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Intramuscular Administration of Zinc Metallothionein to Preslaughter Stressed Pigs Improves Anti-oxidative Status and Pork Quality

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ABSTRACT: This study was conducted to determine the effects of exogenous zinc-metallothionein (Zn-MT) on anti-oxidative function and pork quality. After feeding a corn-soybean meal-based diet for two weeks, 48 pigs (Duroc × Landrace × Chinese Black Pig) were assigned randomly to four groups. Pigs in Group 1 were maintained under non-stress conditions, whereas pigs in Groups 2, 3 and 4 were aggressively handled for 25 min to produce stress. Pigs in Groups 1, 2, 3, and 4 received intramuscular administration of saline (control group; CON), 0 (negative control group; NCON), 0.8 (low dose group; LOW), and 1.6 (high dose group; HIGH) mg rabbit liver Zn-MT per kg body weight, respectively. Pigs were slaughtered at 3 and 6 h post-injection. Zn-MT treatment increased ($p<0.05$) the activities of superoxide dismutase (SOD) and glutathione-peroxidase (GSH-PX) while decreasing the concentration of malondialdehyde (MDA) in liver. These responses were greater ($p<0.05$) at 6 h than at 3 h post Zn-MT injection. Zn-MT treatment increased ($p<0.05$) hepatic SOD mRNA levels in a time and dose-dependent manner and decreased ($p<0.05$) serum glutamate-pyruvate transaminase and lactate dehydrogenase activities (indicators of tissue integrity). Zn-MT administration decreased ($p<0.05$) lactate concentration and increased ($p<0.05$) pH and water-holding capacity in the *longissimus thorasis* meat. Collectively, our results indicate that intramuscular administration of Zn-MT to pre-slaughter stressed pigs improved tissue anti-oxidative ability and meat quality. (**Key Words :** Stress, Metallothionein, Anti-oxidative Enzyme, Superoxide Dismutase Gene Expression, Pork Meat Quality)

INTRODUCTION

Pre-slaughter stress is a major factor affecting pork quality (Rosenvold and Andersen, 2003). Stress results in a decrease in intracellular anti-oxidant ability and an increase in the production of reactive oxygen species (including free radicals), which, in turn, damage proteins, lipids, nucleic acids, tissues, and organs (Yang, 1989). Because altered glycogen/glucose metabolism and compromised membrane integrity in muscle cells, dark-firm-dry (DFD) and pale soft exudation (PSE) meat are common problems in pork production worldwide (Rosenvold and Andersen, 2003; Zhang et al., 2003). Unfortunately, rapid chilling cannot prevent inferior quality pork meat brought about by high pre-slaughter stress. Although dietary supplementation with nutrient antioxidants, such as vitamin E, vitamin C and Se, has the potential to improve meat quality, the findings have been highly variable (Rosenvold and Andersen, 2003; Kil et al., 2006), with no effect reported from many studies.

Pigs exhibit high plasma levels of cortisol and catecholamines (epinephrine and norepinephrine) in response to stress (Li et al., 2003). Cortisol inhibits the intracellular synthesis of glutathione (a major antioxidant) (D'souza et al., 1998) and, therefore, reduces its

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Table 1. Ingredient and nutrient composition of the experimental diet (as-fed percentage basis)

Ingredients	
Corn	67.31
Soybean meal	24.20
Fish meal	6.00
Limestone	0.50
Monocalcium phosphate	1.00
Vitamin premix[a]	0.04
Choline chloride	0.08
Trace mineral premix[b]	0.30
NaCl	0.20
L-lysine.HCl	0.31
Methionine	0.06
Total	100.00
Nutritional level (Calculated/as fed basis)	
DE (MJ/kg)	13.33
CP (%)	17.72
Ca (%)	0.71
Avail. P. (%)	0.48
Lys (%)	1.05
Met (%)	0.32
Dry matter (%)	89.90

[a] Provided the following per kilogram of complete feed: 11,000 IU vitamin A; 1,100 IU vitamin D_3; 22 IU vitamin E; 4 mg menadione as dimethylpirimidinol bisulfate; 0.03 mg vitamin B_{12}; 28 mg d-pantothenic acid; and 33 mg niacin.

[b] Provided the following per kilogram of complete feed: 165 mg Zn ($ZnSO_4$), 165 mg, Fe ($FeSO_4$), 33 mg Mn ($MnSO_4$), 16.5 mg Cu ($CuSO_4$), 297 μg CaI_2, and 297 μg Se (Na_2SeO_3).

concentrations in porcine tissues, e.g. skeletal muscle, liver, and kidney (Chen and Wang, 1997). In addition, the auto-oxidation of catecholamines produces free oxygen radicals and free phenoxy radicals (Fang et al., 2002). Thus, under stress conditions, a marked increase in the formation of highly active oxidants, coupled with a substantial decline in anti-oxidant ability, results in oxidative stress, which leads to altered glycogen/glucose metabolism and cell membrane damage in muscle (Yang, 1989; Wu et al., 2004). Thus, it is very beneficial to identify a new, alternative approach to improve pork quality in pre-slaughter stressed pigs.

Metallothionein (MT), a single-chain polypeptide of 61 amino acid residues, occurs widely in animal cells and is an endogenous antioxidant (Thornalley and Vasak, 1985). This peptide contains N-acetylmethionine and alanine as its N- and C-terminal residues, respectively, as well as a high proportion of cysteine residues (Sato and Bremner, 1993). Metallothionein has multiple functions, including detoxification of heavy metals and scavenging of free radicals (Bremner, 1987; Fang et al., 2002), thereby preventing lipid peroxidation, reducing protein and DNA damage, and improving immune function (Cai et al., 2000; Jin et al., 2001). Indeed, the induction of MT synthesis in response to oxidative stress is part of the cellular antioxidant mechanism (Sato and Bremner, 1993).

At present, little is known about the efficacy of using exogenous MT to reduce oxidative stress in pre-slaughter pigs or enhance meat quality. To our knowledge, this is the first study to determine the effect of exogenous MT administration on anti-oxidant function in pigs and on pork quality. We hypothesized that intramuscular administration of zinc-metallothionein (Zn-MT) to pre-slaughter stressed pigs will be effective in improving anti-oxidative function and pork quality. This hypothesis was tested using rabbit liver Zn-MT.

MATERIALS AND METHODS

Diets and animals

The experiment was carried out in accordance with the Chinese guidelines for animal welfare and approved by the animal welfare committee of the Institute of Subtropical Agriculture, The Chinese Academy of Sciences. Forty-eight barrows (Duroc×Landrace×Chinese Black Pig) with an average body weight (BW) of 85 kg (±1.2 kg) were housed in an environmentally controlled room with slatted floors in a total confinement building. All pigs had free access to drinking water and a typical corn- and soybean meal-based diet (Table 1), which was formulated to meet or exceed the National Research Council (NRC)-recommended nutrient requirements of young swine (1998). After a two-week feeding period, pigs were assigned randomly into four groups. Pigs in Group 1 were maintained under non-stress conditions, whereas pigs in Groups 2, 3 and 4 were aggressively handled for 25 min to produce stress. Pigs in Groups 1, 2, 3, and 4 received intramuscular administration (in the neck) of saline (control group; CON), 0 (negative control group; NCON), 0.8 (low dose group; LOW), and 1.6 (high dose group; HIGH) mg rabbit liver Zn-MT per kg body weight, respectively. Rabbit liver Zn-MT used in this experiment was a frozen dry powder extract containing 95% protein and was provided by Changsha Lugu Biological Co. Ltd (Changsha, China). Six pigs from each group were slaughtered at 3 and 6 h post Zn-MT injection.

Sample collection and preparation

At slaughter, blood samples were collected at exsanguination from the anterior vena cava. The blood was collected in EDTA-coated tubes and then immediately centrifuged at 3,000 rpm for 15 min at 4°C for collecting serum for further chemical analysis. The liver was removed post mortem and then frozen in liquid nitrogen as quickly as possible. The liver samples were stored at -80°C until total RNA isolation (Yang et al., 2005). To prepare the liver extract, 0.2 g liver was washed in cold 0.9% NaCl saline and then ground in a vitreous grinder with 1 ml of ice-cold saline. The homogenates were centrifuged at 3,000 rpm at 4°C for 15 min. The supernatant was collected for enzyme

and metabolite assays.

Following slaughter, hot carcasses were chilled for 24 h. Intact loins from the right side were collected and vacuum-packaged for laboratory analyses (Yin et al., 2001).

Enzyme and metabolite assays

Enzyme activities and metabolite levels in serum were assayed using the following kits. Glutathione-peroxidase, lipid-peroxide and MDA (a product of lipid peroxidation) reagent kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Superoxide dismutase (SOD) and glutathione-peroxidase (GSH-PX) activities were assayed with kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's manuals. Measurements of enzyme activities of SOD, lactate dehydrogenase (LDH), glutamate-oxaloacetate transaminase (GOT), glutamate-pyruvate transaminase (GPT), and concentrations of lactate (LA) were performed using a Synchron CX4 Pro (Beckman Coulter, Fullerton, CA, USA).

Quantification of mRNA levels for superoxide dismutase

The primers were designed using the DNAMAN 4.15 design primer (Lynnon Biosoft, Vandruil, Quebec, Canada) according to the gene sequence in Genebank (http://www.ncbi.nlm.nih.gov/) (SOD:E06791, GAPDH: AF017079). For the SOD gene, the sense primer (5'-ATTCATGGCGACGAAGGC) and antisense primer (5'-TCAATTACACCACAGGCCA) amplify a 453bp region from 2 to 454bp of the SOD cDNA; for GAPDH gene, the sense primer (5'-TGAACGGATTTGGCCGCAT) and antisense primer (5'-TTCTCCATGGTCGTGAAGA) amplify a 298 bp region from 358 to 655 bp of the GAPDH cDNA (Tang et al., 2005).

The BIOXYTECH® SOD-525™ kit for total RNA extraction was purchased from OXIS Co. (Tokyo, Japan). The Read-to-Go RT-PCR was purchased from Amersham Pharmacia Biotech. Gene Ruler™ 100_{bp} DNA Ladder was purchased from MBI Fermntas. The A_{260}/A_{280} of total RNA was between 1.8 and 2.0.

The RT-PCR was done by a one-step reaction with Read-to-Go RT-PCR beads. Briefly, RNA (250 ng) was used for reverse transcription into cDNA at 42°C for 30 min using oligo (dT) $_{12-18}$ primers. Amplifications were performed at 95°C for 30 s, 57°C for 30 s, 72°C for 1 min and repeated for 30 cycles, and finally at 72°C for 10 min using the PCR System 5.331 machine (Eppendorf, Hamburg, Germany). PCR products (10 μl) were separated using 100 V electrophoresis at 50 min on 2% agarose gels containing ethidium bromide, and then digitally imaged. The bands were observed by UV Transilluminator (Ultra-violet Products Ltd., Cambridge, CB4 1TG, UK). The relative abundance of SOD mRNA levels was expressed on the basis of GAPDH mRNA levels.

Pork quality characterization by assays of lactate, pH, and water-holding capacity

At 48 h post mortem, loins of the last breast vertebra were removed and pH was determined on a 1.0 cm loin chop from each *longissimus thorasis* muscle. The pH was determined using a 5-g sample homogenized with 25 ml of distilled water (NPPC, 1994) and a pH meter (Orin model 720A) fitted with a Ross sure-flow combination 81-72 electrode (Orion Research Inc., Boston, MA, USA).

Pork meat color was determined using a Minolta CR 300 (Minolta Camera Co., LTD, Osaska, Japan) and Illuminant D65. An 8-cm loin chop obtained from the anterior end of each *lumborum* muscle section was weighed, placed in a Whirlpark bag, suspended in a 4°C cooler for 24 h, and then reweighed. Water-holding capacity (WHC) was determined, according to the method described by Chen and Wang (1997), which involved stressing with 35 kg of press.

Statistical analyses

All data from the experiment were subjected to analysis of variance using the General Linear Model (GLM) procedure of SAS (SAS, 2000) according to a completely randomized 3×2 factorial design. The treatment effects were

Table 2. Concentrations of malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione-peroxidase (GSH-PX) activities, as well as SOD mRNA levels in the livers of pigs from the control (CON), negative control (NCON), low dose of zinc metallothionein treatment (LOW) and high dose of zinc metallothionein treatment (HIGH) groups

Item	Treatment				Time (h)		SEM[1]	Significance[1]		
	CON	NCON	LOW	HIGH	3	6		Treatment	Time	Treatment× time
Liver levels										
MDA (nmol/mg protein)	1.8^{c}	3.9^{a}	3.1^{ab}	2.6^{b}	3.2	2.5	0.01	*	*	*
SOD (U/mg pro)	212.5^{a}	104.1^{c}	151.8^{b}	185.3^{b}	151.1	174.5	1.39	*	*	*
GSH-PX(U/mg protein)	54.5^{a}	41.0^{b}	49.2^{a}	50.2^{a}	46.0	50.6	0.18	*	*	*
Hepatic mRNA levels										
SOD/GAPDH	2.3^{b}	2.4^{ab}	2.6^{ab}	2.8^{a}	2.3	2.8	0.012	*	*	*

[1] Pooled standard error of the mean (n = 36); * $p<0.05$.

[a, b] Values sharing different superscript letters within a row are different ($p<0.05$).

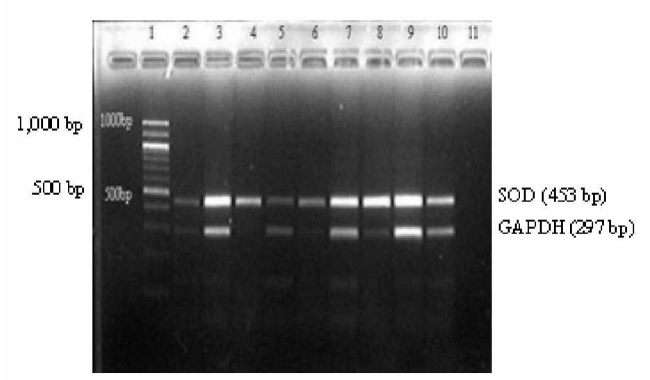

Figure 1. Effects of the zinc metallothionein treatment at 3 h post injection on hepatic SOD mRNA levels in pigs. Lane 1, DNA markers; lanes 2-4, the low dose group (n = 3); lanes 5-7, control group (n = 3); and lanes 8-10, the high dose group (n = 3).

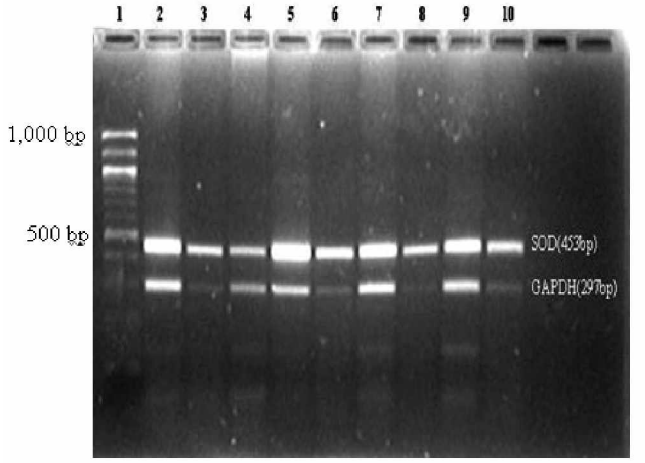

Figure 2. Effects of the zinc metallothionein treatment at 6 h post injection on hepatic SOD mRNA levels in pigs. Lane 1, DNA markers; lanes 2-4, the control group (n = 3); lanes 5-7, the low dose group (n = 3); and lanes 8-10, the high dose group (n = 3).

examined using orthogonal polynomial contrasts. Differences between treatment groups were further compared by the Tukey's test. Probability values ≤0.05 were taken to indicate statistical significance.

RESULTS AND DISCUSSION

Effects of Zn-MT on anti-oxidative function

The effects of pre-slaughter stress and Zn-MT injection on hepatic activities of SOD and GSH-PX and on hepatic concentrations of MDA (an indicator of lipid peroxidation; Fang et al., 2002) are summarized in Table 2. The hepatic concentrations of MDA were increased ($p<0.05$), whereas hepatic SOD activity was decreased ($p<0.05$) in response to the pre-slaughter stress. Hepatic GSH-PX activity did not differ ($p>0.05$) between unstressed and stressed pigs. Zn-MT administration increased ($p<0.05$) the activities of SOD and GSH-PX, while decreasing the concentration of MDA in the liver, when compared with stressed pigs receiving no Zn-MT treatment. The highest responses occurred at the dose of 1.6 mg Zn-MT/kg BW. Furthermore, the effect of the Zn-MT treatment was greater ($p<0.05$) at 6 h than at 3 h post administration. Consistent with the increase in hepatic SOD activity, Zn-MT treatment enhanced ($p<0.05$) the hepatic mRNA levels for SOD, and the effect was greater ($p<0.05$) at 6 h than 3 h post administration (Table 2, Figures 1 and 2). Zn-MT treatment increased hepatic mRNA levels for SOD probably by increasing gene transcription, mRNA stability or both. The underlying mechanism remains to be elucidated.

Pre-slaughter stress decreased ($p<0.05$) serum SOD activity, but increased ($p<0.05$) serum LDH, GOT and GPT activities. A reduction in SOD activity may reflect oxidation of the protein by free oxygen radicals (Fang et al., 2002), while increases in the activities of LDH, GOT and GPT are indicators of damage to tissues, e.g. liver, kidney, and skeletal muscle (Zhou, 2000). Remarkably, Zn-MT treatment effectively increased ($p<0.05$) serum SOD activity and decreased ($p<0.05$) serum activities of GOT and GPT (Table 3). Indeed, serum activities of GOT or GPT were similar between non-stressed pigs and the stressed pigs receiving a higher dose of Zn-MT (Table 3). These results demonstrate, for the first time, the efficacy of intramuscular Zn-MT administration in restoring anti-oxidant ability in pre-slaughter stressed pigs.

The potent anti-oxidant ability of MT is consistent with its molecular properties. Each MT molecule binds 5 to 7 Zn molecules, and contains 24% cysteine, 15% lysine and 10%

Table 3. Serum enzyme activities of superoxide dismutase (SOD, U/ml), lactate dehydrogenase (LDH, U/100 ml), glutamate-oxaloacetate transaminase (GOT, U/100 ml) and glutamate-pyruvate transaminase (GTP, U/100 ml) for pigs from the negative control (NCON), control (CON), low dose zinc metallothionein treatment (LOW) and high dose zinc metallothionein treatment (HIGH) groups

Item	Treatment				Time (h)		SEM[1]	Significance[1]		
	CON	NCON	LOW	HIGH	3	6		Treatment	Time	Treatment×time
SOD	149.0[a]	74.4[d]	90.4[c]	128.4[b]	118.8	102.5	4.45	*	#	#
LDH	600.1[c]	1047.5[a]	768.6[b]	729.2[b]	753.5	827.5	6.34	*	*	*
GOT	60.2[b]	93.5[a]	95.1[a]	72.2[b]	81.6	83.0	0.88	*	#	#
GTP	70.0[b]	93.9[a]	100.0[a]	75.2[b]	83.6	90.0	1.76	*	#	#

[1] Pooled standard error of the mean (n = 36); * $p<0.05$; # $p>0.05$.

[a, b] Values sharing different superscript letters within a row are different ($p<0.05$).

Table 4. Lactate concentration (LA, μg/100 mg tissue), pH, color, and water-holding capacity (WHC, %) in the *longissimus thorasis* meat of pigs from the control (CON), negative control (NCON), low dose of zinc metallothionein treatment (LOW) and high dose of zinc metallothionein treatment (HIGH) groups

Item	Treatment				Time (h)		SEM	Significance		
	CON	NCON	LOW	HIGH	3	6		Treatment	Time	Treatment×time
LA	99.8[c]	161.5[a]	139.3[b]	131.0[b]	139.3	138.0	1.98	*	#	#
pH	6.24[a]	5.52[b]	6.07[a]	6.08[a]	5.96	5.99	0.062	*	#	#
Color	2.93	2.43	2.91	2.92	2.69	2.90	0.071	#	#	#
WHC	73[a]	65[b]	67[ab]	69[ab]	68	70	1.003	*	#	#

* Pooled standard error of the mean (n = 36); # Approaching significance ($p>0.05$).

[a, b, c] Values sharing different superscript letters within a row are different ($p<0.05$).

aspartic acid. The thionein moiety residue of the MT has 20 times more sulfhydryl group numbers than GSH-PX. Sulfhydryl groups confer a high degree of reactivity towards free radicals and electrophies, thereby removing reactive oxygen species (ROS) (Zhang et al., 1999; Zhou et al., 2003). Furthermore, MT is an acute phase protein, whose concentration can change substantially in response to oxidative stress (Cai et al., 2000). Adel and Ruiter (1989) found that DNA degradation was totally inhibited by 13 μmol/L MT, compared with 10 mmol/L GSH-PX. Zhang et al. (1999) also reported that 60% of hydroxyl radicals could be scavenged by 0.6 μmol/L ZnMT-2, while 7.7 μmol/L GSH-PX was required to exert the same function. Additionally, feeding Zn-MT to 6.5-month-old Kunming rats increased hepatic SOD activity and decreased ($p<0.05$) lipid peroxide levels (Zhou et al., 2003).

The effects of Zn-MT on pork quality

Results of this study indicate that pre-slaughter stress reduced pH value in pork meat to 5.52, compared with a pH value of 6.04 for unstressed pigs (Table 4). Thus, the aggressive handling of pigs for 25 min provides a useful model to evaluate the effect of intramuscular Zn-MT administration on anti-oxidant function and pork quality. According to the standard of NPPC (1994), pork with a pH <5.6 is defined as PSE meat, pork with a pH value between 5.61 and 5.79 is deemed as questionable, and pork with a pH value of >5.8 is classified as normal.

We could not detect a statistical difference in meat color scores between the four treatment groups of pigs (Table 4). Meat color, determined by the chemical configuration and quantity of myoglobin, reflects physiological, biochemical and microbial variation, and is used as an important sensory index to estimate meat quality (Rosenvold and Andersen, 2003). However, the values of color scores for Zn-MT-treated pigs were numerically similar to those for unstressed pigs ($p>0.05$), and were all within the normal range (3 to 4) (NPPC, 1994). Color scores were lower ($p<0.05$) for pigs from the NCON group than the other groups (Table 4). This result indicates a positive effect of Zn-MT on meat quality, which may result from a reduction in myoglobin oxidation by free radicals (Zhang and Fu, 2003).

Poor on-farm handling increases the susceptibility to pre-slaughter stress (D'Souza et al., 1998). This undesired practice resulted in lower muscle glycogen stores early *post mortem* and lower pH values at 24 h *post mortem*, as well as a higher incidence of PSE meat, compared with pigs that were handled correctly on the farm. As noted above, stress hormones enhance the per-oxidation of lipid, thereby producing a large amount of free radicals. The lipid oxidation in muscle increases the permeability of cell membrane, causing massive loss of intracellular water and other substances (Fang et al., 2002). In addition, lipid per-oxidation can denature protein (Wu et al., 2004), therefore decreasing the water-holding capacity of meat (D'Souza et al., 1998). Further, a large amount of liquid and water losses results in tougher meat. Thus, increased levels of oxidants cause serious tissue damage and an unfavorable WHC.

Pre-slaughter stress increased ($p<0.05$) concentrations of LA and decreased pH and WHC values in meat (Table 4). Importantly, Zn-MT treatment decreased ($p<0.05$) LA levels and increased ($p<0.05$) pH and WHC values in meat, when compared with stressed pigs receiving no Zn-MT treatment (Table 4). Except for LA, better responses in all the measured parameters occurred at the dose of 1.6 mg/kg BM at 6 h post administration. As a result of the decline in LA production, meat pH and WHC values were increased ($p<0.05$) to the levels similar to those for unstressed pigs (Table 4). Of note, the pH values of the meat from Zn-MT-treated pigs were in the normal range (NPPC, 1994), while those for the stressed pigs receiving no Zn-MT treatment were similar to the PSE pork, according to the standards of NPPC (1994). There was a negative correlation between blood LDH activity and pork quality parameters (pH, color and WHC) (Yang et al., 1990; 1993). This finding suggests an important role for oxidant-induced tissue damage in causing poor meat quality.

The results of the present study are significant for the following reasons. First, oxidative stress is a major mechanism responsible for the undesired characteristics of the low quality meat. Second, through enhancing an anti-oxidant capacity, acute Zn-MT treatment to pre-slaughter

stressed pigs was highly effective in improving pork quality. Thus, our findings not only advance the fundamental knowledge about the role of free radicals in meat quality but also have important implications for improving the economic returns of pork producers.

CONCLUSION

Pre-slaughter stress caused oxidative stress in pigs, reduced pH and WHC values in muscle, and yielded pale soft exudation meat. Intramuscular administration of Zn-MT to stressed pigs at 3 to 6 h before slaughter improved their antioxidant function and meat quality. This provides a new, simple, practical and effective means to prevent the production of poor quality meat in the pork industry.

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