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Effects of a short-term supranutritional selenium supplementation on redox balance, physiology and insulin related metabolism in heat stressed pigs

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Summary

Heat stress (HS) disrupts redox balance and insulin-related metabolism. Supplementation with supranutritional amounts of selenium (Se) may enhance glutathione peroxidase (GPX) activity and reduce oxidative stress, but may trigger insulin resistance. Therefore, the aim of this experiment was to investigate the effects of a short-term high Se supplementation on physiology, oxidative stress and insulin-related metabolism in heat-stressed pigs. Twenty-four gilts were fed either a control (0.20 ppm Se) or a high Se (1.0 ppm Se yeast, HiSe) diet for 2 weeks. Pigs were then housed in thermoneutral (20°C) or HS (35°C) conditions for 8 days. Blood samples were collected to study blood Se and oxidative stress markers. An oral glucose tolerance test (OGTT) was conducted on day 8. The HS conditions increased rectal temperature and respiration rate (both $p < 0.001$). The HiSe diet increased blood Se by 12% ($p < 0.05$) and ameliorated the increase in rectal temperature ($p < 0.05$). Heat stress increased oxidative stress as evidenced by a 48% increase in plasma advanced oxidized protein products (AOPP) ($p < 0.05$), which may be associated with the reductions in plasma biological antioxidant potential (BAP) and erythrocyte GPX activity (both $p < 0.05$). The HiSe diet did not alleviate the reduction of plasma BAP or increase of AOPP observed during HS, although it tended to increase erythrocyte GPX activity by 13% ($p = 0.068$). Without affecting insulin, HS attenuated lipid mobilization, as evidenced by a lower fasting NEFA concentration ($p < 0.05$), which was not mitigated by the HiSe diet. The HiSe diet increased insulin AUC suggesting it potentiated insulin resistance, although this only occurred under TN conditions ($p = 0.066$). In summary, HS induced oxidative stress and attenuated lipid mobilization in pigs. The short-term supranutritional Se supplementation alleviated hyperthermia, but did not protect against oxidative stress in heat-stressed pigs.

Introduction

Heat stress compromises the efficiency of pig production. Physiological responses to HS including reduced gut blood flow and increased core temperature both contribute towards disrupting the redox status and triggering oxidative stress (Cottrell et al., 2015). Oxidative stress may be associated with HS-induced metabolic disorders. Heat stress has a direct effect on increasing fat deposition in pigs (Christon 1988; Kouba et al., 2001; Wu et al., 2016) which may be due to the inhibition of lipolysis or lipid mobilisation (Pearce et al., 2013a). Although the mechanism is unknown, the elevated blood insulin that have been observed in the heat-stressed ruminants may be a reason for the reduced lipid mobilisation (Baumgard and Rhoads 2012; 2013), as insulin is an anti-lipolytic hormone (Wray-Cahen et al., 2012). Since oxidative stress can cause insulin resistance (Houstis et al., 2006), it may be responsible for the elevated insulin in heat-stressed animals. Moreover, oxidative stress occurs in the tissues of heat-stressed pigs (Pearce et al., 2013b; Montilla et al., 2014; Liu et al., 2016). Therefore, oxidative stress may be involved in the pathophysiology of metabolic disorders in pigs during HS, thus an alleviation of oxidative stress may normalize insulin action and lipid mobilisation.

Selenium is incorporated into glutathione peroxidase (GPX) which is an antioxidant enzyme. The current nutrient requirements of swine (National Research Council 2012) recommends 0.2 ppm Se for growing pigs under thermoneutral conditions. However, as a nutritional strategy to reduce HS, it is unknown if a short-term supplementation with supranutritional amounts of Se before and during a heat event can alleviate physiology and oxidative stress in heat-stressed pigs, as it does in sheep (Chauhan et al., 2014; Chauhan et al., 2015). However, pigs are more insulin sensitive than ruminants (Dunshea and D'Souza, 2003; Pethick et al., 2005) and a cautionary note is that supranutritional Se supplementation (0.5-3.0 ppm) can adversely impact on insulin homeostasis in pigs (Liu et al., 2012; Pinto et al., 2012) possibly by inhibiting the expresion and function of the proteins participating in the insulin signalling. Therefore, the aims of the study were to investigate the effects of dietary Se supplementation as a means of mitigating the physiological responses and oxidative stress, and to explore its role in insulin-related metabolism in pigs exposed to HS. Our primary hypothesis was that supplementation with 1.0 ppm Se for 2 weeks before and during HS can mitigate physiological responses and oxidative stress in the

heat-stressed pigs with a secondary hypothesis that an alleviation of oxidative stress may normalize insulin action and lipid mobilization.

Material and Methods

Animals and experimental design

All procedures were approved by an animal ethics committee of The University of Melbourne (protocol number: 1212687) and adhered to the Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition, 2013).

The experiment was a 2 × 2 factorial design with two diets and two environmental conditions. Female Large White × Landrace pigs (n = 24, 25 ± 2 kg, mean ± SD) were acclimatised in individual pens for 7 days then randomly allocated to one of the two diets. The control diet contained 0.2 ppm Se and was formulated to meet NRC (2012) specifications **(Table 1)**. The high Se (HiSe) diet was identical to the control diet with the exception of an addition of 0.04% Se-enriched yeast (Alltech, Lexington, KY, USA) to elevate a final Se dietary concentration to 1.0 ppm. After 14 days, six pigs from each dietary treatment were allocated to either TN conditions (20°C, 35-45% relative humidity) or HS conditions (35°C, 09:00 – 17:00; 28°C, 17:00 – 09:00, 35 - 45% relative humidity) for 8 days. To eliminate the confounding effects of dissimilar feed intake between TN and HS, all pigs received a restricted feed allowance throughout the experiment including acclimatisation period that was approximately 75% of voluntary feed intake under TN conditions. The amount of feed offered was predicted as the feed intake under this magnitude of heat load, based on the break-line linear model developed by Huynh *et al.* (2005). The pair-fed protocol described previously (Pearce et al., 2013a) was not followed in the present experiment, because the pair-fed protocol could result in a greater starting body weight of our selected thermoneutral pigs when they lagged one day behind the heat-stressed pigs. All the pigs were fed twice daily at 09:00 and 17:00, and any individual feed refusals were recorded at 09:00 daily. Water was supplied via nipple drinker *ad libitum*.

Physiological monitoring

All pigs were monitored for physiological signs of HS including rectal temperature and respiration rate at 09:00, 13:00, and 16:00 during the 8-day thermal exposure. Respiration rate

(breaths/min) was counted visually within 20 s and rectal temperatures measured with a digital thermometer (Fast-Read, Livingstone Pty Ltd., Rosebery, NSW, Australia). As a precaution, pigs were removed from the HS room if the rectal temperature exceeded 41 °C until their rectal temperature returned below 40 °C. Two pigs were removed for 1 h due to hyperthermia on the first day of thermal exposure but were returned to the room without incident.

Blood sampling and blood gas measurement

Blood samples were collected at 09:00 and 15:00 on the day 7 during the thermal exposure period via venipuncture from the jugular vein and collected in three different 10 mL vacutainers (no-preservatives, sodium heparin and EDTA coated, BD vacutainer, BD, North Ryde, NSW Australia). Fresh blood from non-coated vacutainers were immediately loaded into an automatic blood gas analyzer (EPOC, Alere, Waltham, MA, USA) for measurement of blood gas and biochemical parameters such as partial pressure of CO_2 (pCO_2) and O_2 (pO_2), pH, bicarbonate, hematocrit. The whole blood (1 mL) was taken from each heparinized vacutainer for preparation of erythrocyte lysates according to the method described by Bernabucci et al. (2002). Blood collected in the sodium heparin and EDTA coated vacutainers were centrifuged at 2000 × g at 4°C for 10 min. Approximately 0.5 mL of the "buffy coat" containing white blood cells (WBC) were aspirated carefully from EDTA coated vacutainers and mixed with 1 mL RNA stabilizing solution (RNAlater, Life Technologies Pty Ltd., Mulgrave, VIC, Australia) stored at 4°C for 12 h before freezing at -20°C until RNA extraction. Plasma samples from heparinized vacutainers were stored at -20°C pending analysis.

Selenium measurement in diet and blood

The blood samples collected on the day 7 and three representative samples from each diet were used for determine the Se concentrations. Blood (0.2 mL) or fresh feed samples (0.2 g) were added in a 100 mL Kjeldhal digestion tube for digesting organic matters. Nitric acid (70%, 2 mL) was mixed with the blood or feed sample in each digestion tube and kept at room temperature overnight. The digestion tubes were heated up to 60 °C and maintained for 1 h, then heating temperature was increased to 110 °C and maintained for 5 h. The digestion tubes were cooled down to room temperature before adding 4 mL hydrogen peroxide and being heated up to 80 °C

for 1 h. Afterwards the digestion tubes were cooled to room temperature again and the digested sample solution was transferred the into a volumetric flask. Finally, the total volume was fixed up to 10 mL by adding 10% hydrochloric acid. Then the digested samples were filtered through 541 Waterman paper (Sigma-Aldrich Pty. Ltd, Sydney, NSW, Australia). Selenium concentration in the digested blood or feed solution was measured by an inductively coupled plasma optical emission spectrometer (ICPOES, 4500DV, Perkin Elmer, Waltham, MA) equipped with a charge coupled solid state detector and an auto sampler. Three replicate readings were made for each sample. The analytical wavelength was chosen 196.032 nm for detecting Se. The digested sample solution was mixed with 0.4% $NaBH_4$ in 0.05 M NaOH at a flow rate of 1.5 mL/min in a hydride generator prior to introduction into nebulizer. There were 2 s flush time between samples at rate 2.5 mL/min and 30 s wash time at rate 1.5 mL/min. A calibration curve was established by using 5, 10, 15 and 20 ppb Se standards.

Oxidative stress biomarkers

An aliquot of heparinised plasma was used for evaluating a panel of oxidative stress biomarkers (FREE Carpe Diem, Diacron International, Grosseto, Italy) as detailed by (Celi et al., 2010). Reactive oxygen metabolites (ROM), biological antioxidant potential (BAP), and thiol groups (SHp) were quantified. The ROM assay measures metabolites of reactive oxygen species (ROS) in the plasma and expressed as an equivalent as H_2O_2 (mg dL^{-1}), and ROM assay has been successfully used in pigs for quantifying oxidative stress (Brambilla et al., 2002). The BAP assay measures the ability of plasma to reduce ferric to the ferrous form and quantifies the biologically active antioxidants in plasma including bilirubin, uric acid, vitamins C and E and proteins. The results of the BAP test were expressed as µmol of iron reduced by 1 L plasma. The SHp assay measures thiolic antioxidants such as lipoic acids and glutathione. Advanced oxidized protein products (AOPP) were quantified according to the method of Witko-Sarsat et al., (1998), and the values were expressed as µM of chloramine T equivalents. Glutathione peroxidase activity was assayed in erythrocyte lysates using a commercial kit (Cayman, Ann Arbor, MI, USA). The GPX activities were expressed in units per mL of red blood cells (RBC) (Bernabucci et al., 2002) where a unit is defined as the amount of GPX oxidize 1 nmol NADPH to $NADP^+$ per min in 25°C.

Gene expression in white blood cells

White blood cell samples which were collected on day 7 were separated from RNAlater after centrifugation at 1,000 *g* at 20 °C for 1 min. Total RNA was then extracted using acid-phenol and chloroform method according to the manufactures manual (RiboPure, Life Technologies Australia Pty Ltd., Mulgrave, VIC, Australia). The concentration and RNA quality index (an overall score by evaluating 28s/18s RNA ratio and 5s RNA concentrations) of extracted RNA were determined using an Experion RNA analysis system (Bio-Rad Laboratories, Inc. Hercules, CA, USA). Then 0.8 µg RNA templates from each sample was reversely transcribed into cDNA in triplicates according to the protocol of Superscript III First-Strand Synthesis kits (Life Technologies Australia Pty Ltd. Mulgrave, VIC, Australia). The synthesized cDNA samples were stored at -20°C. Sequences of primer sets for swine 18S ribosome RNA (*r18s*), heat shock protein 70 (*HSP70*), hypoxia induced factor-1α (*HIF-1 α*) were either designed in NCBI nucleotide database or referenced from others (**Supporting Table 1**). Reactions (25 µL per well) were prepared based on the manufacturer's instruction using SYBR GREEN qPCR Supermix Universal kit (Life Technologies Pty Ltd. Mulgrave, VIC, Australia), 100 nM of each forward and reverse primer were added in each reaction. Each sample was run in triplicates and SYBR green fluorescence was quantified in iQ5 Real Time PCR Detection System (Bio-Rad Laboratories Inc. Hercules, CA, USA). Each PCR plate included a standard curve (five 10-fold dilutions of a pooled cDNA), non-template negative controls, and blank controls to determine amplification efficiency of PCR. For the PCR protocols, after denaturing 95°C for 10 min, 45 cycles consisting of three stages were applied. For *r18s* and *HSP70*, the protocol of (95°C for 30 s, 60 °C for 30 s, 72°C for 30 s) was used; for *HIF-1α,* a protocol of (95°C for 30 s, 55°C for 30 s, 72°C for 30 s) was followed. A melting curve was included after the 45 amplification cycles to verify the amplification of a single PCR product. Gene expression was calculated and expressed as fold change (Livak and Schmittgen 2001).

Oral glucose tolerance test and metabolite analysis

All pigs were fasted for 18 h commencing at 18:00 on day 7 during the thermal exposure period before receiving a simplified oral glucose tolerance test (OGTT) at 12:00 on day 8 (i.e. after 3 h exposure to 35°C). Each pig was given 50 g of pelleted feed mixed with anhydrous D-dextrose

(BDH, VWR, Tingalpa, QLD, Australia; 2 g/kg of fasting BW). Blood samples (5 mL) were sampled and collected as described above (EDTA anticoagulant) at 0, 30, 60 and 120 min, and plasma were separated as previously described then frozen at -20°C until analysis. Plasma glucose was assayed by a commercial kit (Infinity, Thermo Fisher Scientific, Scoresby, VIC, Australia) with an inter- and intra-variation of 3.7% and 6.6 %, respectively. Plasma NEFA concentrations were quantified using the NEFA C kit (Wako Pure Chemical Industries Ltd., Kawagoe, Japan) modified with a 5-fold dilution of reagents A and B in phosphate-buffered saline. The intra- and inter-variation of NEFA measurement were 4.3% and 7.0 %, respectively. Plasma insulin concentrations were quantified by a double anti-body radioimmunoassay (Tindal et al., 1978) with all samples processed in a single assay with an intra-variance of 4.0 %. Plasma glucose, NEFA and insulin area under the curve (AUC) were calculated using the trapezoidal rule with fasting concentrations used as baseline for subtraction.

Statistical analysis

Data were analysed using linear mixed models in GenStat 15th edition (VSN international, Hemel Hempstead, UK). For physiological parameters, temperature (TN and HS), diet (control and HiSe), day (seven days) and time (09:00, 13:00, and 16:00) were set as fixed effects. Results for physiological responses were reported as line graphs using the mean and SEM of the temperature × diet × time interaction. For blood Se, blood gas, oxidative stress markers, and gene expression parameters, temperature, diet, time (09:00 and 15:00) were set as fixed effects, and the results were presented in tables using mean and SEM of temperature × diet interactions. Glucose, insulin and NEFA data during the OGTT were analysed with temperature, diet, time set as fixed effects. To determine the effects of temperature and diet on basal glucose, basal insulin, basal NEFA and areas under the curve in response to OGTT, data were analysed with temperature and diet set as fixed effects. Pigs were used as random effect in all statistical models. Means were considered to differ significantly when $p \leq 0.05$, and a trend was identified when $p \leq 0.10$. Duncan multiple range test was used as a *post-hoc* multiple comparison if $p \leq 0.10$ for the interaction between dietary treatment and temperature.

Results

Physiology and feed intake

Restricting the feed allowance ensured that there was no effect of temperature ($p = 0.96$) or diet ($p = 0.71$) on ADFI which was approximately 1.45 kg across all treatments (data was not shown).

Heat stress increased rectal temperature (38.2°C vs 39.5°C for TN and HS, $p < 0.001$) and there was an interaction between temperature and time ($p < 0.001$) such that rectal temperature rapidly increased from 09:00 to 13:00 then reached a plateau until 16:00 in the heat-stressed pigs, whereas under TN conditions the pigs had relatively stable rectal temperature across the day. The HiSe diet reduced rectal temperature compared with the control diet (38.94 °C and 38.74 °C for the control and HiSe, $p < 0.001$). In particular, pigs offered HiSe diet had lower rectal temperatures than control diet under HS conditions at 16:00 (40.05°C and 39.66 °C for the control and HiSe, $p < 0.05$) **(Fig. 1 A)**.

Heat stress markedly increased respiration rate (31 vs 119 breaths/min for TN vs HS, $p < 0.001$). Respiration rate increased between 09:00 and 13:00 before plateauing until 16:00 under HS conditions (50, 148 and 159 breaths/min for 09:00, 13:00 and 16:00), whereas respiration rate was constant over the day under TN conditions as indicated by an interaction between time and temperature ($p < 0.001$). The HiSe diet tended to have a main effect to reduce respiration rate (75 and 70 breaths/min for control and HiSe $p = 0.087$), whereas respiration rates did not differ between dietary treatments at any time points under any environmental conditions **(Fig. 1 B).**

Blood gas parameters

Blood pCO_2, bicarbonate decreased whereas pH increased during HS (all $p < 0.001$). Blood pO_2 was not affected by HS. Pigs fed the HiSe diet had lower blood bicarbonate than the pigs consuming control diets under TN conditions, whereas the pigs fed on the two diets had similar bicarbonate concentration during HS conditions, as evidenced by an interaction between temperature and diet ($p = 0.006$). The other blood gas parameters were not significantly affected by HiSe diet. **(Table 2)**.

Selenium concentration and erythrocyte GPX activity

The measured Se concentration was 0.24 ± 0.064 ppm and 0.88 ± 0.130 ppm (mean ± SD) in control diet and HiSe diet, respectively. The HiSe diet increased blood Se from 151 to 174 ng/mL ($p = 0.037$) whereas HS did not affect blood Se concentrations (**Fig. 2 A**). Heat stress reduced GPX activity from 17.7 to 15.1 unit/mL RBC ($p = 0.019$) whereas the HiSe diet tended to increase GPX activity from 15.4 to 17.4 unit/mL RBC ($p = 0.068$) (**Fig. 2 B**).

Oxidative stress parameters and relevant gene expression

Heat stress more than doubled the expression of *HSP70* (2.16 vs 4.46, $p < 0.001$, **Table 3**) and *HIF-1α* (1.21 vs 2.78, $p = 0.032$) in white blood cells. The HiSe diet did not affect *HSP70* or *HIF-1α* expression. Heat stress decreased plasma ROM concentrations (49.3 vs 47.4 mg dL^{-1}, $p = 0.039$) whereas the HiSe did not affect ($p = 0.20$) plasma ROM concentrations. Plasma BAP was reduced by HS (2.92 vs 2.76 mmol L^{-1}, $p = 0.024$), whereas HiSe diet did not affect BAP. Heat stress increased plasma AOPP concentrations (18.5 vs 27.9 µmol L^{-1}, for TN vs HS, $p = 0.029$), whereas HiSe diet did not affect plasma AOPP concentrations. Neither HS nor HiSe diet supplementations affected plasma SHp concentrations.

Basal glucose, insulin, NEFA and oral glucose tolerance test (OGTT)

Figure 3 (A, B, C) shows the effect of HS and time on plasma glucose, insulin and NEFA responses to the OGTT. As expected, plasma glucose concentration changed significantly in relative to time after glucose ingestion ($p < 0.001$). Specifically, glucose increased from a baseline of 5.48 mM and peaked at 7.68 mM at 30 min after glucose ingestion before returning close to baseline at 60 min. While there was no overall effect of HS on plasma glucose during the OGTT ($p = 0.47$), there was an interaction ($p = 0.001$) between time and HS such that plasma glucose concentration of the pigs under HS conditions tended to be less than those under TN at 60 min, but became greater than those under TN at 120 min. Plasma glucose was not affected by HiSe diet. Plasma insulin concentrations changed along with time in relative to glucose ingestion ($p < 0.001$), such that insulin increased rapidly from 3.23 µU/mL at 0 min, peaked at 96.8 µU/mL at 30 min then sharply decreased to 18.2 µU/mL at 60 min before further gradually decreasing to 9.20 µU/mL at 120 min. While there was no overall effect of HS on plasma insulin during the OGTT ($p = 0.23$), there was a tendency of an interaction ($p = 0.086$) between time and

HS such that plasma insulin of HS pigs tended to be less than TN pigs at 30 and 60 min. The HiSe diet tended to increase plasma insulin concentration from 29.9 to 33.7 μU/mL ($p = 0.075$), and there was a tendency of interaction between HS and diet ($p = 0.076$) such that the increase of plasma insulin in the pigs fed HiSe was significant under TN conditions but not HS conditions. In response to the increase in plasma insulin, plasma NEFA concentrations decreased from 183 μM to 58 μM at 30 min, then remained lower until 120 min ($p < 0.001$). While there was no overall effect of HS on plasma NEFA during the OGTT ($p = 0.82$), there was an interaction ($p = 0.011$) between time and HS such that plasma NEFA concentrations of the pigs under HS conditions was lower than those under TN conditions at 0 min. Plasma NEFA concentrations was not affected by HiSe diet.

As **Table 4** illustrated, neither HS nor the HiSe diet affected the basal glucose concentration, glucose AUC, basal insulin concentration. The pigs fed the HiSe diet had a greater plasma insulin AUC than control pigs under TN conditions but not during HS conditions as indicated by an interaction between temperature and diet ($p = 0.066$). Heat stress reduced fasting plasma NEFA concentrations (0.23 vs 0.14 mM, $p=0.047$), but attenuated the reduction in plasma NEFA (-17.3 vs -8.3, for plasma NEFA AUC, $p = 0.037$) in response to OGTT.

Discussion

Heat stress comprehensively and negatively affects animals in various aspects including physiology, acid-base balance, oxidative stress and metabolism. The primary hypothesis tested was that a short term (14 days before HS and 7 days during HS) supranutritional amount of Se (1.0 ppm) supplementation can alleviate physiological response and reduce oxidative stress in the heat-stressed pigs. These results suggested that the current Se supplementation increased blood Se concentration by 12%, however, GPX activity was only marginally increased and therefore did not alleviate the HS-induced oxidative stress. However, the HiSe diet did partially alleviate some of the physiological responses to HS as evidenced by a lower rectal temperature, although it did not mitigate the increased respiration rate and respiratory alkalosis that occurred during HS. The secondary hypothesis tested in this study was that oxidative stress triggers hyperinsulinemia such that lipid mobilization is attenuated in the heat-stressed pigs, and that an alleviation of oxidative stress could mitigate these metabolic consequences. The present data showed that HS did attenuate lipid mobilization without increasing fasting insulin or insulin

AUC response to OGTT, therefore the mechanism for the compromised lipid mobilization in the heat-stressed pigs is possibly independent of circulating insulin concentrations.

The HiSe diet partially mitigated the increase in rectal temperature observed in response to HS. Similarly, rectal temperature was ameliorated in heat-stressed sheep that received 5 mg/day Se solution injection (Alhidary et al., 2012) as well as in the heat-stressed sheep supplemented with 1.0 ppm Se plus 200 IU/kg VE (Chauhan et al., 2014; Chauhan et al., 2015). Although the exact mechanism remains unknown, it may be related with the effect of Se in sparing the release stress hormones for catabolism such as cortisol, therefore the total heat production can be reduced (Chauhan et al., 2014)

Increased respiration rate during HS triggered respiratory alkalosis which was incompletely compensated as evidenced by a slight increase in blood pH from 7.41 to 7.45. The direct reason for the increased pH in the heat-stressed pigs is the reduction of carbonic acid formation due to loss of CO_2 via respiration. As a compensatory mechanism to prevent alkalemia, blood bicarbonate concentrations decreased via increased renal excretion. These data are in agreement with previous findings in heat-stressed growing pigs (Patience et al., 2005; Liu et al., 2016) and sheep (Chauhan et al., 2015). The HiSe diet did not mitigate respiratory alkalosis, because it did not ameliorate the increased respiration rate and prevent the loss of blood CO_2. The pO2 in venous blood remained unchanged, however, leukocyte HIF-1a mRNA abundance, a marker of hypoxia, increased in the heat-stressed pigs. This controversy implies that the increase of *HIF-1a* mRNA abundance may be triggered by another factor such oxidative stress, because oxidative stress can activate the promoter of *HIF-1a* (Khatri et al., 2004; Bonello et al., 2007).

Heat stress triggered oxidative stress in pigs, as evidenced by a 48% increase in plasma AOPP. However, the increased oxidative stress in the heat-stressed pigs was not via excessive ROS production, because the plasma ROS level as measured as ROM did not increase but tended to decrease in the heat-stressed pigs. The response of plasma ROM concentration to HS is surprising, because *HSP70 mRNA* abundance increased in leukocytes which represents the body was experiencing an overall hyperthermia. Cellular hyperthermia has been reported to increase ROS production (Zuo et al., 2000; Mujahid et al., 2005). Given that the electron transport chain is the main source of ROS production, the ostensible reduction of ROS maybe a consequence of reduced overall activity of electron transport chain in the heat-stressed pigs. Conversely, an increased ROM concentration in plasma (Bernabucci et al., 2002; Chauhan et al., 2015; Chauhan

et al., 2016b) and exhaled breath condensate (Chauhan et al., 2016a) was reported during HS in ruminants, which might be explained by the different intermediate metabolism between pigs and ruminants. Specifically, the basal ROM concentration in pigs found in the present study and the study of Brambilla et al., (2002) is over 40 mg dL^{-1} H_2O_2, which was two or three times higher than ruminants (around 8-16 mg dL^{-1} H_2O_2) as measured by the same method (Bernabucci et al., 2002; Chauhan et al., 2015; Chauhan et al., 2016b). This is in accordance with higher glucose turnover and insulin sensitivity and responsiveness in pigs compared with ruminants (Cote et al., 1982). In other words, HS may inhibit whole body generation of ROS in pigs but increase ROS production in ruminants.

The compromised overall blood antioxidant defence system during HS, as evidenced by the reductions in both BAP and GPX activity, may be the reason for the increased oxidative stress. Biological antioxidant potential represents the biological active antioxidants such as bilirubin, uric acid, vitamins C and E, and it was reduced by 5.3 % during HS. The reduction in BAP is consistent with previous observations in the heat-stressed ruminants (Bernabucci et al., 2002; Chauhan et al., 2014), suggesting a compromised antioxidant system. Similarly, erythrocyte GPX activity was reduced by 17% during HS, which is also responsible for the compromised antioxidant defence system and the increased oxidative stress, because GPX is an antioxidant enzyme which neutralizes free radicals into water and participates in the regeneration of VE and vitamin C (Rooke et al., 2004). Moreover, our previous study showed that HS reduced GPX activity by 18% in the small intestines of heat-stressed pigs (Liu et al., 2016). The reduction in the components of the antioxidant defence system are often the consequence of the increased oxidative stress (Celi and Gabai 2015), and the current study suggests HS may have direct negative impacts on the regeneration, stability, or biological function of antioxidants in blood. The compromised antioxidant defence system explained the increased plasma AOPP concentration during HS in pigs. An increase in AOPP, as a consequence of oxidative stress, has also been found in sheep exposed chronic HS conditions (Chauhan et al., 2014; Chauhan et al., 2015; Chauhan et al., 2016b). Proteins become dysfunctional after oxidation and therefore an increase of AOPP is associated with many metabolic disorders (Celi and Gabai 2015). The HiSe diet used in the present study only tended to enhanced erythrocyte GPX activity by 13%, and the magnitude of the enhancement in GPX activity did not mitigate the reduction of BAP or increase of AOPP in plasma observed during HS. The duration (2 weeks pre-heat supplementation and 1

week supplementation over the thermal exposure) of Se supplementation may not be sufficient for 1.0 ppm Se reaching the maximum deposition and functionality, because an experiment has shown an increase dietary Se from 0.3 ppm to 3.0 ppm did not increase plasma GPX activity until 16 weeks (Liu et al., 2012). However, supplementing with a lower concentration of Se (0.46 ppm) of Se-enriched probiotic maximized erythrocyte GPX activity in piglets in 2 weeks (Gan et al., 2013). Similarly, plasma Se was maximized after 14 days, whereas, muscular Se was maximized after 4 weeks of supplemental protein-bound Se (1.0 ppm vs 0.14 ppm) in milk fed pigs (Uglietta et al., 2008). Another possibly is that the overall antioxidant defence system may have been limited by a lack of a synergistic effect of Se and VE in the current study, because the reduced form of glutathione participated in the regeneration of VE from oxidized α-tocopherol (Rooke et al., 2004). A 14-day combined supplementation with Se (1.2 ppm) and VE (100 IU/kg) successfully increased BAP and mitigated HS-induced oxidative stress in heat-stressed sheep (Chauhan et al., 2014). Similarly, 1.0 ppm Se and 250 IU/kg VE alleviated the increase of blood AOPP in heat-stressed sheep (Chauhan et al., 2016b). Future studies are required to test the effects of a prolonged supplementation of Se and VE combination on oxidative stress biomarkers.

Previous studies have shown that an increase in blood oxidized protein markers such as AOPP (Koçak et al., 2007) and protein carbonyl (Ruskovska and Bernlohr 2013) correlated with impaired insulin sensitivity in diabetic patients, because increased free radicals can decrease phosphorylated Akt protein abundance which impairs insulin signalling and triggers insulin resistance (Houstis et al., 2006). However, the insulin resistance or hyperinsulinemia was not detected in the heat-stressed pigs by the OGTT used in the present study, even though the blood AOPP concentration was markedly increased by 48 % by HS. An explanation to this apparent contradiction is that HS not only increased oxidative stress but also up-regulated HSP70 expression which has been shown to facilitate insulin signalling (Gupte et al., 2009; Gupte et al., 2011). Therefore, the HS-induced HSP70 up-regulation may have compensated or even overwhelmed any detrimental effects of oxidative stress on insulin sensitivity. In this context, a recent study showed that a 7-day HS condition increased HSP70 (Pearce et al., 2013a) as well as insulin receptor substrate-1 protein abundance in skeletal muscle and improved insulin sensitivity (Sanz Fernandez et al., 2015b) in growing pigs. Noticeably, Se supplemented pigs had increased insulin AUC under TN conditions, which indicates that a supranutritional amount of Se potentiated insulin resistance. Likewise, compared with the pigs fed adequate level (0.2-0.3 ppm)

Se, a prolonged supranutritional Se supplementation can interfere insulin homeostasis. For example, supplementing 0.5 ppm Se for 8 weeks increased blood Se by 25% and numerically increased fasting insulin by 44% (Pinto et al., 2012). Supplementing 3.0 ppm Se for 16 weeks increased blood Se by 140%, decreased hepatic Akt protein abundance, and increased insulin concentration by 50% in pigs (Liu et al., 2012). Therefore, high dietary Se should be avoided under TN conditions or conditions where oxidative stress is not anticipated. However, the HiSe diet did not affect insulin response to OGTT during HS conditions, suggesting Se has been utilized as a selenoprotein in GPX to counteract the HS-increased oxidative stress.

While HS attenuated lipid mobilization, it was not via elevated insulin concentrations which is at odds with the original hypothesis. Although the exact mechanism is not clear, the reduced NEFA concentrations is a consistent observation in heat-stressed pigs (Pearce et al., 2013a; Sanz Fernandez et al., 2015a) and ruminants (O'Brien et al., 2010; Wheelock et al., 2010; Baumgard et al., 2011). Heat stress may attenuate lipid metabolism via different modes of actions. For example, prolonged HS may directly desensitize β-adrenergic receptor or activate adenosine A1 receptor, thereby affecting adipocyte sensitivity to lipolytic signals (Faylon et al., 2015). Or on the other hand, the reduced NEFA concentration might be due to the increased fatty acid esterification and enhanced glyceroneogenesis after adipocytes were heat-stressed (Qu et al., 2016). In the present study, the reduction in plasma NEFA in response to elevated insulin during the OGTT was less in the heat-stressed pigs, because the heat-stressed pigs had lower fasting NEFA concentrations thus had less potential to decrease. Due to the limitations of the OGTT used in the present experiment, such as the limited number of sampling points and relatively stressful snare restraining during the blood sampling, the effects of Se and HS on insulin sensitivity need to be confirmed by intravenous glucose tolerance test using catheterized pigs with more descriptive blood sampling points.

In conclusion, HS alters physiological parameters, increased oxidative stress biomarkers, and attenuated lipid mobilization independent of insulin resistance. The short-term supplementation of high Se (1.0 ppm) partially mitigated hyperthermia, but did not alleviate the HS-induced oxidative stress, respiratory alkalosis, or the attenuated lipid mobilization. Improved nutritional strategies such as a combined supplementation with supranutritional Se and VE needs to be tested in future studies.

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Table 1 Composition of control diet*

Ingredients	% of fed basis
Wheat	83.2
Canola meal	19.4
Soybean meal	2.3
Tallow	1.47
Limestone	0.7
DL-Methionine	0.07
Lysine	0.55
Threonine	0.18
Vitamin Blend†	0.03
Mineral Blend‡	0.06
Salt	0.23
Phytase	0.012
Calculated values	
Digestible energy, kcal/kg	3343
Crude protein, %	18.5
Total phosphorus, %	0.57
Calcium, %	0.70
Lysine, %	1.14
Selenium, ppm	0.20

*Control diet is formulated to NRC 2012 standard

† Supplied per kg of diet: vitamin A, 1486 IU; vitamin D_3, 297 IU; vitamin E 17.5 IU; vitamin K, 0.4 mg; vitamin B-1, 0.6mg; vitamin B-2, 2.0 mg; vitamin B-6 1.2 mg; vitamin B-12 2.0 mg; Niacin, 8.0 mg; pantothenic acid, 6 mg

‡ Supplied per kg of diet: copper, 18.6mg; cobalt, 0.5mg; manganese, 28.8 mg; zinc, 50.9 mg; iron, 65.2 mg; iodine, 0.50 mg; selenium; 0.20 mg; chromium, 186.3 mg

Table 2 Blood gas in the pigs fed control or high selenium diet under thermoneutral or heat stress conditions

Parameters	20 °C	35 °C	SEM	p-values

	Control	HiSe	Control	HiSe		T*	D†	T×D
pCO_2, mmHg	59.2^{c}	55.0^{bc}	45.5^{a}	49.4^{ab}	2.68	<0.001	0.94	0.067
pO_2, mmHg	37.4	46.0	41.4	42.0	7.43	0.98	0.39	0.45
Bicarbonate, mM	36.9^{c}	34.6^{b}	32.2^{a}	33.2^{ab}	0.83	<0.001	0.28	0.006
pH	7.40	7.42	7.46	7.44	0.03	<0.001	0.56	0.34

Within a row means without common superscript differ ($p < 0.05$). Values were pooled day and time factors

* Temperature

† Diet

Table 3 Plasma oxidative stress biomarkers and leukocyte mRNA expression in the pigs fed control or high selenium diet under thermoneutral or heat stress condition

Parameters	20 °C		35 °C		SEM	p-values		
	Control	HiSe	Control	HiSe		T*	D†	T×D
HSP70 fold change	1.79	2.53	4.78	4.41	1.104	0.006	0.95	0.38
HIF-1α fold change	1.24	1.59	2.54	3.00	1.465	0.042	0.92	0.80
ROM‡, mg dL^{-1}	54.4	49.8	48.3	46.9	3.70	0.09	0.26	0.55
BAP§, mmol L^{-1}	2.96	2.87	2.71	2.81	0.963	0.028	0.87	0.18
SHp¶, µmol L^{-1}	440	430	449	432	33.5	0.82	0.58	0.88
AOPP**, µmol L^{-1}	21.3	15.7	28.7	27.1	5.82	0.029	0.39	0.63

Values were pooled across two blood sampling time points (09:00 and 15:00)

* Temperature

† Diet

‡ Reactive oxygen metabolites, units expressed as mg dL^{-1} H_2O_2

§ Biological antioxidants potential

¶ Thiol group

** Advanced oxidize protein products

604 Table 4 Metabolites of OGTT in growing pigs fed on control or high selenium diet under
605 thermoneutral or heat stress conditions

Parameters	20 °C		35 °C		SEM	p-values		
	Control	HiSe	Control	HiSe		T*	D†	T×D
Glucose (fasting), mM	5.49	5.27	5.25	5.91	0.487	0.57	0.53	0.21
Glucose AUC‡	89	160	127	101	99.1	0.88	0.75	0.50
Insulin (fasting), μU mL^{-1}	2.85	3.25	2.25	4.06	1.574	0.93	0.34	0.54
Insulin AUC	3257^{a}	4409^{b}	3551^{ab}	3349^{ab}	493.2	0.29	0.31	0.066
NEFA(fasting), mM	0.19	0.27	0.16	0.12	0.064	0.047	0.59	0.18
NEFA AUC	-11.9^{bc}	-22.7^{ab}	-9.0^{c}	-7.7^{c}	5.68	0.037	0.25	0.15

606 Within a row means without common superscript differ ($p < 0.05$).
607 * Temperature
608 † Diet
609 ‡ Area under the curve
610

611 Fig. 1 (A) Rectal temperature and (B) respiration rate of pigs fed control or high selenium diet
612 exposed to thermoneutral or heat stress conditions

613 Pigs were fed either a control (Se 0.2 ppm) or high selenium (HiSe, 1.0 ppm selenium) diet and
614 exposed to thermoneutral (20 °C) or heat stress condition (35°C from 09:00 to 17:00; 28°C from
615 17:00 to 09:00). The error bars are the SEM for the interaction of temperature × diet × time. The
616 p-values for the effects diet, temperature, time, diet × temperature, diet × time, temperature ×
617 time, and temperature × diet × time were <0.001, <0.001, <0.001, 0.23, 0.030, <0.001 and 0.81
618 for rectal temperature; 0.087, <0.001, <0.001, 0.13, 0.85, <0.001, and 0.62 for respiration rate.

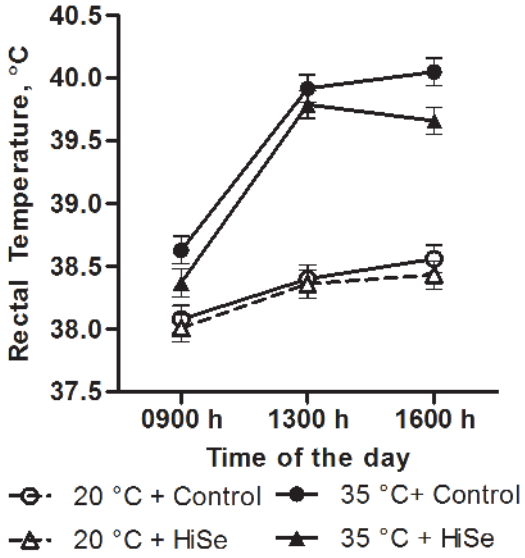
619 Fig. 2 (A) Blood selenium and (B) erythrocyte GPX activity of pigs fed control or high selenium
620 diet exposed to thermoneutral or heat stress condition

621 Pigs were fed either (NRC 2012 recommended, Se 0.2 ppm), high selenium (HiSe, 1.0 ppm
622 selenium) diet for 14 days then exposed to thermoneutral (20°C) or heat stress (35°C, 09:00 -
623 17:00; 28°C rest of the day) condition for 8 days. Blood samples were taken on the day 7 of

624 thermal exposure. The values were expressed as mean and SEM (n=6), and the values without
625 common superscript differ ($p < 0.05$).
626

627 Fig. 3 (A) Glucose, (B) insulin and (C) NEFA during oral glucose tolerance test

628 (A) glucose, (B) insulin, (C) NEFA of the pigs were fed the control diet (0.2 ppm selenium) or
629 supranutritional selenium (1.0 ppm) and exposed to thermoneutral (20°C) or heat stress
630 conditions (35°C from 09:00 to 17:00, 28°C in rest of the day) (n=6). The p - values for the
631 effects of temperature, diet, time, temperature × time, diet × time, temperature × diet, and
632 temperature × diet × time are 0.47, 0.40, <0.001, 0.001, 0.54, 0.70 and 0.59 for glucose; 0.23,
633 0.075, <0.001, 0.086, 0.29, 0.076 and 0.47 for insulin; and 0.15, 0.55, <0.001, 0.011, 0.55, 0.29,
634 and 0.10 for NEFA.



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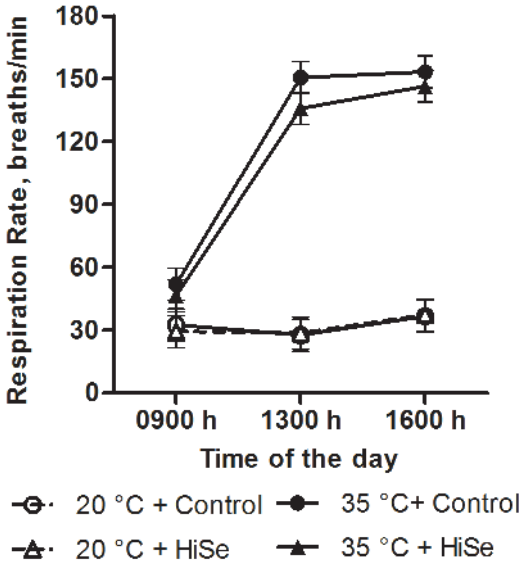

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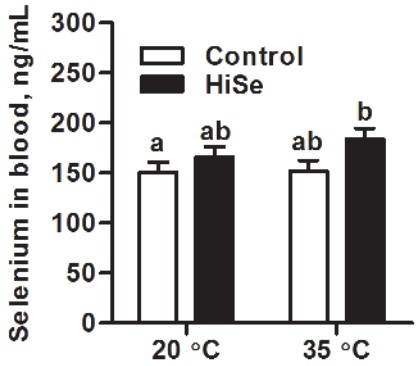

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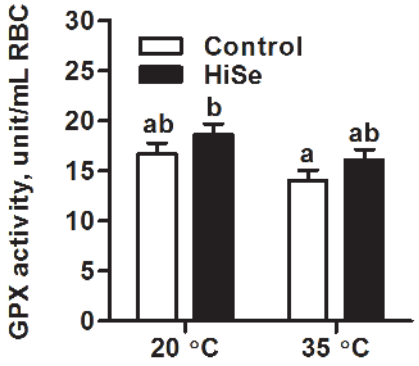

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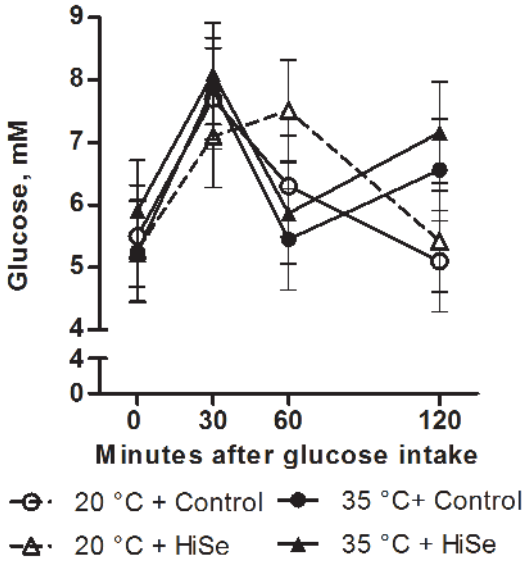

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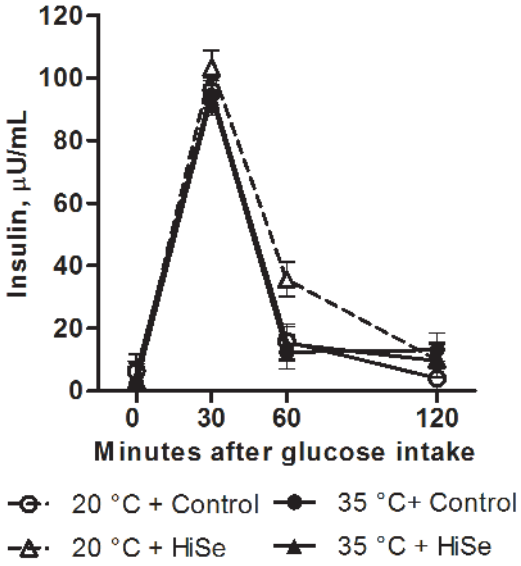

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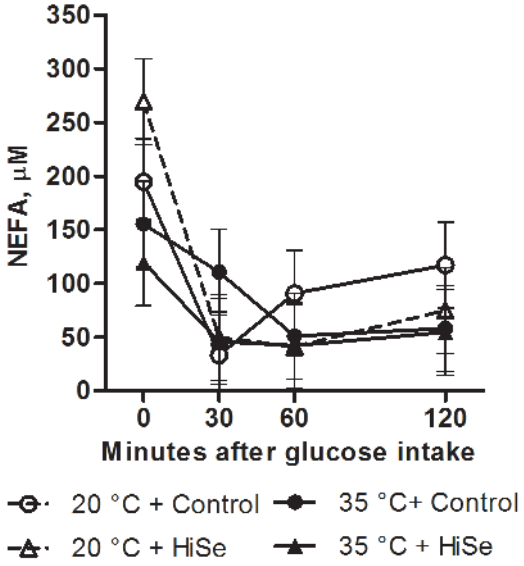

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