

Effect of N-acetyl cysteine on enterocyte apoptosis and intracellular signalling pathways' response to oxidative stress in weaned piglets

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Abstract

N-acetyl cysteine (NAC) has been widely used for preventing reactive oxygen species-induced damage. However, little is known as to whether dietary NAC supplementation would alleviate intestinal injury in weaned piglets. The present study evaluated the effect of NAC on enterocyte apoptosis and intracellular signalling pathways' response to weaning stress. The control piglets were normally suckling, and piglets in the weaning and NAC groups were fed the basal diet and basal + NAC diet from 14 to 25 d of age, respectively. Compared with the control piglets, weaning increased cortisol concentrations ($P < 0.05$), decreased superoxide dismutase and glutathione peroxidase activities ($P < 0.05$), increased malondialdehyde content ($P < 0.05$) in serum and enhanced enterocyte apoptosis index (AI) and concentrations of caspase-3, caspase-8 and caspase-9 ($P < 0.05$). Gene expression analyses indicated that weaning induced apoptosis via Fas signalling and mitochondrial pathways in weaned piglets. Dietary NAC supplementation decreased ($P < 0.05$) cortisol concentrations and the AI, increased ($P < 0.05$) antioxidant status in serum and alleviated histopathological changes in the intestine. It also inhibited *Fas*, caspase-3, caspase-8 and integrin $\alpha\beta6$ ($\alpha\beta6$) gene expressions in the NAC-treated piglets. However, no significant decrease ($P > 0.10$) in caspase-3, caspase-8 and caspase-9 concentrations was observed in the NAC group compared with the weaning group. In conclusion, weaning may induce enterocyte apoptosis via the activation of Fas-dependent and mitochondria-dependent apoptosis. Although NAC had no effect on caspase concentrations, it was clearly beneficial for preserving morphological integrity in weaned piglets via the regulation of cell apoptosis and the inhibition of Fas-dependent apoptosis and $\alpha\beta6$ expression.

Key words: Piglets; Intestinal morphology; N-acetyl-L-cysteine; Apoptosis

Apoptosis is thought to be essential for epithelial turnover in intestinal mucosa and tissue homeostasis in intestinal epithelium. Abnormal apoptosis would result in intestinal mucosal barrier damage and gastrointestinal disorders⁽¹⁾. In swine production, weaning is a critical process for piglets due to dramatic changes in diets and environment. The obvious intestinal morphological changes induced by weaning stress are villous shedding, villous shortening and crypt hyperplasia⁽²⁾. Indeed, reactive oxygen species (ROS)-induced apoptosis has been widely studied, which is also an important mechanism for many chemotherapeutic agents. Oxidative stress results from the overproduction of ROS, which are also known as mediators of damage to cell structures, including lipids and membranes, proteins and DNA⁽³⁾. The effect of ROS is balanced by the antioxidant action of antioxidant enzymes as well as non-enzymatic antioxidants. The most

efficient enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase⁽⁴⁾. Non-enzymatic antioxidants are represented by vitamin C, vitamin E, glutathione (GSH) and so on⁽⁵⁾. Reducing oxidative stress by supplementation of non-enzymatic antioxidants, such as L-arginine⁽⁶⁾, GSH⁽⁷⁾ and vitamin C⁽⁸⁾, could potentially reduce ROS-induced damage and inhibit apoptosis.

We have demonstrated previously that weaning increases the concentrations of NO and H₂O₂ in serum in post-weaning piglets and that feeding an antioxidant-containing diet could prevent free radical-induced damage and suppress oxidative stress⁽⁹⁾. However, the relationships among weaning-induced free radical generation, apoptosis and intestinal mucosal damage are still unclear in pigs. Therefore, elucidation of the mechanisms of weaning-induced apoptosis might help in

Abbreviations: Bcl-2, B-cell lymphoma-2; GSH, glutathione; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; NAC, N-acetyl cysteine; ROS, reactive oxygen species; SOD, superoxide dismutase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling.

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understanding the physiological regulation of the apoptotic process in pig intestinal mucosa.

GSH, responsible for the detoxification of ROS, is the primary metabolic precursor for intestinal epithelial cells⁽¹⁰⁾. Intracellular GSH depletion induces chromosomal DNA fragmentation, which is associated with apoptosis and necrosis⁽¹¹⁾. Glutamine administration can protect against gut injury, and one of the possible ways of action is related to the preservation of GSH content^(12,13). N-acetyl cysteine (NAC), a well-known thiol antioxidant and GSH precursor, has been demonstrated to be beneficial for preventing ROS-induced damages^(14,15). Recently, it has been proved that NAC prevents exercise-induced intestinal lymphocyte apoptosis by maintaining intracellular GSH contents and reducing mitochondrial membrane depolarisation⁽¹⁴⁾. Administration of NAC before ischaemia in experiments was effective in the prevention of bacterial translocation and oxidative stress⁽¹⁶⁾. Moreover, NAC is a clinically proven safe dietary supplement that may be useful in clinical therapy for ROS-mediated diseases⁽¹⁷⁾. These beneficial properties of NAC suggest that it may be considered as a useful additive to diets for the prevention of weaning stress.

In the present study, we attempted to characterise the relationship between apoptosis and intestinal mucosal damage induced by weaning in piglets. We also aimed to investigate whether NAC can effectively prevent weaning-induced intestinal damage in piglets. Particular attention was afforded to changes in serum antioxidant activities, intestinal morphology, and enterocyte apoptosis and functions in an attempt to identify any intracellular signalling changes regulated by weaning and NAC treatment. To our knowledge, this is the first study in piglets to simultaneously evaluate weaning-induced apoptosis and the potential wound-healing function of NAC through the regulation of cell apoptosis in response to weaning stress.

Materials and methods

Animals and experiment design

The experiment was approved by the Shanghai Jiaotong University Institutional Animal Care and Use Committee. A total of 150 14-d-old piglets (Duroc × Landrace) from fifteen litters were randomly divided by litter into control, weaning and NAC groups with five litters per group. The piglets were kept with the sow in conventional farrowing pens and suckled until 21 d of age. From 14 to 25 d of age, piglets in the control and weaning groups had *ad libitum* access to the basal diet and the NAC-treated piglets were fed the basal diet supplemented with 500 mg/kg of NAC. The dosage of supplemental NAC was chosen based on the study by Hou *et al.*⁽¹⁸⁾ with 35-d-old piglets. Dietary ingredients, analysed energy and nutrient contents of the basal diets (as-fed basis) with 14.48 MJ/kg digestible energy and 20.50% crude protein (N × 6.25) have been described previously⁽⁹⁾. At 21 d of age, piglets of the weaning and NAC groups were weaned and moved from the farrowing pens to nursery pens without mixing any litters. The control piglets were retained in the far-

rowing pens to suckle until the 25 d experiment. Temperature in the farrowing and nursery pens was maintained at about 30°C.

Sample collection

At 25 d of age, three piglets from each litter, resulting in a total of fifteen piglets per treatment, were selected and blood samples were collected from the anterior vena cava. The selected piglets had a body weight that was close to the average for the litter. Serum was separated by centrifugation at 3500 g for 15 min at 4°C and stored at -20°C until analysis for cortisol and antioxidative physiological measures. One piglet was randomly chosen from each litter, resulting in a total of five piglets per treatment. The piglets were anaesthetised by intramuscular injection of sodium pentobarbital (50 mg/kg body weight) and then euthanised, and gut samples were collected. The entire small intestine was carefully removed and placed on ice. Some pieces, approximately 2 cm in length, were resected from the middle portion of the jejunum and fixed in 4% neutral buffered formalin for the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay. In addition, some specimens of jejunum sections were fixed with 2.5% glutaraldehyde for transmission electron microscopy examination. Other segments of the jejunum were rinsed thoroughly with physiological saline and opened longitudinally to expose the intestinal epithelium. The mucosal layer was harvested by gentle scraping of the epithelium with a glass slide, as described by Noda *et al.*⁽¹⁹⁾. Then, the mucosal specimens were frozen in liquid N₂ and stored at -70°C. Half of the specimens were used for the evaluation of apoptotic proteins with ELISA and the remaining half were used for quantitative RT-PCR analyses.

Serum cortisol content

Serum concentrations of cortisol were quantified using a Coat-A-Count Cortisol kit (Diagnostic Product Corporation) according to the manufacturer's protocol. Unknown concentrations were compared against the cortisol kit standard and expressed as µg/l of cortisol. This value was then converted and expressed as ng/ml of cortisol. The optical density at 450 nm was measured after colouration. All assays were performed in duplicate. The minimum detection limit was 7 ng/ml, and the use of the cortisol kits for porcine samples has been validated⁽²⁰⁾.

Antioxidative physiological analyses

The activities of SOD and GSH-Px and the content of malondialdehyde (MDA) in serum were determined to evaluate the antioxidant ability and lipid peroxidation in pigs, using assay kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute). In brief, the activity of total SOD was determined by monitoring the inhibition of nitro blue tetrazolium reduction. One unit of SOD activity was defined as the amount that reduced the absorbance at 550 nm by 50% in 1 ml serum, and data were expressed as

units/ml serum. The activity of GSH-Px was determined by measuring the reduction of GSH per min on the basis of its catalysis. The final result was expressed as a decrease of 1 $\mu\text{mol/l}$ of GSH per 5 min for 0.1 ml serum at 37°C after the subtraction of the non-enzymatic reaction, and data were expressed as units/ml serum and then converted to units/ μl . MDA content was analysed with 2-thiobarbituric acid, and data were expressed as nmol/ml serum. All absorbance levels were measured using a UV-visible spectrophotometer (Tongfang, Inc.).

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling staining

Jejunum samples, fixed in 4% neutral buffered formalin, were embedded in paraffin and sectioned. The specimens were stained with haematoxylin and eosin for histological examination. Mucosal length (villus height plus crypt depth) was analysed with a light microscope (Nikon). Fragmented DNA was stained using the TUNEL assay method with some modifications using a FragEL™ DNA Fragmentation Detection Kit (Merck). In brief, the specimens were de-waxed and incubated with 20 $\mu\text{g/ml}$ proteinase K for 20 min at room temperature and then reacted with the terminal deoxynucleotidyl transferase enzyme for 60 min at 37°C. The specimens were then incubated with anti-digoxigenin peroxidase at room temperature for 30 min followed by a thorough washing with PBS. Finally, the specimens were incubated with diaminobenzidine solution and counterstained with methyl green. A minimum of twenty crypts were randomly selected for apoptotic index analysis, and the number of apoptotic cells was calculated. The apoptotic index was determined by dividing the number of apoptotic cells by the total number of cells in the crypt column and multiplying by 100.

Specimen preparation for electron microscopy

Mucosal specimens of jejunum sections were fixed with 2.5% glutaraldehyde in 0.1 mol/l cacodylate buffer (pH 7.2) for 2 h at 4°C. These specimens were then washed three times with the same buffer and post-fixed with osmium tetroxide for 2.5 h at 4°C. They were later washed with 0.1% sodium acetate, stained with 1% uranyl acetate, dehydrated with ethanol and embedded in Spurr's low-viscosity resin. Representative areas were sectioned and stained with toluidine blue. Ultra-thin sections were prepared and double stained with uranium and lead. A Hitachi H-600 transmission electron microscope (Hitachi) was used to visualise the ultrastructure of the enterocytes and the distribution of epithelial cell microvilli.

Concentrations of caspase in the jejunum

Relative amounts of caspase-3, caspase-8 and caspase-9 in the homogenates of the jejunum of the selected piglets were determined using specific ELISA kits (R&D Systems, Inc.) according to the manufacturer's instructions. Each individual sample was analysed in triplicate. In brief, individual jejunum samples were homogenised in ten volumes of PBS (pH 7.0) on ice and centrifuged at 3000 *g* for 15 min. The supernatant was decanted and stored. Protein concentrations in the supernatant were determined using a BCA protein assay kit (Pierce). Caspase concentrations were determined using a spectrophotometer (450 nm) on a microtitre plate reader.

Quantitative RT-PCR

Total RNA was extracted from jejunal tissues using TRIzol reagent (Invitrogen) following the manufacturer's instructions and purified using DNase I (Invitrogen) for 15 min. The yield

Table 1. Primer sequences used for quantitative RT-PCR

| | GenBank accession no. | Sequence (5'–3') | Product (bp) |
|---|-----------------------|--|--------------|
| Reference genes | | | |
| <i>ACTB</i> | DQ452569 | F: GGACCTGACCGACTACCTCAT R: GGGCAGCTCGTAGCTCTTCT | 181 |
| <i>GAPDH</i> | DQ178130 | F: AAGGAGTAAGAGCCCTGGA R: TCTGGGATGGAACTGGAA | 140 |
| <i>B²M</i> | NM_213978 | F: TTCACACCGCTCCAGTAG R: CCAGATACATAGCAGTTCCAGG | 166 |
| Gene names | | | |
| <i>Bax</i> | XM_003127290 | F: CCGAAATGTTTGCTGACG R: AGCCGATCTCGAAGGAAGT | 154 |
| <i>Bcl-2</i> | NM_214285 | F: ACCTGAATGACCACCTAGAGC R: TCCGACTGAAGAGCGAAC | 180 |
| <i>Caspase-3</i> | NM_214131 | F: GTGGGACTGAAGATGACA R: ACCCGAGTAAGAAATGTG | 190 |
| <i>Caspase-8</i> | NM_001031779 | F: AGACAAGGGCATCATCATCGG R: GGTTTACCAAGAAGGGAACGG | 102 |
| <i>Caspase-9</i> | XM_003127618 | F: AATGCCGATTTGGCTTACGT R: CATTGTGCTTGGCAGTCAGGTT | 195 |
| <i>Fas</i> | NM_213839 | F: TGATGCCCAAGTGACTGACC R: GCAGAATTGACCCTCACGAT | 103 |
| <i>$\alpha\text{v}\beta\text{6}$</i> | EF432729 | F: AAGTTCAAGTTCGCCAGAC R: AAAAGAGCCAAAGCCAG | 167 |

ACTB, β -actin; F, forward primer; R, reverse primer; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *B²M*, β -2-microglobulin; *Bax*, B-cell lymphoma-2-associated X protein; *Bcl-2*, B-cell lymphoma-2; *$\alpha\text{v}\beta\text{6}$* , integrin $\alpha\text{v}\beta\text{6}$.

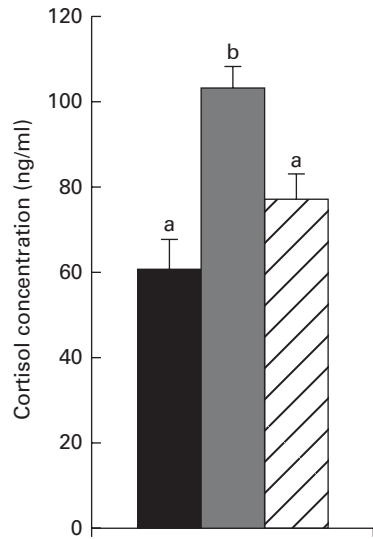


Fig. 1. Serum cortisol concentrations in piglets of the control, weaning and N-acetyl cysteine (NAC) groups at 25 d of age. Values are means, with their standard errors represented by vertical bars. ^{a,b}Mean values with unlike letters were significantly different ($P < 0.05$). Control group (■), piglets suckled from 14 to 25 d of age. Weaning group (■), piglets weaned at 21 d of age and fed the basal diet. NAC group (▨), piglets weaned at 21 d of age and fed the basal diet supplemented with 500 mg/kg of NAC.

and quality of RNA were assessed using a spectrophotometer (Bio-Rad Laboratories, GmbH), considering the ideal absorbance ratio ($1.8 \leq A_{260}/A_{280} \leq 2.0$). The integrity of RNA was checked by electrophoresis on a 1.5% agarose gel. A volume equivalent to 1 μ g of total RNA was reverse-transcribed using random primers according to the manufacturer's protocol (TaKaRa). The resulting complementary DNA was diluted and used as a PCR template to evaluate gene expression. Quantitative RT-PCR was conducted on an Eppendorf Mastercycler ep Realplex Real-Time Quantitative PCR System (Eppendorf) using SYBR Premix Ex Taq™ kits (TaKaRa) under the following conditions: pre-denaturation at 95°C for 30 s and forty cycles of 95°C for 5 s, 60°C for 30 s and 72°C for 15 s. A dissociation curve was constructed at the end of the reaction to ensure that only one amplicon was formed. Primers for the genes of interest were designed based on the pig (*Sus scrofa*) sequence (Table 1) using primer design software (Primer Premier 5.0; PREMIER Biosoft International). Amplification efficiencies were calculated based on the slope of the line ($E = 10^{(-1/\text{slope})} - 1$), considering an ideal value range ($0.95 \leq E \leq 1.05$). All experiments were repeated in triplicate. The geometric means of the relative abundances were normalised against porcine β -actin, glyceraldehyde 3-phosphate dehydrogenase and β -2-microglobulin genes using the $2^{-\Delta\Delta C_t}$ method⁽²¹⁾. Relative gene expressions in the weaned and NAC-treated piglets represented the comparison *v.* suckling piglets and were reported as a fold change from the value of the suckling piglets.

Statistical analyses

All data are presented as means with their standard errors. Differences among the examined groups were determined

using one-way ANOVA followed by Tukey's test. Values with $P < 0.05$ were considered as statistically significant. Trends were reported when $0.10 < P < 0.05$.

Results

Serum cortisol content

As shown in Fig. 1, cortisol concentrations were increased ($P < 0.05$) after weaning compared with the control piglets. Feeding the NAC-containing diet decreased cortisol concentrations ($P < 0.05$) compared with the weaned piglets. No significant differences in cortisol concentrations were found between the control and NAC-treated piglets ($P > 0.10$).

Oxidative stress induced by weaning

The activities of SOD and GSH-Px and the content of MDA in serum are presented in Table 2. The activities of GSH-Px in serum were significantly lower ($P < 0.05$) in the weaned piglets than in the control piglets. The activities of SOD also showed a trend to be lower ($P = 0.055$) in the weaning group compared with the control group. The content of MDA, the product of lipid peroxidation, was significantly increased ($P < 0.05$) in the piglets of the weaning group compared with those of the control group. Supplementing the diet with NAC increased the activities of SOD and GSH-Px ($P < 0.05$) compared with the weaning group and lessened ($P < 0.05$) the MDA content to levels that were not different from those observed in the control piglets.

Light microscopy findings

Representative histological graphs of haematoxylin and eosin-stained intestine samples of the control, weaning and NAC groups are shown in Fig. 2(E). The control specimens showed normal and typical leaf-like villus crypts. In contrast, villus height in the jejunum was significantly decreased ($P < 0.05$) in the weaning group, whereas villus width showed a small decrease ($P < 0.05$) in the weaned piglets

Table 2. Serum superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities and malondialdehyde (MDA) content in piglets of the control, weaning and N-acetyl cysteine (NAC) groups at 25 d of age

(Mean values with their pooled standard errors, $n = 5$)

| Items | Control* | Weaning† | NAC‡ | Pooled SE |
|---------------------------------|--------------------|--------------------|--------------------|-----------|
| SOD§ (units/ml serum) | 72.23 ^b | 47.79 ^c | 98.36 ^a | 13.61 |
| MDA (nmol/ml serum) | 4.52 ^b | 7.08 ^a | 4.58 ^b | 0.964 |
| GSH-Px (units/ μ l serum) | 1.28 ^a | 0.56 ^b | 1.27 ^a | 0.185 |

^{a,b,c}Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

* Piglets suckling from 14 to 25 d of age.

† Piglets weaned at 21 d of age and fed the basal diet.

‡ Piglets weaned at 21 d of age and fed the basal diet supplemented with 500 mg/kg of NAC.

§ One unit of SOD activity was defined as the amount required to inhibit the reduction in nitro blue tetrazolium by 50% of maximum inhibition in 1 ml serum.

|| One unit of GSH-Px activity was defined as a decrease of 1 μ mol/l of GSH per 5 min for 0.1 ml serum at 37°C after subtraction of the non-enzymatic reaction.

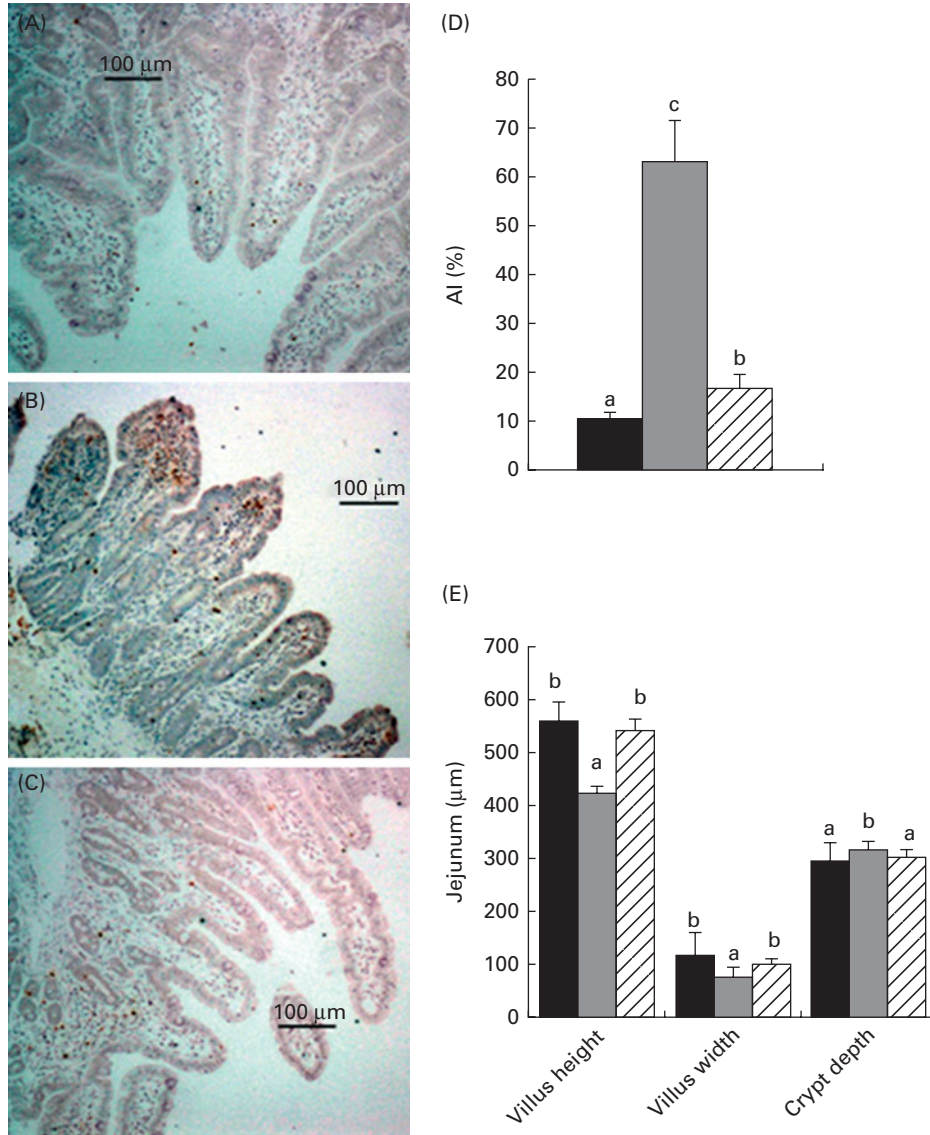


Fig. 2. Light micrographs of the jejunum of the (A) control, (B) weaning and (C) *N*-acetyl cysteine (NAC) groups stained using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling method. Magnifications: $\times 100$. Apoptotic nuclei are stained dark brown. Values are means, with their standard errors represented by vertical bars. ^{a,b,c} Mean values with unlike letters were significantly different ($P < 0.05$). (D) Apoptotic index (AI). (E) Histological evaluation of the jejunum of the control, weaning and NAC groups stained with haematoxylin and eosin. Control group (■), piglets suckled from 14 to 25 d of age. Weaning group (■), piglets weaned at 21 d of age and fed the basal diet. NAC group (▨), piglets weaned at 21 d of age and fed the basal diet supplemented with 500 mg/kg of NAC.

relative to the control piglets. In addition, the weaned piglets showed larger crypt depths ($P < 0.05$) than the control piglets. Feeding the NAC-containing diet increased ($P < 0.05$) villus height and villus width ($P < 0.05$) and decreased the crypt depths ($P < 0.05$) in the jejunum compared with the weaned piglets. The increased number of apoptotic cells (apoptotic nuclei are stained dark brown) with significant localisation of apoptotic cells in the upper tip of the intestinal villi (Fig. 2(A)–(C)) was revealed by the TUNEL assay in the weaned piglets. Moreover, the apoptotic index was higher in the weaned piglets ($P < 0.05$) than in the control piglets (Fig. 2(D)). A few apoptotic cells were present at the villus tips in the jejunum of the NAC group, and the apoptotic

index ($P < 0.05$) in the piglets fed the NAC diet was decreased compared with those of the weaning group.

Electron microscopy findings

Electron microscopic examination of the jejunal mucosa is shown in Fig. 3. In the weaning group, some enterocytes showed necrotic injuries characterised by serious damage to the plasma membrane, complete loss of junction specialisations between adjacent cells and swollen mitochondria without crests. Some other detached cells had characteristic features of apoptosis with vacuolated cytoplasm, cell compaction, condensed chromatin and fragmented mitochondria. No apoptotic features were found in the intestinal epithelia of the

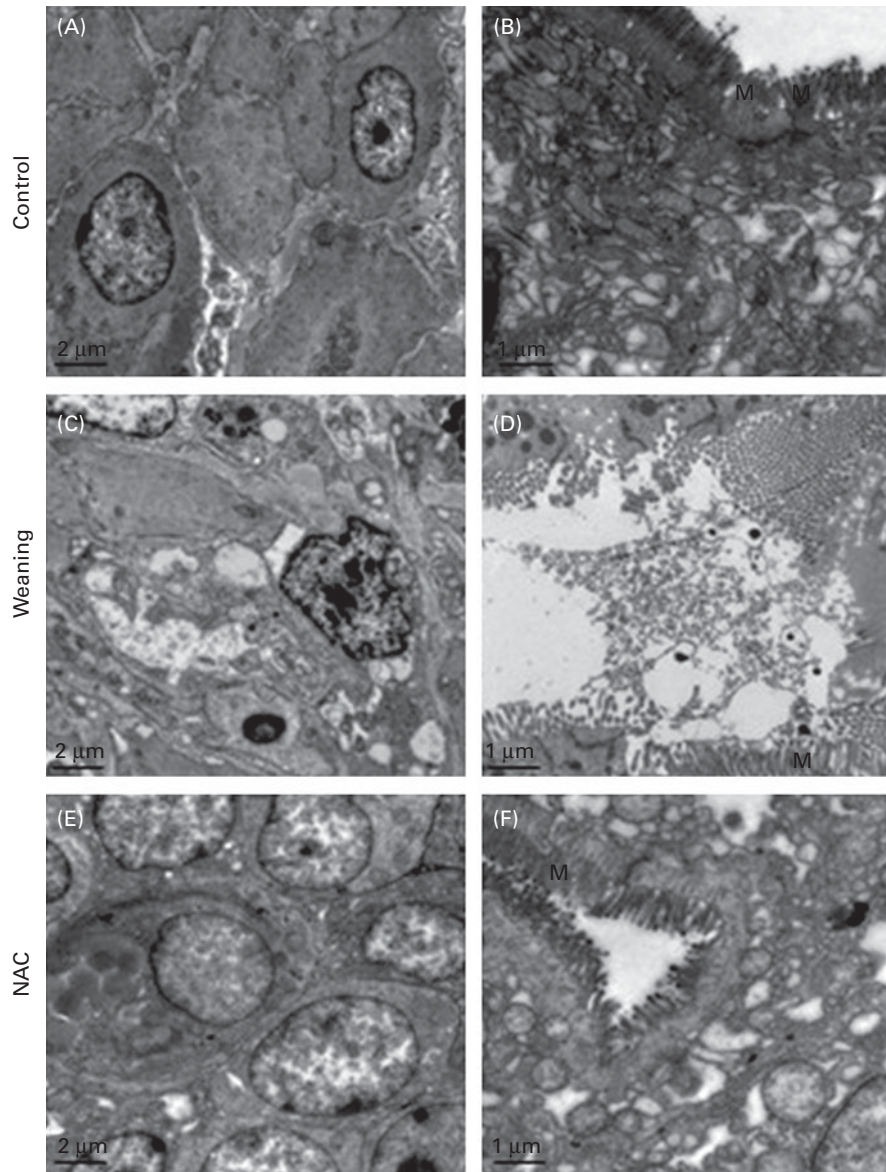


Fig. 3. Examination of jejunal mucosal injury by transmission electron microscopy. Control, piglets suckled from 14 to 25 d of age. Weaning, piglets weaned at 21 d of age and fed the basal diet. NAC, *N*-acetyl cysteine group, piglets weaned at 21 d of age and fed the basal diet supplemented with 500 mg/kg of NAC. (A) Most of the enterocytes were well connected with intact adherence, while several cells showed chromatin agglutination and mitochondrial ridge damage. (B) Microvilli were well developed. (C) Some epithelial cells showed compaction and segregation of chromatin against the nuclear envelope, disrupted basement membrane, autophagosomes and swollen mitochondria. Some enterocytes showed vacuolated cytoplasm, degenerated cell organelles (mitochondria and endoplasmic reticulum) and autophagosomes. (D) Microvilli loss. (E) Cell linkage was tight, but several enterocytes showed chromatin agglutination and presented signs of blebbing. (F) The microvilli were well developed and similar to those of the control piglets. Original magnification: $\times 5000$ (A, C, E); $\times 10\,000$ (B, D, F). M, microvilli.

control group except severe chromatin agglutination and mitochondrial ridge damage. The microvilli were well developed in the control piglets. In the NAC group, the microvilli were well developed, and the enterocyte ultrastructure was similar to that of the control group.

ELISA for caspase content

As shown in Table 3, the concentrations of caspase-3, caspase-8 and caspase-9 were strongly increased ($P < 0.05$) in the jejunum of the weaned piglets when compared with the control piglets, showing an increased apoptosis due to

the weaning treatment. The concentrations of caspase-3 and caspase-9 tended to decrease ($P = 0.092$ and $P = 0.075$) in the NAC-treated group compared with the weaning group. Compared with the control piglets, concentrations of caspase-3, caspase-8 and caspase-9 were still strongly increased ($P < 0.05$) after the NAC-containing diet treatment.

Quantitative RT-PCR analysis of apoptosis genes

Results for quantitative RT-PCR analysis are shown in Fig. 4. A significant increase ($P < 0.05$) in *caspase-3*, *caspase-8*, *Fas* and integrin $\alpha\beta 6$ (*av\beta 6*) expressions was observed in the weaned

Table 3. Jejunal tissue caspase concentrations in piglets of the control, weaning and *N*-acetyl cysteine (NAC) groups at 25 d of age (Mean values with their pooled standard errors, *n* 5)

| Items | Control* | Weaning† | NAC‡ | Pooled SE |
|---------------------------|--------------------|--------------------|--------------------|-----------|
| Caspase-3 (μg/mg protein) | 0.317 ^b | 0.650 ^a | 0.521 ^a | 0.084 |
| Caspase-8 (μg/mg protein) | 0.283 ^b | 0.524 ^a | 0.444 ^a | 0.045 |
| Caspase-9 (ng/mg protein) | 0.142 ^b | 0.302 ^a | 0.234 ^a | 0.038 |

^{a,b} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

* Piglets suckled from 14 to 25 d of age.

† Piglets weaned at 21 d of age and fed the basal diet.

‡ Piglets weaned at 21 d of age and fed the basal diet supplemented with 500 mg/kg of NAC.

piglets compared with the control piglets. No significant differences in the pro-apoptotic factor B-cell lymphoma-2 (*Bcl-2*)-associated X protein and *caspase-9* expressions ($P > 0.10$) were observed among the three groups, while weaning decreased ($P < 0.05$) the expression of the anti-apoptotic factor *Bcl-2* in the weaned piglets compared with the control piglets. The NAC-containing diet decreased ($P < 0.05$) the expressions of *caspase-3*, *caspase-8*, $\alpha v \beta 6$ and *Fas* compared with the weaning group. Compared with the weaning group, the expression of *Bcl-2* was increased ($P < 0.05$) in the NAC group.

Discussion

Cortisol is the primary glucocorticoid released during stress, which is normally increased for at least the first 24 h post-weaning in pigs, revealing the occurrence of a stress response⁽²²⁾. Antioxidant supplementation is able to decrease cortisol concentrations under stress^(22,23). In the present study, the increased cortisol concentrations after weaning suggest

that the piglets experienced stress at weaning. NAC, a well-known antioxidant⁽¹⁴⁾, tended to decrease cortisol concentrations after weaning, indicating that it has the potential to counteract weaning stress.

Stress, especially oxidant stress, that is caused by reactive nitrogen species and/or ROS is known to play a major role in the pathogenesis of tissue injury in many diseases, and the removal of free radicals may offer a treatment choice. SOD enzymes, as the major cellular antioxidants, can directly scavenge the excessive free radicals such as OH^- , H_2O_2 and O^{2-} by working in conjunction with other H_2O_2 -removing enzymes, such as GSH-Px⁽²⁴⁾. GSH, a potent endogenous antioxidant, is a first line of defence against free radicals and is often depleted as a consequence of the increased status of oxidative stress and inflammation⁽¹¹⁾. Lipid peroxidation, a free radical-related process, is a chain reaction leading to the oxidation of fatty acids, disrupting membrane structure and producing toxic metabolites such as MDA⁽²⁵⁾. In the present study, we observed higher lipid peroxidation and reduced SOD and GSH-Px activities in the weaned piglets compared with the control piglets, which is in agreement with previous findings in intestinal ischaemia–reperfusion injury in rats⁽²⁶⁾. We also demonstrated that weaning induced free radical generation, including NO and H_2O_2 , and decreased O_2 concentrations in serum⁽⁹⁾. Hence, in response to the increased production of free radicals after weaning, antioxidant enzymes (SOD and GSH-Px) may be broken down by free radicals. In addition, studies have reported that NAC has protective effects on the occurrence of oxidative stress resulting from ischaemia–reperfusion by preventing MDA, SOD and GSH-Px changes in animal models^(27,28). As a potent antioxidant, NAC directly scavenges H_2O_2 , OH^- and hypochloric acid *in vitro*⁽²⁹⁾ and exerts antioxidative effects by facilitating the

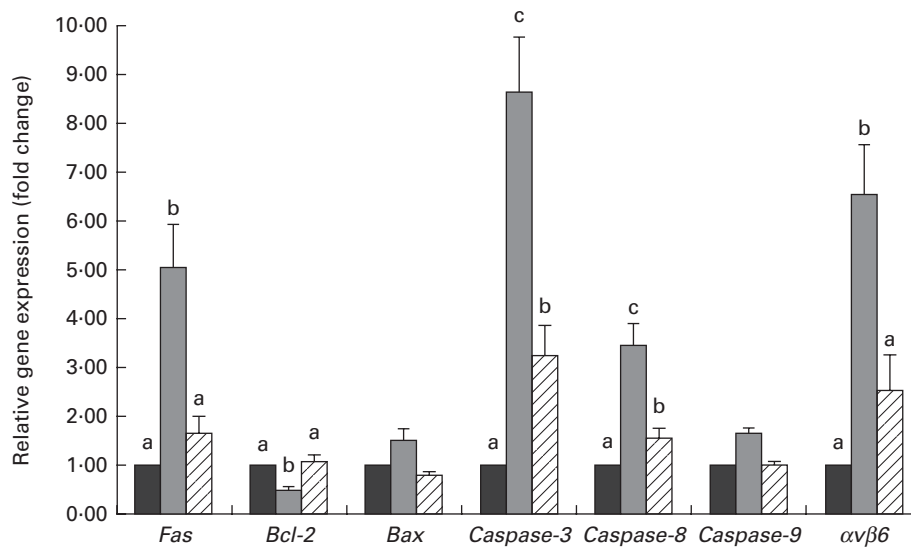


Fig. 4. Relative expression of apoptosis-related genes in piglets of the control, weaning and *N*-acetyl cysteine (NAC) groups at 25 d of age. Values are means, with their standard errors represented by vertical bars. ^{a,b,c} Mean values with unlike letters were significantly different ($P < 0.05$). mRNA levels in the weaning and NAC groups are presented as the multiples of gene expression level in the control group, which was set as 1.0. Control group (□), piglets suckled from 14 to 25 d of age. Weaning group (■), piglets weaned at 21 d of age and fed the basal diet. NAC group (▨), piglets weaned at 21 d of age and fed the basal diet supplemented with 500 mg/kg of NAC. *Bcl-2*, B-cell lymphoma-2; *Bax*, Bcl-2-associated X protein; $\alpha v \beta 6$, integrin $\alpha v \beta 6$.

generation of intracellular antioxidants, such as GSH⁽³⁰⁾. In the present study, administration of the NAC-containing diet to the weaned piglets significantly reduced lipid peroxidation and restored the SOD and GSH-Px activities to near-control levels; this is probably because of its free radical-scavenging activity or endogenous synthesis of GSH by NAC^(29,30). The high SOD activities in the NAC-treated piglets than in the control piglets might be due to the restoration of GSH by NAC and the early NAC supplementation for 12 d (from 14 to 25 d of age). It could be that the increased SOD and GSH-Px activities in serum may reflect the up-regulation of the antioxidant defence system in response to weaning stress in the NAC-treated piglets. These results suggest that NAC may have a protective effect on oxidative stress resulting from weaning in the piglet models.

As apoptosis plays a crucial role in free radical-induced gastrointestinal damage^(31,32), we examined enterocyte apoptosis and histopathological changes in the intestine in response to weaning stress and determined whether NAC supplementation could improve the integrity of intestinal mucosa in weaned piglets. Confirmation of apoptosis was based on the findings obtained from both morphological and biochemical measurements. The TUNEL assay and ELISA procedure confirmed an increased apoptosis in the intestinal mucosa of the weaned piglets. However, cell death occurs by two distinctly different pathways: programmed cell death (apoptosis) or necrosis⁽³³⁾. In the present study, transmission electron microscopy was used to distinguish apoptosis and necrosis, since necrosis is associated with histological signs of inflammation, whereas apoptosis is not. In the present study, concurrent necrosis and apoptosis were observed in the jejunum of the weaned piglets. These data provide an explanation for the clinical observation that piglets that are weaned tend to develop intestinal inflammation easily⁽³⁴⁾. This would also explain why there is an increase of pro-inflammatory cytokines in the intestine of the weaned piglets⁽³⁵⁾. These findings obtained from the TUNEL assay were confirmed by the noted increase in caspase-3, caspase-8 and caspase-9 concentrations through the ELISA method. Besides, we also found that the mucosal histology of piglets in the NAC group was preserved better than that of the piglets in the weaning group. Here, it seems that the potential wound-healing function of the NAC-containing diet is related to the preserved morphological integrity of intestinal mucosa.

There are two major apoptotic pathways: the intrinsic (mitochondria-dependent apoptosis) and the extrinsic (Fas-dependent apoptosis) pathways. The intrinsic pathway is mitochondria mediated and mainly characterised by the activation of caspase-9⁽³⁶⁾, whereas the extrinsic pathway involves caspase-8, which is activated through the activation of membrane death receptors, such as Fas⁽³⁷⁾. Both these pathways converge to a common execution phase of apoptosis that requires proteolytic activation of caspase-3, triggering a cascade of caspase activation⁽³⁸⁾. In the present study, the increased *caspase-3*, *caspase-8* and *Fas* expressions and the decreased anti-apoptotic *Bcl-2* expressions in piglets of the weaning group suggest that both mitochondria-dependent apoptosis and Fas-dependent apoptosis are induced in

epithelial cell apoptosis following the weaning treatment. The expressions of *caspase-3*, *caspase-8*, *Bcl-2* and *Fas* were reversed by the NAC-containing diet treatment. Thus, it appears that the *caspase-3*, *caspase-8*, *Bcl-2* and *Fas* genes may be regulated via redox-sensitive mechanisms in intestinal epithelial cells. However, no significant effect was observed on the expressions of *Bcl-2 associated X protein* and *caspase-9* in the NAC-treated piglets. This likely reflects the prolonged kinetics of apoptosis in epithelial cells. In addition, the intrinsic pathway is essential for the ROS-mediated apoptosis⁽³⁹⁾, but is not exclusive to this death pathway. In fact, ROS-mediated apoptosis is shared between mitochondria-dependent apoptosis and Fas-dependent apoptosis^(15,39). Evidence has proved that ROS scavengers, such as GSH, NAC and SOD, can cause the inhibition of Fas-induced apoptosis in different cell types^(15,40,41). In the present study, we observed that an increase in *Fas*, *caspase-8* and *caspase-3* expressions coincided with decreases in the GSH-Px and SOD activities in the weaned piglets, and these were reversed by the NAC-containing diet. These results suggest that the ability of NAC to inhibit cell death in the weaned piglets might be more inclined to decrease apoptosis via Fas-dependent apoptosis.

Integrin $\alpha\beta6$ is a well-known cell surface receptor and is not expressed constitutively by healthy adult epithelial cells, but is rapidly enhanced upon stimulation such as cell injury, cancer and inflammation, and its expression is maintained until wound closure is complete⁽⁴²⁾. High expression of $\alpha\beta6$ in colon cancer is associated with a more aggressive disease outcome, leading to the spread and development of cancer⁽⁴³⁾. In the present study, we showed a dramatic increase in the expression of $\alpha\beta6$ in the weaned piglets, which is consistent with the previous studies of gut challenged with nematodes⁽⁴⁴⁾. The high expression of $\alpha\beta6$ in the weaned piglets might provide an explanation for the clinical observation that weaned piglets with small-intestinal inflammation tend to have worse outcomes than diarrhoea⁽⁴⁵⁾. Furthermore, on blocking the function of $\alpha\beta6$ in colon cancer cells, apoptosis is enhanced and signalling involves the mitochondrial pathways⁽⁴⁶⁾. Enhanced mitochondrial ROS production is classically reported during cell apoptosis. Mitochondrial dysfunction promotes cancer cell migration through the induction of integrin $\alpha\beta5$ via an increase in mitochondria-generated ROS⁽⁴⁷⁾. In the case of weaned piglets, damage of the intestinal epithelium has been observed due to weaning stress via enhanced free radical generation⁽⁹⁾. Hence, we think that the increased apoptosis in the weaned piglets is associated with the expression of $\alpha\beta6$. Additionally, $\alpha\beta6$ is up-regulated during tissue remodelling, which occurs in epithelial repair as well as in cancer, but the timing and topology of the expression of $\alpha\beta6$ are important for normal repair⁽⁴⁸⁾. Partial inhibition of $\alpha\beta6$ may prevent pulmonary fibrosis without exacerbating inflammation⁽⁴⁹⁾. Here, the down-regulated expression of $\alpha\beta6$ in the NAC-treated piglets might indicate that the potential wound-healing function stimulated by NAC might be associated with the regulation of $\alpha\beta6$. However, the expression of $\alpha\beta6$ was still higher than that in the control piglets, as well as high caspase-3, caspase-8 and caspase-9 concentrations were

observed using the ELISA method. These data may reflect that further studies should be conducted to more completely determine the optimal dosage and time of NAC.

In conclusion, weaning could increase enterocyte apoptosis by activating both the intrinsic and extrinsic apoptotic pathways in post-weaning piglets. The NAC-containing diet used in the present study could preserve the morphological integrity of intestinal mucosa and inhibit cell death by reducing apoptosis via Fas-dependent apoptosis and $\alpha\beta6$ expression in weaned piglets. The signalling mechanisms still remain to be elucidated.

Supplementary material

To view supplementary material for this article, please visit <http://dx.doi.org/10.1017/S0007114513001608>

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