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Weaning-induced cell cycle arrest through up-regulation of p21 and p27 in the jejunum of piglets

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Abstract

This study was aimed at investigating the effects of weaning on jejunal redox status, morphology and expression of cell cycle regulators in piglets. A total of 120 new-born piglets from 12 litters were divided into two groups: the control (suckled normally) and weaning group (weaned at day 21). Six piglets from each group were slaughtered at day 22, day 25 and day 28. Results showed that weaning increased the serum hydrogen peroxide (H_2O_2) and malondialdehyde **(**MDA), decreased inhibitory hydroxyl radical (IHR) concentrations and superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities at day 25. Furthermore, the jejunal nitric oxide (NO) level was increased at days 22 and 28, while H_2O_2 concentration was increased at days 22, 25 and 28. Results of jejunum morphology showed weaning decreased villous height and width, and increased crypt depth at day 25. Relative mRNA and protein expression showed that Smad4 was decreased at day 25 and then increased at day 28. The expression of p21 and p27 were significantly increased by weaning at days 25 and 28, but cyclin D and cyclin E showed no significant differences compared with control group. In conclusion, weaning increased the serum and jejunal reactive oxygen species (ROS) production, inhibited jejunum development and cell cycle progression through down-regulation of Smad4 and up-regulation of p21 and p27 of piglets, which further explained weaning-induced stress syndrome.

Keywords: Proliferation, intestine, reactive oxygen species, weaning
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Introduction

Weaning is a systemic response that involves physiological, environmental and psychological changes in piglets, which lead to disturbances of intestinal barrier function and poor growth performance (Heo *et al*., 2013). Previous studies found that oxidative stress caused by weaning may be an important factor that affects intestinal dysfunction in piglets (Zhu *et al*., 2012; Yin *et al*., 2014). In addition, a previous study indicated that weaning stress induced overproduction of reactive oxygen species (ROS) and eventually contributed to apoptosis and inhibited cell proliferation in the small intestines of piglets (Zhu *et al*., 2014). In fact, eukaryotic cell proliferation is precisely regulated by cell cycle, which is controlled by a regulatory network. Cell cycle progression consists of four sequential phases: gap (G0/G1), synthesis (S), gap (G2), and mitosis (M), which are regulated by cyclins/cyclin-dependent kinase (CDK) and CDK inhibitory proteins (CKIs) such as p21 and p27 (Hunter, 1995). Cyclin D is the central regulator in the G1 phase, while cyclin E plays a critical role in inducing the G1→S transition (Hartwell & Kastan, 1994; Doonan & Kitsios, 2009). Negative regulators, such as p21 and p27, restrain the expression of these cyclins and the activities of cyclins/CDK complexes, inducing cell cycle arrest and consequently inhibiting cell proliferation (Cheng *et al*., 1999).

Intracellular redox status such as ROS is directly involved in cell division and cell cycle regulation (Delgado *et al*., 2015; Lallès *et al*., 2007). ROS can modulate cell cycle regulatory proteins and cell progress. High doses of ROS may cause growth arrest and cell death (Martindale *et al*., 2002). Oxidant-reduced expression of cyclin D1 has been found in fibroblast cells owing to the effects of H_2O_2 exposure (Barnouin *et al.,* 2002). A dramatic decrease in cyclin E-CDK2 activity induced by oxidants is described in lung alveolar

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epithelial cells. These results suggest that oxidants may block G1 entry into S phase by decreasing the cyclin D expression and activating cyclin E-CDK2 complexes due to CKI binding (Corroyer *et al*., 1996). Recent research using microarray analysis suggested that weaning may cause cell cycle progress change in the intestinal epithelial cells (Zhu *et al*., 2014). However, so far, studies about the changes of cell cycle regulatory proteins such as cyclins and CKIs in weaned piglets are scarce.

Therefore, this study aimed to explore the redox status, morphology change, and altered expression of cell cycle regulatory proteins in the jejunum of post-weaning piglets.

Materials and Methods

All protocols used in this experiment were approved by Guidelines for the Care and Use of Laboratory Animals, established by Shanghai Jiao Tong University Institutional Animal Welfare and Use Committee. About 120 new-born piglets (Duroc × Landrace × Large White) from 12 litters were randomly assigned by litter into a control group or a weaning group, resulting in six litters per group. Each litter was treated as a replicate. Piglets in the control group stayed with the sows in the farrowing unit and suckled until slaughtered, while the piglets in the weaning group were weaned at 21 days old. From 7 to 28 days old, all of

Table 1 Dietary composition and nutrient contents of the basic diet (dry basis)

Item	Amount
Ingredients (%)	
Corn	41.18
Whey powder	15.00
Extruded soybean	11.22
Lactose	8.75
Soybean meal, peeled	7.00
Soybean meal, fermented	5.00
Fish meal	5.00
Plasma protein	4.00
Monocalcium phosphate	0.90
Limestone	0.50
Vitamin premix[1]	0.50
Mineral/vitamin premix[2]	0.50
Sodium chloride	0.35
Choline	0.10
Calculated nutrient content	
DE (MJ/kg)	14.48
CP (N×6.25, %)	20.50
Calcium (%)	0.85
Total phosphorus (%)	0.67
Available phosphorus (%)	0.55
Lysine (%)	1.55
Methionine (%)	0.42
Methionine + cysteine (%)	0.83
Tryptophan (%)	0.27
Threonine (%)	1.01

[1] Provided/kg of mixed diet: vitamin A, 12 000 IU/kg; vitamin D3, 3 200 IU/kg; vitamin K3, 2.5 mg; vitamin E, 80 mg; vitamin B1, 2.5 mg; vitamin B2, 6.5 mg; vitamin B6, 5 mg; vitamin B12, 0.05 mg; niacin, 45 mg; D-pantothenic acid, 20 mg. [2] Provided/kg of mixed diet: folic acid, 1.5 mg; biotin, 0.15 mg; Fe, 150 mg as ferrous sulphate; Cu, 125 mg as copper sulphate; Zn, 200 mg as zinc oxide; Mn, 30 mg as manganese oxide; I, 0.3 mg as potassium iodide; and Se, 0.3 mg as sodium selenite

the piglets were allowed free access to a commercial creep-feed. The dietary ingredients and nutrition levels are shown in Table 1.

All piglets were kept in nursery pens and had free access to water during the experimental period. The room temperature was maintained constantly at approximately 25 ºC. At days 22, 25, and 28, one piglet (similar average bodyweights (BW)) from each litter was randomly selected and bodyweight was measured. The selected pigs were euthanized through an overdose of sodium pentobarbital intramuscularly (50 mg/kg BW). Blood samples were collected from the anterior vena cava, and serum was collected after centrifugation (3500 *g*, 15 min, 4 ºC). Samples were stored at -20 ºC pending antioxidant capacity determination. In parallel, the entire small intestines were carefully removed. Approximately one centimetre of jejunal was collected from the middle part of the jejunum. After washing in PBS, the samples were soaked in 4% triformol in PBS for the intestinal morphological assay. Meanwhile, the jejunal specimens were frozen in liquid nitrogen and stored at -80 ºC for quantitative real time-PCR and western blot analyses.

The activities of superoxide dismutase (SOD, EC 1.15.1.1) and glutathione peroxidase (GSH-Px, EC 1.11.1.9) as well as the malondialdehyde (MDA) concentration and inhibition capacity of hydroxyl radicals (IHR) in the serum were determined by using the enzymatic colorimetric methods according to the manufacturers' instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Briefly, SOD activity was determined with the hydroxylamine method by monitoring the inhibition of reduction of nitro blue tetrazolium and absorbance at 550 nm was recorded. GSH-Px activity was monitored with the 5,5-dithiobisp-nitrobenzoic acid method, spectrophotometrically at 412 nm. The concentration of MDA, which is an indicator of lipid peroxidation, was analysed with the thiobarbituric acid (TBA) method with an absorbance at 532 nm. H_2O_2 was analysed with the molybdic acid method at an absorbance of 405 nm. The concentration of IHR was detected with the Fenton's reaction method and the changes of absorbance were read at 550 nm. All absorbance levels were determined by spectrophotometric assays (Thermo Fisher Scientific, CA, USA).

The jejunal specimens were soaked in 4% neutral buffered formalin, then embedded in paraffin wax. The paraffin blocks were serially sliced into 5 μm thickness. They were then stained with routine haematoxylin-eosin for examination, including villus height, width and crypt depth of 20 well-oriented villi in each sample using an image processing and analysis system (Olympus Co., Tokyo, Japan).

A free-radical analyser (TBR 4100, World Precision Instruments, WPI) was used to record the NO and H_2O_2 levels in tissues. Electrodes of ISO-HOP-100 and ISO--NOPF-100 were used to monitor the NO and H_2O_2 in the intestine. Nitric oxide (NO) concentrations were determined using the NO electrode and the S-nitroso-N-acetyl-DL-penicillamine (0.2 to 500 nmol/L) combined with $CuCl_2$ catalysing was used to construct the calibration curves by the generation of a known amount of NO in the solution (Zhang *et al*., 2000). For the H_2O_2 measurements, the H_2O_2 electrode calibrated with known concentrations of H_2O_2 and H_2O_2-equilibrated solutions from WPI were used.

Table 2 Primer sequences used for quantitative RT-PCR

Gene	Sequence (5'–3')	Product (bp)	Reference
β-actin	F: GGACCTGACCGACTACCTCAT R: GGGCAGCTCGTAGCTCTTCT	181bp	[27]
GAPDH	F: AAGGAGTAAGAGCCCCTGGA R: TCTGGGATGGAAACTGGAA	140bp	[27]
Smad4	F: CGGTGTTGATGACCTTCG R: GGCAATAGGCATGGTATGA	200 bp	DQ_90438890
p21	F: GACCTGGGATGACCTGGGAG R: CGGCGTTTGGAGTGGTAGAA	221 bp	XM_003361105.2
p27	F:GTGAGAGTGTCTAACGGGAGC R:GGGTCTGTAGTAGAACTCGGGCAAG	219 bp	NW_003541232
Cyclin D	F:TTACCTGGACCGCTTCTTG R:GAGGCTTGATGGAGTTGTCG	192 bp	EF_908776.1
Cyclin E	F:AGAAGGAAAGGGATGCGAAGG R:AGTGGCGAGGCTCTGGATG	299 bp	XM_005653265.1

p21 and p27: cyclin-dependent kinase (CDK) inhibitory proteins

The total RNA from jejunal tissues was extracted with TRIzol reagent (Invitrogen, USA) and quantified by spectrophotometry (Nano Drop Lite, Thermo Scientific). Ratios of OD260 and OD280 between 1.8 and 2.0 were considered high quality. The PCR reaction was performed in a volume of 20 μL with an ABI Plus 7500 System (Applied Biosystems, USA) by SYBR Premix Ex Taq kits (TaKaRa, Japan). The PCR program was performed as follows: 95 °C for 30 sec, cycled for 40 cycles at 95 °C for 5 sec, and 60 °C for 30 sec. Internal controls of β-actin and glyceraldehyde 3-phosphate dehydrogenase (GADPH) were used to normalize target-gene expression using the equation $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001). The primers designed for RT-PCR are listed in Table 2**.** Agarose gel electrophoresis was used to verify the amplification products.

For western blot analysis, samples were homogenized in RIPA lysis buffer (KeyGEN Biotech, Nanjing, China) and protein concentrations in the jejunal tissues were measured by BCA analysis. Protein (30 mg) was loaded and separated by SDS-polyacrylamide gels electrophoresis. The proteins were then transferred to nitrocellulose membranes. Mouse monoclonal cyclin D, cyclin E, Smad4, p27 and p21 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA) were diluted 1 : 200 and incubated with the membrane for one hour. After washing, anti-mouse IgG labelled with horseradish peroxidase was incubated and diluted to 1 : 500. The results were visualized with the ECL detection system (Amersham Biosciences).

All data are presented as means with standard error. Statistical analysis was performed with the independent sample *t*-test method to compare values between the control and weaning groups, using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistical significance.

Results

Compared with the control group, the BWs of piglets were not significantly different ($P > 0.05$) in weaning group at day 22. But weaning significantly decreased ($P < 0.05$) the BW of piglets at day 25 and day 28 (Figure 1).

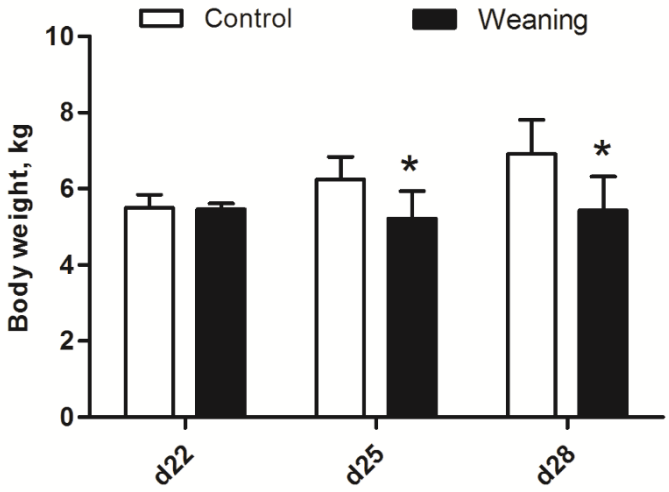


Figure1 Bodyweights of piglets during the whole weaning period
*Means are significantly different between the control and weaning groups ($P < 0.05$)

The effects of weaning on the oxidative stress parameters in serum of piglets are shown in Figure 2. Compared with the control group, the concentration of serum H_2O_2 in the weaning group was significantly increased ($P < 0.05$) at day 25. There were no significant differences ($P > 0.05$) between these two groups at days 22 and 28. The results of antioxidant enzymes showed that the activities of serum SOD showed no difference ($P > 0.05$) between these two groups. Compared with control group, the activity of serum GSH-Px was significantly decreased ($P < 0.05$) in the weaning group at day 25, but not different ($P > 0.05$) at days 22 and 28. The concentration of IHR was significantly decreased ($P < 0.05$) in the weaning group at days 25 and 28, but not different ($P > 0.05$) at day 22. Results of oxidative injury showed that weaning significantly increased ($P < 0.05$) the serum MDA at days 25 and 28. No significant difference ($P > 0.05$) about concentration of MDA was observed at day 22 between the two groups.

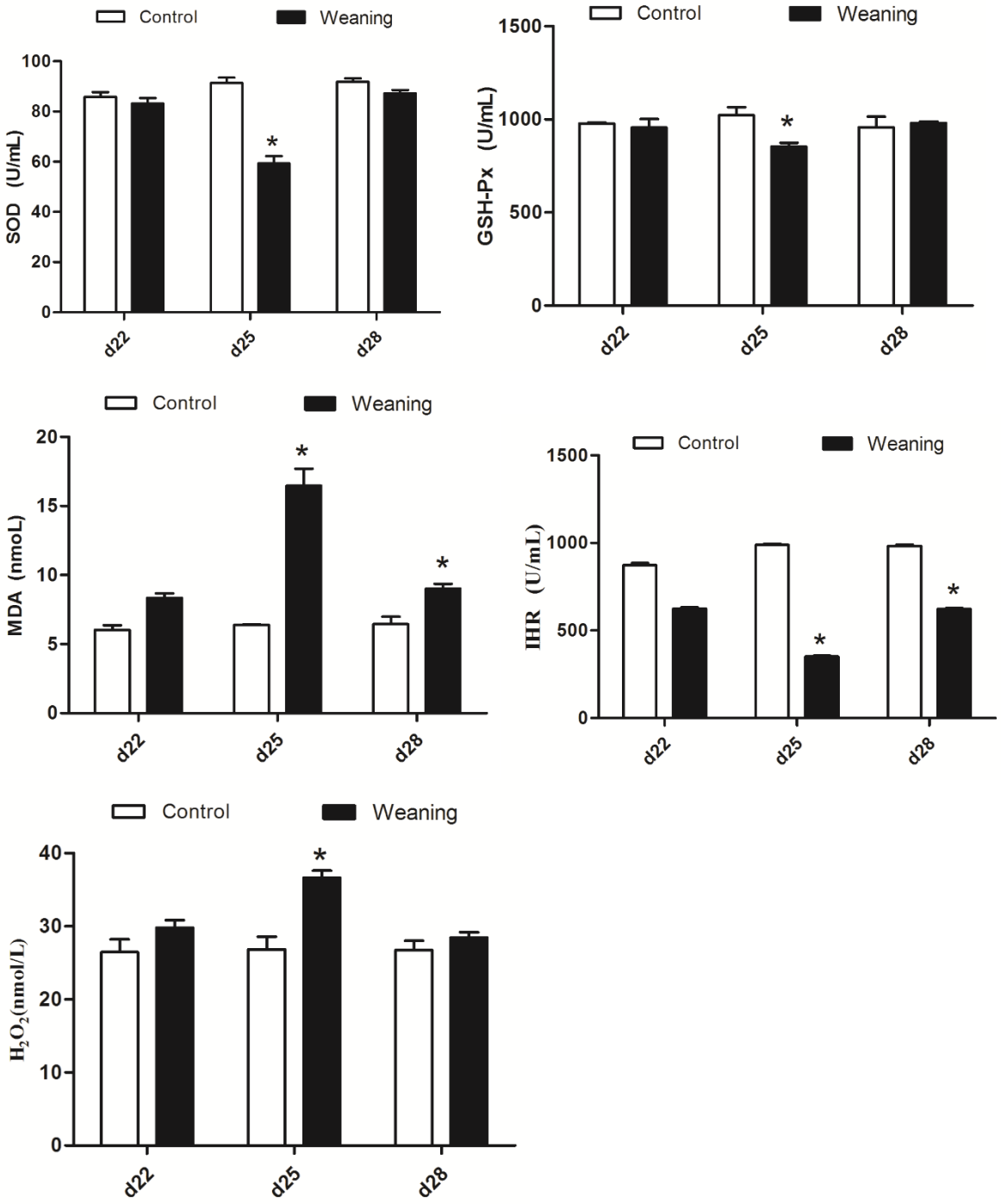


Figure 2 Serum oxidative stress parameters (SOD: superoxide dismutase; GSH-Px: glutathione peroxidase; MDA: malondialdehyde; IHR: inhibitory hydroxyl radical and H_2O_2: hydrogen peroxide) in piglets after weaning

*Means are significantly different between the control and weaning groups (P <0.05)

As shown in Figure 3, compared with the control group, the concentration of NO in the jejunum was significantly increased (P <0.05) in the weaning group at s 22 and 28. No significant difference (P >0.05) was observed at day 25. The concentration of H_2O_2 in the jejunum was significantly increased (P <0.05) in the weaning group at days 22, 25 and 28.

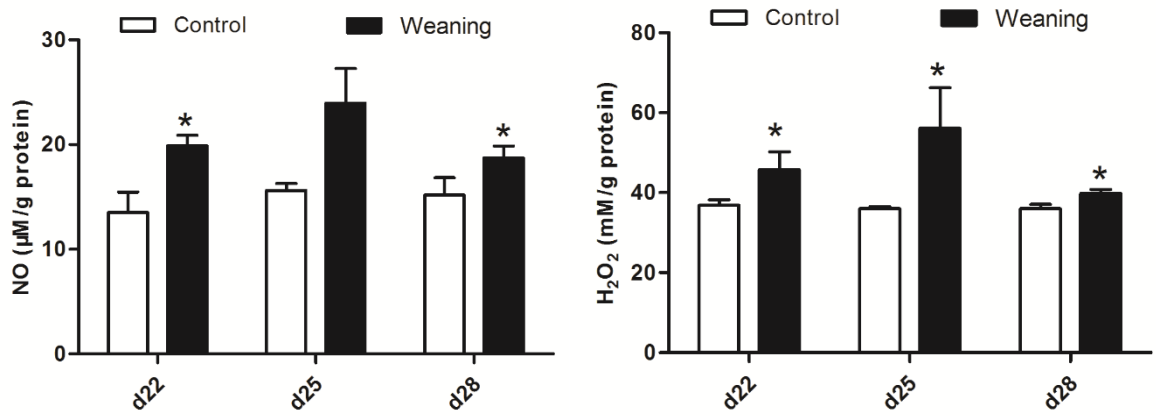


Figure 3 Concentrations of nitric oxide（NO） and hydrogen peroxide（H_2O_2） in jejunum of piglets after weaning
*Means are significantly different between the control and weaning groups (P <0.05)

As shown in Figure 4 and Table 3, compared with the control group, weaning significantly decreased (P <0.05) the villus heights and width, increased (P <0.05) the crypt depths at day 25. No significant differences (P >0.05) of morphology were observed at day 22 and day 28 between the two groups.

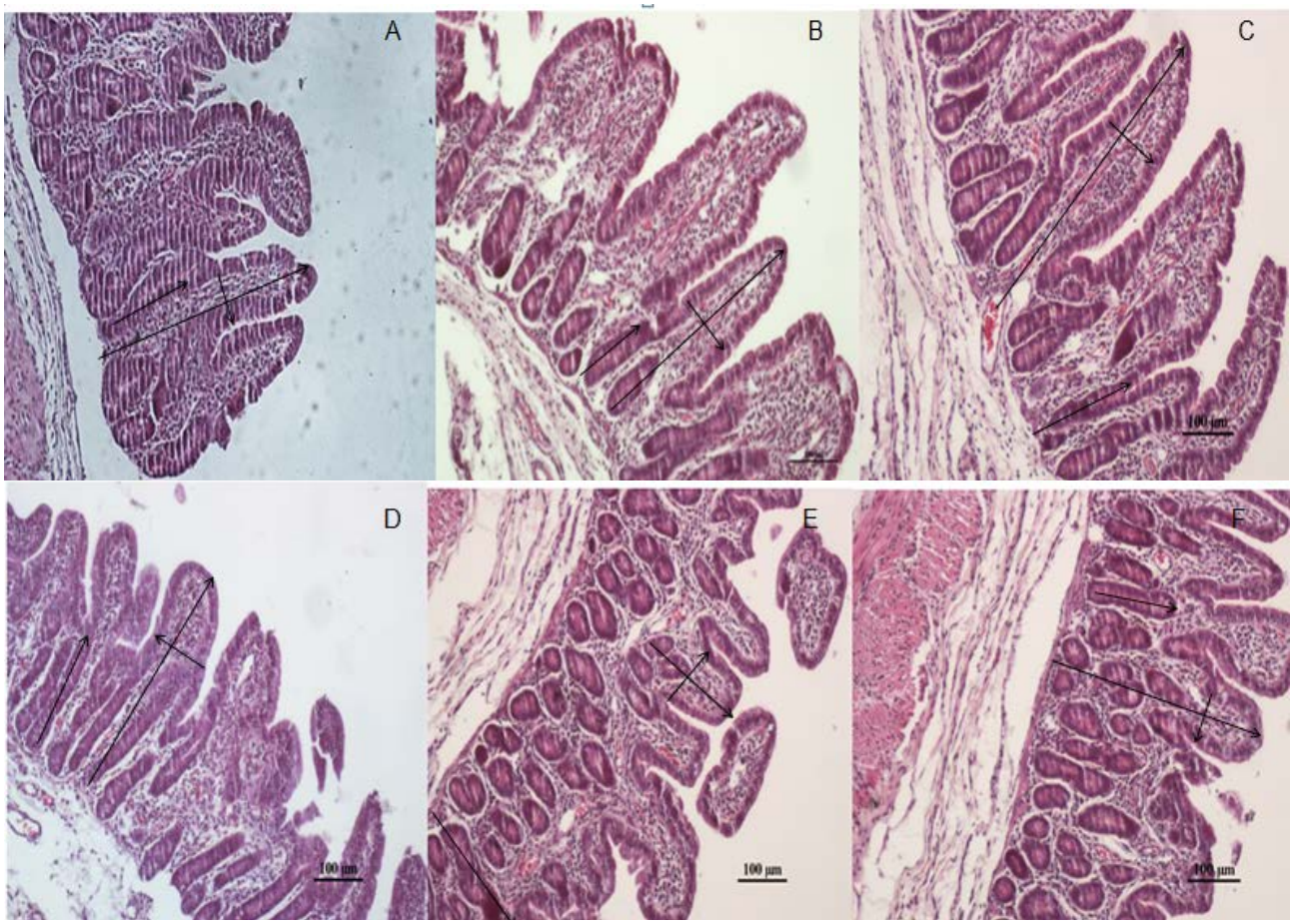


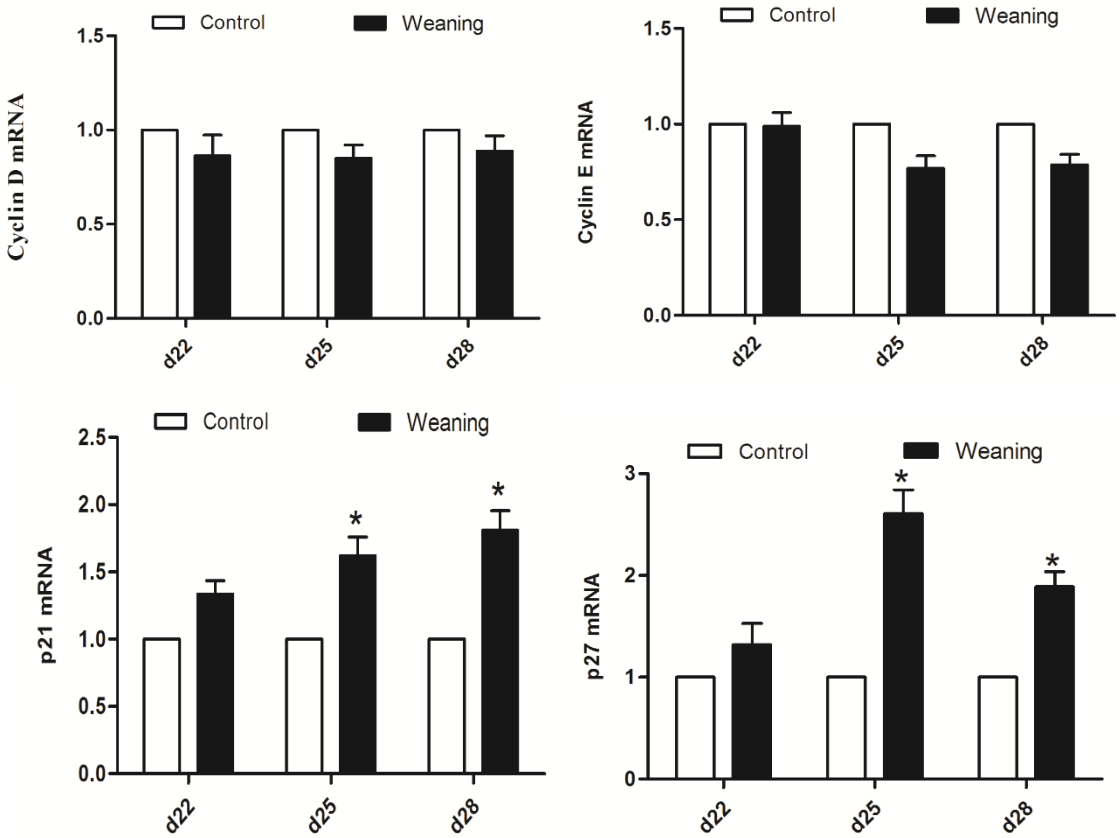
Figure 4 Histological analysis of jejunum of piglets after weaning
(A-C) Histological changes in the control group at day 22, day 25 and day 28, respectively
(D-F) Histological changes in the weaning group at day 22, day 25 and day 28, respectively

Table 3 Histological evaluation of jejunal tissues after weaning

	Control	Weaning
day 22 (μm)		
Villus height	349.84 ± 19.91	344.12 ± 15.71
Villus width	101.63 ± 7.37	91.91 ± 8.17
Crypt depth	116.78 ± 9.10	124.45 ± 10.48
day 25		
Villus height	351.07* ± 19.89	304.25 ± 19.96
Villus width	102.91* ± 12.37	86.08 ± 5.92
Crypt depth	120.95 ± 9.48	153.79* ± 12.7
day 28		
Villus height	356.52 ± 28.64	333.2 ± 27.16
Villus width	105.28 ± 8.05	98.92 ± 5.52
Crypt depth	117.89 ± 5.82	123.88 ± 12.99

*Means are significantly different between the control and weaning groups at the same day (P <0.05)

The mRNA expression of cyclin D, cyclin E, p21, p27 and Smad4 are presented in the jejunum (Figure 5). The mRNA expression levels of cyclin D and cyclin E were not significantly different (P >0.05) between the two groups. Compared with the control group, relative mRNA expressions of p21 and p27 were significantly higher (P <0.05) in the weaning group at day 25, but not different at days 22 and 28 (P >0.05). The relative mRNA expression of Smad4 was significantly decreased in the weaning group at day 25 (P <0.05), but was increased (P <0.05) at day 28.



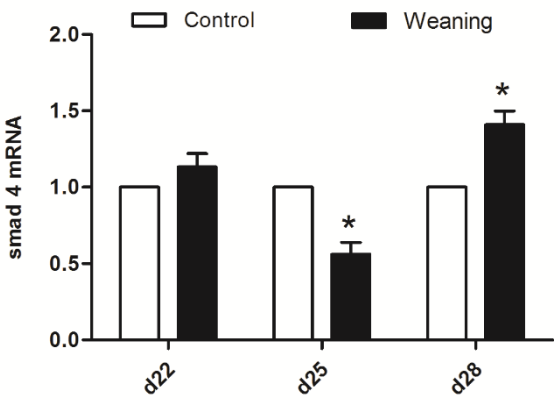
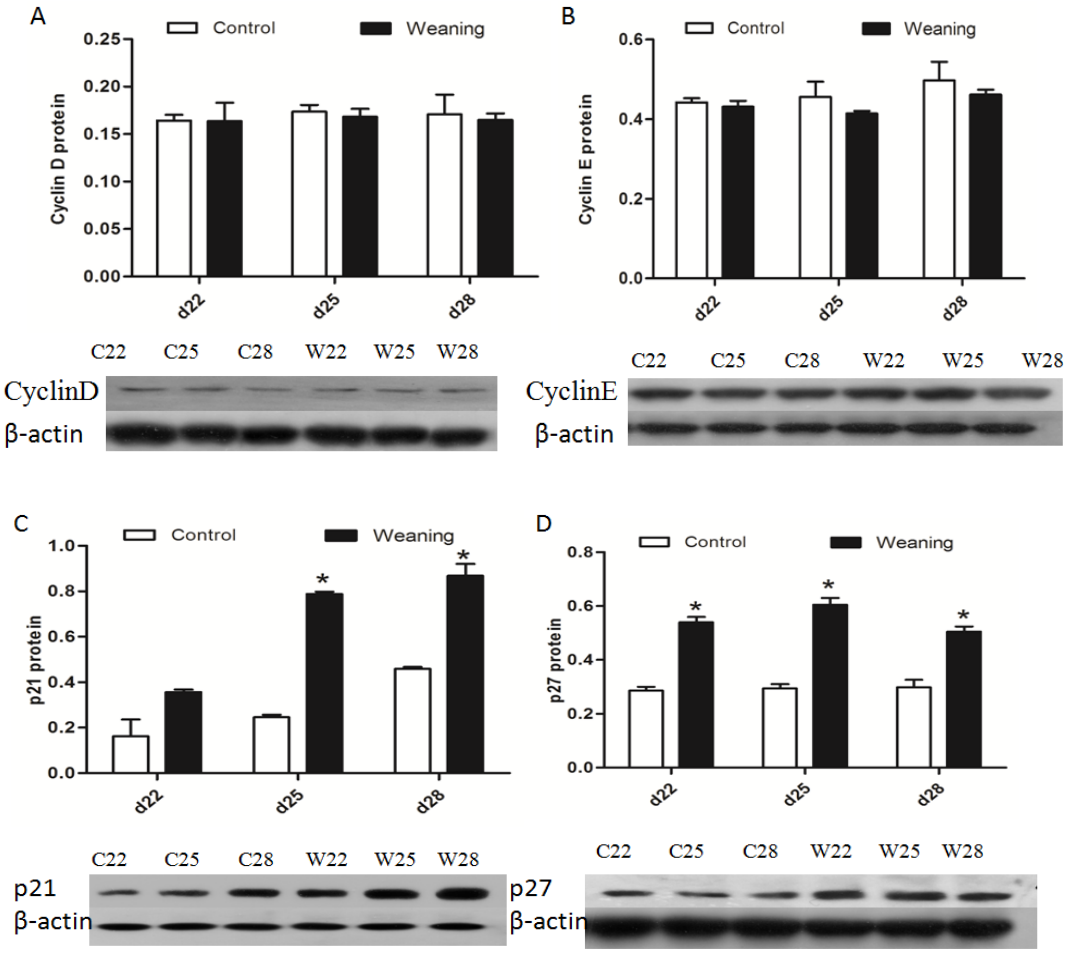


Figure 5 Relative mRNA expression of cell cycle regulators in jejunum of piglets after weaning
p21 and p27: cyclin-dependent kinase (CDK) inhibitory proteins
*Means are significantly different between the control and weaning groups (*P* <0.05)

Protein expression levels of cell cycle regulators in the jejunum are shown in Figure 6. Protein expression levels of cyclin D and cyclin E were not significantly different (*P* >0.05) between these groups. Compared with the control group, p21 and p27 expressions were significantly increased (*P* <0.05) in the weaning group at day 25 and 28. Furthermore, the protein expression of p27 was significantly increased (*P* <0.05) at day 22. The expression levels of Smad4 in the weaning group were significantly decreased (*P* <0.05) at day 25 but increased significantly (*P* <0.05) at day 28.



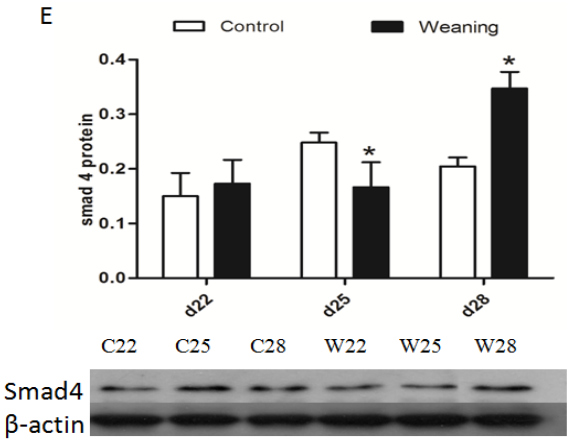


Figure 6 Protein expression of cell cycle regulators in jejunum of piglets after weaning
*Means are significantly different between the control and weaning groups (P <0.05)
C, control group; W, weaning group. (A) Cyclin D, (B) Cyclin E, (C) p21, (D) p27, (E) Smad4

Discussion

Early weaning has been reported to induce mucosal shedding and villous atrophy, resulting in barrier dysfunction and disorders in the small intestines of piglets (Pluske *et al*., 1997). In the present study, results showed that the concentrations of free radicals were increased, and the activities of antioxidant enzymes were decreased in weaning piglets. Oxidative stress parameters demonstrated the intracellular redox state was imbalanced and more seriously so in the weaning piglets at day 25. This is quite critical because excessive ROS production can produce significant damage to cell structure (Zhu *et al*., 2013). The decreased villus height, width and increased crypt depth were found in the jejunum of weaning piglets in the current study. In agreement with earlier reports, the morphological analyses of the current result indicated that weaning stress could result in villus shortening and crypt hyperplasia in the intestines of pig. Many researchers found that the upregulation expression of cell cycle inhibitory protein such as p21 and p27 could lead to cell cycle arrest which can cause growth retardation (Burch *et al*., 2005). Thus, the findings of the current study indicated that ROS-induced cell cycle arrest can be a mechanism resulting in the loss of jejunum development in post-weaning piglets.

In mammalian cells, intracellular ROS are reported to modulate expression of cell cycle regulators such as cyclin D and cyclin E (Martinez *et al*., 2002). The transcription of cyclin D1 can be regulated by the transcription factors such as Ap-1 and Sp1 interacting with binding sites in the promoter region in response to oxidants (Menon *et al*., 2007). In the study by Burch *et al.* (2004), oxidative stress can also inhibit entry of Fra-1 (it may be the most potent activator in regulating cyclin D1 expression) into the nucleus and block cyclin D1 expression. Hence, the redox state could significantly influence transcription and expression of cyclin D and cyclin E.

The activation of MAPK signalling pathway enhances the growth-inhibitory function of p21 and p27 in response to oxidative stress (Bianco *et al*., 2011). Chung *et al.* (2002) observed that H_2O_2 induced up-regulation of p21, which leads to G2/M phase arrest in H1299 cells. In addition, high ROS levels can lead to adverse cellular events such as inhibition of proliferation and autophagy via the p53/p21 axis, which can induce cell cycle arrest (Gudkov & Komarova, 2003; Gongpan *et al*., 2016). Zhu *et al.* (2012) reported that the redox-active transcription factor, p53, was up-regulated in the small intestines of post-weaning piglets. It is indicated that up-regulation of p53 contributed to p21 protein accumulation with G_0/G_1 phase cell cycle arrest in MCF-7cells (Agrawal *et al*., 1996). Active development of stem cell in intestines is also controlled precisely by Smad signalling pathways. Smad4 is found to be a key transcript modulator in gastrointestinal epithelial regeneration (Lee & Bae, 2002; Cui & Chang, 2016). Additionally, p21 acts as a downstream effector of Smad4 (Bauer *et al*., 2016). Datto *et al* (1995) reported that growth arrest was regulated by the reduction of Smad4, which increased the expression of p21 in intestinal stem cells. Furthermore, Wang *et al.* (2011) reported that knockdown of Smad4 significantly inhibited proliferation of porcine granulosa cell and resulted in G0/G1 arrest, suggesting the important regulation role of Smad4 in cell growth and development. In the present study, results of qRT-PCR and western blot analysis showed that Smad4 were significantly decreased in the jejunum of weaning piglets at day 25, while p21 and p27 expressions were significantly increased. In addition, the expression of Smad4 in the weaning group increased at day 28 probably

suggested that the inhibitory role was gradually relieved, caused by weaning. Our histological evaluation results also suggested that villus height and crypt depth of weaning piglets have recovered to the preweaning values. A study has also reported that the overproduction of ROS can regulate the protein expression of p27, which inhibits S-phase entry and induces the DNA-damage (Fikaris *et al*., 2006). In some reports, the transcription of p27 also could be mediated by forkhead box O (Foxo) (Agrawal *et al*., 1996). As the transcription factor, Foxo3a, enters the nucleus and controls the expression of genes involved in cell cycle withdrawal in response to oxidative stress (Sedding, 2008), DNA-damage and cell cycle withdrawal are associated with the loss of intestinal development. These findings are in accordance with the current study.

Yin *et al.* (1999) reported that p21 could inactivate cyclin D/CDK complexes following H_2O_2 stimulation. Cheng *et al.* (1999) suggested that tensile forces mediate growth inhibition in the G0/G1 phase through decreasing the expression of cyclin D and cyclin E in murine fibroblasts. Sheaff *et al.* (1997) demonstrated that increase of oxidants also induced p27 to bind to cyclin E/CDK2 complex, and inhibited cyclin E activity, consequently leading to cell arrest at G1/S phase. In the present study, cyclin D and cyclin E protein abundance and mRNA expression in the intestinal mucosa are slightly lower in weaned piglets than in the control piglets. Similarly, Yin *et al.* (2016) indicated that cyclin A was reduced in weaning pigs, and they suggested that cell cycle arrest occurred. In the present study, p21 and p27 at the mRNA and protein levels were significantly increased in the weaning group compared with the control group at day 25. In accordance with the present finding, an earlier study in mice showed that Barnouin *et al.* (2002) observed a transient multi-phase cell cycle arrest through reduction of cyclin D and p21 up-regulated expression induced by H_2O_2. Here, the intestinal morphological changes induced by weaning decreased villus height, decreased width, and crypt hyperplasia. The results of the present study demonstrated that the intestinal epithelial cell proliferation in weaning piglets was regressed, probably because p21 and p27 inhibited the activities of cyclin E and cyclin D complexes and mediated intestinal epithelial cell cycle arrest.

Conclusion

In conclusion, weaning induces overproduction of ROS, which may contribute to cell cycle arrest through the up-regulation of p21 and p27. Smad4 also mediates the up-regulation of p21.The p21 and p27 may inhibit the activities of cyclin D and cyclin E complexes and suppress cell cycle progression at G_1, thereby inhibited intestinal epithelial cell proliferation and caused the loss of development in weaning piglets. Further studies are needed to investigate how ROS acts as signalling molecules regulating proliferation and growth arrest in weaning piglets.

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Conflict of interest

The authors declare that they have no competing interests.

References

Agrawal, D., Hauser, P., Mcpherson, F., Dong, F. & Garcia, A., 1996. Repression of p27kip1 synthesis by platelet-derived growth factor in BALB/c 3T3 cells. Mol. Cell Bio. 16, 4327-4336.

Barnouin, K., Dubuisson, M.L., Child, E.S., de Mattos, S.F., Glassford, J., Medema, R.H., Mann, D.J. & Lam, E.W.F., 2002. H_2O_2 induces a transient multi-phase cell cycle arrest in mouse fibroblasts through modulating cyclin D and p21Cip1 expression. J. Biol. Chem. 277, 13761-13770.

Bauer, J., Staudacher, J.J., Krett, N.L. & Jung, B., 2016. Commentary: Activin and TGFbeta use diverging mitogenic signaling in advanced colon cancer. J. Rare Dis. Res. Treat. 1, 43-45.

Bianco, M.R., Berbenni, M., Amara, F., Viggiani, S., Fragni, M., Galimberti, V., Colombo, D., Cirillo, G., Papa, M., Alberghina, L. & Colangelo, A.M., 2011. Cross-talk between cell cycle induction and mitochondrial dysfunction during oxidative stress and nerve growth factor withdrawal in differentiated PC12 cells. J. Neurosci. Res. 89, 1302-1315.

Blagosklonny, M.V. & Pardee, A.B., 2002. The restriction point of the cell cycle. Cell Cycle 1, 102-109.

Burch, P.M. & Heintz, N.H., 2005. Redox regulation of cell-cycle re-entry: cyclin D1 as a primary target for the mitogenic effects of reactive oxygen and nitrogen species. Antioxid. Redox. Signal. 7, 741-751.

Burch, P.M., Yuan, Z., Loonen, A. & Heintz, N.H., 2004. An extracellular signal-regulated kinase 1- and 2-dependent program of chromatin trafficking of c-Fos and Fra-1 is required for cyclin D1 expression during cell cycle re-entry. Mol. Cell Biol. 24, 4696-4709.

Cheng, M., Olivier, P., Diehl, J.A., Fero, M., Roussel, M.F., Roberts, J.M. & Sherr, C.J., 1999. The p21Cip1 and p27Kip1 CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts. EMBO J. 18, 1571-1583.

Corroyer, S., Maitre, B., Cazals, V. & Clement, A., 1996. Altered regulation of G1 cyclins in oxidant-induced growth arrest of lung alveolar epithelial cells. Accumulation of inactive cyclin E-CDK2 complexes. J. Biol. Chem. 271, 25117-25125.

Chung, Y.W., Jeong, D.W., Won, J.Y., Choi, E.J., Choi, Y.H. & Kim, I.Y., 2002. H_2O_2-induced AP-1 activation and its effect on p21 WAF1/CIP1-mediated G2/M arrest in a p53-deficient human lung cancer cell. Biochem. Biophys. Res. Commun. 293, 1248-1253.

Cui, S. & Chang, P.Y., 2016. Current understanding concerning intestinal stem cells. Wrld J. Gastroenterol. 22, 7099-7110.

Datto, M.B., Li, Y., Panus, J.F., Howe, D.J., Xiong, Y. & Wang, X.F., 1995. Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. Proc. Natl. Acad. Sci., USA. 92, 5545-5549.

Delgado, M.E., Grabinger, T. & Brunner. T., 2015. Cell death at the intestinal epithelial front line. FEBS. J. 283, 2701-2709.

Doonan, J.H. & Kitsios, G., 2009. Functional evolution of cyclin-dependent kinases. Mol. Biotechnol. 42, 14-29.

Fikaris, A.J., Lewis, A.E., Abulaiti, A., Tsygankova, O.M. & Meinkoth, J.L., 2006. Ras triggers ataxia-telangiectasia-mutated and Rad-3-related activation and apoptosis through sustained mitogenic signaling. J. Biol. Chem. 281, 34759-34767.

Gongpan, P., Lu, Y., Wang, F., Xu, Y. & Xiong, W., 2016. AS160 controls eukaryotic cell cycle and proliferation by regulating the CDK inhibitor p21. Cell Cycle 15, 1733-1741.

Gudkov, A.V. & Komarova, E.A., 2003. The role of p53 in determining sensitivity to radiotherapy. Nat. Rev. Cancer 3, 117-129.

Hartwell, L.H. & Kastan, M.B., 1994. Cell cycle control and cancer. Science 266, 1821-1828.

Heo, J.M., Opapeju, F.O., Pluske, J.R., Kim, J.C., Hampson, D.J. & Nyachoti, C.M., 2013. Gastrointestinal health and function in weaned pigs: A review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. J. Anim. Physiol. Anim. Nutr. 97, 207-237.

Hunter, T., 1995. Protein kinases and phosphatases: The yin and yang of protein phosphorylation and signaling. Cell 80, 225-236.

Lallès, J.P., Bosi, P., Smidt, H. & Stokes, C.R., 2007. Nutritional management of gut health in pigs around weaning. Proc. Nutr. Soc. 66, 260-268.

Lee, K.Y. & Bae, S.C., 2002. TGF-beta-dependent cell growth arrest and apoptosis. J. Biochem. Mol. Biol. 35, 47-53.

Livak, K.J. & Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and 2 method. Methods 25 ,402-408.

Martindale, J.L. & Holbrook, N.J., 2002. Cellular response to oxidative stress: signaling for suicide and survival. J. Cell Physiol. 192, 1-15.

Martinez Munoz, C., Post, J.A., Verkleij, A.J., Verrips, C.T., & Boonstra, J., 2002. The effect of hydrogen peroxide on the cyclin D expression in fiborblasts. CMLS. 58, 990-996.

Pluske, J.R., Hampson, D.J. & Williams, I.H., 1997. Factors influencing the structure and function of the small intestine in the weaned pig: a review. Livest. Prod. Sci. 51, 215-236.

Menon, S.G., Sarsour, E.H. & Kalen, A.L., 2007. Superoxide signalling mediates N-acetyl-L-cysteine-induced G1 arrest: regulatory role of cyclin D1 and manganese superoxide dismutase. Cancer Res. 67, 6392-6399.

Sedding, D.G., 2008. FoxO transcription factors in oxidative stress response and ageing--a new fork on the way to longevity? Biol. Chem. 389, 279-283.

Sheaff, R.J., Groudine, M., Gordon, M., Roberts, J.M. & Clurman, B.E., 1997. CyclinE-CDK2 is a regulator of p27Kip1. Genes Dev. 11, 1464-1478.

Wang, W., Chen, X., Li, X., Wang, L., Zhang, H., He, Y., Wang, J., Zhao, Y., Zhang, B. & Xu, Y., 2011. Interference RNA-based silencing of endogenous SMAD4 in porcine granulosa cells resulted in decreased FSH-mediated granulosa cells proliferation and steroidogenesis. Reproduction 141, 643-651.

Yang, H., Wang X., Xiong, X. & Yin, Y., 2016. Energy metabolism in intestinal epithelial cells during maturation along the crypt-villus axis. Sci. Rep. 6, 31917-31923.

Yin, J., Wu, M.M., Xiao, H., Ren, W.K., Duan, J.L., Yang, G., Li, T.J. & Yin, Y.L., 2014. Development of an antioxidant system after early weaning in piglets. J. Anim. Sci. 92, 612-619.

Yin, Y., Solomon, G., Deng, C. & Barrett, J.C., 1999. Differential regulation of p21 by p53 and Rb in cellular response to oxidative stress. Mol. Carcinog. 24, 15-24.

Zhu, L., Cai, X., Guo, Q., Chen, X., Zhu, S. & Xu, J., 2013. Effect of N-acetyl cysteine on enterocyte apoptosis and intracellular signalling pathways' response to oxidative stress in weaned piglets. Br. J. Nutr. 110, 1938-1947.

Zhu, L.H., Zhao, K.L., Chen, X.L. & Xu, J.X., 2012. Impact of weaning and an antioxidant blend on intestinal barrier function and antioxidant status in pigs. J. Anim. Sci. 90, 2581-2589.

Zhu, L.H., Xu, J.X., Zhu, S.W., Cai, X., Yang, S.F., Chen, X.L. & Guo, Q., 2014. Gene expression profiling analysis reveals weaning-induced cell cycle arrest and apoptosis in the small intestine of pigs. J. Anim. Sci. 92, 996-1006.