Selenium and Vitamin E together improve intestinal epithelial barrier function and alleviate oxidative stress in heat stressed pigs

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What is the central question of this study?

Oxidative stress may play a role in compromising intestinal epithelial barrier integrity in pigs subjected to heat stress, but it is unknown if an increase of dietary antioxidants (selenium and Vitamin E) could alleviate gut leakiness in heat stressed pigs.

What is the main finding and its importance?

Levels of dietary selenium (1.0 ppm) and Vitamin E (200 IU kg⁻¹), greater than usually recommended for pigs, reduced intestinal leakiness caused by heat stress. This finding suggests that oxidative stress plays a role in compromising intestinal epithelial barrier integrity in heat stressed pigs and also provides a nutritional strategy for mitigating these effects.

ABSTRACT

Heat stress compromises intestinal epithelial barrier integrity of mammals through mechanisms that may include oxidative stress. Our objective was to test whether dietary supplementation with antioxidants, selenium (Se) and Vitamin E (VE), protects intestinal epithelial barrier integrity in heat stressed pigs. Female growing pigs (n=48) were randomly assigned to four diets containing from 0.2 ppm Se and 17 IU kg⁻¹ VE (control, NRC recommended), to 1.0 ppm Se and 200 IU kg⁻¹ VE for 14 days. Six pigs from each dietary treatment were then exposed to either thermoneutral 20°C or heat stress (35 °C, 09.00-17.00/ 28°C, overnight) conditions for two days. Trans-epithelial electrical resistance (TER) and FITC-dextran (4 kDa; FD4) permeability were measured in isolated jejunum and ileum using Ussing chambers. Rectal temperature, respiration rate, and intestinal *HSP70* mRNA abundance increased (all $P < 0.001$), and respiratory alkalosis occurred, suggesting that pigs were heat stressed. Heat stress also increased FD4 permeability and decreased TER (both $P < 0.01$). These changes were associated with changes indicative of oxidative stress, a decreased glutathione peroxidase (GPX) activity and an increased glutathione disulfide (GSSG): glutathione (GSH) ratio (both $P < 0.05$). With increasing dosage of Se and VE, *GPX-2* mRNA ($P = 0.003$) and GPX activity ($P = 0.049$) linearly

increased, and the GSSG:GSH ratio linearly decreased ($P = 0.037$) and the impacts of heat stress on intestinal barrier function were reduced ($P < 0.05$ for both TER and FD4 permeability). In conclusion, an increase of dietary Se and VE mitigated heat stress impacts on intestinal barrier integrity, associated with a reduction in oxidative stress in pigs.

Abbreviations

FD4, fluorescein isothiocyanate–dextran 4k Da; GPX-2, glutathione peroxidase-2; GSH, glutathione; GSSG, glutathione disulphide; HIF-1 α , hypoxia induced factor-1 α ; HS; heat stress; HSP70, Heat shock protein 70; IL-8, interleukin-8; ROS, reactive oxygen species; RPL32, ribosome protein L32; Se, selenium; TER, trans-epithelial electrical resistance; TJ, tight junction; TN, thermoneutral; TNF- α , tumor necrosis factor- α ; VE, vitamin E;

1 INTRODUCTION

Heat stress compromises intestinal epithelial barrier integrity in pigs (Pearce *et al.*, 2012; Pearce *et al.*, 2013a; Pearce *et al.*, 2013b; Cui & Gu, 2015) and the mechanism may involve oxidative stress. Blood is redistributed away from splanchnic tissue to peripheral tissue to maximize the radiant heat dissipation when pigs are in a hot environment (Collin *et al.*, 2001), thus hyperthermia, hypoxia, and even inflammation may occur in the gastrointestinal tract (Lambert *et al.*, 2002; Lambert, 2009; Zuhl *et al.*, 2014), which can all trigger oxidative stress. Although dependent on the duration and intensity of heat stress, markers of intestinal oxidative stress have been reported to increase in the intestine of heat stressed rats (Hall *et al.*, 1999; Oliver *et al.*, 2012) and pigs (Pearce *et al.*, 2013b). Oxidative stress can disrupt tight junctions (TJ) (Rao, 2008) and decrease viability of epithelial cells (Vergauwen *et al.*, 2015). Therefore, oxidative stress may play a role in the etiology of the compromised intestinal barrier integrity in the heat stressed pig, and dietary alleviation of oxidative stress may protect against heat stressed induced intestinal barrier dysfunction.

Two common dietary antioxidants include selenium and vitamin E. Selenium (Se) forms selenoprotein such as glutathione peroxidase which is the enzyme that catalyzes the reduction of hydrogen peroxide to water. In this process, monomeric reduced glutathione (GSH) is oxidized to

form glutathione disulphide (GSSG). Vitamin E (VE) is a lipid soluble antioxidant capable of reducing free radicals particularly lipid hydroperoxides. Selenium and VE synergistically participate in neutralizing free radicals (Rooke *et al.*, 2004). The recent version of “Nutrient Requirements of Swine” recommends 0.2 ppm Se and 11 IU kg⁻¹ VE as adequate levels for growing pigs (body weight 25-50 kg) in a normal physiological state (National Research Council, 2012). However, it is unknown whether additional dietary Se and VE above recommended requirements are beneficial in counteracting the heat stress impacts on the intestine. Therefore the objective of this experiment was to evaluate effects of increasing the levels of dietary Se and VE as a nutritional strategy to mitigate the heat stress impacts on intestinal barrier integrity in pigs. We hypothesize that increased dietary Se and VE may protect intestinal barrier integrity from heat stress damage by alleviating oxidative stress.

METHODS

Ethical Approval

All procedures were approved by the Animal Ethics Committee of the Faculty of Veterinary and Agricultural Sciences, the University of Melbourne, Australia (protocol number: 1413093) and the Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition, 2013) was followed. Authors also acknowledge the ethical principle of Experimental Physiology, and the experiment was conducted in compliance with the animal ethic checklist as detailed by Grundy (2015)

Animal origin, feeding and experimental design

The experiments utilized 48 female growing pigs (Large White × Landrace, 20 ± 3 kg, mean ± SD) and followed a 4 × 2 factorial design (n=6 per treatment). Pigs were assigned into one of the four dietary treatments, with selenium yeast (Selplex[®], Alltech, KY, USA) and synthetic α-tocopherol (Rovimix[®] E50, DSM Nutritional Products, MD, USA) added to diets. The control diet was formulated mainly with wheat and canola meal, and it contained 14 MJ/kg digestible energy and 18.3% crude protein. The control diet contained Se 0.2 ppm and 17 IU kg⁻¹ VE as recommended by National Research Council (NRC 2012) as meeting the nominal requirements of this class of pig. The other three diets contained increased concentrations of Se and VE to reach i) 0.3 ppm Se and 50 IU kg⁻¹ VE, ii) 0.5 ppm Se and 100 IU kg⁻¹ Se, or iii) 1.0 ppm Se and 200 IU kg⁻¹ VE, respectively. After a 14-day supplementation, six pigs from each dietary group were allocated to either 1) a climate-controlled

room set at 20°C, humidity 35% as the thermoneutral (TN) condition or 2) a heat stress (HS) condition with a room set to 35°C, 9.00-17.00 h, and 28°C, rest of the day, humidity 35% for two days. Pigs continued their respective diets during the two-day thermal exposure. Given heat stress can reduce voluntary feed intake, and to eliminate the confounding effects of dissimilar feed intake between TN and HS conditions on intestinal blood flow (Collin *et al.*, 2001) and barrier function (Pearce *et al.*, 2013b), all pigs were pair-fed at a rate of 75% of *ad libitum* intake (2.5 times of maintenance energy requirement) which is the predicted voluntary feed intake under this heat load (Huynh *et al.*, 2005). Water was supplied via nipple drinkers *ad libitum*.

Physiological monitoring

Physiological responses to heat stress were assessed by quantifying each pig's respiration rate and rectal temperature which were monitored daily at 9.00, 11.00, 13.00, 15.00 and 17.00 h each of the two days thermal exposure. Researchers were blinded about dietary treatments to minimize bias on physiological recordings. As a precaution, the pig was removed from the heat chamber if its rectal temperature exceeded 41°C, and the pig was then rested under the thermoneutral condition until its temperature was below 40°C. During the experiment only one pig was removed for 1 h due to hyperthermia and then returned to the heat chamber without incident.

Blood sampling, euthanasia, and tissue preparation

At the end of the two-day climate challenge, pigs were sedated with intramuscular injection of Ketamine (13 mg·kg⁻¹ body weight) and Xylazine (1.0 mg·kg⁻¹ body weight). After confirming the level of anesthesia, by lack of response to a toe pinch and touching adjacent to the eye, pigs were killed by cardiac injection of pentobarbitone sodium (162.5 mg kg⁻¹ body weight. Lethabarb, Virbac Animal Health, Australia). Euthanasia was confirmed by an absence of breathing, heart rate (detected with a stethoscope) and palpebral reflex. Thereafter, a midline abdominal incision was made to access the GI tract, and a 5 mL blood sample was taken from the portal vein with heparinized vacutainers (BD vacutainer® Australia) from each pig. Fresh blood was immediately loaded into an automatic blood gas analyzer (EPOC®, Alere, US) for detecting blood gas and chemistry parameters: partial pressure of CO₂ (P_{CO2}), total CO₂ concentration (ctCO₂), partial pressure of O₂ (P_{O2}), pH, bicarbonate, lactate, and hematocrit percentage. Sections (20 cm) of proximal jejunum and distal ileum were collected and fresh tissues were rinsed and transported in oxygenated PBS (0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2) to Ussing chamber equipment for assessing mucosal integrity. Jejunum

and ileum were collected from the same site of each pig and snap frozen in liquid nitrogen then stored at -80°C for biochemical and RNA analysis.

Intestinal barrier integrity measurements

Ussing chamber sliders (exposed area of 0.71 cm²) with fresh mucosa (muscle layer was removed) were inserted into two-part chambers (EasyMount Diffusion Chambers, Physiologic Instruments, San Diego, USA) that contained physiological saline (115 mM NaCl, 25 mM NaHCO₃, 2.4 mM K₂HPO₄, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 0.4 mM KH₂PO₄, pH 7.4) at 37°C and gassed with carbogen (5% CO₂, 95% O₂). Each chamber half contained 5 mL of the Krebs bicarbonate buffer, with the serosal bath having an additional 10 mM glucose to provide an energy substrate and the mucosal bath containing an additional 10 mM mannitol to maintain osmotic balance across the mucosa on the tissues. Each chamber had a set of four electrodes (two voltage sensing and two current passing electrodes) installed on opposite sides of the tissue and connected to the amplifier through agar bridges. A Multichannel voltage-current clamp (Physiologic Instruments, model VCC MC6) was used to record short circuit current. Tissues were allowed to equilibrate for 20-30 min in the chambers before measurements were made. Trans-mucosal voltage was stepped to five graded levels under a voltage clamp conditions and the corresponding currents were measured, TER was calculate by Ohm's law and multiplied the exposed area.

The paracellular probe, fluorescently labeled dextran (FITC-dextran; molecular mass = 4kDa, FD4, 1 mg mL⁻¹; Sigma-Aldrich, St Louis, MO, USA), was added to the mucosal compartment. Subsequently 200 µL of solutions was collected from the both sides of the tissue sheet at 1, 30 and 60 min for quantifying mucosal FD4 permeability. The FD4 fluorecence was measured using a fluorescence reader (FlexStation II; Molecular Devices, Sunnyvale, CA, USA). The apparent permeability co-efficient (PaPP) of FD4 was calculated by the following equation given by Pearce *et al.* (2013a): $PaPP = dQ / (dt \times A \times C_0)$, where dQ/dt = transport rate (mg/min); A = area of the membrane (cm²); C_0 = initial concentration in the donor chamber (mg/cm³). Carbachol (100µm; Sigma, Sydney, Australia) was added at the end of the experiment to check tissue viability. Values of TER and FD4 permeability of jejunum and ileum were obtained in duplicates for each pig.

Oxidative stress biomarkers

Frozen jejunum and ileum samples were pulverized in liquid nitrogen and homogenized in Tris-HCL buffer (pH 7.5), then supernatants were collected after centrifuging (1000 g, 15 min, 4°C) for measuring glutathione peroxidase activity (GPX), reduced glutathione (GSH), and oxidized glutathione (GSSG) concentration by commercial kits (Cayman, USA). Antioxidant enzyme activities and glutathione concentrations were normalized in relation to the total protein concentration (Pierce™ BCA kit, Thermo Fisher) of the tissue homogenates.

Gene abundance

Total RNA was isolated from 0.2 g pulverized jejunum and ileum tissue according to the manufactures manual (Purelink™, Life Technologies). The concentration and quality of total RNA were verified using an Experion RNA analysis kit (Bio-Rad Laboratories, Inc.). RNA was stored at -80°C until reverse transcription. The total RNA (0.8 µg) was reverse transcribed using SuperScript™ III First-Strand Synthesis (Invitrogen™, Life Technologies) and the synthesized single-strand cDNA was stored at -20°C until used for Q-PCR. Sequences of primer sets for swine ribosome protein L32 (*RPL32*), hypoxia induced factor-1α (*HIF-1α*), interleukin-8 (IL-8), and tumor necrosis factor-α (*TNF-α*) were obtained from Pearce *et al.* (2013b), Heat shock protein 70 (*HSP70*) was referenced from Chauhan *et al.* (2014). Sequences of primers can be found in supporting information **Table S1**. A total volume of 25 µL reaction mix was prepared based on the manufacturer's instruction using SYBR® GreenER™ qPCR Supermix Universal (Invitrogen™, Life Technologies); 100 nM of each forward and reverse primer were applied in each reaction. Each sample was run in triplicate and fluorescence was quantified in iQ5 Real Time PCR Detection System (Bio-Rad Laboratories, Inc.). Each sample plate included a standard curve (five 10-fold dilutions of a pooled cDNA), a non-template negative control, and a blank to determine amplification efficiency of the respective primer pair. The abundances of the mRNA were normalized to *RPL32* according the method described by Livak and Schmittgen (2001).

Statistics

Data were analyzed by ANOVA using Genstat 16th Version (VSNi Ltd, UK). For physiological parameters, treatment factors included "temperature", "diet", "time" and "day". For TER, FD4 permeability, blood gas parameters, OS indices and mRNA fold changes, effects of "temperature", "diet" and "intestinal site (jejunum or ileum)" were analyzed. Linear and quadratic response of the Se and VE dosages (defined as 1, 2, 4, 8 for the four antioxidant dosages) of "diet" were in both

statistical models. Duncan multiple range test was used for multiple comparison *post-hoc* when the effects of heat stress and antioxidant are both significant. Data were expressed as mean $\pm$ standard error of the mean (SEM). $P \leq 0.05$ was considered significant and $P \leq 0.10$ was considered as a trend. Statistical analysis showed that although the baseline parameters were different between the jejunum and ileum, the interaction of temperature $\times$ diet $\times$ site (jejunum or ileum) was not significant for TER, dextran permeability, *GPX* mRNA, *GPX* activity, or GSSG:GSH ratio, so the data were pooled across the intestinal sites. The separate data for jejunum and ileum are provided in the supplementary data.

RESULTS

Physiological parameters and feed intake

As expected, compared to the thermoneutral pigs, heat stress increased the respiration rate from 25 to 158 breaths $\cdot$ min $^{-1}$ ($P < 0.001$). There was an interaction between temperature and time of heat stress ($P < 0.001$, **Fig. 1 (A)**), such that thermoneutral pigs had stable respiration rate, and by comparison, the respiration rate of the heat stressed pigs increased dramatically from 54 to 200 breaths $\cdot$ min $^{-1}$ between 09.00 and 13.00 h and was then maintained around this rate until the end of thermal exposure time. Dietary antioxidants did not affect respiration rate (Diet (linear), $P=0.95$; Diet (quadratic), $P = 0.66$), and there was no interaction with temperature (Temperature $\times$ Diet (linear), $P = 0.72$; Temperature $\times$ Diet (quadratic), $P = 0.52$).

Heat stress increased rectal temperature from 38.3 °C to 39.6 °C ($P < 0.001$). There was an interaction between temperature and time of heat stress ($P < 0.001$, **Fig. 1 (B)**), such that the pigs in thermoneutral conditions exhibited a small change of rectal temperature over time (38.2 °C at 11.00 h, 38.3 °C at 13.00 h, 38.5 °C at 17.00 h), whereas the heat stressed pigs markedly increased rectal temperature from 38.5°C to 39.2°C from 9.00 h to 11.00 h and reached a plateau of 40.0 °C at 13.00 h which was maintained until 17.00 h, as evidenced by an interaction between time and temperature ($P < 0.001$). There was no dietary effect of on rectal temperature, and neither were interactions of temperature and diet significant (Temperature $\times$ Diet (linear), $P = 0.96$; Temperature $\times$ Diet (quadratic), $P = 0.66$).

The design of this experiment was to pair feed the pigs to remove any confounding effect of reduced feed intake due to HS. The feed intake averaged 1.10 kg/d and was not influenced by temperature and dietary antioxidants supplementation (Temperature, $P = 0.57$; Diet (linear), $P = 0.60$; Diet (quadratic) = 0.22; Temperature $\times$ Diet (linear) = 0.96; Temperature $\times$ Diet (quadratic) = 0.70). Therefore pigs under heat stress and thermoneutral conditions received an equal dose of antioxidants, and the impacts of dissimilar feed intake on intestinal barrier function were avoided.

Blood gas parameters

In portal venous blood, heat stress decreased total CO_2 (40.8 vs 37.4 mM, $P = 0.025$) and bicarbonate (39.8 vs 35.5 mM, $P < 0.001$). P_{CO_2} , P_{O_2} , pH, lactate and hematocrit were not affected by heat stress. Selenium and VE supplementation linearly increased bicarbonate (36.7, 37.3, 37.3 and 38.8 mM) going from the low to high doses (Diet (linear), $P = 0.041$; Diet (quadratic), $P = 0.68$). The heat stressed pigs fed on Se 0.3 ppm + VE 50 IU kg^{-1} and Se 1.0 ppm + VE 200 IU kg^{-1} had similar concentrations of blood bicarbonate compared with the pigs housed under thermoneutral conditions. Other blood gas indices in the portal vein blood were not affected by antioxidant supplements (**Table 1**).

Intestinal epithelial barrier integrity

Heat stress reduced TER from 60.4 to 46.7 $\Omega \text{ cm}^2$ ($P = 0.009$). Selenium and VE supplementation linearly increased TER with increasing dosages (45.9, 51.5, 56.1 and 62.4 $\Omega \text{ cm}^2$ for the dosages from low to high; Diet (linear), $P = 0.016$; Diet (quadratic), $P = 0.56$), and the pigs fed on the highest dosage of Se and VE had higher TER than those on the control diet (62.4 vs 45.9 $\Omega \text{ cm}^2$, $P < 0.05$). As **Fig. 2 (A)** shows, Se and VE supplementation exhibited a similar effect in increasing TER under thermoneutral and heat stress conditions, as the interaction between temperature and dietary treatment was not significant (Temperature $\times$ Diet (linear), $P = 0.56$; Temperature $\times$ Diet (quadratic), $P = 0.37$). The TER of the highest dosage of Se and VE group was higher than control diet within heat stress condition (56.7 vs 36.6 $\Omega \text{ cm}^2$, $P < 0.05$). Jejunum had lower TER than ileum (41.0 vs 67.0 $\Omega \text{ cm}^2$, $P < 0.001$), as shown in **Fig 2 (B)**.

Basal short circuit current was greater in small intestine samples from heat stressed pigs. This tended to be resolved by the higher doses of Se and VE, but the changes did not reach statistical significance (see supplementary **Fig. S1**).

Compared with thermoneutral control, intestinal permeability to FD4 was elevated by heat stress from 13.8 to 18.9 cm min⁻¹×10⁻³ (P = 0.005). Selenium and Vitamin E supplementation decreased FD4 permeability linearly and quadratically with the increasing dosages (23.2, 15.9, 12.9 and 13.4 cm min⁻¹×10⁻³ for from low to high Se and VE dosages; Diet (linear), P = 0.002; Diet (quadratic), P = 0.016). As **Fig. 3 (A)** illustrated, the trend for antioxidants supplementation to decrease FD4 permeability was similar in both environmental conditions because the interactions of temperature and diet were not significant (Temperature × Diet (linear), P = 0.35; Temperature × Diet (quadratic), P = 0.92). Within the control diet treatment, heat stressed pigs had higher FD4 permeability than thermoneutral pigs (13 vs 30 cm min⁻¹×10⁻³, P < 0.05). The FD4 permeability of the second highest dosage of antioxidants was lower than control diet during thermoneutral condition (P < 0.05), and the pigs fed on two highest level of Se and VE had lower permeability than control fed pigs during heat stress (both P < 0.05). As shown in **Fig. 3 (B)**, jejunum had a larger FD4 permeability than ileum (19.8 vs 13.0 cm min⁻¹×10⁻³, P = 0.007).

A statistical data set for showing the barrier integrity in jejunum and ileum separately can be found in supporting information **Table S2**.

GPX mRNA, activity, and GSSG:GSH ratio

Glutathione peroxidase-2 mRNA abundance tended to be reduced by heat stress from 1.58 to 1.22 (P = 0.071), however, Se plus VE linearly increased *GPX-2* mRNA abundance along with increased dosages (0.97, 1.35, 1.50, 1.78; Diet (linear), P = 0.007; Diet (quadratic), P = 0.39). As **Fig 4 (A)** shows, the highest dosage of Se and VE had higher *GPX-2* mRNA abundance than control diet (P < 0.05) during heat stress condition, whereas *GPX-2* abundance did not differ among treatments during thermoneutral condition. **Fig 4 (B)** shows that *GPX-2* mRNA abundance was similar between ileum and jejunum.

Glutathione peroxidase activity was reduced by heat stress from 111 to 91 unit mg⁻¹ protein (P = 0.004), conversely, GPX activity was linearly enhanced with increasing dosages of dietary antioxidants (90.3, 100.0, 103.5, and 109.9 unit mg⁻¹ protein; Diet (linear), P = 0.049; Diet (quadratic), P = 0.49). As **Fig. 5 (A)** demonstrated, the trend of dietary antioxidants in improving GPX activity was not influenced by ambient temperature (Temperature × Diet (linear), P = 0.54; Temperature × Diet (quadratic), P = 0.74). The highest Se and VE group had higher GPX activity than control diet (104.2 vs 78.4 unit mg⁻¹ protein, P < 0.05) during heat stress condition, whereas the GPX

activity remained similar among dietary treatments during thermoneutral condition. Ileum had higher GPX activity than jejunum (120 vs 82 unit mg⁻¹ protein, P < 0.001), as shown in **Fig 5 (B)**.

The ratio of GSSG: GSH was increased by heat stress from 0.026 to 0.037 (P < 0.001). Selenium and VE supplementation linearly decreased GSSG:GSH ratio with the increasing dosages (0.034, 0.034, 0.035 and 0.022; Diet (linear) P = 0.005; Diet (quadratic) P = 0.39). As **Fig. 6 (A)** shows, the dietary antioxidants reduced GSSG:GSH ratio regardless of ambient temperature (Temperature × Diet (linear), P = 0.25; Temperature × Diet (quadratic), P = 0.24). Only within control diet, heat stress pigs had a higher GSSG:GSH ratio than thermoneutral pigs (P < 0.05). As **Fig. 6 (B)** shows, jejunum had higher GSSG:GSH ratio than ileum (P < 0.001).

A statistical data set for showing the oxidative stress biomarkers in jejunum and ileum separately can be found in supporting information **Table S2**.

Stress gene abundance

Heat stress greatly increased *HSP70* mRNA abundance (0.86 vs 3.56, P < 0.001), but antioxidant supplementation did not significantly decrease *HSP70* mRNA abundance (P = 0.13). However, heat stress and antioxidants did not alter *HIF-1α*, *IL-8*, or *TNF-α* mRNA expression (**Table 2**). A statistical data set for showing the expression of stress genes in jejunum and ileum separately can be found in supporting information (**Table S3**).

DISCUSSION

In this study we used two measures to assess gut leakiness following heat stress, the *ex vivo* trans-epithelial electrical resistance (TER) and FD4 flux. We related this to the oxidative stress in the tissue, assessed by the ratio of oxidized to reduced glutathione (GSSG:GSH ratio), supplemented by functional measurement of glutathione peroxidase (GPX) activity and measurement of *GPX-2* mRNA abundance. Glutathione peroxidase is important for the removal of free radicals that accumulate during oxidative stress, the major form associated with the intestinal mucosa being GPX-2 (Brigelius Flohe *et al.*, 2012). During oxidative stress, intracellular reduced GSH is depleted and its oxidized form (GSSG) increases (Sentellas *et al.*, 2014). This is reflected in our study, in which the GSSG:GSH ratio in the intestine of heat stressed pigs on the normal recommended diet (Se 0.2 ppm, VE 17 IU

kg⁻¹) was almost twice that of pigs kept in thermoneutral conditions on the same diet. This incapacity of the intestine to manage oxidative stress was accompanied by a lower *GPX-2* mRNA abundance and a reduced GPX activity in heat exposed compared to non-exposed pigs. Other studies also point to hyperthermia causing oxidative stress, which has been reported to increase oxidized lipids (Pearce *et al.*, 2013b) and proteins (Oliver *et al.*, 2012) in the small intestine. Under the normal recommended diet (Se 0.2 ppm, VE 17 IU kg⁻¹), the intestinal mucosal barrier was also compromised, the trans-epithelial electrical resistance was 34% less and permeability to FD4 was 57% greater in heat stressed compared to thermoneutral pigs.

In heat stressed pigs, there was a dose-dependent linear reduction in the GSSG:GSH ratio as Se was increased to 1.0 ppm and VE was increased to 200 IU kg⁻¹. At this dose, the GSSG:GSH ratio in the mucosa of heat stressed pigs was similar to the ratio in thermoneutral pigs on a normal Se/ VE diet. Thus this dose appears to reverse the oxidative stress. At the same high dose, *GPX-2* mRNA abundance was similar in thermoneutral and heat stressed pigs, and about 1.6 times the level in intestine from thermoneutral pigs on the normal Se/ VE diet. GPX activity was also greater with the highest Se/ VE dose, but to a lesser degree than the gene abundance difference. Thus there is a direct, but non-linear, relation between increased gene abundance, increased enzyme activity, and lowered oxidative stress as measured by the GSSG:GSH ratio.

Accompanying the reduced oxidative stress, there was a 61% greater TER, that is, reduced gut leakiness, in the heat stressed pig fed on highest versus the lowest dose of Se plus VE. However, there was only a very small positive difference between the lowest and highest dose in the intestines of pigs kept under thermoneutral conditions, suggesting that Se plus VE does not directly affect the TER when there is no strong indication of oxidative stress. We therefore conclude that it is the reduction of oxidative stress that protects the mucosa. Similarly 1.0 ppm Se and 300 mg kg⁻¹ alleviated the increase of epithelial conductance in heat stressed rats (Maseko *et al.*, 2014). On the other hand, the permeability of the mucosa to FD4 was reduced by 53% in heat stressed pigs when Se plus VE was increased from the lowest to the highest dose, and by 44% in pigs kept under thermoneutral conditions. This suggests that Se plus VE may reduce the movement of large molecules across the mucosa even in under thermoneutral conditions. Under thermoneutral conditions, the Se/ VE supplementation tended to increase transepithelial resistance and increase *GPX-2* activity. The effects in thermoneutral conditions are probably a reflection of the intestinal lining always being exposed to micro-organism and potentially injurious substances and thus always

being in a state of mild inflammation and immune vigilance (Artis, 2008; Furness *et al*, 2013). Thus, the results suggest that dietary supplementation may improve the barrier function of the intestine even in conditions of mild threat. Pig feed is normally supplemented with both Se and VE because these antioxidants work effectively together. Whether one or other might contribute more under conditions of heat stress is not known.

The present results are consistent with other studies that have reported association between oxidative stress, hyperthermia and loss of intestinal barrier function (Lambert, 2009; Pearce *et al.*, 2013a; Pearce *et al.*, 2013b). A factor that could link heat stress to reduced intestinal barrier function is inflammation. However, we did not find any increases in *IL-8* or *TNF- α* mRNA abundance, which might suggest that inflammation was not a significant factor. However, we did not look for histopathological changes or a greater range of cytokines, which might have revealed changes. Moreover, gut leakiness might lead to a systemic inflammatory response (Pearce *et al.*, 2013b), which was not investigated. Previous studies have also found that heat stress did not increase inflammatory markers in the intestine of pigs (Pearce *et al.*, 2013b; Gabler & Pearce, 2015). The rectal temperature was increased by 1.3 °C in the heat stressed pigs and *HSP70* mRNA abundance was up-regulated as reported in previous studies (Yu *et al.*, 2010; Cui & Gu, 2015), indicating a generalized stress to the intestine.

We observed that the jejunum had a higher FD4 permeability and lower TER than the ileum. This difference of barrier integrity may be associated with their different oxidative conditions; for example, we found that the jejunum has lower GPX activity and higher GSSG:GSH ratio compared with ileum. Similarly, Degroote *et al.* (2012) found the mucosa of the proximal small intestine has a 2-fold higher GSSG:GSH ratio than the distal small intestine in weaning piglets, although in contrast to the present results they found that the GPX activity of proximal small intestine was higher.

In addition to our main objective of this study, we found heat stress triggered respiratory alkalosis which affected acid-base balance in portal vein blood. Respiratory alkalosis was caused by the loss of blood CO₂ concentration due to the increased respiration rate in heat stressed pigs, and the blood bicarbonate decreased corresponding to reduced formation of carbonic acids, so that blood pH could be maintained. The whole process is a typical respiratory alkalosis which was compensated by metabolic acidosis. It is unknown if the compensated respiratory alkalosis had any impacts on intestinal barrier function because of limited literature. Interestingly, 0.3 ppm Se + 50 IU kg⁻¹ VE and 1.0 ppm Se + 200 IU kg⁻¹ VE prevented the drop of bicarbonate in response to respiratory alkalosis,

the effects possibly due to effect of VE, because our previous study showed VE (200 IU kg⁻¹) but not Se (1.0 ppm) prevented a decrease in bicarbonate in heat stressed pigs (Liu *et al.*, 2014). However, the mechanism remains unknown. Besides, hematocrit was unaffected in current experiment, although the pigs had free access to water. We conclude that there was not a significant dehydration or sufficient excess water intake to have significantly influenced hematocrit.

In conclusion, heat stress augmented intestinal oxidative stress and decreased barrier integrity. High levels of dietary Se and VE reduced both oxidative stress and leakiness. It is suggested that elevating Se and VE in pig diets could mitigate against deleterious effects of hot weather on the gut.

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Competing interests:

All authors, no conflicts of interest.

Author contributions:

FL, JJC, LRR, FWK, DW, JBF, PC, BJL, DMB, NKG and FRD developed the conception and designed the experiment. FL, FWK, DW conducted daily feeding, physiological monitoring in live animal phase. JJC, FWK, DW, RVP, JBF, PC participated in blood and tissue samplings. FL, LRR, RVP, LJF quantified intestinal barrier integrity in Ussing chamber. FL, JJC and PC conducted lab analysis. FL, FRD, JJC, JBF participated in the statistical analysis. All the authors participated in drafting or revising the manuscript. The final version of the manuscript was approved by all authors

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Supporting information

Table S1. Primers for Q-PCR

Table S2. Barrier function and oxidative stress markers for jejunum and ileum. (statistical data for Fig. 2, Fig. 3, Fig. 4, Fig. 5 and Fig. 6)

Table S3. mRNA abundance of stress and inflammatory genes for jejunum and ileum (statistical data for Table 2)

Fig. S1. Short circuit currents of the intestines of pigs that were fed with different levels of selenium and Vitamin E and subjected to a 20 °C ambient environment or 35°C.

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Tables

Table 1. Blood gas and acid balance in portal vein blood

	20°C				35°C				SEM	T ¹
Selenium (ppm)	0.2	0.3	0.5	1.0	0.2	0.3	0.5	1.0		
Vitamin E (IU kg ⁻¹)	17	50	100	200	17	50	100	200		
P _{CO2} (mmHg)	66.9	61.0	69.7	58.0	68.1	56.2	60.3	61.6	6.55	0.6
ctCO ₂ (mM)	41.2	40.3	42.9	38.6	36.4	37.9	37.0	38.4	1.87	0.01
P _{O2} (mmHg)	27.7	27.8	23.0	29.6	25.4	31.8	28.3	31.2	3.25	0.3
HCO ₃ (mM)	39.2 ^{bc}	38.5 ^{bc}	40.7 ^c	40.9 ^c	34.3 ^a	36.2 ^{ab}	35.1 ^a	36.6 ^{ab}	1.76	<0.0
pH	7.38	7.41	7.39	7.43	7.32	7.43	7.38	7.40	0.040	0.4
Lactate (mM)	4.03	2.30	3.40	3.39	3.30	2.23	3.01	2.79	0.692	0.3
Hematocrit (%)	30.8	32.2	29.8	31.5	29.7	28.8	29.3	29.3	1.90	0.1

^{a b c} Within a row means without common superscript differ (P < 0.05).

¹Temperature;

²Linear effects of diet;

³Quadratic effects of diet

Table 2. mRNA expression of stress and inflammatory genes

	20°C				35°C					P-values				
Selenium (ppm)	0.2	0.3	0.5	1.0	0.2	0.3	0.5	1.0	SE M	T ¹	D ² (L) ²	D ³ (Q) ³	T×D (L)	T×D (Q)
Vitamin E (IU kg ⁻¹)	17	50	100	200	17	50	100	200						
<i>HSP70</i> fold change	1.20	0.90	0.77	0.55	4.40	4.40	3.25	2.12	1.02	<0.01	0.13	0.86	0.35	0.99
<i>HIF-1α</i> fold change	1.04	1.37	1.37	1.28	1.02	1.14	1.14	1.21	0.13	0.13	0.25	0.17	0.93	0.34
<i>IL-8</i> fold change	1.32	1.25	1.21	1.27	1.01	1.05	1.09	0.95	0.27	0.21	0.82	0.98	0.93	0.68
<i>TNF-α</i> fold change	1.56	1.29	1.39	1.45	1.24	1.24	1.20	1.12	0.24	0.20	0.77	0.76	0.81	0.71

¹Temperature; ²Linear effects of diet; ³Quadratic effects of diet;

Figure captions

Figure 1. Respiration rate (A) and rectal temperature (B) of growing pigs that were fed on diets containing different levels of selenium (Se, ppm) and vitamin E (VE in IU kg⁻¹). Pigs were maintained under thermoneutral (TN, 20 °C) or a heat stress (HS, 35°C) ambient environment (n=6 per group). Each point is the average data for the two days of HS or TN conditions. SEM for respiration rate and rectal temperature were 9.14 breaths min⁻¹ and 0.138°C, respectively. There were no significant

differences between dietary treatments at any time for the TN or HS groups. Significance was observed for the effects of time ($P<0.001$), temperature ($P<0.001$) and the interaction ($P<0.05$). For both respiration rate and rectal temperature, all HS time points were different from the corresponding TN time points (brackets marked a). There were no differences in respiration rate with time under TN conditions (bracket marked b). Rectal temperature under TN conditions increased with time such that at 8 hr (*) it was no different from the first time point in HS pigs, but was significantly greater than for TN at 0.1 and 2 h.

Figure 2. (A) Trans-epithelial electrical resistance (TER, $\Omega \text{ cm}^2$) of the intestines of pigs that were fed on different levels of selenium (Se, ppm) and Vitamin E (VE, IU kg^{-1}) when being subjected to a 35°C or 20 °C ambient environment (values pooled across jejunum and ileum, and expressed as mean $\pm$ SEM, $n=6$ per group). (B) Trans-epithelial electric resistance of jejunum and ileum (values are expressed as mean $\pm$ SEM, $n=24$ per group). Multiple comparison was conducted by Duncan's multiple range test, “#” indicates that the values differ ($P < 0.05$).

Figure 3. (A) FITC-dextran (4kDa) permeability of the intestines of pigs that were fed on different level of selenium (Se, ppm) and Vitamin E (VE, IU kg^{-1}) when being subjected to a 35°C or 20 °C ambient environment (values pooled across jejunum and ileum, and expressed as mean $\pm$ SEM, $n=6$ per group); (B) FITC-dextran permeability of jejunum and ileum (values are expressed as mean $\pm$ SEM, $n=24$ per group). Units of permeability are $\text{cm min}^{-1} \times 10^{-3}$. Multiple comparison was conducted by Duncan's multiple range test, “#” indicates that the values differ ($P < 0.05$).

Figure 4. (A) Glutathione peroxidase-2 (GPX-2) mRNA fold change in the intestines of pigs that were fed on different levels of selenium (Se, ppm) and Vitamin E (VE, IU kg^{-1}) when being subjected to a 35°C or 20 °C ambient environment (values pooled across jejunum and ileum, and expressed as mean $\pm$ SEM, $n=6$ per group); (B) GPX-2 mRNA fold change in the jejunum and ileum (values are

expressed as mean $\pm$ SEM, n=24 per group). Multiple comparison was conducted by Duncan's multiple range test, “#” indicates that the values differ ($P < 0.05$).

Figure 5. (A) Glutathione peroxidase (GPX) activity (units mg^{-1} protein) in the intestines of pigs that were fed on different levels of selenium (Se, ppm) and Vitamin E (VE, IU kg^{-1}) when being subjected to a 35°C or 20 °C ambient environment (values pooled across jejunum and ileum, and expressed as mean $\pm$ SEM, n=6 per group); (B) GPX activity of jejunum and ileum (values are expressed as mean $\pm$ SEM, n=24 per group). Multiple comparison was conducted by Duncan's multiple range test, “#” indicates that the values differ ($P < 0.05$).

Figure 6. (A) The ratio of oxidized glutathione (GSSG) to reduced glutathione (GSH) in the intestines of pigs that were fed on different level of selenium (Se, ppm) and Vitamin E (VE, IU kg^{-1}) when being subjected to a 35°C or 20 °C ambient environment (values were pooled across jejunum and ileum, and expressed as mean $\pm$ SEM, n=6 per group); (B) GSSG:GSH ratio of jejunum and ileum (values are expressed as mean $\pm$ SEM, n=24 per group). Multiple comparison was conducted by Duncan's multiple range test, “#” indicates that the values differ ($P < 0.05$).

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