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ORIGINAL RESEARCH

X/XO or H_2O_2 induced IPEC-J2 cell as a new in vitro model for studying apoptosis in post-weaning piglets

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Abstract We previously demonstrated that intestinal epithelial cell apoptosis in weaned piglets is much more serious than that observed in sucking piglets and is related to oxidative stress during weaning. It is difficult to study the apoptosis mechanisms only using in vivo methods because of the limit of existing research technology. An in vitro cellular system is required for piglet intestinal epithelial cell apoptosis research. In this study, a non-tumorigenic epithelial cell line, IPEC-J2 cells, was employed as a cell model. Hydrogen peroxide and xanthine/xanthine oxidase (X/XO) were both used and compared for apoptosis modeling. The concentrations of hydrogen peroxide and XO were selected and verified using cell viability analysis, the comet assay and flow cytometry. Intracellular ROS were measured using fluorescent probes. Additionally, the expression levels of the apoptosis-related genes Fas, Bcl-2, P53, Caspase 3, Caspase 8, and Caspase 9 were analyzed using quantitative RT-PCR. The results indicated the optimal modeling method is a final concentration of 0.5 mM H_2O_2 incubated with IPEC-J2 cells for 1 h at 37 °C in 5 % CO_2 for hydrogen peroxide-induced apoptosis modeling, and a final concentration of 250 μM X/50 U/L XO incubated with IPEC-J2 cells for 6 h at 37 °C in 5 % CO_2 for X/XO-induced apoptosis modeling. For the apoptotic pathway, the X/XO modeling method is more similar to 21 days weaning piglets. Therefore, we suggest that X/XO modeling with IPEC-J2 cells be used as an in vitro cell culture model for weaning piglet intestinal epithelial cell apoptosis.

Keywords Apoptosis · Oxidative stress · Cell model · IPEC-J2 · Weaning

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Introduction

Weaning in piglets is a stressful event associated with gastrointestinal disorders, diarrhea, and increased disease susceptibility (Boudry et al. 2004). Our previous research showed that weaning may induce intestinal epithelial cell apoptosis via the activation of Fas-dependent and mitochondria-

dependent apoptosis (Zhu et al. 2013), which satisfactorily explained some phenomena during piglets' early weaning, such as intestinal barrier function damage (Zhu et al. 2013, 2012) and intestinal flora change (Xu et al. 2014). However, it is difficult to study apoptosis mechanisms only using in vivo methods because of the limit of existing research technology. By in vitro method, we can easily observe the phenomenon of apoptosis by flow cytometry or study the signal transduction pathway by gene knockout method. We can also easily examine apoptosis induced by specialized microbes to intestinal epithelial cell (Koh et al. 2008), or the relationship between apoptosis and nutrients transport (Mustafin et al. 2010). Additionally, apoptosis is believed to be an important regulatory mechanism in intestine maturation (Vachon et al. 2000). In vitro research would provide new insights into the understanding of the balance between intestinal cell proliferation and apoptosis. Hence, a highly suitable in vitro cellular system is required for piglet intestinal epithelial cell apoptosis research.

In our previous study, Caco-2, HT-29, IEC-6 and some other intestinal epithelial cell lines have been used as cell models for oxidative stress studies (Cai et al. 2014). However, both, Caco-2 and HT-29, are tumor cells (Langerholc et al. 2011), and IEC-6 is a cell line from rat, whose digestive tube construction is very different from that of swine. As a type of non-tumorigenic epithelial cell line, IPEC-J2 cells were isolated from neonatal piglet mid-jejunum in 1989 at the University of North Carolina. This cell line secretes mucins, and produces cytolins and chemokines (Geens and Niewold 2011; Brosnahan and Brown 2011). Importantly these cells are stable and easy to be cultured. Therefore, we chose the IPEC-J2 cell line for apoptosis modeling.

Evidences have shown that oxidative stress is one of the major factors causing cell apoptosis (Simon et al. 2000; Sinha et al. 2013). We previously proved that weaning stress is the primary cause of the promotion of free radical generation (Zhu et al. 2012) and is associated with an increase in free radicals (Zhu et al. 2013); therefore, we established the apoptosis model using ROS induction. There are many ways to induce ROS in cell culture media, including X-ray irradiation (Teruya et al. 2013), hypoxic/re-oxygenation (Wasa et al. 2005) or certain drugs (Li et al. 2012; Zhang et al. 2011); however, the most common and conventional method to model weaning in piglets should be hydrogen peroxide and/or xanthine/xanthine oxidase (X/XO) induction of apoptosis (Cai et al. 2014).

In this study, hydrogen peroxide and X/XO were both used and compared for apoptosis modeling. The concentrations of hydrogen peroxide and XO were selected by flow cytometry. Intracellular free radical and cell viability are also important criteria in the selection of the apoptosis model. DNA modifications were examined genes Fas, Bcl-2, Bax, Caspase-3, Caspase-8, Capase-9 was measured using quantitative RT-PCR. N-acetyl cysteine (NAC), which is a well-known thiol antioxidant and GSH precursor (Aruoma et al. 1989), was used as the positive antioxidant control in this study.

Materials and methods

Cell line and cell culture

The IPEC-J2 cell line was a gift from Dr. Wei Zhanyong (College of Animal Science and Veterinary Medicine, Henan Agricultural University, Zhengzhou, Henan, China). IPEC-J2 cells were grown in Dulbecco's modified Eagle's medium and in Ham's F-12 medium (DMEM/F12, Hyclone, Logan, UT, USA), which was supplemented with 5 % fetal bovine serum (BIOIND, Kibbutz Beit Haemek, Israel), 5 μg/mL insulin, 5 ng/mL epidermal growth factor (Sigma, St. Louis, MO, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, Grand Island, NY, USA) at 37 °C in 5 % CO_2 in 25 cm^2 tissue culture flasks. The cell culture media were regularly changed (Cai et al. 2013). Cell cultures were tested using the Hoechst stain assay (Beyotime Institute of Biotechnology, Shanghai, China) and were found to be free of mycoplasma contamination (supplementary materials 1). Keratin-18 is a biomarker of epithelial cell, and this characterization is confirmed by immunofluorescence method in this study (supplementary materials 2). Some cytokines and chemokines expression is confirmed by PCR method in this study. The data were shown in supplementary materials 2.

Cell model

To select the optimal H_2O_2 and/or XO concentration, IPEC-J2 cells were seeded into 96-well plates or into 6-well plates at a density of 5.0×10^5 cells/mL. Cells were allowed to adhere for 24 h before treatment. Then, final concentrations of 0–4 mM (0, 0.1, 0.5, 1, 2 or 4 mM) H_2O_2 or 250 μM X and 0–70 U/L XO (0, 10, 30, 50, or 70 U/L) were added in wells for apoptosis modeling. H_2O_2 or X/XO were incubated with cells for 1 and 6 h according to Paszti-Gere et al. (2011) and Rao et al. (1999), respectively. Content of intracellular ROS, cell apoptosis rate, and cell viability were measured as following:

Intracellular free radical analyzed using a fluorescent probe technique

The total content of intracellular ROS was measured based on the ROS-mediated conversion of non-fluorescent 2′,7′-dichlorfluorescein-diacetate (DCFH-DA, Beyotime Institute of Biotechnology, China) into DCFH (Zha et al. 2007). Intracellular O_2^- was detected with dihydroethidium (DHE, Beyotime; Martins De Lima-Salgado et al. 2011). Cells with different treatments were incubated with DCFH-DA/DHE probes for 30 min and then washed twice with PBS. The fluorescence was read with a fluorescence microplate reader (Bio-TEK, Winooski, VT, USA) and observed in situ, as described in the operation manual. An increase in the fluorescence intensity compared with the control group showed an increase in intracellular ROS/O_2^-.

Apoptosis rate of IPEC-J2 analyzed by flow cytometry

The detection was performed using an Annexin V-FLUOS Staining Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. Cells were washed twice with PBS, detached with trypsin, and then incubated with Annexin V-FITC/PI buffer. Flow cytometric analysis was immediately performed using a BD FACSCalibur (BD, USA) to detect apoptosis.

Cell viability analysis

Cell viability was determined using WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) dye (Qcbio Science and Technologies, Shanghai, China) according to the manufacturer's instructions. Briefly, all wells were washed twice after modeling to remove all oxidizing agents. Then, 100 μL DMEM without phenol red and 10 μL WST-8 were added. Cells were incubated at 37 °C for 2 h, and the absorbance was determined at 450 nm using a microplate reader (Bio-TEK, USA).

Comet assay

The alkaline comet assay was performed using a CometAssay Kit purchased from Trevigen Inc. (Gaithersburg, MD, USA) according to the manufacturer's instructions, with some modifications. All operations were identical to those procedures previously described (Cai et al. 2013). Digital images were analyzed using CometScore^{TM} software. Comets appearing in randomly selected fields were measured ($n = 50$), and only overlapping comets were omitted. The tail length, % DNA in the tail, and comet area were used to describe the DNA damage in cells.

Quantitative RT-PCR

Total RNA isolated by TransZol UP reagent (TransGen Biotech, Beijing, China) was reverse transcribed to cDNA using random primers according to the manufacturer's instructions (Takara, Otsu, Shiga, Japan). The qualities of total RNA samples were assessed using both a Nanodrop Lite (Thermo, Waltham, MA, USA) and 0.8 % agarose gels. PCR primers and conditions for Fas, Bcl-2, Bax, Caspase-3, Caspase-8, and Caspase-9 were identical to the conditions previous described (Zhu et al. 2013). In brief, reactions start in a 20 μL system included 1 μl cDNA, 0.4 μl forward primer, 0.4 μl reverse primer, 8.2 μl ddH_2O and 10 μl SYBR Premix Ex Taq (Tli RNaseH Plus) (Takara, Japan), and primer sequences, please, refer to supplementary materials 1. The PCR program was performed as predenaturation 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. Beta-actin (β-actin) and beta-2-microglobulin (B^2M) genes were selected as reference genes for $2^{-\Delta\Delta Ct}$ analysis (Livak and Schmittgen 2001). All the products were sequenced (supplementary materials 3) by Sunny Biotech (Shanghai, China) and then the sequence were BLASTed with all the gene sequence in Gene bank, the results showed the sequence cloned in this study was consistent with our design.

Statistical analysis

All experimental results were from at least three separate experiments, and all data were expressed as the mean ± SEM. ANOVA analyses were used for comparisons of more than two groups, and Student's t-tests were used for comparisons of two groups using SPSS version 17.0 software (SPSS Inc, Chicago, IL, USA). Differences were considered significant at $P < 0.05$.

Results

Determination of H_2O_2 and XO concentrations in IPEC-J2 cell apoptosis modeling

The concentrations of H_2O_2 and of XO in IPEC-J2 cell apoptosis modeling were selected according to the intercellular radical level, cell viability, and Annexin V/PI flow cytometry analysis. Figure 1 shows the total intercellular free radical and superoxide anion levels with different concentrations of H_2O_2 and/or X/XO modeling. The data showed that the total intercellular free radical level increased with 0.1–1 mM H_2O_2 or 250 μM X/10–50 U/L XO but decreased when incubated with over 2 mM or 50 U/L XO. The cell viability of IPEC-J2 decreased with the concentrations of H_2O_2 or XO increase, but no significant difference was detected when concentrations of H_2O_2 were below 0.1 mM or XO below 10 U/L compared with the control. However, a sharp decline in cell viability for H_2O_2 was observed at concentrations over 1 mM or over 50 U/L for XO (Fig. 2). A microscopic inspection showed that IPEC-J2 cells incubated with over 3 mM H_2O_2 for 1 h or with 250 μM X/100 U/L XO for 6 h (data not shown) caused acute injury, with some cells no longer adhering. The results of intracellular free radical and cell viability analyses suggested that 0.5–1 mM H_2O_2 for 1 h or 250 μM X/30–50 U/L XO for 6 h may be a suitable modeling concentration. Additionally, the exact concentration must be confirmed by flow cytometry analysis (Fig. 3).

As shown in Fig. 3, the number of viable cells (annexin−, PI−) decreased with increasing concentrations of H_2O_2 (0–2 mM) and/or XO (0–70 U/L), whereas the number of apoptosis cells (both early apoptosis and late apoptosis; annexin+ ; quadrants 1

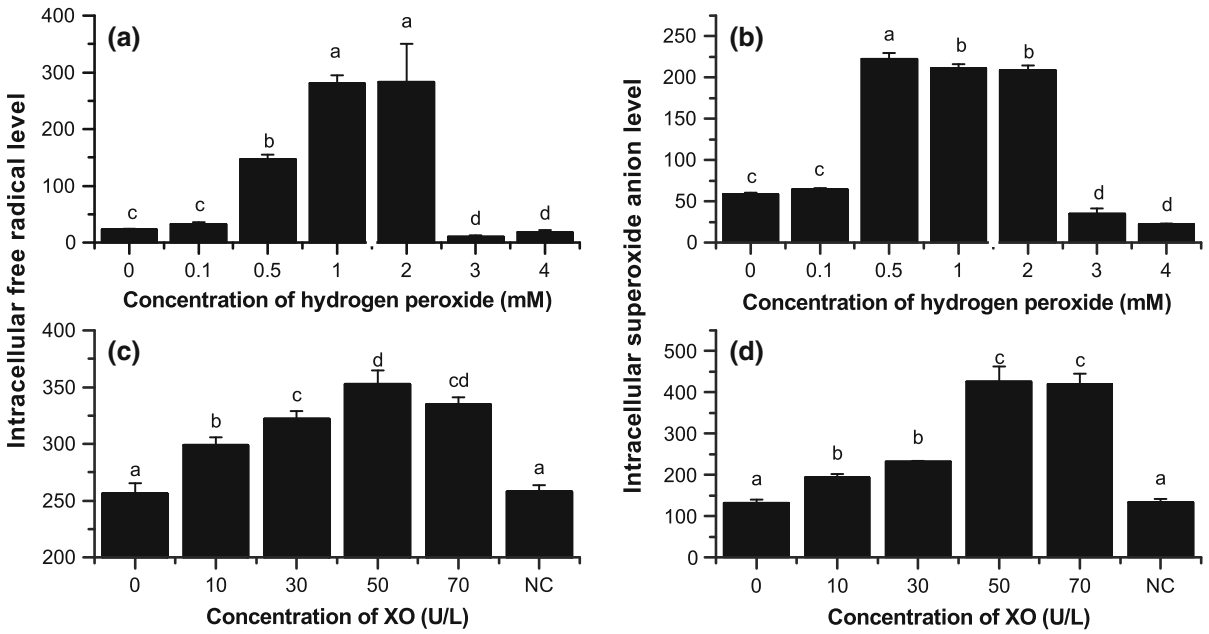


Fig. 1 Total intercellular free radical and superoxide anion levels in IPEC-J2. Radicals were detected using a fluorescent probe method. **a** the total intracellular free radical level for IPEC-J2 under different concentration of H_2O_2; **b** the intracellular superoxide anion level for IPEC-J2 under different concentration of H_2O_2; **c** the total intracellular free radical level for IPEC-J2 under 250 μM X and different concentrations of XO; **d** the intracellular superoxide anion level for IPEC-J2 under 250 μM X and different concentrations of XO. *Different letters* indicate significant differences ($P < 0.05$, n = 3)

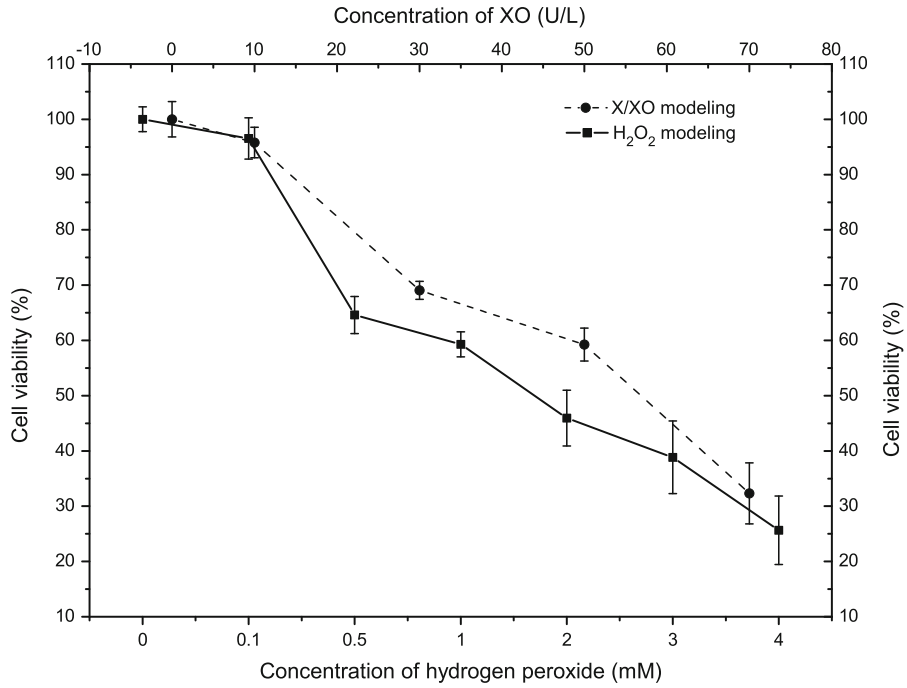


Fig. 2 Cell viability of IPEC-J2 cells as tested by WST-8 dye. The data were calculated as a percentage of control cells. For ANOVA analysis results, refer to S4

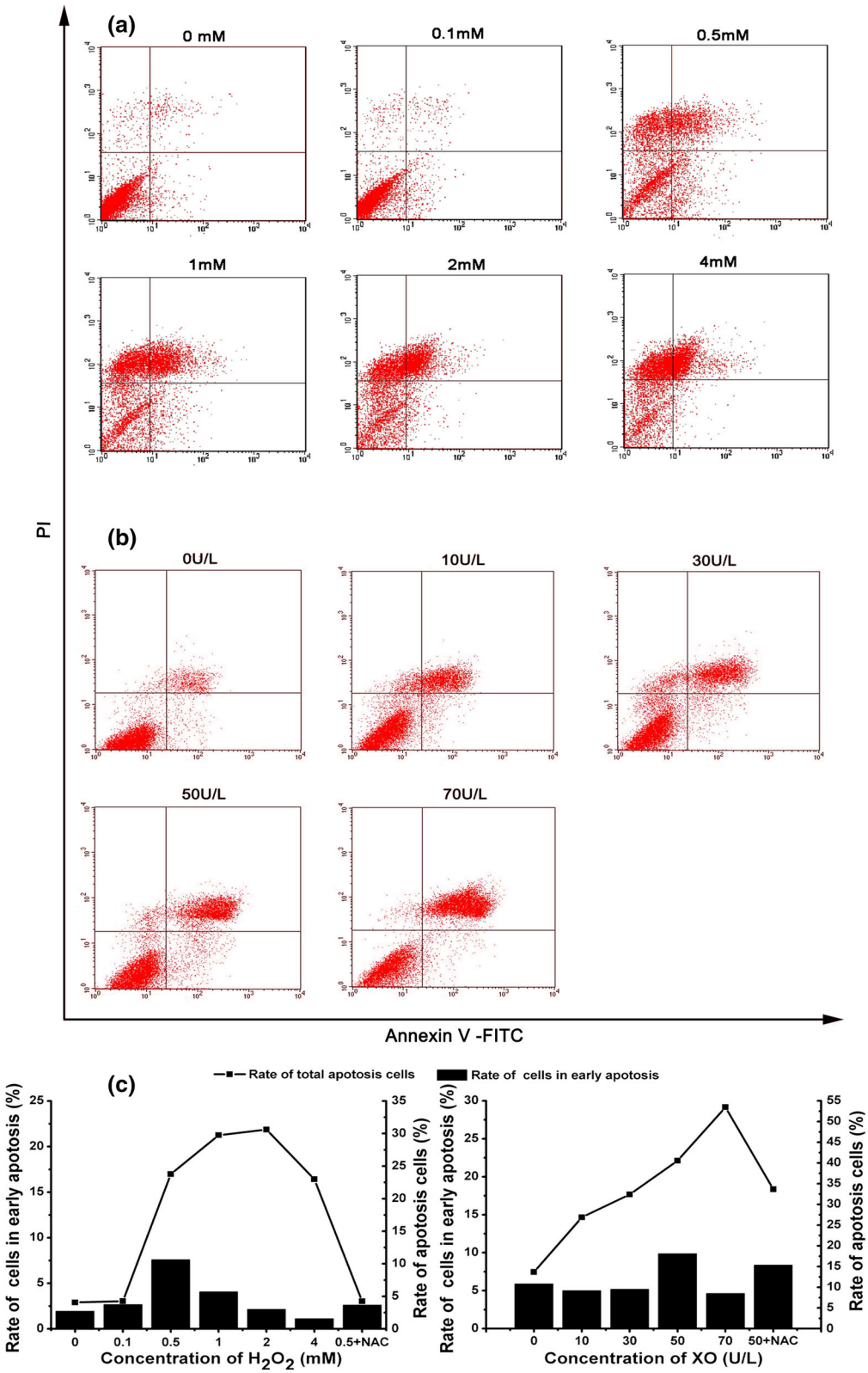
and 4) increased. This result is consistent with the results of intracellular free radicals and of cell viability. However, a higher concentration of H_2O_2 (4 mM) caused the apoptosis rate to decrease because 4 mM H_2O_2 for 1 h (Fig. 3a) caused most of the IPEC-J2 cells to become necrotic (annexin−, PI+ ; quadrants 1). Then, the early apoptotic cells were calculated because early apoptosis is the most noticeable status in cancer, aging and developmental biology research. The highest early apoptosis rates in H_2O_2 modeling and in X/XO modeling were observed at 0.5 mM H_2O_2 for 1 h and at 250 μM X/50 U/L XO for 6 h, respectively (Fig. 3c).

Considering all the results (intracellular free radicals, cell viability and Annexin V/PI staining), 0.5 mM H_2O_2 for 1 h or 250 μM X/50 U/L XO for 6 h share the highest early apoptosis rates, relatively high total apoptosis rates, significant differences in intracellular free radicals and cell viability, and stable adherence; therefore, we selected final concentrations of 0.5 mM H_2O_2 incubated with IPEC-J2 cells for 1 h at 37 °C in 5 % CO_2 for hydrogen peroxide-induced apoptosis modeling and 250 μM X/50 U/L XO incubated with IPEC-J2 cells for 6 h at 37 °C in 5 % CO_2 for X/XO-induced apoptosis modeling.

Model examination

The modeling methods were examined using morphological observation (Fig. 4a), an intracellular total free radical (Fig. 4b) and superoxide anion probe (Fig. 4c), and a comet assay (Fig. 4d; Table 1). NAC, which is a recognized anti-apoptosis reagent, was used as the positive control. The effect of NAC on cell viability (Fig. 4e) and Annexin V/PI staining (Fig. 3c) during apoptosis modeling were also examined in this study.

Figure 4a shows that both H_2O_2 and X/XO modeling caused some IPEC-J2 cell vacuole formation, which is a typical morphological feature of cell apoptosis, and the NAC treated groups showed cell conditions similar to control cells. Figures 4b, c confirmed that the ROS levels in the modeling groups are much higher than those levels in the control groups, which indicated that the apoptosis of modeling group cells was related to oxidative stress. In a comet, the head is undegraded DNA, and fragmented DNA is shown as the tail. Thus, the comet length, DNA in the tail, and comet area are usually used to describe a comet image for DNA fragmentation. The data in Table 1 (typical image is shown as Fig. 4d) showed


(a)
0 mM
0.1mM
0.5mM
1mM
2mM
4mM
(b)
0U/L
10U/L
30U/L
50U/L
70U/L
PI
Annexin V -FITC
(c)
Rate of total apotosis cells
Rate of cells in early apotosis
Rate of cells in early apotosis (%)
Rate of apotosis cells (%)
0 0.1 0.5 1 2 4 0.5+NAC
Concentration of H2O2 (mM)
0 10 30 50 70 50+NAC
Concentration of XO (U/L)

◀**Fig. 3** Apoptosis of IPEC-J2 cells as determined by flow cytometry after coincubation with H_2O_2 or with X/XO. The apoptotic rates were analyzed by Annexin V/PI staining. **a** Apoptosis of IPEC-J2 cells incubated with different concentrations of H_2O_2; **b** apoptosis of IPEC-J2 cells incubated with 250 μM X and with different concentrations of XO for 6 h; **c** rate of early apoptotic IPEC-J2 cells (annexin+, PI−; quadrants 4) or total apoptotic cells (annexin+; quadrants 1, 4)

either H_2O_2 or X/XO modeling caused a longer comet length, more DNA in the tail and a larger comet area in IPEC-J2 cells, which indicated that modeling caused IPEC-J2 cells DNA fragmentation and that apoptosis modeling was successful. Additionally, NAC treatments in all indexes for both H_2O_2 and X/XO modeling showed no differences compared with the control group.

Expressions of apoptosis relative genes

Relative expression of apoptosis genes in IPEC-J2 cells in the H_2O_2- or X/XO-induced apoptosis model is shown in Fig. 5 by $2^{-\Delta\Delta Ct}$ way. β-actin and B^2M genes were both selected as reference genes. A fold change over 1.5 or <0.67 was believed to indicate up-regulation or down-regulation relative to the β-actin or B^2M gene, respectively. The data show that H_2O_2 modeling up-regulated caspase-3, capase-8 and caspase 9 gene expression and down-regulated Bcl-2 gene expression. X/XO modeling up-regulated Fas, P53, caspase 3, and caspase 8 gene expression and down-regulated Bcl-2 gene expression; however, no significant differences were observed relative to β-actin for P53 and caspase 9 gene expression. There is no difference in the gene expression of all apoptosis genes in NAC-treated cells compared with control cells.

Discussion

Apoptosis, which is also known as programmed cell death, may be the most popular word in cancer, aging and development research (Ulukaya et al. 2011). Currently, apoptosis is believed to play an important role in early weaning piglet weaned stress syndrome (Zhu et al. 2013, 2012). A result of gene microarray analysis showed that 83 cellular process- related genes are differentially expressed in weaning piglets and that many of these genes were related to apoptosis, such as TNF, ATM, BAD, and c-JUN (Zhu et al. 2014). Additionally, a follow-up study by qRT-PCR confirmed this result (Zhu et al. 2013). Studies by Wang et al. (2009) and by Huguet et al. (2007) also supported this result. Thus, apoptosis in weaning piglet intestine became a new target for studying piglet weaned stress syndrome.

There are many other factors in addition to weaning that cause apoptosis in intestinal epithelial cells, such as ischemia–reperfusion, inflammatory bowel disease, bacterial infection and radiation (Ramachandran et al. 2000). Concerning health status, old intestinal epithelium cells will be completely replaced by newly generated cells (Günther et al. 2013). To study the mechanism of these cells, many apoptosis cell models have been established. Desjardins and MacManus (1995) induced apoptosis in human adenocarcinoma colon cell line (HT29) by teniposide (VM26), Yan and Polk (2002) induced HT29 cell apoptosis by tumor necrosis factor (TNF), and Harnois et al. (2004) induced HT29 cell apoptosis by cytochalasin D. However, similar to weaning, most of these apoptosis models are believed to be related to oxidative stress (Zhu et al. 2012). Buccigrossi et al. used HT29 and Caco-2 cells for a Tat-induced apoptosis study, and 10 mM H_2O_2 for 5 min was used as the control in the paper by Buccigrossi et al. (2011). Nevertheless, HT29 and Caco-2 cells are both tumorigenic cell lines. It is well known that oxidative stress-induced apoptosis in normal and tumor cells may occur via distinctly different mechanisms (Wang et al. 2004). In addition, many studies used rat original non-tumorigenic intestinal cell lines, such as IEC-6; however, the digestion metabolism of rodents has many differences compared with piglet (Sasser and Jarboe 1980). Therefore, although many in vitro apoptosis cell models exist, a new oxidative stress-induced model using swine non-tumorigenic intestinal epithelial cells remains required.

Many apoptosis detection methods have been described, for example, in situ TUNEL, nucleosomal ELISA, morphological analysis by electron microscopy, comet assay, and flow cytometry, each with its own advantages and disadvantages (Gavrieli et al. 1992). As a type of in vitro cell culture model, the comet assay and Annexin V/PI flow cytometry assay are believed to be both easy and accurate for apoptosis detection. Additionally, these assays detect apoptosis

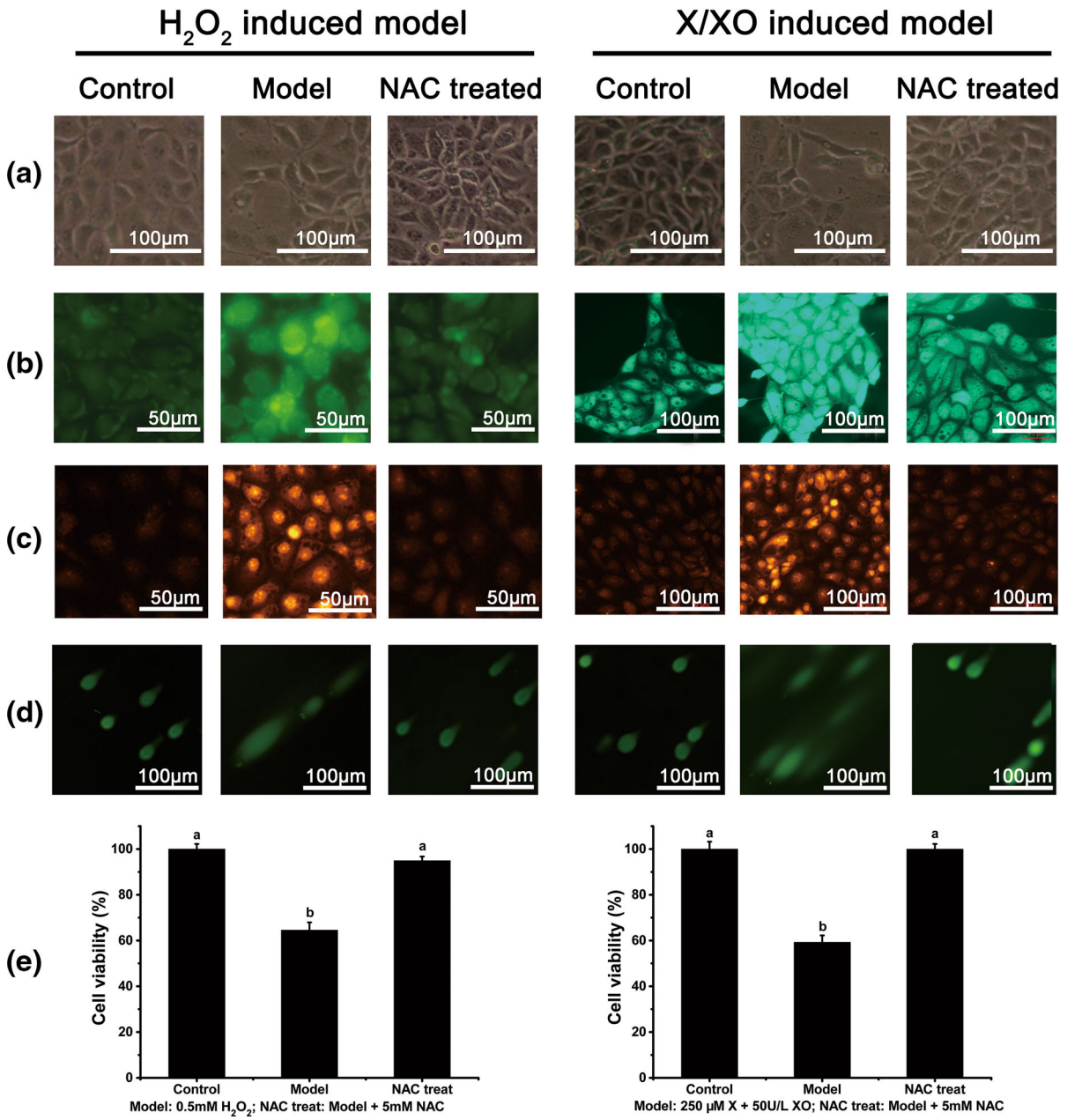


Fig. 4 Modeling method analysis. In all experiments, the control group was normal IPEC-J2 cells; the model groups included modeling with 0.5 mM H_2O_2 for 1 h or with 250 μM X/50 U/L XO for 6 h, and in NAC groups (anti-apoptosis controls), 5 mM NAC was added at the same time for modeling. **a** Cells of different treatments were observed using a 100× inverted microscope. **b** Total intracellular free radicals were detected using a DCFH-DA probe for different treatment cells, and the images were captured using a fluorescence microscope with identical exposure. *Higher fluorescence intensity* indicates a higher degree of intracellular free radicals. **c** Superoxide anions were detected using a DHE probe for different treatments cells, and the images were captured using a fluorescence microscope with identical exposure. *Higher fluorescence intensity* indicates a higher degree of superoxide anions. **d** Comet assay image captured by fluorescence microscope; a *longer tail* indicates more serious DNA damage. For details concerning the statistical analysis, please refer to Table 1. **e** Cell viability of different treatments as analyzed by WST-8 dye. *Different letters* indicate significant differences ($P < 0.05$, n = 3)

Table 1 DNA fragmentation of IPEC-J2 cells as tested by the comet assay

	H_2O_2 modeling			X/XO modeling		
	Control	Model	NAC treat	Control	Model	NAC treat
Comet length (px)	120.68 ± 4.69^b	227.78 ± 9.45^a	127.1 ± 13.04^b	85.52 ± 2.93^b	162.62 ± 3.94^b	92.96 ± 3.84^b
DNA in tail (%)	2.37 ± 0.44^b	15.68 ± 1.51^a	3.44 ± 0.54^b	2.40 ± 0.37^b	14.40 ± 0.79^a	2.79 ± 0.37^a
Comet area (px)	$12{,}112 \pm 830^b$	$40{,}600 \pm 2650^a$	$12{,}171 \pm 890^b$	$8{,}179 \pm 432^b$	$24{,}865 \pm 1156^a$	$8{,}302 \pm 626^b$

Comet images were analyzed using CometScore™ software. Values marked with different letters in each model in the same line differ significantly ($P < 0.01$, n = 3)

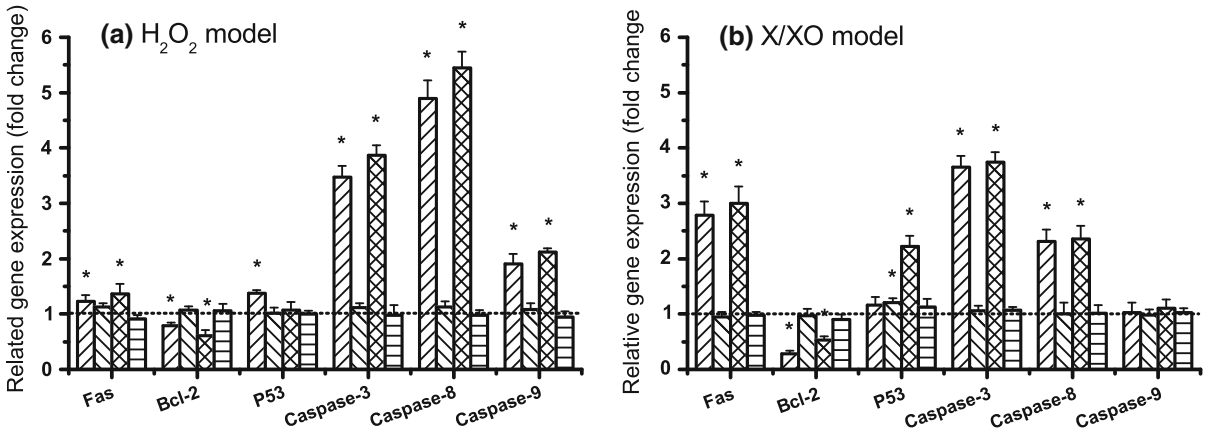


Fig. 5 Apoptosis related gene expression of IPEC-J2 cells in **a** H_2O_2- or **b** X/XO-induced model. mRNA levels in the models or in NAC-treated cells are presented as the fold change in the gene expression levels compared with control cell levels, which were set as 1.0. *Striped bar* represents the gene expression of model cells relative to the β-actin gene; *Striped bar* represents the gene expression of NAC-treated cells relative to the β-actin gene; *Cross striped bar* represents the gene expression of model cells relative to the B^2M gene; *Dashed bar* represents the gene expression of NAC-treated cells relative to the B^2M gene. *Asterisk* denotes significantly different gene expression compared with the reference gene ($P < 0.05$)

in different ways: the comet assay detects DNA fragmentation, and Annexin V/PI stain based on cell membrane permeability and on phosphatidylserine distribution. Therefore, the comet assay and flow cytometry were selected to analyze the apoptosis cell model, and cell viability was detected as a supplementary analysis.

To study the possible apoptotic pathways and the correlation between the cell models and weaning piglets, we analyzed the gene expression of the apoptosis-related genes Fas, Bcl-2, P53, Caspase 3, Caspase 8, Caspase 9 and compared this expression with the expression of the same genes in 21 days weaned piglets (euthanized at 25 days of age). Researchers usually classify apoptotic pathways into extrinsic and intrinsic according to whether the major factors that induce apoptosis are extracellular or intracellular (Ulukaya et al. 2011; Zhu et al. 2013). Fas is one of the typical membrane death receptors that mediate the extrinsic pathway, and Caspase 8 is the key downstream gene, whereas caspase 9 mediates the intrinsic pathway. Caspase 3 is activated by both caspase 8 and caspase 9, and is believed to be an important gene in apoptotic pathways (Zhu et al. 2013). Bcl-2, which plays a pivotal role in regulating apoptosis activating factor-1 (Apaf-1) and in preventing the activation of down-stream caspase (Adams and Cory 1998). Additionally, p53 gene, which plays a role in apoptosis and tumor suppressor, too (Ramachandran et al. 2000). The results of qRT-PCR showed that the H_2O_2 and X/XO models might be induced via both extrinsic and intrinsic pathways; however, H_2O_2-induced IPEC-J2 cell apoptosis was not mediated via Fas/FasL.

We compared the gene expression patterns (Fig. 5) with our previous research (Zhu et al. 2013, 2012), and

Table 2 Regulation trends of apoptosis-related genes in weaning piglets (in vivo) or in the cell model (in vitro)

Genes	Weaning piglets*	H_2O_2 model**	X/XO model**
Fas	↑	–	↑
Bcl-2	↓	↓	↓
P53	↑	–	↑
Caspase 3	↑	↑	↑
Caspase 8	↑	↑	↑
Caspase 9	–	↑	–

The control groups of in vivo and in vitro test are suckling piglet and PBS treated cell, respectively

* The data for Fas, Bcl-2, Caspase 3, Caspase 8, Caspase 9 are from reference (Zhu et al. 2013), and data for P53 are from reference (Zhu et al. 2012)

** The fold change compared with both reference genes was considered

↑ denotes gene up-regulation, ↓ denotes gene down-regulation, – denotes unchanged gene expression

the regulation trends of Fas, Bcl-2, P53, Caspase 3, Caspase 8, and Caspase 9 genes in weaning piglet in vivo or in the cell model in vitro are summarized in Table 2. In addition, Pearson correlation coefficients were calculated (S5). Results showed that a high positive and significant correlation exists between 21 days weaning piglets and the X/XO model (Pearson correlation $= 0.946$, $P = 0.04$) and that a positive but not significant correlation exists between 21 days weaning piglets and the H_2O_2 model (Pearson correlation $= 0.499$, $P = 0.31$). This result suggests that although both H_2O_2 and X/XO models are suitable for apoptosis research but that the mechanism of the X/XO model is more similar to weaning piglets.

The establishment of an in vitro apoptosis cell model is only the first step of the process. More work is required to continue, including ensuring the correct pathway for the apoptosis of intestinal epithelial cells in weaning piglets, determining the relation between intestinal epithelial cell apoptosis and cell cycle change, and examining the influence of intestinal epithelial cell apoptosis on the intestinal microenvironment. Better understanding these factors will help us to solve the problem of weaned stress syndrome in early weaning piglets.

Of course, in vitro model also have some disadvantages in studying weaning piglet apoptosis in vivo. For example, oxidative stresses that cause apoptosis are both by endogenous and exogenous nature, however, the in in vitro models oxidative stress is only exogenous. What's more, the apoptosis in vivo during weaning is not only causing by oxidative stress, some other factor such as enterotoxin (Johnson et al. 2009) will also induce apoptosis in intestinal epithelial cell. It need us to develop a more thorough cell model combined with the in vivo study to improve our research.

In conclusion, we established an oxidative stress-induced swine intestinal epithelial cell apoptosis model using H_2O_2 or X/XO. The optimal modeling methods are a final concentration of 0.5 mM H_2O_2 incubated with IPEC-J2 cells for 1 h at 37 °C in 5 % CO_2 for hydrogen peroxide-induced apoptosis modeling and a final concentration of 250 μM X/50 U/L XO incubated with IPEC-J2 cells for 6 h at 37 °C in 5 % CO_2 for X/XO-induced apoptosis modeling. For the apoptotic pathway, the X/XO modeling method is more similar to 21 days weaning piglets. Therefore, we suggest that X/XO modeling by IPEC-J2 cells can be used as an in vitro cell culture model for weaning piglet intestinal epithelial cell apoptosis.

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