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Original Research Article

Low-protein diets supplemented with glutamic acid or aspartic acid ameliorate intestinal damage in weaned piglets challenged with hydrogen peroxide

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ABSTRACT

Glutamic acid (Glu) and aspartic acid (Asp) are acidic amino acids with regulatory roles in nutrition, energy metabolism, and oxidative stress. This study aimed to evaluate the effects of low-protein diets supplemented with Glu and Asp on the intestinal barrier function and energy metabolism in weaned piglets challenged with hydrogen peroxide (H_2O_2). Forty piglets were randomly divided into 5 groups: NC, PC, PGA, PG, and PA ($n = 8$ for each group). Pigs in the NC and PC groups were fed a low-protein diet, while pigs in the PGA, PG, or PA groups were fed the low-protein diet supplemented with 2.0% Glu +1.0% Asp, 2.0% Glu, or 1.0% Asp, respectively. On day 8 and 11, pigs in the NC group were intraperitoneally injected with saline (1 mL/kg BW), while pigs in the other groups were intraperitoneally administered 10% H_2O_2 (1 mL/kg BW). On day 14, all pigs were sacrificed to collect jejunum and ileum following the blood sample collection in the morning. Notably, low-protein diets supplemented with Glu or Asp ameliorated the intestinal oxidative stress response in H_2O_2-challenged piglets by decreasing intestinal expression of genes ($P < 0.05$) (e.g., manganese superoxide dismutase [*MnSOD*], glutathione peroxidase [*Gpx*]-*1*, and *Gpx-4*) encoding oxidative stress-associated proteins, reducing the serum concentration of diamine oxidase ($P < 0.05$), and inhibiting apoptosis of the intestinal epithelium. Glu and Asp supplementation attenuated the upregulated expression of energy metabolism-associated genes (such as hexokinase and carnitine palmitoyltransferase-1) and the H_2O_2-induced activation of acetyl-coenzyme A carboxylase (ACC) in the jejunum and adenosine monophosphate-activated protein kinase−acetyl-ACC signaling in the ileum. Dietary Glu and Asp also ameliorated intestinal barrier damage as indicated by restored intestinal histology and morphology. In conclusion, low-protein diets supplemented with Glu and Asp protected against oxidative stress-induced intestinal dysfunction in piglets, suggesting that this approach could be used as a nutritional regulatory protectant against oxidative stress.

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1. Introduction

Glutamic acid (Glu) and Aspartic acid (Asp) are regarded as functional amino acids. Accumulated findings have demonstrated

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the roles of amino acids in nutrition, antioxidative functions, intestinal barrier remodeling, and metabolism in healthy and pathological models (Birsoy et al., 2015; Duan et al., 2016; Wang et al., 2017). Generally, Glu and Asp are mainly utilized in the intestine to yield adenosine triphosphate (ATP) for enterocytes (Nakamura et al., 2013; Rezaei et al., 2013). Approximately 35% of the total energy produced in the intestine is estimated to be derived from dietary Glu (Stoll et al., 1999), thus highlighting the pivotal role of this amino acid in intestinal homeostasis. Both Glu and Asp are required to synthesize proteins and other biologically active molecules, including glutamine (Gln), glutathione (GSH), arginine, purines, and pyrimidines (Blachier et al., 2009; Lane and Fan, 2015; Wu et al., 2013). Furthermore, Glu has various beneficial functions in lipid and nitrogen metabolism (Boutry et al., 2012; Chen et al., 2014), intestinal barrier function (Jiao et al., 2015), antioxidative ability (Wu et al., 2014a), and protects animals from damage caused by exposure to toxins (Duan et al., 2014; Wu et al., 2014b). Meanwhile, Asp could restore the intestinal barrier and improve intestinal and liver energy metabolism in piglets challenged with lipopolysaccharide (LPS) (Kang et al., 2015; Pi et al., 2014). Asp also plays essential roles in cell viability and proliferation, including in a model of mitochondrial electron transport chain dysfunction (Birsoy et al., 2015). However, although Glu and Asp have long been considered as non-essential nutrients in mammals, their regulatory roles in nutrition, metabolism, and even oxidative stress remain unclear.

The intestine provides an open interface for luminal microbes and antigens and is constitutively exposed to multiple specific or non-specific stimuli or stressors. The host intestinal barrier forms a sophisticated defensive line against the invasion of bacteria and exogenous antigens to maintain intestinal homeostasis (Camilleri et al., 2012; Guttman and Finlay, 2009). However, studies of animals (e.g., pigs) have shown that the intestinal barrier function is prone to be disturbed in response to an exogenous stimulus such as hydrogen peroxide (H_2O_2) (Duan et al., 2016; Yin et al., 2015a).

The mechanism by which oxidative stress damages the intestinal barrier function has been widely investigated. Many compelling investigations have revealed that oxidative stress compromises intestinal cell proliferation and mucosa development, disrupts intestinal tight junction protein expression, distribution, and organization, and increases intestinal apoptosis (Bhattacharyya et al., 2014; Duan et al., 2015; Nathan and Cunningham-Bussel, 2013; Shen et al., 2006). Intriguingly, some researchers have focused on the relationship between the intestinal barrier and intestinal energy metabolism (Le Drean and Segain, 2014). Glover et al. (2013) revealed a fundamental link between cellular bioenergetics and the mucosal barrier. Under conditions of energy stress, activation of the liver kinase B1/adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) signaling pathway prevents cell apoptosis, implying that disturbed intestinal energy metabolism in oxidative stress may contribute to intestinal barrier dysfunction (Shaw et al., 2004). Therefore, Glu and Asp might serve as intestinal energy sources to alleviate oxidative stress-induced intestinal injuries.

Given the versatile functions of Glu and Asp, we hypothesized that dietary Glu and Asp supplementation would alleviate intestinal damage. Therefore, we aimed to explore the effects of these amino acids on various markers of oxidative stress, such as abnormalities in antioxidative-associated gene expression (i.e., superoxide dismutase, catalase, and glutathione peroxidase), apoptosis, energy metabolism, and intestinal barrier function, in weaned piglets challenged with H_2O_2.

2. Materials and methods

All experiments were performed under the relevant guidelines and regulations and were approved by the Laboratory Animal Welfare Commission of the Institute of Subtropical Agriculture, Chinese Academy of Sciences.

2.1. Animals and experiment design

This study was designed as described in our earlier publication (Duan et al., 2016). Forty weaned piglets (Landrace × Large White, 20 castrated males and 20 females, BW = 10.96 ± 0.61 kg) were randomly divided into 5 groups: 1) control group (NC); 2) H_2O_2 group (PC); 3) PC + Glu + Asp group (PGA); 4) PC + Glu group (PG); 5) PC + Asp group (PA). Pigs in the NC and PC groups were fed a low-protein diet containing 18% crude protein and other nutrients according to the 2012 requirements of the National Research Council (Appendix Table 1). Pigs in the PGA, PG, and PA groups were fed the low-protein diet supplemented with 2.0% Glu +1.0% Asp, 2.0% Glu, or 1.0% Asp, respectively. Pigs in the NC group were intraperitoneally injected with saline, i.e., 1 mL/kg BW once on day 8 and repeated on day 11, whereas pigs in the other 4 groups were intraperitoneally injected with10% H_2O_2, i.e., 1 mL/kg BW once on day 8 and repeated on day 11. The H_2O_2 dosage (Duan et al., 2016; Yin et al., 2015a) and Glu and Asp supplementation dosages were based on our previous work (Kang et al., 2015; Yin et al., 2015). The contents of Glu and Asp and other amino acids in diets were measured, and Glu and Asp were added to replace L-alanine (Ala).

The piglets were housed individually in an environmentally controlled facility with hard plastic slatted flooring maintained at an ambient temperature of 25 ± 2 °C and had free access to diets and drinking water. The experiment duration was 14 days. All blood samples were collected from the jugular vein on the morning of day 14 after a 12-h fasting period. Subsequently, the piglets were sacrificed, and approximately samples of 1 cm long from the upper part of the jejunum and lower part of the ileum were collected after flushed with normal saline. The blood samples were centrifuged at 2,000×*g* for 10 min to separate the serum. All samples were snap-frozen in liquid nitrogen and stored at −80 °C before further processing.

2.2. Serum diamine oxidase

The serum diamine oxidase (DAO) concentrations were measured using a Porcine Diamine Oxidase ELISA kit (Shanghai Bluegene Biotech Co., Ltd., Shanghai, China) according to the manufacturer's recommendation.

2.3. Morphological analysis

Hematoxylin and eosin (H&E) staining was used to observe the morphology of the intestinal sections, according to our previously published protocol (Chen et al., 2019). The jejunal and ileal tissue samples were fixed with 10% neutral buffered formalin at 4 °C, dehydrated in a gradient ethanol series, and embedded in paraffin wax according to routine histological methods. Six-micrometer thick sections were then cut from the embedded tissue blocks and stained with H&E. Imaging was performed using a light microscope (Nikon, Tokyo, Japan).

2.4. Real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated from snap-frozen jejunal and ileal tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase I (Invitrogen) according to the manufacturer's instructions. For

each sample, the RNA quality was evaluated by 1% agarose gel electrophoresis and gel staining with 10 μg/mL ethidium bromide. First-strand cDNA synthesis was performed using oligo (dT) 20 primers and Superscript II reverse transcriptase (Invitrogen, USA). Real-time polymerase chain reaction was performed according to our previously published protocol (Chen et al., 2018). The target gene-specific primers used in this study (Appendix Table 2) were designed using Primer 5.0, according to the *Sus scrofa* gene sequence (http://www.ncbi.nlm.nih.gov/pubmed/). Primers specific for β-actin mRNA (housekeeping gene) were also used to provide a reference to normalize the target gene transcript levels. The relative expression was expressed as a ratio of the target gene to the housekeeping gene using formula $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct = (Ct^{Target} - Ct^{\beta-Actin})$ of treatment $- (Ct^{Target} - Ct^{\beta-Actin})$ of control (Livak and Schmittgen, 2001).

2.5. Western blot analysis

The antibodies used in this study were purchased from the following suppliers: caspase-3 (ab4051), occludin (ab31721), claudin1 (ab15098), and zonula occludens (ZO)-1 (ab59720) (Abcam, Inc., Cambridge, MA, USA); B-cell lymphoma (Bcl)-2 (sc-783), Bcl-2-associated X protein (Bax) (sc-493), and AMPKα1/2 (H-300; sc-25792) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA); and phosphorylated (p)-AMPK (#5256), adenosine monophosphate-activated protein kinase—acetyl-coenzyme A carboxylase (ACC) (#3676), p-ACC (#3661), apoptotic peptidase activating factor (Apaf)-1, cytochrome (Cyt)-c (#11940), P53 (#2524), and β-Actin (#4970) (Cell Signaling Technology, Inc., Danvers, MA, USA).

Approximately 100 mg of each intestinal sample was homogenized in a 1.5-mL microcentrifuge tube containing ice-cold radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Beijing, China) supplemented with phosphatase inhibitor and protease inhibitor cocktail tablets (Roche Diagnostics Ltd., Shanghai, China), according to the manufacturer's protocol. After thorough mixing of the samples and centrifugation (12,000×*g* for 10 min at 4 °C), the supernatants were collected. The total protein contents were measured using the Enhanced BCA Protein Assay Kit (Beyotime Institute of Biotechnology, China) according to the manufacturer's instructions. The Western blot (WB) analysis was conducted as previously described (Yin et al., 2014).

2.6. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining analysis

In all groups of pigs, intestinal apoptosis was detected by TUNEL staining with a commercially available kit (KGA7075, KeyGEN TECH, Jiangsu, China) based on the manufacturer's protocol. Briefly, fixed and embedded tissue sections were deparaffinized with xylene, rinsed with phosphate-buffered saline (PBS), and hydrated in a gradient ethanol series for 5 min per step. The washed sections were treated with proteinase K for 30 min, rinsed with PBS, and immersed in a pre-mixed buffer containing terminal deoxynucleotidyl transformerase and fluorescein isothiocyanate (FITC)-streptavidin in a humid chamber for 60 min at 37 °C. The samples were again washed with PBS and stained with 4′,6-diamidino-2-phenylindole (DAPI) for 10 min at 37 °C to label the nuclei. Imaging was performed using an Olympus fluorescent microscope (Olympus Life Science, Tokyo, Japan).

2.7. Statistical analysis

All statistical analyses were performed using SPSS 21.0 software (IBM, Inc., Armonk, NY, USA). Levene's test was used to analyze the normality and constant variance of the data, which were then subjected to a one-way analysis of variance followed by the Duncan (D) multiple comparisons test. $P < 0.05$ was considered to indicate a significant difference, and *P*-value between 0.05 and 0.1 was considered to indicate a tendency toward significance. Data are expressed as the means ± standard errors of the mean (SEM).

3. Results

3.1. Supplementation with Glu and Asp alleviated intestinal oxidative stress in piglets challenged with H_2O_2

Challenge with H_2O_2 tended to decrease the jejunal abundance of *MnSOD* mRNA ($P < 0.1$, Fig. 1A) but tended to increase the ileal abundance (Fig. 1B). Glu and Asp supplementation reduced both the jejunal and ileal abundance of *MnSOD* mRNA (Fig. 1A). Challenge with H_2O_2 also induced the jejunal and ileal expression of *Gpx-1* mRNA (Fig. 1), whereas ileal *Gpx-1* mRNA expression was decreased in the PGA, PG, and PA groups and jejunal *Gpx-1* expression was decreased in the PGA group relative to the PC group. Further, the challenge with H_2O_2 downregulated the catalase mRNA abundance in the jejunum and upregulated it in the ileum (Fig. 1). Similarly, Glu and Asp supplementation, either alone or combination, decreased the ileal catalase mRNA abundance, whereas the combination of Glu and Asp increased this mRNA abundance relative to the PC group (Fig. 1). Collectively, Glu and Asp supplementation may ameliorate the intestinal oxidative stress response induced by the H_2O_2 challenge but may exhibit a segmental effect, especially for catalase.

3.2. Supplementation with Glu and Asp attenuated intestinal apoptosis in piglets challenged with H_2O_2

TUNEL staining revealed apparent intestinal apoptosis in the jejunum (Appendix Fig. 1A) and ileum (Fig. 2A) in response to the H_2O_2 challenge. Increased jejunal (Appendix Figs. 1B and 3A) and ileal (Fig. 2B, Appendix Figs. 2A, and Fig. 3B) caspase-3 mRNA expression and protein cleavage were observed after H_2O_2 challenge. The ileum exhibited increased levels of cleaved caspase-3 (Fig. 2B and Appendix Fig. 2A) and Bax (Fig. 2B) and a reduced level of Bcl-2 (Fig. 2B) under the H_2O_2 challenge. In contrast, the jejunum exhibited no obvious changes in the cleaved-caspase-3 protein levels (Appendix Fig. 1B) or caspase-3 mRNA abundance (Appendix Fig. 3A) and varying Bax levels across the treatment groups (Appendix Fig. 1B). Therefore, we focused on the regulatory roles of Glu and Asp in ileal apoptosis in the piglets.

Dietary supplementation with Glu or Asp alone significantly attenuated ileal apoptosis, evidenced by decreased TUNEL staining and reduced cleaved caspase-3 protein levels (Fig. 2B and Appendix Fig. 2A), and downregulated caspase-3 mRNA expression (Appendix Fig. 3B). We next used WB to detect the Cyt-C, Apaf-1, and caspase-8 protein levels in the ileum (Fig. 2C, Appendix Figs. 2A and 3B). Although the H_2O_2 challenge had no apparent effects on the Cyt-C and Apaf-1 protein levels, this challenge increased the caspase-8 level. Dietary supplementation with Glu or Asp decreased the protein levels of both Cyt-C and caspase-8 (Fig. 2C). However, we failed to identify a causal relationship between apoptosis and the Jun N-terminal kinase (JNK) and p53 pathway (Fig. 2D).

3.3. Supplementation with Glu and Asp restored intestinal energy metabolism in piglets challenged with H_2O_2

Challenge with H_2O_2 increased the jejunal abundance of isocitrate dehydrogenase β (*ICDHβ*) mRNA (Fig. 3A) and the ileal

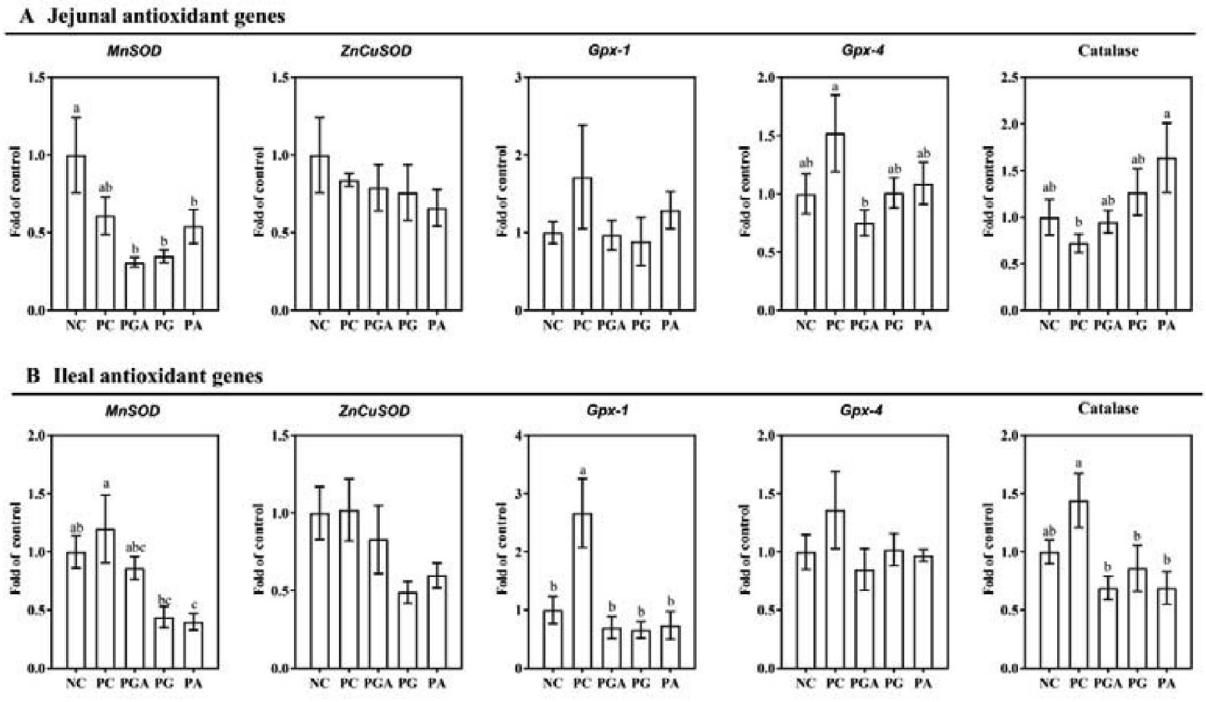

Fig. 1. Dietary supplementation with Glu and Asp regulated intestinal antioxidative gene expression in piglets challenged with H_2O_2. (A) Abundances of mRNA (*MnSOD*, *ZnCuSOD*, *Gpx-1*, *Gpx-4*, and catalase) encoding anti-oxidative proteins in the jejunum. (B) Abundances of mRNA (*MnSOD*, *ZnCuSOD*, *Gpx-1*, *Gpx-4*, and catalase) encoding anti-oxidative proteins in the ileum. The data are presented as means ± standard errors of the means (n = 8). [a, b] Bars with different superscript letters are significantly different ($P < 0.05$). NC, control group; PC, H_2O_2-challenged group fed the basal diet; PGA, H_2O_2-challenged group fed the basal diet +2% Glu +1% Asp; PG, H_2O_2-challenged group fed the basal diet +2% Glu; PA, H_2O_2-challenged group fed the basal diet +1% Asp. *MnSOD* = manganese superoxide dismutase; *ZnCuSOD* = zinc/copper superoxide dismutase; *Gpx* = glutathione peroxidase.

abundances of hexokinase (*Hexok*) (Fig. 3B) and carnitine palmitoyltransferase-1 (*CPT-1*) mRNA (Fig. 4 A), but had no significant effect on the expression of other glucose metabolism-related genes [e.g., citrate synthase (*CS*), pyruvate dehydrogenase (*PDH*), and phosphoenolpyruvate carboxykinase 1 (*PCK-1*)] (Fig. 3) or lipid metabolism-related genes [e.g., *ACC* and fatty acid synthase (*FAS*)] (Fig. 4A and B). Dietary supplementation with a combination of Glu and Asp restored the abundances of *Hexok* (Fig. 3) and *CPT*-1 mRNA (Fig. 4) in the jejunum and ileum.

Challenge with H_2O_2 enhanced the level of p-ACC but decreased the level of p-AMPK (Fig. 5A) in the jejunum and activated the AMPK—ACC pathway in the ileum of the piglets, as demonstrated by the elevated levels of p-AMPK and p-ACC (Fig. 5B and Appendix Fig. 2B). However, dietary supplementation with Glu and Asp might inhibit the AMPK—ACC signaling pathway (Fig. 5A and B and Appendix Fig. 2B) in the ileum and the ACC signaling pathway in the jejunum.

3.4. Supplementation with Glu and Asp repaired the ileal barrier function in piglets challenged with H_2O_2

Challenge with H_2O_2 also impaired the intestinal barrier, as evidenced by the images of H&E-stained intestinal tissues, which revealed sloughing of intestinal epithelial cells in the villus cavities in the jejunum and ileum (Fig. 6A). The challenge was also associated with increased serum DAO concentrations (Fig. 6B). Unsurprisingly, dietary supplementation with Glu and Asp partly repaired the intestinal barrier, as evidenced by a more intact morphology in the jejunum and ileum (Fig. 6A) and decreased serum DAO concentrations (Fig. 6B). However, WB revealed similar levels of the tight junction proteins claudin-1, occludin, and ZO-1 between the groups (Fig. 6C to D).

4. Discussion

The amino acids Glu and Asp have recently garnered attention because of their essential functions in nutrition, antioxidative effects, and mycotoxin-induced damage repair (Duan et al., 2016; Jiao et al., 2015). We previously demonstrated that both Glu and Asp alleviate the oxidative stress response, including growth suppression, in piglets challenged with H_2O_2 and diquat (Duan et al., 2016; Yin et al., 2015b). This study demonstrated that dietary supplementation with Glu and Asp regulated apoptosis, energy metabolism, and the epithelial barrier function in piglets' intestines challenged with H_2O_2.

Physiologically, Glu and Asp are metabolized extensively in the intestine. The carbon backbones derived from these amino acids are oxidized to yield ATP, whereas nitrogen is used mainly to synthesize other amino acids such as Ala, Gln, and citrulline (Nakamura et al., 2013). Glu is also a component of GSH, a vital antioxidant and immune regulator. Glu is primarily extracted (95%) during the first pass through the small intestine in pigs (Brosnan and Brosnan, 2013; Burrin and Stoll, 2009). Asp plays an essential role in cell viability and proliferation. Many compelling studies have shown that Glu and Asp can attenuate the oxidative stress response and intestinal damage in pigs challenged with H_2O_2 (Duan et al., 2016), mycotoxin (Duan et al., 2014), deoxynivalenol (Wu et al., 2014a), and diquat (Yin et al., 2015b). Dietary Glu supplementation increased the expression of *CuSOD* and *Gpx-1* mRNA in *Cyprinus carpio* var. *Jian*, whereas Asp reversed catalase and *Gpx-1* gene expression in the LPS-induced oxidative

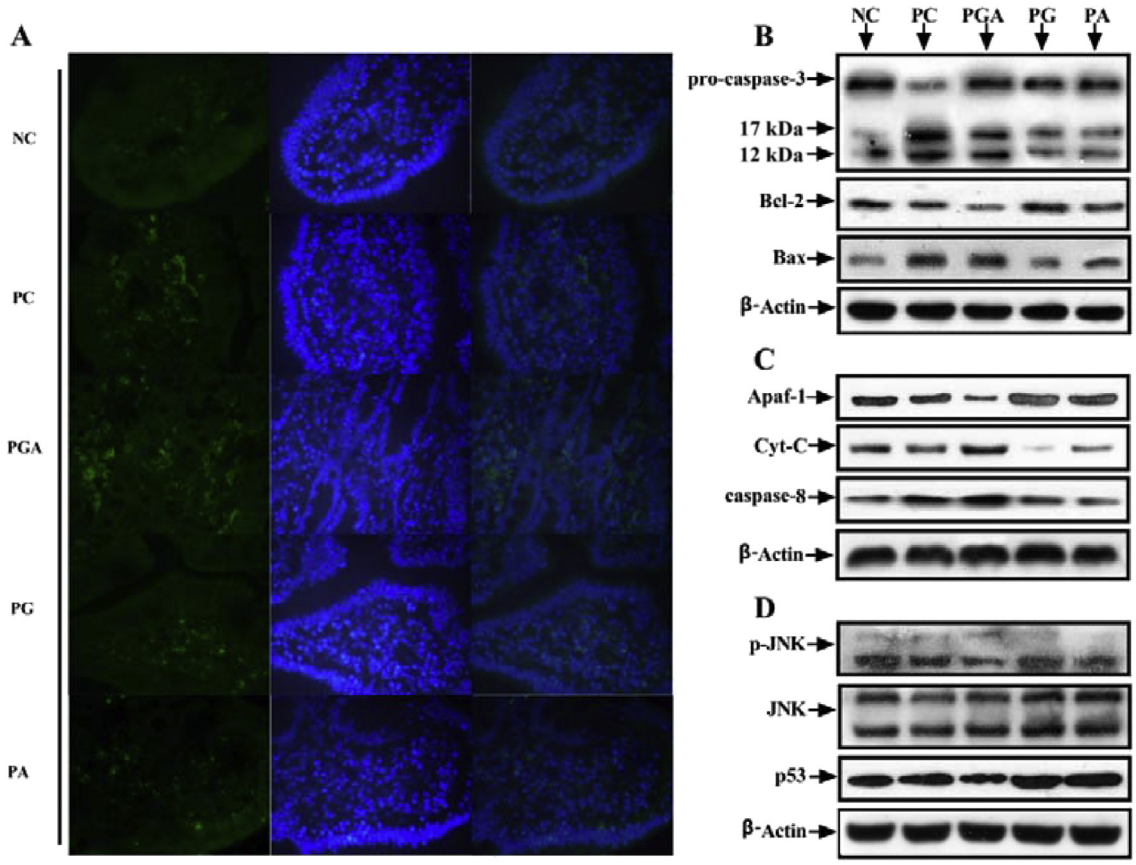

Fig. 2. Dietary supplementation with Glu and Asp decreased ileal apoptosis in piglets challenged with H_2O_2. (A) Representative images of transferase-mediated dUTP nick end-labeling (TUNEL)-stained ileal tissues to determine the degree of apoptosis. (B to D) Green speckles represent apoptosis-positive cells. The Western blot was to detect the protein levels of cleaved and pro-caspase-3, Bcl-2, Bax, Apaf-1, Cyt-c, caspase-8, p-JNK, JNK, and p53. Beta-actin was used as a loading control. NC, control group; PC, H_2O_2-challenged group fed a basal diet; PGA, H_2O_2-challenged group fed the basal diet +2% Glu +1% Asp; PG, H_2O_2-challenged group fed the basal diet +2% Glu; PA, H_2O_2-challenged group fed the basal diet +1% Asp. Bcl-2 = B-cell lymphoma 2; Bax = Bcl-2-associated X protein; Apaf-1 = apoptotic peptidase activating factor-1; Cyt-c = cytochrome C; JNK = Jun N-terminal kinase.

injury of ovine intestinal epithelial cells and upregulated catalase and *Gpx* gene abundances in young grass carp (Wang et al., 2015; Zhang et al., 2019; Zhao et al., 2019). These findings demonstrated the regulatory roles of Glu and Asp in oxidative stress-associated gene expression and corroborated the findings in this study.

Dietary supplementation with Glu and Asp attenuated the intestinal oxidative stress response and intestinal barrier damage in H_2O_2-challenged piglets, as evidenced by the restored intestinal histology and morphology, decreased serum DAO concentrations, and reduced intestinal apoptosis observed in this study. Plausibly, the intestinal barrier was impaired by H_2O_2, a common ROS that can induce an oxidative stress response in tissues challenged with a supra-physiological dosage. Emerging evidence indicates that oxidative stress disturbs the intestinal barrier function by disrupting the expression, organization, and distribution of tight junction proteins (Shen et al., 2006) and inducing apoptosis (Baregamian et al., 2011), and both of these phenomena profoundly contribute to intestinal barrier disorders (Roth et al., 2005). However, it remains unclear how Glu and Asp maintain intestinal barrier. Recently, Xiao et al. (2014) showed that Glu could prevent intestinal atrophy in a mouse model of total parenteral nutrition. In that study, Glu stimulated the expression of taste-sensing receptors (e.g., T1R3, mGluR5, and G-protein subunit G) to reactivate the luminal sensing signaling pathways, promoted intestinal epithelial cell proliferation via phosphorylated-protein kinase B/mammalian target of rapamycin signaling, and modulated intestinal mucosa development, trans-epithelial electrical resistance, permeability, and the barrier function (Xiao et al., 2014). In another study, Glu supplementation prevented jejunal atrophy in piglets during the first week postweaning and improved the D-xylose-absorbing capacity of the small intestine (Liu et al., 2002). Asp could enhance intestinal integrity and energy status via NF-κB and p38 signaling (Pi et al., 2014; Wang et al., 2017). Glu and Asp promoted the expression of *Gpx-4*, *CuZnSOD*, and catalase mRNA in the testis and epididymis of H_2O_2-challenged piglets but did not affect *Gpx-1* (Tang et al., 2020). Thus, we can ascribe Glu and Asp's beneficial effects to their regulatory roles in the contexts of nutrient sensing, cell proliferation, and energy maintenance. Furthermore, Glu and Asp are substrates of Gln, which specifically regulates the intestinal barrier (Wang et al., 2016). Thus, the protective roles of Glu and Asp may also involve the synthesis of Gln. However, further studies are needed to support this hypothesis.

Studies of the intestine have revealed an association of barrier function with energy metabolism (Le Drean and Segain, 2014) and a fundamental link between cellular bioenergetics and the mucosal barrier (Glover et al., 2013). Generally, cellular energy metabolism is controlled by AMPK, an energy sensor that

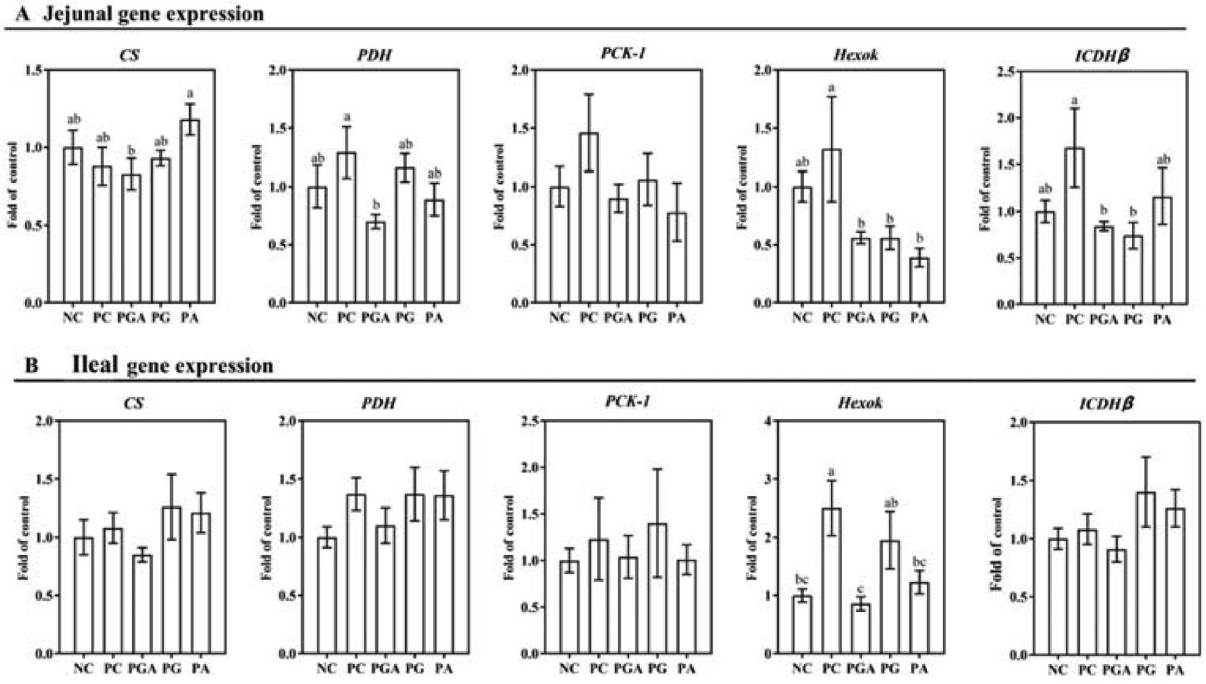

Fig. 3. Dietary supplementation with Glu and Asp regulated intestinal glucose metabolic gene expression in piglets challenged with H_2O_2. (A) The abundance of mRNA (*CS, Hexok, PDH, ICDHβ, PKC-1*) encoding glucose metabolic proteins in the jejunum. (B) The abundance of mRNA (*CS, Hexok, PDH, ICDHβ, PKC-1*) encoding glucose metabolic proteins in the ileum. The data are presented as means ± MES ($n = 8$). $^{a, b, c}$ Bars with different superscript letters are significantly different ($P < 0.05$). NC, control group; PC, H_2O_2-challenged group fed the basal diet; PGA, H_2O_2-challenged group fed the basal diet +2% Glu +1% Asp; PG, H_2O_2-challenged group fed the basal diet +2% Glu; PA, H_2O_2-challenged group fed the basal diet +1% Asp. *CS* = citrate synthase; *PDH* = pyruvate dehydrogenase; *PCK-1* = phosphoenolpyruvate carboxykinase 1; *Hexok* = hexokinase; *ICDHβ* = isocitrate dehydrogenase β.

regulates energy availability in various host tissues (Lage et al., 2008). The ratio of AMP to ATP regulates the status of AMPK: a higher ratio activates AMPK, whereas a lower ratio maintains inactivation (Mihaylova and Shaw, 2011). Under conditions of stress (e.g., LPS treatment), ATP will be depleted, leading to an increased AMP-to-ATP ratio, which leads to AMPK activation and ultimately affects the intestinal energy status and barrier function (Pi et al., 2014). However, these adverse effects can be reversed by dietary Asp supplementation (Pi et al., 2014). Thus, in this study, we tested whether supplementing low-protein diets with Glu and Asp could restore intestinal metabolism via the AMPK–ACC signaling pathway. As expected, dietary Glu and Asp supplementation attenuated the increases in *Hexok* and *CPT-1* expression and AMPK–ACC signaling pathway activation in the jejunum and ileum in response to the H_2O_2 challenge. *Hexok* and *CPT-1* are rate-limiting enzymes in glycolysis and mitochondrial β-oxidation, respectively (Thupari et al., 2002; Wolf et al., 2011). These observations suggest that intestinal metabolism may shift to glycolysis and fatty acid mitochondrial β-oxidation, as observed in some immune cells (e.g., macrophages) under inflammatory conditions (O'Neill and Hardie, 2013) and cancer cells under metabolic stress (Sun et al., 2015). However, we only explored the gene expression of some proteins related to metabolism and AMPK–ACC signaling and cannot conclude definitively that intestinal cells undergo such a metabolic switch.

Massive oxidative stress-mediated depletion of cellular ATP might be the most likely cause of the observed effects on the AMPK–ACC signaling pathway. This phenomenon can increase the AMP-to-ATP ratio, allowing AMP to bind directly to its γ regulatory subunits and promoting AMPK activation (Mihaylova and Shaw, 2011; Oakhill et al., 2011). However, it remains unclear how Glu and Asp regulate metabolic gene expression and inhibit AMPK–ACC signaling under oxidative stress conditions. Previous studies suggest three perspectives that support our findings. Firstly, Glu and Asp are major sources of ATP. When supplied by the diet, both amino acids can be absorbed and metabolized into ATP to restore the intestinal energy status, which may inactivate the AMPK–ACC signaling pathway. Indeed, Kang et al. (2015) reported that Asp could restore the intestinal ATP level and AMP-to-ATP ratio in LPS-challenged piglets. Secondly, Glu and Asp are required for the synthesis of tricarboxylic acid cycle intermediates and other bioactive molecules, such as nucleic acids and GSH; therefore, dietary supplementation may enhance molecular synthesis and alleviate oxidative stress, as we reported previously (Duan et al., 2016; Jiao et al., 2015). Thirdly, Glu critically links glucose metabolism to incretin/cyclic AMP action and amplifies insulin secretion (Gheni et al., 2014), and Asp plays an essential role in the mitochondrial electron transport chain (Birsoy et al., 2015). Therefore, we speculate that Glu and Asp may regulate glucose metabolism, insulin secretion, and intestinal cell proliferation to maintain intestinal health. We also observed that the protein abundance of p-AMPK is segment-specific in the jejunum and ileum in H_2O_2-challenged piglets, consistent with a previous report that different p-AMPK levels between the intestinal segments might be associated with differences in the mucosal pH (Klinger, 2020). However, further studies are needed to determine how Glu and Asp modulate intestinal energy metabolism.

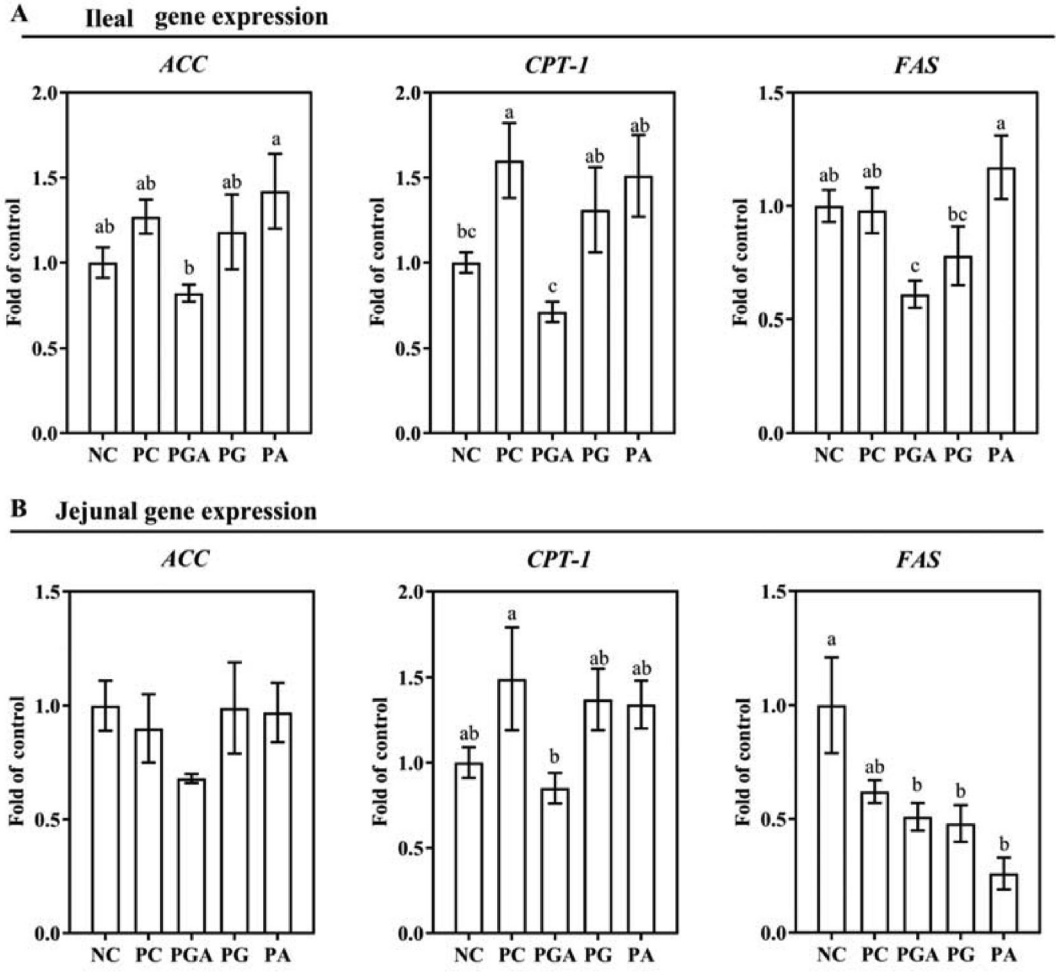

Fig. 4. Dietary supplementation with Glu and Asp regulated intestinal lipid metabolic gene expression in piglets challenged with H_2O_2. (A) Abundances of mRNA (*ACC, CPT-1, FAS*) encoding lipid metabolic proteins in the ileum. (B) Abundances of mRNA (*ACC, CPT-1, FAS*) encoding lipid metabolic proteins in the jejunum. The data are presented as means ± standard errors of the means ($n = 8$). [a, b, c] Bars with different superscript letters are significantly different ($P < 0.05$). NC, control group; PC, H_2O_2-challenged group fed the basal diet; PGA, H_2O_2-challenged group fed the basal diet +2% Glu +1% Asp; PG, H_2O_2-challenged group fed the basal diet +2% Glu; PA, H_2O_2-challenged group fed the basal diet +1% Asp. *ACC* = acetyl-coenzyme A carboxylase; *CPT-1* = carnitine palmitoyltransferase-1; *FAS* = fatty acid synthase.

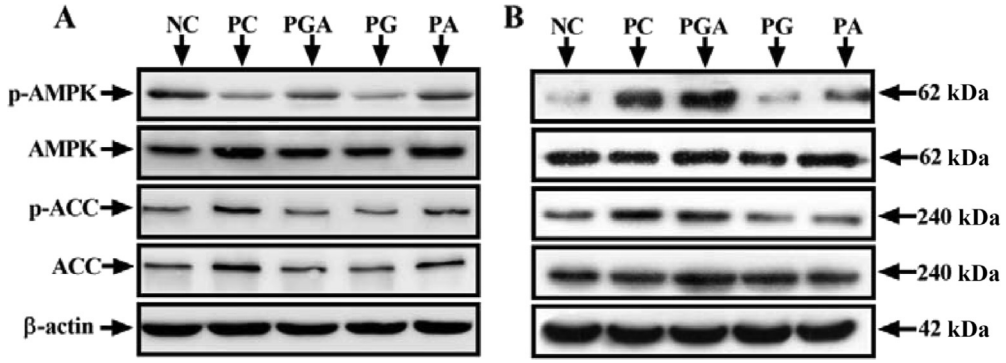

Fig. 5. The Western blot of AMPK and ACC protein levels in the jejunum (A) and ileum (B). Dietary supplementation with Glu and Asp affected the AMPK–ACC signaling pathway in the intestine of piglets challenged with H_2O_2. Beta-actin was used as a loading control. The data are presented as means ± standard errors of the means ($n = 8$). NC, control group; PC, H_2O_2-challenged group fed the basal diet; PGA, H_2O_2-challenged group fed the basal diet +2% Glu +1% Asp; PG, H_2O_2-challenged group fed the basal diet +2% Glu; PA, H_2O_2-challenged group fed the basal diet +1% Asp. AMPK = adenosine 5′-monophosphate-activated protein kinase; ACC = acetyl-coenzyme A carboxylase.

5. Conclusion

Low-protein diets supplemented with Glu and Asp appeared to attenuate oxidative stress-induced intestinal barrier dysfunction and altered the intestinal energy metabolic gene expression in H_2O_2-challenged piglets. Our results suggest that Glu and Asp may be used as nutritional regulators to protect animals against oxidative stress.

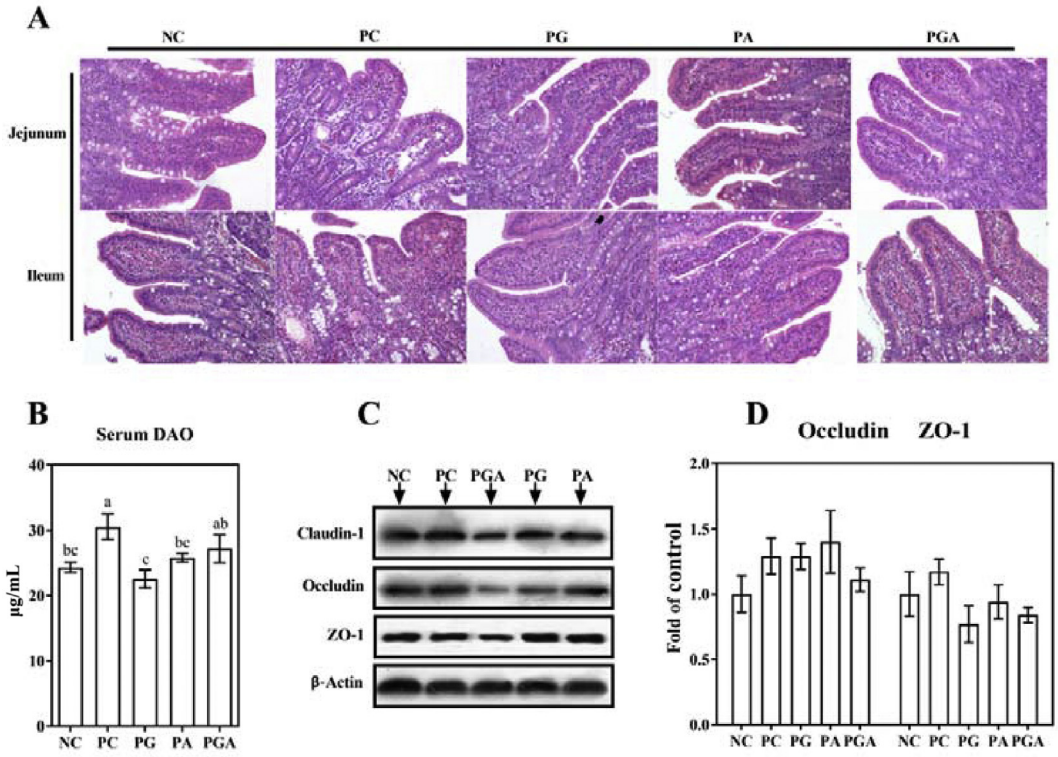

Fig. 6. Dietary supplementation with Glu and Asp enhanced the intestinal barriers in piglets challenged with H_2O_2. (A) Representative hematoxylin and eosin-stained sections of the jejunum and ileum. (B) Serum diamine oxidase (DAO) concentrations. (C) The Western blot to detect tight junction protein levels (claudin-1, occludin, ZO-1) in the ileum. (D) Abundances of occludin and ZO-1 mRNA in the ileum. Beta-actin was used as a loading control. The data are presented as means ± standard errors of the means ($n = 8$). $^{a, b, c}$ Bars with different superscript letters are significantly different ($P < 0.05$). NC, control group; PC, H_2O_2-challenged group fed the basal diet; PGA, H_2O_2-challenged group fed the basal diet +2% Glu +1% Asp; PG, H_2O_2-challenged group fed the basal diet +2% Glu; PA, H_2O_2-challenged group fed the basal diet +1% Asp. DAO = diamine oxidase; ZO-1 = zonula occludens-1.

Author contributions

Shuai Chen: methodology, investigation, writing - original draft; **Xin Wu**: investigation, visualization; **Jielin Duan**: validation, formal analysis; **Pan Huang**: formal analysis, writing - review and editing; **Tiejun Li**: conceptualization, writing - review and editing; **Yulong Yin**: supervision, resources; **Jie Yin**: conceptualization, writing - review and editing. The data used or analyzed in the current study are available from the corresponding author upon reasonable request.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that might inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix

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