	0.46 (0.04)^{b}	3	0.62 (0.04)^{c}	3	0.95 (0.06)^{d}

M. longissimus dorsi (LD), *M. semitendinosus* (MST), *M. semimembranosus* (MSM), *M. biceps brachii* (MBB), *M. quadriceps femoris* (MQF), *M. psoas major* (MPM), diaphragm, myocardium (left and right ventricle [LV and RV], septum). Values are shown as Mean (SD). The regressions (R *lm*) were repeated such that all diets were used as control. Values in a row not connected by the same letter are significantly different ($P < 0.05$).

4.3. Se, Mo and Mg concentrations in plasma before and after LPS injection

Plasma Se concentrations increased significantly from the start of the study until day 46 (prior to LPS challenge) in pigs receiving diets supplemented with Se. A low plasma-Se-concentration, considered Se deficient [74], was shown for the control.

Although decreasing after LPS challenge in pigs fed Se supplemented diets, plasma-Se-concentrations did not reach levels considered Se deficient. In pigs, serum- or plasma-Se-concentrations between 0.005-0.06 mg/kg are classified as deficient [74]. In line with our study, other authors have shown a declining Se status during infections in humans, rodents and pigs [63,64,75–78], which might be due to a changed pattern of selenoprotein synthesis and endothelial binding of SelP1 [79–82]. The stable plasma-Se-concentrations observed during our LPS study in pigs receiving the control diet, however, did probably reflect reduced selenoprotein synthesis [83], since these animals already were low in Se when challenged with LPS.

The increase of plasma-Mo-concentrations shortly after LPS-application in the Se-supplemented groups might be related to the turnover of Se compounds and selenoproteins for liberation of Se via the transselenation pathway [73]. This support the above suggested lowered selenoprotein synthesis in control pigs. Mo-containing enzymes catalyze basic metabolic reactions in the sulfur (S) cycle [18]. As Se and S compounds have similar chemical/physical properties, they will largely follow the same metabolic routes [19].

The higher plasma-Mg-concentrations observed in pigs fed Se yeast and SeMet enriched diets versus pigs fed NaSe and control might relate to the antioxidant properties of Mg [21,22]. It counteracts the development of ROS-related diseases [84] and is essential for the synthesis of reduced GSH [85–88]. Sugimoto *et al.* showed that Mg decreases the cytokine production [89].

4.4. Se concentrations in tissues

Se concentrations in the porcine liver and kidney mirrored the Se-supplementation differently from all the muscles including the myocardium due to the relative high amount of SeCys in liver and kidney [90,91]. Incorporation of selenomethionine in non-selenoproteins led to higher muscle-Se-concentrations in the pigs fed diets supplemented with organic Se compared with both NaSe-fed and control pigs, being highest in SeMet pigs. In comparison to pure SeMet formulations Se yeast contains different Se compounds whereof selenomethionine accounted for 54–74% [92–94]. This might explain the significant different Se concentrations observed in the skeletal muscle samples from Se yeast compared with SeMet fed pigs.

In most of Scandinavia, some other parts of Europe and parts of China, local produced grain is low in Se [95]. Years after the replacement of imported Se-rich grain from the USA and Canada with more homegrown grains, the Norwegian population has showed a considerable decrease in serum-Se concentration [96]. Offering Se rich meat from animals fed organic Se sources, in particular selenomethionine, is one possibility to increase the Se status in Norwegian consumers.

4.5. Conclusions

SelenoW could be a good molecular marker of Se status as its expression was lower in skeletal muscle of the control animals fed a diet low in Se. NaSe at a dietary level of 0.3 mg Se/kg might have induced additional oxidative stress in the fast growing pig as indicated by the higher expression of several seleno- and non- selenogenes compared with control, SeMet and Se yeast. This, together with the higher plasma Mg concentrations in pigs fed organic Se, possibly reflect that organic Se positively influenced the pigs' immune response and antioxidant capacity. Future studies might focus on the impact of non-toxic levels of NaSe, Se yeast and SeMet on antioxidative active compounds like e.g. GSH or thiol groups. In addition, more knowledge is crucial to understand the relations between selenoproteins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jtemb.2018.03.003.

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