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Impact of weaning and an antioxidant blend on intestinal barrier function and antioxidant status in pigs[1]

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ABSTRACT: The objective of this experiment was to investigate the influence of weaning stress and an antioxidant blend on gut health and free radical metabolism in postweaning pigs. A total of 96 pigs from 12 litters were randomly divided by litter to 3 groups with 4 litters each. The control group and the weaning group were fed the basal diet, and the antioxidant group was fed the basal diet supplemented with an antioxidant blend. The control group was suckling normally during the experimental period and the other 2 treatments were weaned at 21 d of age. Morphology in different parts of the intestines was used as a measure of intestinal barrier function. Activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and the concentrations of malondialdehyde (MDA), NO, H_2O_2, and O_2 were measured in serum. Activities of the digestive enzymes, including sucrase, maltase, amylase, lipase, and pepsin, were measured at 24 d of age for all treatments. Gene expressions of free radicals, digestive enzymes, or antioxidant enzymes were selected for quantitative reverse transcription-PCR analyses. Results showed that weaning resulted in reductions ($P < 0.05$) in the villus height and width, and activity of digestive enzymes. Activity of SOD decreased ($P < 0.05$) and the concentrations of MDA, NO, and H_2O_2 increased ($P < 0.05$) after weaning. The expression results indicated that the genes related to the antioxidant enzymes and digestive enzymes were down regulated ($P < 0.05$) after weaning. Tumor protein 53, which regulates reactive oxygen-species generation, tended to increase ($P < 0.10$) in the weaning group. The concentration of PPARγ coactivator-1α (*PGC-1α*), which plays an important protective role against oxidative stress by regulating the expression of mitochondrial antioxidants, was reduced ($P < 0.05$) in weaning pigs and increased ($P < 0.01$) in antioxidant pigs compared with the control pigs. Results indicated that intestinal dysfunction occurred after weaning and there was an inhibition of the antioxidant system. The antioxidant blend has the potential to prevent free radical-induced damage and suppress oxidative stress by modulating the expressions of tumor protein 53 and *PGC-1α* genes.

Key words: antioxidant, gut health, oxidative stress, reactive oxygen species

INTRODUCTION

Weaning pigs is a stressful event associated with gastrointestinal disorders, causing diarrhea, and increased disease susceptibility (Boudry et al., 2004). However, the mechanisms that link weaning stress and intestinal disease are unknown. Overproduction of reactive-oxygen species and reactive-nitrogen species may result in oxidative stress (Valko et al., 2007) and changes in the antioxidant defense systems, including superoxide dismutase (**SOD**), catalase, and glutathione peroxidase (**GSH-Px**; Han et al., 2009, 2011). Supplementation of natural antioxidants, such as vitamin E, vitamin C, tea polyphenols, and probiotics, has been practiced to relieve stress in livestock husbandry (Bonnette et al., 1990; Eicher et al., 2006; West et al., 2008; Deng et al., 2010). However, results of clinical and laboratory studies indicate that, rather than working independently, antioxidants interact in a complex network to recycle and regenerate each

other, and protect against stress-induced diseases (Sies et al., 2005). Although stress is thought to play a role in postweaning intestinal disease, weaning-induced free-radical metabolism in weaning pigs has not been reported previously. The present study was conducted to investigate the effects of weaning stress and an antioxidant blend on free-radical metabolism and gut-barrier function in weaning-induced intestinal dysfunction.

MATERIALS AND METHODS

The experiment was approved by the Shanghai Jiaotong University Institutional Animal Care and Use Committee.

Animals and Experiment Design

A total of ninety-six 14-d old pigs (Duroc × Landrace) from 12 litters were randomly divided by litter to 3 treatment groups with 4 litters per group. The 3 treatment groups were designated as control, weaning, and antioxidant groups. The size of the litters, in which pigs were allotted to each group, were reared as indicated in Table 1. Pigs were kept with their dams in conventional farrowing pens and suckled until 21 d of age. From 14 to 35 d of age, the control pigs and weaning pigs had ad libitum access to the basal diet (Table 2), and the antioxidant pigs were fed the basal diet supplemented with 6.75 g/kg of an antioxidant blend, including 200 mg vitamin C, 100 mg vitamin E, 450 mg tea polyphenols, 1 g lipoic acid, and 5 g microbial antioxidants fermented by *Bacillus*, *Lactobacillus*, photosynthetic bacteria, and beer yeast. The microbial antioxidants were inactivated after fermentation.

When pigs were 21 d of age, weaning pigs and antioxidant pigs were weaned and moved from the farrowing pens to nursery pens without mixing any litters. The control pigs remained suckling in the farrowing pens until the end of the experiment. Room temperature in the farrowing and nursery building was maintained at about 30°C.

Sample Collection

At 21, 28, and 35 d of age, 3 pigs from each litter, resulting in a total of 12 pigs per treatment, were selected, and an amount of approximately 2 mL blood samples were collected by venipuncture method from precaval ein and placed in a tube for 2 h until centrifugation. The selected pigs had a BW that was close to the average

for the litter. Serum was separated by centrifugation at $3,500 \times g$ for 15 min at 4°C and stored at –20°C until analyses for free-radical and antioxidative physiological measures. At 24 d of age, 1 pig was randomly removed from each litter, resulting in a total of 4 pigs per treatment. These pigs were harvested and gut samples were collected. Gut segments (3 cm) were obtained at approximately 10, 50, and 90% of the small intestinal length (i.e., duodenum, jejunum, and ileum sections). Subsequently, the samples were kept in 4% neutral buffered formalin. Jejunum and stomach samples (approximately 2 g) were collected and stored at –80°C for subsequent gene expression and digestive enzyme activities analyses.

Determination of Free Radicals in Serum

A free-radical analyzer [TBR 4100, World Precision Instruments (**WPI**) Sarasota, FL], with software (LabTrax 2; MedTrax Software LLC, Glen Cove, NY), was used to record the NO, H_2O_2, and O_2 electrical potential traces in serum. Electrodes of ISO-NOP, ISO-HPO-2, and ISO-OXY-2 (TBR 4100, WPI) were used to monitor NO, H_2O_2, and O_2, respectively. Nitric oxide concentrations were determined using the NO electrode and calibration curves that were constructed with S-nitroso-N-acetyl-DL-penicillamine (0.2 to 500 nmol/L) in combination with the catalyst $CuCl_2$ to generate a known amount of NO in the solution (Zhang et al., 2000). The calibration curve was constructed by plotting the signal output vs. the concentration of the S-nitroso-N-acetyl-DL-penicillamine added at that time. For H_2O_2 measurements, the H_2O_2 electrode was calibrated with known concentrations of H_2O_2, using H_2O_2-equilibrated solutions from WPI. For O_2 measurements, the O_2 electrode was calibrated by 3-point calibration. Buffer solution with 0% O_2 was prepared by adding a slight excess of sodium dithionite to the buffer. Next, the sensor was exposed serially to 21% O_2 and 100% O_2 solutions. The calibration curve was constructed by plotting the signal output vs. percentage of O_2. Calibrations and all measurements were performed at room temperature. The reaction solution was constantly stirred with a magnetic bar controlled by a stirrer under the vial.

Table 1. Number of pigs in different groups

	Treatment											
Item	Control				Weaning				Antioxidant			
Litter	1	2	3	4	5	6	7	8	9	10	11	12
Number of pigs	9	7	10	7	6	7	9	9	4	10	10	8

Table 2. Dietary ingredient and analyzed energy and nutrient contents of the basic diets (as-fed basis)

Item	Amount
Ingredient, %	
Corn	41.18
Soybean meal, fermented	5.00
Soybean meal, peeled	7.00
Extruded soybean	11.22
Fish meal	5.00
Plasma protein	4.00
Whey powder	15.00
Limestone	0.50
Monocalcium phosphate	0.90
Choline	0.10
Lactose	8.75
Sodium chloride	0.35
Vitamin premix[1]	0.50
Mineral premix[2]	0.50
Analyzed composition	
DE, MJ/kg	14.48
CP (N × 6.25) , %	20.50
Ca, %	0.85
P, %	0.67
Lys, %	0.55
Met, %	1.55
Try, %	0.27
Thr, %	1.01

[1]Provided per kilogram of mixed diet: vitamin A, 12,000 IU/kg; vitamin D_3, 3,200 IU/kg; vitamin K_3, 2.5 mg; vitamin E, 80 mg; vitamin B_1, 2.5 mg; vitamin B_2, 6.5 mg; vitamin B_6, 5 mg; vitamin B_{12}, 0.05mg; niacin, 45 mg; and D-pantothenic acid, 20 mg.

[2]Provided per kilogram of mixed diet: folic acid, 1.5 mg; biotin, 0.15 mg; Fe, 150 mg as ferrous sulfate; Cu, 125 mg as copper sulfate; Zn, 200 mg as zinc oxide; Mn, 30 mg as manganous oxide ; I, 0.3 mg as potassium iodide; and Se, 0.3 mg as selenium selenite.

Antioxidative Physiological Analyses

The antioxidant capacities of serum were determined using assay kits according to the manufacturer instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Superoxide dismutase activity was detected by monitoring the inhibition of nitro blue tetrazolium reduction. The GSH-Px activity was measured with 5, 50-dithiobis-p-nitrobenzoic acid and the change in absorbance at 412 nm was recorded. Malondialdehyde (**MDA**) concentration was analyzed with 2-thiobarbituric acid and the change in absorbance was read at 532 nm. All absorbance levels were measured using a UV-visible spectrophotometer (Tongfang Inc., Shanghai, China).

Digestive Enzyme Determinations

The intestinal and stomach tissues were homogenized in ice-cold deionized water at a ratio of 1:10 (wt/vol) and then centrifuged at 2,200 × *g* for 15 min at 4 ° C, and the supernatant fractions were diluted to an appropriate con-

centration. Enzyme and supernatant-fraction protein determinations were accomplished as described by Kelly et al. (1991a). Amylase (EC 3.2.1.1) activity was determined using the method of Somogyi (1960). The activities of maltase (EC 3.2.1.20) and sucrase (EC 3.2.1.48) were determined according to Dahlqvist (1968). Lipase (EC 3.1.1.3.) activity was assayed using the method described by Tietz and Fiereck (1966). Pepsin (EC 3.4.23.1) activity was determined with the Ryle (1970) assay. One unit of enzyme activity was defined as the hydrolysis of 1 μM of substrate in 1 min.

Intestinal Histomorphometry

The samples of duodenum, jejunum, and ileum were kept in 4% neutral buffered formalin, processed using routine histological methods, and mounted in paraffin blocks. Six-micrometer-thick sections were cut and stained with Masson's trichrome. All specimens were examined under a light microscope (Nikon Eclipse E-400 equipped with a digital camera head [DS-5M] and camera control unit [DS-L1], Nikon, Tokyo, Japan). Villus height, villus width, and crypt depth were measured using an image-analysis system.

Quantitative Reverse Transcription-PCR

Total RNA of jejunum tissues was extracted following the Trizol Reagent instructions (Invitrogen, Carlsbad, CA) and reverse transcribed using random primers according to the manufacturer's protocol (TaKaRa, Otsu, Japan). The resulting cDNA was diluted and used as a PCR template to evaluate gene expression. The reaction was performed in a volume of 10 μL and carried out (ABI Prism 7700 Sequence Detection System; Applied Biosystems, Foster City, CA). A control without template was included in all batches. The PCR conditions were as follows: 95°C for 30 s, 35 cycles of 95°C for 5 s, annealing for 15 s, and 72°C for 10 s, followed by a melting curve. The β-actin gene was used to normalize variations in the amount of starting material. Dissociation curve analysis was done after each real-time experiment to ensure that there was only 1 specific product. Primers for genes of interest were designed based on the pig (*Sus scrofa*) sequence (Table 3) using primer design software (Primer Premier 5.0, PREMIER Biosoft International, Palo Alto, CA). Amplification efficiencies were calculated for each primer pair using 1:4 serial dilution of the cDNA template. The amplification efficiency of each gene was determined to be ³1.9 (Rosenheck et al., 2001). The relative expression was expressed as a ratio of the target gene to the control gene using the formula $2^{-(\Delta\Delta Ct)}$ according to Livak and Schmittgen (2001), where $\Delta\Delta Ct = (Ct_{Target} - Ct_{\beta\text{-actin}})_{treatment} - (Ct_{Target} - Ct_{\beta\text{-actin}})_{control}$. Relative expression

Table 3. Primers used for quantitative reverse transcription-PCR[1]

Gene	Accession No.	Primer,[2] 5'-3'	Size, bp
p53	NM_213824	F: CTGCTTCCTGAAAACAACC	199
		R: AAGGGACAAAGGACGACA	
PGC-1α	AB106108	F: TGTGGATGAAGACGGATTG	167
		R: GTGGTCGGTTGTGAGTCG	
GSH-Px	NM_001115136	F: CAAGTCCTTCTACGACCTCA	184
		R: GAAGCCAAGAACCACCAG	
CAT	NM_214301	F: AACTGTCCCTTCCGTGCTA	202
		R: CCTGGGTGACATTATCTTCG	
SOD1	NM_001190422	F: ACCTGGGCAATGTGACTG	176
		R: TCCAGCATTTCCCGTCT	
SOD2	NM_214127	F: GGACAAATCTGAGCCCTAACG	159
		R: CCTTGTTGAAACCGAGCC	
LPL	NM_21426	F: CTGGCATTGCAGGAAGT	157
		R: GGTGACCCTCTGGTGAAT	
PGA	NM_213873	F: TGCCCTCTGTCTACTGCTCT	130
		R: ATGCCTGTCATGCTACCG	
SI	XM_003132515	F: CCTACTGCTGGACGTTGG	169
		R: CGGGCACAGAGCTGATAT	
GAA	XM_003131141	F: AGGTCACTGGCTACTTTCCG	126
		R: TCACCCACTGTCCCTTGCT	
AMY2	NM_214195.1	F: CATATTCCAGGAGGTGAT	198
		R: AAGACAAGGGCTCTATCAG	
β-actin	DQ452569	F: GGACCTGACCGACTACCTCAT	181
		R: GGGCAGCTCGTAGCTCTTCT	

[1]*p53* = tumor protein 53; *PGC-1α* = PPARγ coactivator-1α; *GSH-Px* = glutathione peroxidase; *CAT* = catalase; *SOD1* = superoxide dismutase 1; *SOD2* = superoxide dismutase 2; *LPL* = lipoprotein lipase; *PGA* = pepsinogen A; *SI* = sucrase-isomaltase; *GAA* = lysosomal alpha-glucosidase; *AMY2* = amylase, alpha 2B.

[2]F = forward; R = reverse.

was normalized and expressed as a ratio to the expression in suckling pigs. Therefore, relative expression of target genes in suckling pigs was 1.0. Relative gene expressions in weaning pigs and antioxidant pigs represented the comparison vs. suckling pigs and reported as a fold change from the value of suckling pigs.

Statistical Analyses

All data were presented as mean ± SD. Comparison of variables was performed using ANOVA (SPSS Inc., Chicago, IL). Pig was the experimental unit. The statistical model included treatment (suckling vs. weaning) and diet (basal vs. basal + antioxidant blend). A probability of $P < 0.05$ was considered significant and differences were considered tendencies if $0.05 < P < 0.10$.

RESULTS

Determination of Free Radicals in Serum

As shown in Table 4, compared with the control group, the concentration of NO was increased ($P < 0.05$) and content of O_2 decreased in the weaning group on d 35 ($P < 0.05$), and the amount of H_2O_2 in the weaning group was increased on d 28 ($P < 0.05$). Compared

with the weaning group, NO was less ($P < 0.05$) in the antioxidant group on d 35. The content of O_2 was increased in the antioxidant group on d 35 ($P < 0.05$). Supplementing the diet with an antioxidant blend decreased H_2O_2 ($P < 0.05$) in the antioxidant group on d 28 compared with the weaning group. However, an increase in NO ($P < 0.05$) in weaning pigs and antioxidant pigs, and an increase in H_2O_2 ($P < 0.05$) in wean-

Table 4. Concentration of free radicals in serum from pigs fed experimental diets

	Treatment		
	Control	Weaning	Antioxidant
Item	(n = 12)	(n = 12)	(n = 12)
	NO, nM		
d 21	6.88 ± 1.00[a]	20.64 ± 0.26[b]	14.27 ± 1.74c
d 28	9.66 ± 0.21	11.93 ± 0.89	10.75 ± 0.05
d 35	9.48 ± 1.56[a]	12.76 ± 0.05[c]	9.85 ± 1.32[a]
	H_2O_2, μM		
d 21	33.19 ± 0.27[a]	55.29 ± 4.89[c]	33.67 ± 5.39[a]
d 28	31.61 ± 0.26[a]	47.25 ± 6.77[c]	27.10 ± 3.68[a]
d 35	25.72 ± 1.32	22.67 ± 0.77	30.28 ± 3.51
	O_2, %		
d 21	23.34 ± 0.70	22.05 ± 0.73	23.76 ± 0.69
d 28	23.70 ± 0.64	22.20 ± 0.02	22.99 ± 0.09
d 35	23.96 ± 0.40[b]	21.13 ± 0.26[a]	22.77 ± 0.23[c]

[a–c] Means within a row without a common superscript differ ($P < 0.05$).

ing pigs were also observed on d 21 compared with the control pigs.

Determination of Serum SOD, GSH-Px, and MDA

As shown in Table 5, compared with the control group, the activity of serum SOD decreased ($P < 0.05$) on d 28 and the content of MDA increased ($P < 0.05$) on d 35 in the weaning group. Supplementing the diet with an antioxidant blend reduced ($P < 0.05$) MDA to quantities that were not different from those observed in the suckling pigs. However, compared with the control group, the activity of SOD in the antioxidant group was less ($P < 0.05$) on d 28. There was no difference in the activity of GSH-Px among the 3 groups.

Digestive Enzyme Assay

Activities of sucrase, maltase, amylase, lipase, and pepsin were reduced after weaning ($P < 0.05$; Table 6). Feeding the antioxidant blend increased the activity of maltase ($P < 0.05$) compared with the weaning group. Weaning in conjunction with feeding the antioxidant blend did not alter the weaning-induced changes in intestinal sucrase, amylase, lipase, and gastric pepsin activity.

Macroscopic Presentation and Histological Evaluation

A decrease ($P < 0.05$) in villus height and width, and an increase ($P < 0.05$) in crypt depth in duodenum, jejunum, and ileum were observed after weaning (Table 7). Feeding the antioxidant blend increased ($P < 0.05$) villus height in jejunum and villus width in jejunum and ileum

Table 5. Antioxidant indices in serum from pigs fed experimental diets

	Treatment		
	Control	Weaning	Antioxidant
Item[1]	(n = 12)	(n = 12)	(n = 12)
	SOD, U/mL		
d 21	151.58 ± 5.54	147.63 ± 2.48	150.56 ± 7.53
d 28	148.23 ± 2.68[a]	77.35 ± 5.26[b]	124.91 ± 3.67[c]
d 35	125.06 ± 5.82	108.14 ± 16.17	115.23 ± 8.73
	GSH-Px, U/mL		
d 21	58.23 ± 2.05	61.01 ± 8.36	64.86 ± 14.29
d 28	65.57 ± 3.80	53.89 ± 6.65	65.31 ± 3.12
d 35	58.05 ± 3.42	55.50 ± 2.47	58.32 ± 5.70
	MDA, nmol/mL		
d 21	2.34 ± 0.44	2.57 ± 0.73	2.66 ± 0.40
d 28	2.13 ± 1.10	2.87 ± 0.52	1.91 ± 0.21
d 35	1.98 ± 0.11[a]	3.67 ± 0.63[b]	2.27 ± 0.32[a]

[a–c] Means within a row without a common superscript differ ($P < 0.05$).
[1] SOD = superoxide dismutase; GSH-Px = glutathione peroxidase; MDA = malondialdehyde.

Table 6. Digestive enzyme activities of gastrointestinal tissue in pigs fed experimental diets

	Treatment		
	Control	Weaning	Antioxidant
Item[1]	(n = 4)	(n = 4)	(n = 4)
Jejunum			
Sucrase	15.08 ± 0.88[a]	12.56 ± 0.58[c]	14.68 ± 0.56[ac]
Maltase	32.61 ± 0.49[b]	24.46 ± 0.62[c]	31.93 ± 1.48[c]
Amylase	0.16 ± 0.01[a]	0.14 ± 0.01[c]	0.15 ± 0.02[ac]
Lipase	75.91 ± 1.56a	55.18 ± 3.45c	63.01 ± 2.13c
Stomach			
Pepsin	37.04 ± 2.43[a]	16.34 ± 3.74[c]	19.09 ± 4.50[c]

[a–c] Means within a row without a common superscript differ ($P < 0.05$).
[1] Enzyme activity, U/mg protein.

compared with the weaning group. As shown in Figure 1, no histological damage was observed in the control group. In weaning pigs, the villus was scattered and desquamated seriously in duodenum, jejunum, and ileum. A greater villus in duodenum, jejunum, and ileum was observed in antioxidant pigs relative to weaning pigs.

Quantitative Reverse Transcription-PCR

After weaning, expression of catalase, sucrase-isomaltase, and *GSH-Px* mRNA abundance was 3.3-fold ($P < 0.05$), 5.5-fold ($P < 0.05$), and 2.4-fold less (P < 0.05), respectively, in weaning pigs compared with control pigs (Figure 2). The mRNA abundance of sucrase-isomaltase and *GSH-Px* were 3.8-fold ($P < 0.05$) and 3.4-fold greater ($P < 0.01$) in pigs fed the antioxidant blend compared with pigs in the weaning group. Abundance of PPARγ coactivator-1α (**PGC-1α**) mRNA was 3.23-fold less ($P < 0.05$) after weaning compared with pigs in the

Table 7. Morphological characteristics in intestinal tissues in pigs fed experimental diets

	Treatment		
	Control	Weaning	Antioxidant
Item	(n = 4)	(n = 4)	(n = 4)
Villus height, μm			
Duodenum	420.00 ± 49.67[a]	270.00 ± 33.65[c]	320.00 ± 18.26[c]
Jejunum	390.00 ± 21.21[b]	270.00 ± 33.67[c]	368.00 ± 22.80[a]
Ileum	390.00 ± 52.92[a]	288.00 ± 48.17[c]	304.00 ± 32.09[c]
Villus width, μm			
Duodenum	108.00 ± 8.37[a]	68.00 ± 13.04[c]	82.00 ± 13.04[c]
Jejunum	104.00 ± 19.49a	70.00 ± 14.14[c]	90.00 ± 8.16[a]
Ileum	110.00 ± 8.16[c]	74.00 ± 11.40[c]	98.00 ± 17.89[a]
Crypt depth, μm			
Duodenum	255.00 ± 43.58[c]	368.00 ± 35.64[a]	298.00 ± 54.49a
Jejunum	255.00 ± 43.59[c]	350.00 ± 21.21[a]	318.00 ± 38.34[a]
Ileum	280.00 ± 46.37[c]	348.00 ± 53.57[a]	300.00 ± 29.44[ac]

[a–c] Means within a row without a common superscript differ ($P < 0.05$).
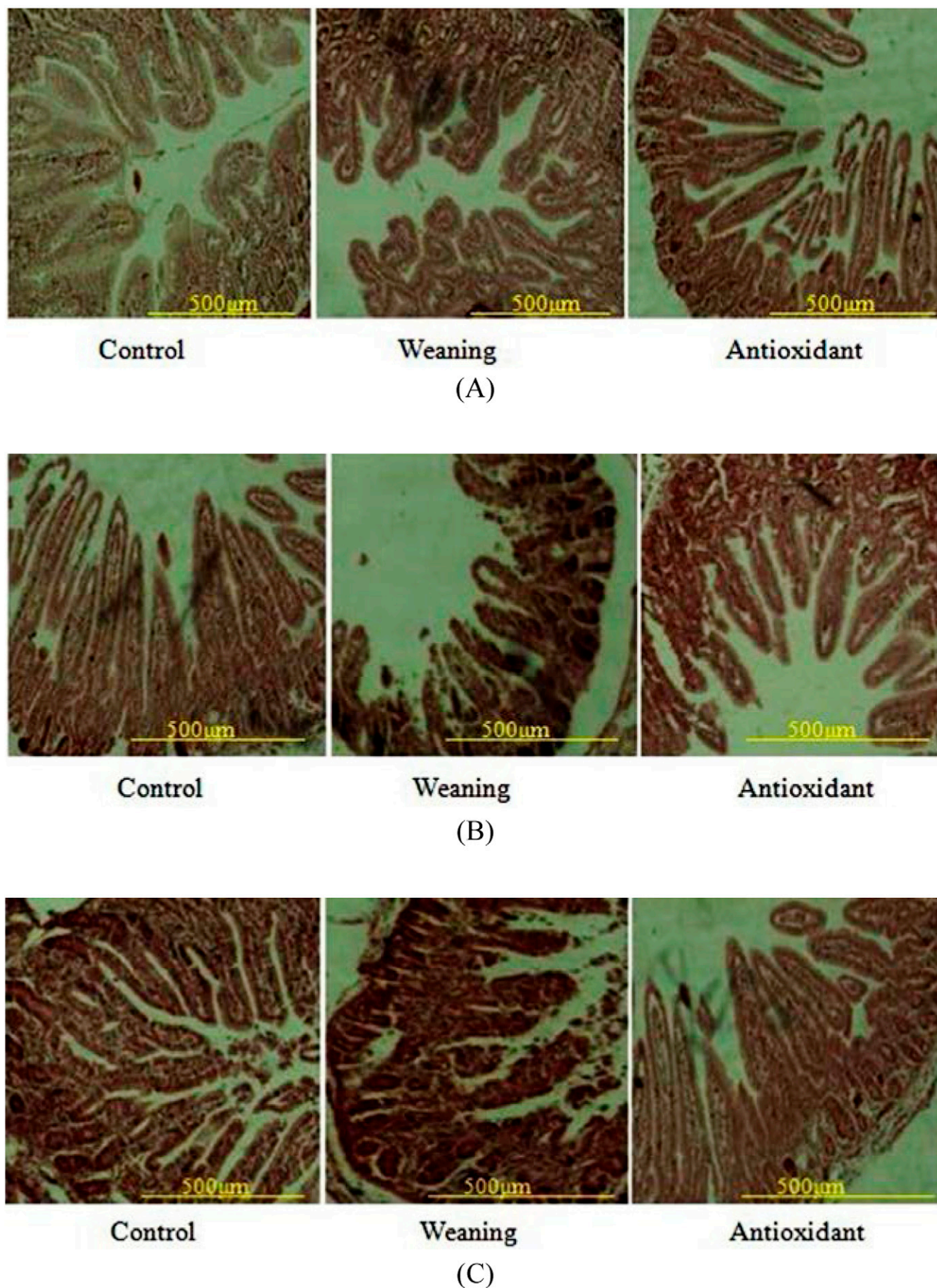
Figure 1. Histological evaluation of intestinal tissues in the 3 groups: A) changes of histology in duodenum; B) changes of histology in jejunum; and C) changes of histology in ileum. See online version for figure in color.
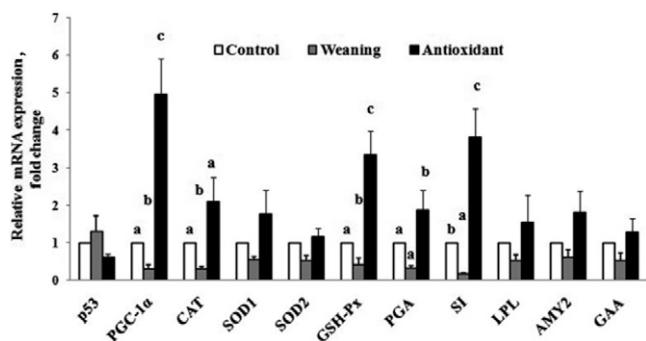
Figure 2. Expression of selected genes quantified by quantitative reverse transcription-PCR. Relative mRNA expression of selected genes in weaning pigs and antioxidant pigs represented the comparison vs. suckling pigs and reported as a fold change from the value of the suckling pigs. The mean expression value was calculated with 3 independent replications. Letters above the bars (a, b, c) indicate statistical significance of gene expression among the 3 treatments by ANOVA ($P < 0.05$). $p53$ = tumor protein 53; PGC-1α = PPARγ coactivator-1α; CAT = catalase; $SOD1$ = superoxide dismutase 1; $SOD2$ = superoxide dismutase 2; GSH-Px = glutathione peroxidase; PGA = pepsinogen A; SI = sucrase-isomaltase; LPL = lipoprotein lipase; GAA = lysosomal alpha-glucosidase; $AMY2$ = amylase, alpha 2B.

control group. In the antioxidant group, the abundance of PGC-1α mRNA increased 4.97-fold ($P < 0.01$) and the pepsinogen A mRNA abundance increased 1.9-fold ($P < 0.05$) compared with the suckling pigs. Abundance of superoxide dismutase 1 in the antioxidant group and tumor protein 53 (**p53**) mRNA in the weaning group tended to be increased ($P < 0.10$) after weaning.

DISCUSSION

Weaning stress in pigs is a critical factor in relation to the compromised intestinal barrier function, increased disease susceptibility, and endocrine disorder causing serious economic losses after weaning. In agreement with earlier reports (Kenworthy, 1976; Kelly et al., 1991a,b), results of the present experiment indicated that weaning can cause villus shortening and crypt hyperplasia, as well as decreases in intestinal digestive enzyme activities. To the best of our knowledge, this is the first study analyzing the quantity of free radicals using electrochemical methods after weaning in pigs.

Reactive-oxygen species from mitochondria and other cellular sources may be toxic byproducts of metabolism with the potential to cause damage to lipids, proteins, and DNA (Valko et al., 2007). Molecular oxygen is essential for the survival of all aerobic organisms. In the process of aerobic energy metabolism, O_2 serves as the final electron acceptor for cytochrome-C oxidase, the terminal enzymatic component of the mitochondrial enzymatic complex that catalyzes the 4-electron reduction of O_2 to H_2O. Partially reduced and highly reactive metabolites of O_2 may be formed during the electron transfer reactions to produce superoxide anion (O_2^-) and H_2O_2. These partially reduced or activated derivatives of oxygen (O_2,

O_2^-, and H_2O_2) are highly reactive and toxic, and can lead to the oxidative destruction of cells (Rao and Berk, 1992). The intestinal tissue from hemorrhagic shock in rats presents an up-regulation of inducible NO synthase expression (Hierholzer et al., 2004). Overproduced NO can react with O_2^- to form peroxynitrite anion (**ONOO$^-$**), which in turn can be protonated to form peroxynitrous acid that is considered a strong oxidizing agent, causing damage to cells. To protect against the potentially damaging effects of free radicals, cells possess several antioxidant enzymes, such as SOD, which reduces O_2^- to H_2O_2, catalase, and GSH-Px, which reduces H_2O_2 to H_2O. The decrease in SOD and O_2 and the increase in NO and H_2O_2 after weaning in our study indicated that an oxidative stress was present in weaning pigs, which caused increased free-radical generation. The increase in NO and H_2O_2 on d 21 after weaning might have been due to the transport and blood collection stress.

Various natural antioxidant extracts have been used to protect pigs from weaning stress in intensive pig production. Fragou et al. (2004) suggested that 100 mg/kg vitamin E needs to be added to the diet for pigs during the initial 2 wk postweaning. Administration of live yeast to weaned pigs improves growth performance, villus height, epithelial cell proliferation, and the numbers of macrophages at various sites of the small intestine (Bontempo et al., 2006). Eicher et al. (2006) also reported that the combination of vitamin C and β-glucan enhanced postweaning growth and reduced tumor necrosis factor-α expression of intestinal and liver tissues, indicating an important immunomodulatory role of a combination treatment. This approach, however, showed only limited effectiveness because the pathogenesis of oxidative tissue injury involves multiple disturbances of cellular physiological processes, including mitochondrial dysfunction (Lu and Gong, 2009). These antioxidants always work in a complex network. They occupy distinct cellular compartments and, among them, there is active recycling (Chan et al., 1999). For example, vitamin C is a hydrophilic antioxidant that acts together with the membrane-localized vitamin E in protecting membrane lipids from peroxidation as it regenerates oxidized vitamin E (Kojo, 2004). It is likely that a combination of a variety of different antioxidants is needed to keep animal cells protected from oxidative stress.

Therefore, we used an antioxidant blend including vitamin C, vitamin E, tea polyphenols, lipoic acid, and microbial antioxidants for the prevention and repair of the complex diseases in pigs that are related to weaning stress. In the present study, the protective effects of the antioxidant blend against free radical-induced oxidative insults were examined in pigs. The specific increase in the activities of digestive enzymes and antioxidants, the decrease of NO and H_2O_2 concentrations, and the improved mor-

phological and histological evaluation of intestinal tissues indicated that the antioxidant blend enhanced the antioxidant defense system, reduced the effects of free radicals, and maintained intestinal barrier function.

Reactive oxygen-species generation induced by endogenous and exogenous stimulation leads to increased oxidative stress in a variety of tissues. There are some endogenous antioxidant systems within cells that neutralize reactive-oxygen species. These antioxidant enzyme systems are critical to maintaining proper cellular function. When the endogenous antioxidant network fails to provide a sufficient compensatory response to restore cellular redox balance, the system becomes overwhelmed (i.e., redox imbalance), leading to activation of stress-sensitive intracellular signaling pathways. One major consequence is production of gene products that cause cellular damage and are ultimately responsible for late complications of disease (Mates, 2000). Alteration of antioxidant enzymes in response to exogenous and endogenous factors leading to oxidative stress depends on many factors, including expression of the genes that encode for these enzymes (Rodriguez et al., 2004).

A major cellular antioxidant is reduced glutathione, which is regenerated most efficiently by glutathione reductase and reduced nicotinamide adenine dinucleotide phosphate. Superoxide dismutase provides the efficient dismutation of O_2^-, leading to the formation of H_2O_2, which is removed by GSH-Px and catalase (Rodriguez et al., 2004). In the absence of an appropriate compensatory response from the endogenous antioxidant network, GSH-Px, catalase, and SOD concentrations are reduced, and oxidative stress ensues. In this experiment, we examined the mRNA expression of genes related to the antioxidant enzymes and digestive enzymes. All the mRNA expressions were consistent with the enzyme activities. In our opinion, weaning stress down regulated the expression of genes for *GSH-Px*, catalase, and sucrase-isomaltase, whereas the mRNA increase of the abundance of these genes in the antioxidant group can be considered a regulation that was enabled by the mixture of antioxidants that were used.

Tumor protein 53 is a redox-active transcription factor that organizes and directs cellular responses in the face of a variety of stresses that lead to genomic instability (Liu and Chen, 2006). Activation of *p53* can induce a range of responses, including cell cycle arrest, DNA repair, apoptosis, and senescence. These responses contribute to tumor suppression either by preventing or repairing genomic damage or through the elimination of potentially oncogenic cells from the proliferating population (Vousden, 2006). Under normal or low cellular stress, decreased concentrations of *p53* induce the expression of antioxidant genes. In severe cellular stress, high concentrations of *p53* promote the expression of genes that contribute to free-

radical formation and *p53*-mediated apoptosis (Sablina et al., 2005). Thus, under normal or low stress conditions, *p53* seems to have an antioxidant role that protects cells from oxidative DNA damage. Although this effect might depend on the concentration of *p53*, other cellular factors likely participate in a cell's final fate. The relative proapoptotic and antiapoptotic functions of *p53* seem to depend at least partly on the cellular *p53* concentration, as well as on other factors, such as *p53* subcellular localization and phosphorylation status (Tomko et al., 2006). In the present study, the greater content of *p53* in weaning pigs indicated that *p53* may play the proapoptosis role in the small intestine after weaning.

The *PGC-1α* gene is a transcriptional coactivator implicated in transducing physiological stimuli into metabolic programs and it stimulates mitochondrial biogenesis and mitochondrial respiration, which generates free radicals. Growing evidence indicates that *PGC-1α* is a broad and powerful regulator of free-radical metabolism that plays an important protective role against stress-induced oxidative stress by regulating the expression of mitochondrial antioxidants, which provide a potential target for the therapeutic manipulation of the important endogenous toxins (St-Pierre et al., 2006; Lu et al., 2010). Over expression of *PGC-1α* increased mitochondrial antioxidant enzyme expression and decreased oxidative stress and cell death (Valle et al., 2005). In this study, the endogenously generated NO, H_2O_2, and the down regulation of expression of antioxidants support the concept that weaning stress stimulated free-radical production and inhibited antioxidant capacity. Results also indicated that supplementing weanling pig diets with an antioxidant blend may counteract some of the detrimental changes that often occur after weaning by modeling *p53* and *PGC-1α* gene expression. These dietary supplements may be used to prevent or suppress oxidative stress.

In conclusion, weaning stress is the main reason for the loss of intestinal barrier function of early-weaned pigs, promotion of free radical generation, limitation of the antioxidant effects, and reduction in digestive enzyme activities. The antioxidant blend that was used in this experiment counteracted some of the detrimental changes that occurred at weaning in pigs by regulating the *p53* and *PGC-1α* gene expression. Further studies should be conducted to more completely determine the mechanism regulating free-radical generation after weaning.

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