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RESEARCH ARTICLE

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Icariin and its phosphorylated derivatives alleviate intestinal epithelial barrier disruption caused by enterotoxigenic *Escherichia coli* through modulate p38 MAPK in vivo and in vitro

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Abstract

The natural product icariin (ICA) and its phosphorylated derivatives (pICA) have been shown to have outstanding anti-inflammatory and antioxidant properties. This study was to explore the protective effects of ICA and pICA on the intestinal epithelium of enterotoxigenic *Escherichia coli* (ETEC)-induced piglet diarrhea and its underlying mechanisms in vivo and in vitro. ETEC K88 increased pro-inflammatory cytokine expression, activated oxidative stress and inhibited antioxidant enzyme activity, induced phosphorylated p38 MAPK gene and protein expression, disrupted intestinal barrier function, and led to diarrhea in piglets. Pretreatment with ICA and pICA effectively alleviated ETEC-induced intestinal barrier dysfunction in vivo and in vitro. Pretreatment with p38 MAPK inhibitor (SB203580) significantly rescued the IPEC-J2 cells barrier function damaged by ETEC challenge. However, pretreatment with p38 MAPK activator (anisomycin) did not alleviated the IPEC-J2 cells barrier function damaged by ETEC challenge. Our data demonstrated that ICA and pICA regulate the inflammatory response and oxidative stress of intestinal epithelial cells by inhibiting the expression of p38 MAPK, thereby alleviating ETEC K88-induced disruption of intestinal barrier function and intestinal permeability. These findings provide new insights into the prevention and treatment of intestinal barrier dysfunction induced by ETEC K88.

KEYWORDS

enterotoxigenic *Escherichia coli* K88, icariin, intestinal barrier function, p38 MAPK, phosphorylated icariin

Abbreviations: ADG, weight gain; BW, body weight; CAT, catalase; DAO, diamine peroxidase; ETEC, enterotoxigenic *Escherichia coli*; FITC-D4, fluorescein isothiocyanate-dextran; GSH-Px, glutathione peroxidase; H_2O_2, hydrogen peroxide; ICA, icariin; IL, interleukin; MDA, malondialdehyde; pICA, phosphorylated icariin; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; T-AOC, total antioxidant capacity; TEER, trans epithelium resistance; TJs, tight junctions; TNF, tumor necrosis factor.

1 | INTRODUCTION

Diarrhea and intestinal diseases in neonates and postweaning piglets caused by enterotoxigenic *Escherichia coli* (ETEC) K88 is a major challenge for the swine industry worldwide.[1-3] As a common pathogen, ETEC adheres to the intestinal mucosal epithelial cells and adjacent mucosal layer-specific glycoprotein receptors through the bacterial surface of the bacteria, releasing enough endotoxin to cause piglet diarrhea, reduce growth performance, increase morbidity and mortality, resulting in an incalculable economic loss.[4,5] The intestinal epithelium has the ability to selectively penetrate to prevent harmful substances in the intestinal lumen from invading intestinal epithelial cells.[6] Tight junctions (TJs) are the primary component of the intestinal epithelium to maintain selective permeability.[7] The TJs mainly include the Claudin, Occludin, and ZO families, which together constitute the main physical barrier against intestinal pathogenic microbial invasion and spread.[8] Increasing evidence suggested that ETEC infection disrupts the TJs structure of the intestinal epithelial barrier by altering the TJs genes and proteins expression, thereby reducing intestinal barrier function and increasing intestinal permeability, ultimately causing diarrhea in animals.[9,10]

Harmful physiological processes such as inflammatory responses and oxidative stress cause intestinal epithelial dysfunction, which ultimately leads to inflammation, metabolism, and intestinal diseases.[11,12] Current evidences suggested that inflammatory responses and oxidative stress may be the primary pathway by which ETEC regulates intestinal barrier function.[13,14] Therefore, relieving ETEC-induced inflammatory response and oxidative stress to enhance intestinal barrier function is a reliable strategy for preventing and treating diarrhea in piglets. Natural products and their derivatives are increasingly used to combat diseases. Icariin (ICA) is a flavonoid derived from epimedium and has a variety of biological activities and pharmacological effects, the main effects of which are anti-inflammatory and antioxidant.[15,16] In addition, phosphorylated derivatives of ICA (pICA) have also been shown to have potent anti-inflammatory and antioxidant properties.

MAPKs signaling pathway is involved in many biological processes, such as regulation of oxidative stress, involvement in inflammation, apoptosis, and cell proliferation, and is closely related to intestinal epithelial barrier function.[17,18] There was study found that phosphorylated p38 MAPK protein level dramatically elevates in ETEC-induced weaned pig diarrhea, suggesting that p38 MAPK has an important contribution to ETEC-induced diarrhea.[19] Our previous study demonstrated that ICA and pICA inhibit duck liver virus-induced tissue damage by regulating the expression of p38 MAPK.[20] Therefore, we hypothesize that ICA and pICA have protective effects on the intestinal epithelial barrier function of ETEC-induced piglet diarrhea by regulating the expression of p38 MAPK. To demonstrate this hypothesis, we explored the protective effects of ICA and pICA on the intestinal barrier of piglets by constructing an ETEC-induced model of in vivo piglet diarrhea and an in vitro IPEC-J2 cell injury model. In addition, the protective mechanism of ICA and pICA on intestinal barrier function in piglets was clarified by specifically blocking and activating the p38 MAPKs signaling pathway.

2 | MATERIALS AND METHODS

2.1 | Ethical approval

All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, Eighth edition, 2011) and was approved by the Laboratory Animal Welfare and Ethics Committee of Southwest University (SWU 20190307). The experimental regulations and methods were approved and then performed according to relevant criterions.

2.2 | Bacterial strains, ICA, pICA, and reagents

The ETEC K88 strain (serotype O149:K91:K88ac), obtained from China Institute of Veterinary Drug Control (Beijing, China), was grown in Luria Broth. ICA (lot no. 20171125, net content 90.00%) was bought from Xi'an Grassroot Chemical Engineering Co. Ltd. (China). pICA was synthesized using the sodium tripolyphosphate-sodium trimetaphosphate method.[21] The synthesized product was purified by preparative HPLC (purity > 90%, detected by molybdenum blue colorimetry, and double checked by HPLC). SB203580 (lot no. GC13595, purity = 98.00%) was purchased from GlpBio Technology (USA). Anisomycin (lot no. SC0132, purity = 99.00%) was obtained from Beyotime Biotechnology (China).

2.3 | Animals and dietary treatments

Seventy-two male piglets (Duroc × Landrace × Large White, 4-d old) were assigned to one of four treatments with six replicate pens of 3 piglets. The pens with plastic mesh flooring were housed in air-conditioned rooms (one treatment per room) to control temperature (30 ± 1°C) and humidity and prevent cross-contamination between treatments. The basal diet (Table S1) was formulated as a powder form without any in-feed antibiotics according to NRC (2012) for 3-5-kg piglets and was freshly mixed with water at 1:3 before

each feeding. All piglets were fed every 2 hours from 800 to 2400 hours and had free access to warm water throughout the 17-day feeding trial. All piglets were randomly divided into four groups: control group, ETEC K88 group, ICA + ETEC K88 group, and pICA + ETEC K88 group. The control group of piglets fed the basal diet for the whole period, and were orally given 10 mL of PBS at 800 hours on Day 15; the ETEC K88 group of piglets fed the basal diet for the whole period, and were orally given 10-mL PBS containing approximately 1×10^8 cfu of ETEC K88 10 mL at 0800 hours on Day 15; ICA + ETEC K88 group piglets were fed basal diet supplemented with 1 g/kg BW of ICA for the whole period, and were orally given 10-mL PBS containing approximately 1×10^8 cfu of ETEC K88 10 mL at 0800 hours on Day 15; pICA + ETEC K88 group piglets were fed basal diet supplemented with 1 g/kg BW of pICA for the whole period, and were orally given 10-mL PBS containing approximately 1×10^8 cfu of ETEC K88 10 mL at 800 hours on Day 15. Piglets were weighed at 800 hours on Days 1, 15, and 18 to calculate body weight (BW) and weight gain (ADG). Animal diarrhea was examined daily to assess its status before and after challenge with ETEC K88. Fecal consistency was assessed visually and classifed at four levels as described by previous report[22]:0, normal; 1, pasty; 2, semiliquid; and 3, liquid. The piglets were considered to have diarrhea when the fecal consistency was at level 2 or 3, and the diarrhea rate was calculated using the number of pig days with diarrhea in each pen as percentage of total pig days during that time interval.

At Day 3 after the ETEC K88 challenge (Day 18), one piglet from each pen was chosen at random, blood and jejunum samples were collected. Blood samples were collected from piglets by vena jugularis, with a minimum amount of stress, into heparinized tubes (5 mL). Plasma samples were then obtained by centrifuging the blood samples at 3000 *g* and 4°C for 10 minutes and were stored at −80°C until analysis. The jejunal segments were collected from the approximately middle positions in the intestinal tracts. The intestinal epithelium was separated from the muscular layers by blunt dissection and then stored at −80°C prior to further analysis.

2.4 | Cell culture

The IPEC-J2 cell line is a nontransformed intestinal epithelial cell line derived from jejunal epithelium isolated from a neonatal, unsucked piglet.[23] Cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% gentamycin, 1% HEPES buffer, and 1% nonessential amino acids with the medium changed every 2-3 days. The incubation condition was 37°C and 5% CO_2 atmosphere.

2.5 | Pretreatment of jejunal tissue and cell samples

One hundred micrograms frozen jejunal mucosa specimen were minced and homogenized in 1 mL of ice-cold cytoplasm RIPA containing the protease inhibitor cocktail complete EDTA free (Roche, Penzberg, Germany). The homogenates were centrifuged at 12 000 *g* for 20 minutes at 4°C and then collected the supernatant. IPEC-J2 cells were disrupted by sonication (VCX105; Sonics, Newtown, CT, USA) in ice-cold PBS and centrifuged at 12 000 rpm for 20 minutes at 4°C to remove debris and collect supernatant. Protein concentration was determined using a BCA Protein Assay kit (Pierce, Rockford, IL, USA), and then diluted to the same concentration for subsequent analysis.

2.6 | Determination of intestinal permeability

The plasma and jejunal endotoxin, diamine peroxidase (DAO), D-lactate, and zonulin were determined using porcine enzyme-linked immunosorbent assay kits (Shanghai Enzyme-linked Biotechnology Co. Ltd, Shanghai, China), according to the manufacturer's instructions.

2.7 | Determination of TEER and FITC-D4

The IPEC-J2 cells monolayer was constructed by seeding 3.75×10^5 cells into each well of a six-well Transwell plate (Corning, Inc, Corning, NY, USA). The insert area was 0.33 cm^2 and the pore size was 0.4 μm. The culture medium was changed every other day. Cells reached confluence on the second day and, on Day 7, the treatments were performed. The value of trans epithelium resistance (TEER) of the IPEC-J2 monolayers reached about 150 Ω•cm^2 7 days after confluence. Fluorescein isothiocyanate-dextran (FITC-D4, 4 kDa, 0.25 mM) measurements were taken for paracellular permeability.[24] FITC-D4 was added to the apical chamber at the end of treatment. After 2 hours, 50-μL medium from the bottom chamber were transferred to a fluorescence measurement plate, and fluorescence intensity was measured at an excitation wavelength of 485 nm and emission wavelength of 530 nm. TEER and FITC-D4 flux values were both expressed as percent of control.

2.8 | Flow cytometry

Intracellular ROS in IPEC-J2 cells were measured with 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Sigma) as a previously reported method.[25] In brief, after dilution to a final concentration of 10 μM with serum-free DMEM, DCFH-DA was added to the cells after the culture medium was removed

and incubated for 30 minutes at 37°C. Next, the cells were washed 3 times with phosphate-buffered saline (PBS). The cells were resuspended in PBS, and the fluorescence intensity was measured for more than 10 000 cells of each sample by a FACSVerse flow cytometer. The level of total intracellular ROS, paralleled by an increase in fluorescence intensity, was calculated as the percentage of control cells.

Mitochondrial ROS in IPEC-J2 cells were measured with MitoSox red mitochondrial superoxide indicator (Invitrogen) as described previously.[26] Briefly, after the culture medium was removed, the cells were washed 3 times with PBS. MitoSox red mitochondrial superoxide indicator, diluted to a final concentration of 4 mM with serum-free DMEM, was added to the cells and incubated for 20 minutes at 37°C in the dark. The cells were then washed 3 times with PBS. The cells were resuspended in PBS and the fluorescence was measured immediately by FACSVerse flow cytometer. The level of mitochondrial ROS corresponded with an increase in fluorescence and was calculated as the percentage of the measured signals for control cells.

2.9 | Determination of oxidative an antioxidative status

Reactive oxygen species (ROS), reactive nitrogen species (RNS), malondialdehyde (MDA), and hydrogen peroxide (H_2O_2) contents were determined using enzyme-linked immunosorbent assay kits (Shanghai Enzyme-linked Biotechnology Co. Ltd, Shanghai, China), according to the manufacturer's instructions.

The activity of superoxide dismutase (SOD) activity, glutathione peroxidase (GPx) activity, catalase (CAT) activity, and total antioxidant capacity (T-AOC) were determined using enzyme-linked immunosorbent assay kits (Shanghai Enzyme-linked Biotechnology Co. Ltd, Shanghai, China), according to the manufacturer's instructions.

2.10 | Determination of inflammatory status

The levels of interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α) were determined using enzyme-linked immunosorbent assay kits (Shanghai Enzyme-linked Biotechnology Co. Ltd, Shanghai, China), according to the manufacturer's instructions.

2.11 | Messenger RNA expression analysis by real-time PCR

Jejunal tissue or cells samples was quickly collected and immediately frozen in liquid nitrogen, and stored at −80°C until RNA isolation. Total RNA was extracted from colon samples with Trizol Reagent (15596026, Invitrogen). Concentration and quality of the RNA were measured by NanoDrop ND-1000 Spectrophotometer (Thermo, USA). Then 2 mg of total RNA were treated with RNase-Free DNase (M6101, Promega, USA) and reverse-transcribed according to manufacturer's instructions. Two microliters of diluted cDNA (1:40, vol/vol) were used for real-time PCR which was performed in Mx3000P (Stratagene, USA). GAPDH, which is not affected by the experimental factors, was chosen as the reference gene. All the primers used in this study were listed in Table 1, were synthesized by Generay Company (Shanghai, China). The method of $2^{-\triangle\triangle Ct}$ was used to analyze the real-time PCR results and gene mRNA levels were expressed as the fold change relative to the mean value of control group.

2.12 | Protein expression analysis by western blotting

Jejunal tissue or cells samples was minced and homogenized in 1 mL of ice-cold homogenization buffer RIPA containing the protease inhibitor cocktail Complete EDTA free (Roche, Penz-berg, Germany). The homogenates were centrifuged at 12 000 rpm for 20 minutes at 4°C and then collected the supernatant fraction. Protein concentration was determined using a BCA Protein Assay kit (Pierce, Rockford, IL, USA). Eighty micrograms of protein extract from each sample were then loaded onto 7.5% and 15% SDS-PAGE gels and the separated proteins were transferred onto the nitrocellulose membranes (Bio Trace, Pall Co, USA). After transfer, membranes were blocked for 2 hours at room temperature in blocking buffer and then membranes were incubated with the following primary antibodies: Claudin-1, Occludin, ZO-1, p38, p-p38, and GAPDH in dilution buffer overnight at 4°C. After several times washes in Tris-buffered saline with Tween (TBST), membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies in dilution buffer for 2 hours at room temperature. Finally, the blot was washed and detected by enhanced chemiluminescence's (ECL) using the LumiGlo substrate (Super Signal West Pico Trial Kit, Pierce, USA), and the signals were recorded by an imaging System (Bio-Rad, USA), and analyzed with Quantity One software (Bio-Rad, USA).

2.13 | Statistical analysis

Data are presented as means ± SEM. Statistical significance was assessed by the independent sample *t* test using SPSS (SPSS v. 20.0, SPSS Inc, Chicago, IL, USA) software packages. Data were considered statistically significant when $P < .05$. Numbers of replicates used for statistics are noted in the Table and Figures.

TABLE 1 Primer sequences used for reverse transcription quantitative real-time PCR

Target genes	Prime forward/reverse	Primer sequence (5′→3′)
GAPDH	Forward	CGTCCCTGAGACACGATGGT
	Reverse	GCCTTGACTGTGCCGTGGAAT
ZO-1	Forward	CTCTTGGCTTGCTATTCG
	Reverse	AGTCTTCCCTGCTCTTGC
Occludin	Forward	GTAGTCGGGTTCGTTTCC
	Reverse	GACCTGATTGCCTAGAGTGT
Claudin-1	Forward	GATTTACTCCTACGCTGGTGAC
	Reverse	CACAAAGATGGCTATTAGTCCC
IL-1β	Forward	CAAGGCAGGTCAGGTTTCGT
	Reverse	GATGGTAGCGGTTGTCTCTGAT
IL-6	Forward	TCAGTTCTCACCTTCCTCCTG
	Reverse	GAACACAGCCGAATGGTCTAC
IL-8	Forward	AGGACCAGAGCCAGGAA
	Reverse	GTGGAATGCGTATTTATGC
TNF-α	Forward	CACCACGCTCTTCTGCCTAC
	Reverse	ACGGGCTTATCTGAGGTTTGAGACG
p38	Forward	TTCGTGGGGCGTGTAGTGT
	Reverse	GGTGCTGATTGCTGGTGTC

TABLE 2 Effect of ICA and pICA on growth performance and diarrhea incidence in piglets with ETEC K88 challenge

	Groups			
Items	CON	K88	ICA + K88	pICA + K88
BW, kg				
1 d	2.53 ± 0.09	2.52 ± 0.10	2.53 ± 0.11	2.54 ± 0.09
15 d	5.44 ± 0.21^{b}	5.42 ± 0.18^{b}	5.88 ± 0.12^{a}	5.89 ± 0.22^{a}
18 d	6.78 ± 0.18^{a}	6.05 ± 0.16^{b}	7.12 ± 0.22^{a}	7.14 ± 0.19^{a}
1-15 d (prechallenge)				
ADG, g	194 ± 11^{b}	193 ± 11^{b}	223 ± 13^{a}	223 ± 12^{a}
Diarrhea rate, %	10.32 ± 0.59^{b}	10.42 ± 0.66^{b}	6.53 ± 0.48^{a}	6.43 ± 0.55^{a}
15-18 d (postchallenge)				
ADG, g	446 ± 24^{a}	210 ± 22^{b}	413 ± 28^{a}	416 ± 31^{a}
Diarrhea rate, %	2.12 ± 0.72^{c}	85.24 ± 11.24^{a}	16.43 ± 1.05^{b}	14.38 ± 1.03^{b}
1-18 d (pre- and postchallenge)				
ADG, g	236 ± 14^{a}	196 ± 22^{b}	255 ± 18^{a}	255 ± 21^{a}
Diarrhea rate, %	10.46 ± 0.85^{b}	22.38 ± 1.58^{a}	8.32 ± 0.66^{b}	8.64 ± 0.73^{b}

Notes: Data were shown as mean ± SEM (n = 6). The result was analyzed by one-way ANOVA with Turkey's test and the variant alphabetical superscript in the same row indicated a significant difference when $P < .05$. ADG, average daily gain.

3 | RESULTS

3.1 | Effect of ICA and pICA on growth performance and diarrhea incidence in piglets with ETEC K88 challenge

As shown in Table 2, there was no difference in the average body weight of all piglets at birth (Day 0) ($P > .05$). Before challenge with ETEC K88 (Day 15), the average body weight of piglets in the ETEC K88 group did not differ from that of the control group ($P > .05$), but was lower than that of the piglets fed the diet supplemented with ICA and pICA ($P < .05$). After challenge with ETEC K88 (Day 18), the weight of piglets in the ETEC K88 group was significantly lower than that of the control group and was also lower than that of piglets fed ICA and pICA ($P < .05$). In

addition, before challenge with ETEC K88 (Days 1-15), the average daily gain and diarrhea rate of piglets in the ETEC K88 group did not differ from that of the control group ($P > .05$), but the average daily gain and diarrhea rate were lower and higher than those of piglets fed ICA and pICA, respectively ($P < .05$). After challenge with ETEC K88 (Days 15-18 and Days 1-18), the average daily gain and diarrhea rate of piglets in the ETEC K88 group were lower than and higher than the control group, ICA + ETEC K88 group and pICA + ETEC K88, respectively ($P < .05$).

3.2 | Effect of ICA and pICA on intestinal epithelial barrier function in piglets with ETEC K88 challenge

Intestinal epithelial barrier function was evaluated by measuring the chemical markers (endotoxin, DAO, D-lactate, and zonulin) in plasma and jejunum and the expression of TJ proteins (ZO-1, occluding and claudin-1) in jejunum. Challenge with ETEC K88 increased plasma and jejunum concentrations of endotoxin, DAO, D-lactate, and zonulin, meanwhile, the concentration of endotoxin, DAO, D-lactate, and zonulin in plasma and jejunum of piglets supplemented with ICA and pICA groups was significantly lower than that of ETEC K88 group ($P < .05$, Figure 1A-H). In addition, challenge with ETEC K88 decreased ZO-1 and occludin gene and protein expression ($P < .05$), but claudin-1 expression did not change in all piglets ($P > .05$), while the gene and protein expression of ZO-1 and occludin of ICA + ETEC K88 and pICA + ETEC K88 group was significantly higher ($P < .05$) than the ETEC K88 group (Figure 1I-N).

3.3 | Effect of ICA and pICA on inflammatory status of jejunum in piglets with ETEC K88 challenge

Inflammatory status of piglets jejunum was evaluated by measuring the gene expression and content of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α). Challenge with ETEC K88 increased the gene expression of IL-1β, IL-6, IL-8, and TNF-α in jejunum; meanwhile, the gene expression of IL-1β, IL-6, IL-8, and TNF-α in jejunum of piglets supplemented with ICA and pICA groups was significantly lower than that of ETEC K88 group ($P < .05$, Figure 2A-D). In addition, the content of IL-1β, IL-6, IL-8, and TNF-α in jejunum of ETEC K88 group was also significantly higher than the control group, whereas the IL-1β, IL-6, IL-8, and TNF-α concentration in ICA and pICA piglets were lower than those in ETEC K88 group ($P < .05$, Figure 2E-H).

3.4 | Effect of ICA and pICA on redox status of jejunum in piglets with ETEC K88 challenge

Redox status of piglet jejunum was evaluated by measuring the chemical markers (ROS, RNS, MDA, H_2O_2, SOD, GPx, CAT, and T-AOC). Challenge with ETEC K88 increased the levels of ROS, RNS, MDA, and H_2O_2 in jejunum; meanwhile, the ROS, RNS, MDA, and H_2O_2 contents in jejunum of piglets supplemented with ICA and pICA groups was significantly lower than that of ETEC K88 group ($P < .05$, Figure 3A-D). In addition, the activities of SOD, GPx, CAT, and T-AOC in jejunum of ETEC K88 group was significantly lower than the control group, whereas the SOD, GPx, CAT, and T-AOC activities in ICA and pICA piglets were higher than those in ETEC K88 group ($P < .05$, Figure 3E-H).

3.5 | Effect of ICA and pICA on p38 MAPK expression of jejunum in piglets with ETEC K88 challenge

As shown in Figure 4, piglets challenged with ETEC K88 had greater p38 MAPK gene expression and phosphorylated p38 MAPK protein expression in jejunum than control piglets ($P < .05$, Figure 4A,B). Besides, the jejunal p38 MAPK gene expression and phosphorylated p38 MAPK protein expression of piglets fed the ICA and pICA was significantly lower than that of the ETEC K88 group ($P < .05$, Figure 4A,B).

3.6 | Effect of ICA and pICA on barrier function in IPEC-J2 cells with ETEC K88 challenge

Barrier function of IPEC-J2 cells was evaluated by measuring the TEER, FITC-D4 flux, and the expression of TJ proteins (ZO-1, occludin, and claudin-1). Challenge with ETEC K88 reduced TEER and increased FITC-D4 flux in IPEC-J2 cells ($P < .05$, Figure 5A,B). Compared with the ETEC K88 group, ICA and pICA pretreatment increased TEER and decreased FITC-D4 flux, respectively ($P < .05$, Figure 5A,B). In addition, challenge with ETEC K88 decreased ZO-1 and occludin gene and protein expression in IPEC-J2 cells ($P < .05$), but claudin-1 expression did not change in all group ($P > .05$), while the gene and protein expression of ZO-1 and occludin of ICA + ETEC K88 and pICA + ETEC K88 groups was significantly higher ($P < .05$) than the ETEC K88 group (Figure 1C-H).

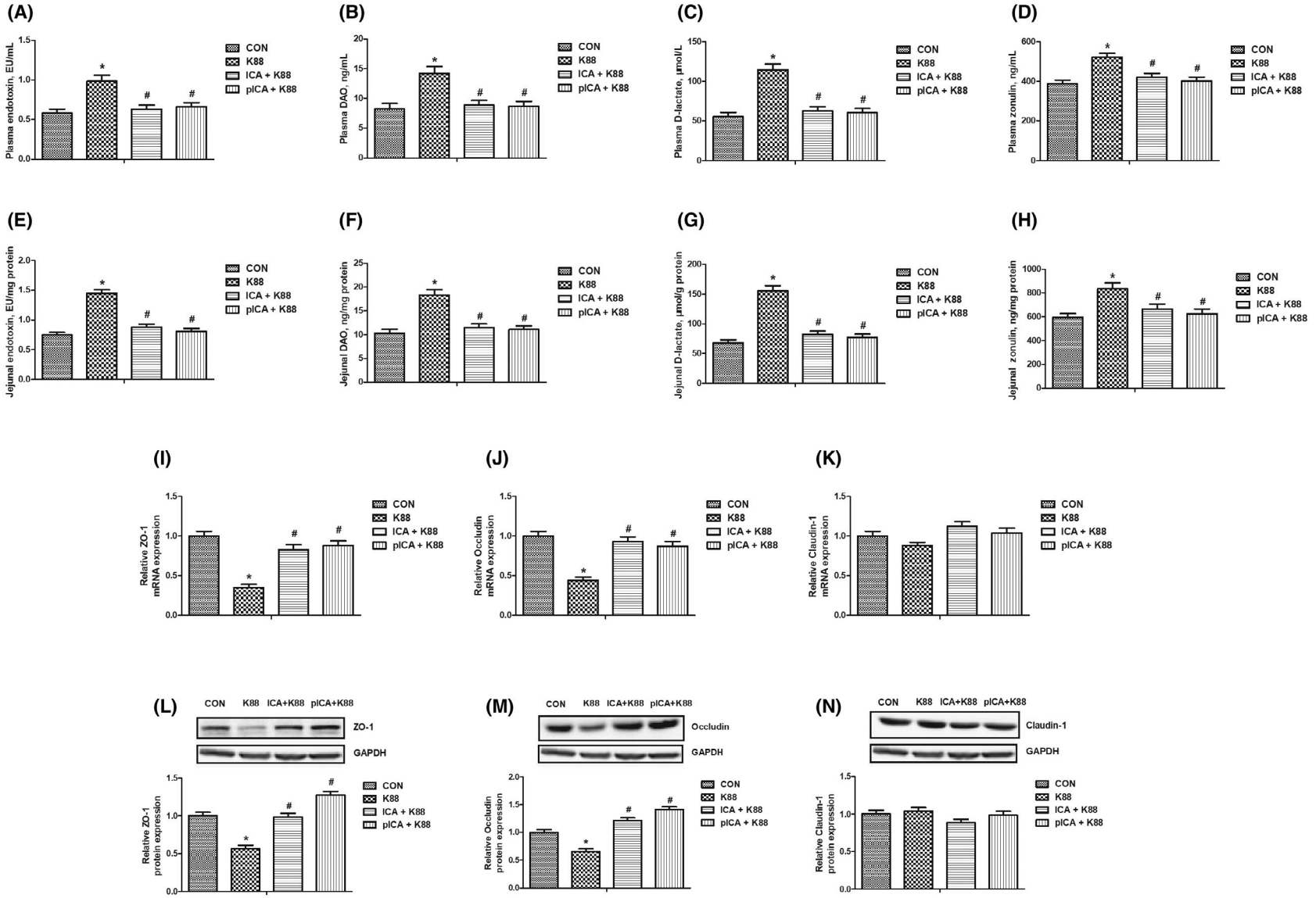

FIGURE 1 Effect of ICA and pICA on intestinal epithelial barrier function in piglets with ETEC K88 challenge. (A) Endotoxin, (B) DAO, (C) D-lactate, and (D) Zonulin contents in plasma of piglets. (E) Endotoxin, (F) DAO, (G) D-lactate, and (H) Zonulin contents in jejunum of piglets. The gene expression of (I) ZO-1, (J) Occludin, and (K) Claudin-1 in jejunum of piglets. The protein expression of (L) ZO-1, (M) Occludin, and (N) Claudin-1 in jejunum of piglets. Data are represented as means ± SEM, n = 6, $^{*}P < .05$ vs CON group; $^{\#}P < .05$ vs ETEC K88 group

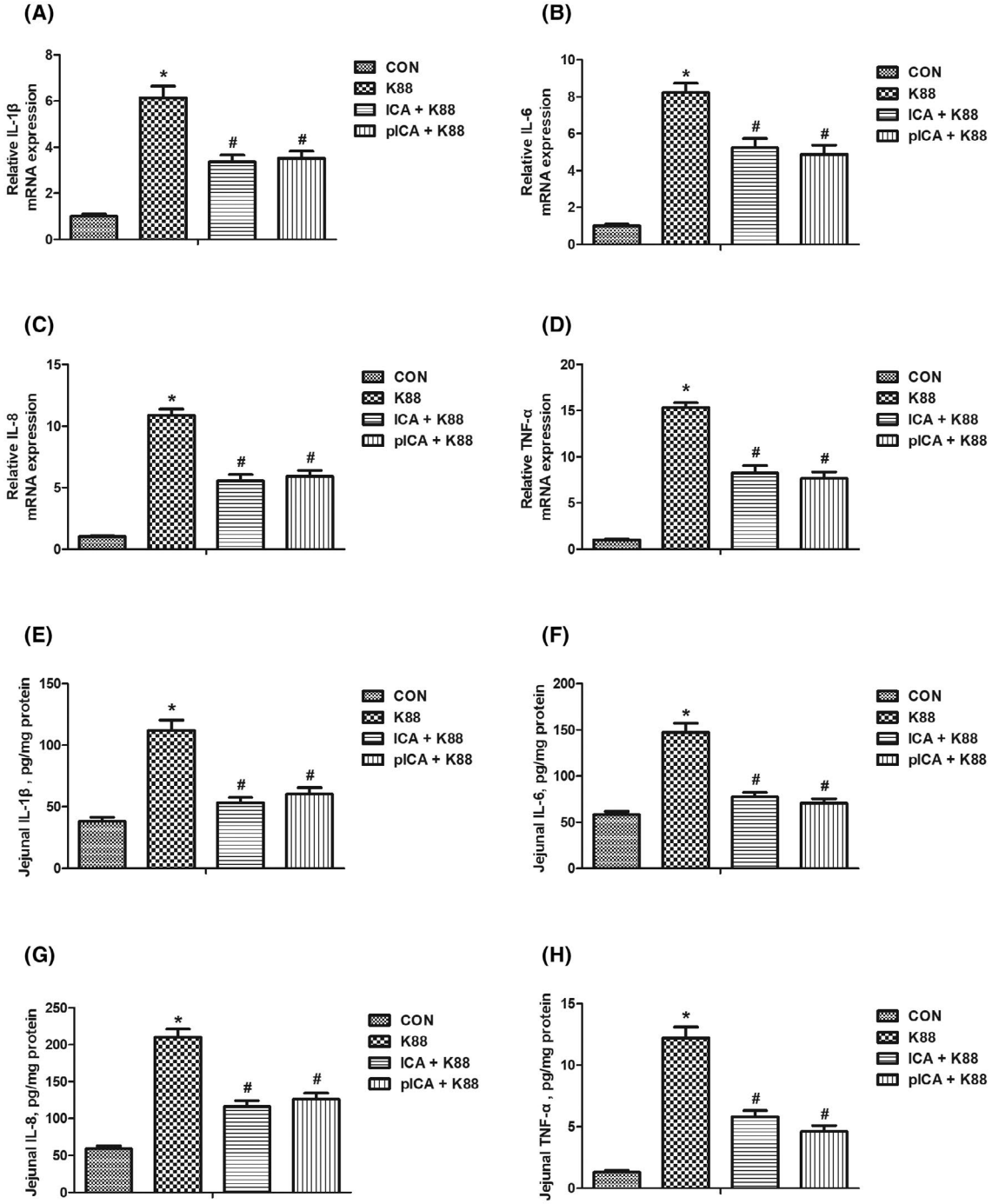

FIGURE 2 Effect of ICA and pICA on inflammatory status of jejunum in piglets with ETEC K88 challenge. The gene expression of (A) IL-1β, (B) IL-6, (C) IL-8, and (D) TNF-α in jejunum of piglets. The content of (E) IL-1β, (F) IL-6, (G) IL-8, and (H) TNF-α in jejunum of piglets. Data are represented as means ± SEM, n = 6, $^{*}P < .05$ vs CON group; $^{\#}P < .05$ vs ETEC K88 group

3.7 | Effect of ICA and pICA on inflammatory status in IPEC-J2 cells with ETEC K88 challenge

Inflammatory status of IPEC-J2 cells was evaluated by measuring the gene expression and content of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α). Challenge with ETEC K88 increased the gene expression of IL-1β, IL-6, IL-8, and TNF-α in IPEC-J2 cells; meanwhile, the gene expression of IL-1β, IL-6, IL-8, and TNF-α in ICA and pICA pretreatment groups was significantly lower than that of ETEC K88 group ($P < .05$, Figure 6A-D). In addition, the

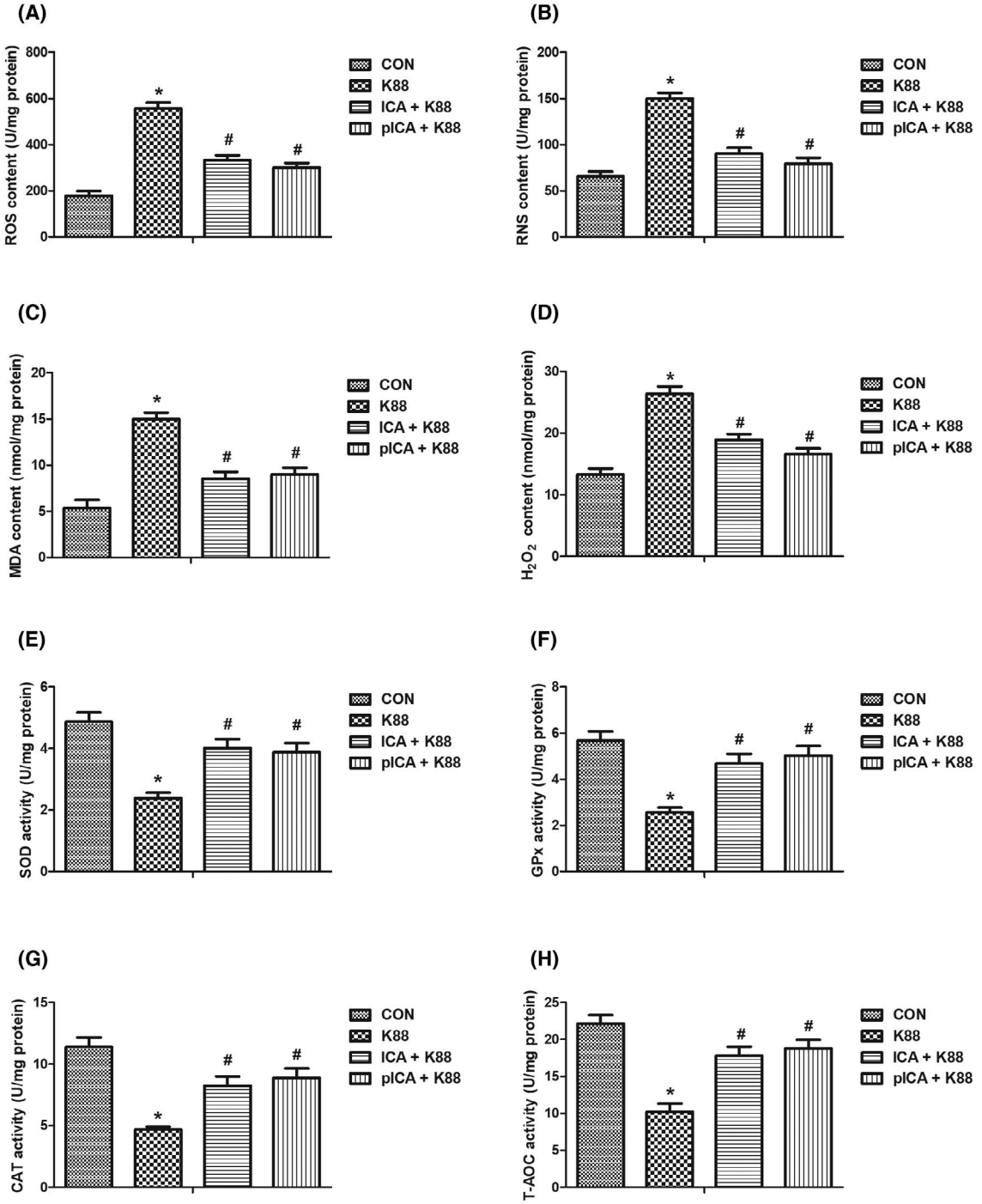

FIGURE 3 Effect of ICA and pICA on redox status of jejunum in piglets with ETEC K88 challenge. The content of (A) ROS, (B) RNS, (C) MDA, and (D) H_2O_2 in jejunum of piglets. The activity of (E) SOD, (F) GPx, (G) CAT, and (H) T-AOC in jejunum of piglets. Data are represented as means ± SEM, n = 6, $^*P < .05$ vs CON group; $^{\#}P < .05$ vs ETEC K88 group

content of IL-1β, IL-6, IL-8, and TNF-α in IPEC-J2 cells of ETEC K88 group was also significantly higher than the control group, whereas the IL-1β, IL-6, IL-8, and TNF-α concentration in ICA and pICA pretreatment groups were lower than those in ETEC K88 group ($P < .05$, Figure 6E-H).

3.8 | Effect of ICA and pICA on redox status in IPEC-J2 cells with ETEC K88 challenge

Redox status of IPEC-J2 cells was evaluated by measuring the mitochondrial and intracellular ROS levels, and

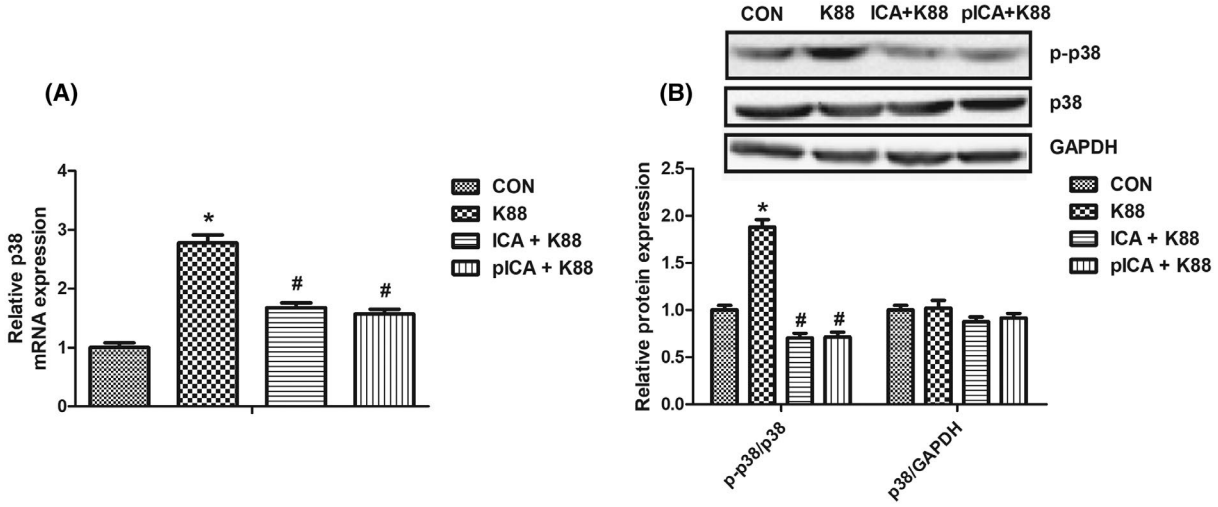

FIGURE 4 Effect of ICA and pICA on p38 MAPK expression of jejunum in piglets with ETEC K88 challenge. The gene (A) and protein (B) expression of p38 in jejunum of piglets. Data are represented as means ± SEM, n = 6, $^{*}P < .05$ vs CON group; $^{\#}P < .05$ vs ETEC K88 group

chemical markers (MDA, H2O2, SOD, GPx, CAT, and T-AOC). Challenge with ETEC K88 increased the levels of mitochondrial and intracellular ROS, MDA, and H_2O_2 in IPEC-J2 cells; meanwhile, the mitochondrial and intracellular ROS, MDA, and H_2O_2 contents in ICA and pICA pretreatment groups were significantly lower than that of ETEC K88 group ($P < .05$, Figure 7A-D). In addition, the activities of SOD, GPx, CAT, and T-AOC in IPEC-J2 cells of ETEC K88 group was significantly lower than the control group, whereas the SOD, GPx, CAT, and T-AOC activities in ICA and pICA pretreatment groups were higher than those in ETEC K88 group ($P < .05$, Figure 7E-H).

3.9 | Effect of ICA and pICA on p38 MAPK expression in IPEC-J2 cells with ETEC K88 challenge

As shown in Figure 8, IPEC-J2 cells challenged with ETEC K88 had greater p38 MAPK gene expression and phosphorylated p38 MAPK protein expression than control group ($P < .05$, Figure 8A,B). Besides, the p38 MAPK gene expression and phosphorylated p38 MAPK protein expression of IPEC-J2 cells in the ICA and pICA pretreatment groups were significantly lower than that of the ETEC K88 group ($P < .05$, Figure 8A,B).

3.10 | Effect of p38 MAPK inhibitor on barrier function, oxidative stress, and inflammatory status in IPEC-J2 cells with ETEC K88 challenge

As shown in Figure 9, compared with the ETEC K88 group, p38 MAPK inhibitor (SB203580) group significantly increased TEER, reduced FITC-D4 flux, reduced mitochondrial and intracellular ROS levels, and reduced IL-8 and TNF-α concentrations ($P < .05$, Figure 9A-F). Besides, compared with the ETEC K88 group, p38 MAPK inhibitor (SB203580) group significantly increased the mRNA and protein expression of ZO-1 and occludin ($P < .05$, Figure 9G-J).

3.11 | Effect of co-treatment with p38 MAPK activator and ICA/pICA on barrier function, oxidative stress, and inflammatory status in IPEC-J2 cells with ETEC K88 challenge

As shown in Figure 10, compared with the control group, ETEC K88 group, ICA + p38 MAPK activator (anisomycin) + ETEC K88 group and pICA + p38 MAPK activator (anisomycin) + ETEC K88 group significantly decreased TEER, increased FITC-D4 flux, increased mitochondrial and intracellular ROS levels, increased IL-8 and TNF-α concentrations, and reduced the mRNA and protein expression of ZO-1 and occludin ($P < .05$, Figure 10A-J). However, compared with the ETEC K88 group, ICA + p38 MAPK activator (anisomycin) + ETEC K88 group and pICA + p38 MAPK activator (anisomycin) + ETEC K88 group has no changed in barrier function, oxidative stress, and inflammatory status.

4 | DISCUSSION

ETEC K88-induced piglet diarrhea is a high-risk factor for the economic loss of the pig industry.[27] In the past few decades, researchers have never stopped exploring the pathogenesis of ETEC K88-induced diarrhea in piglets. To date, inflammatory responses and oxidative stress have been

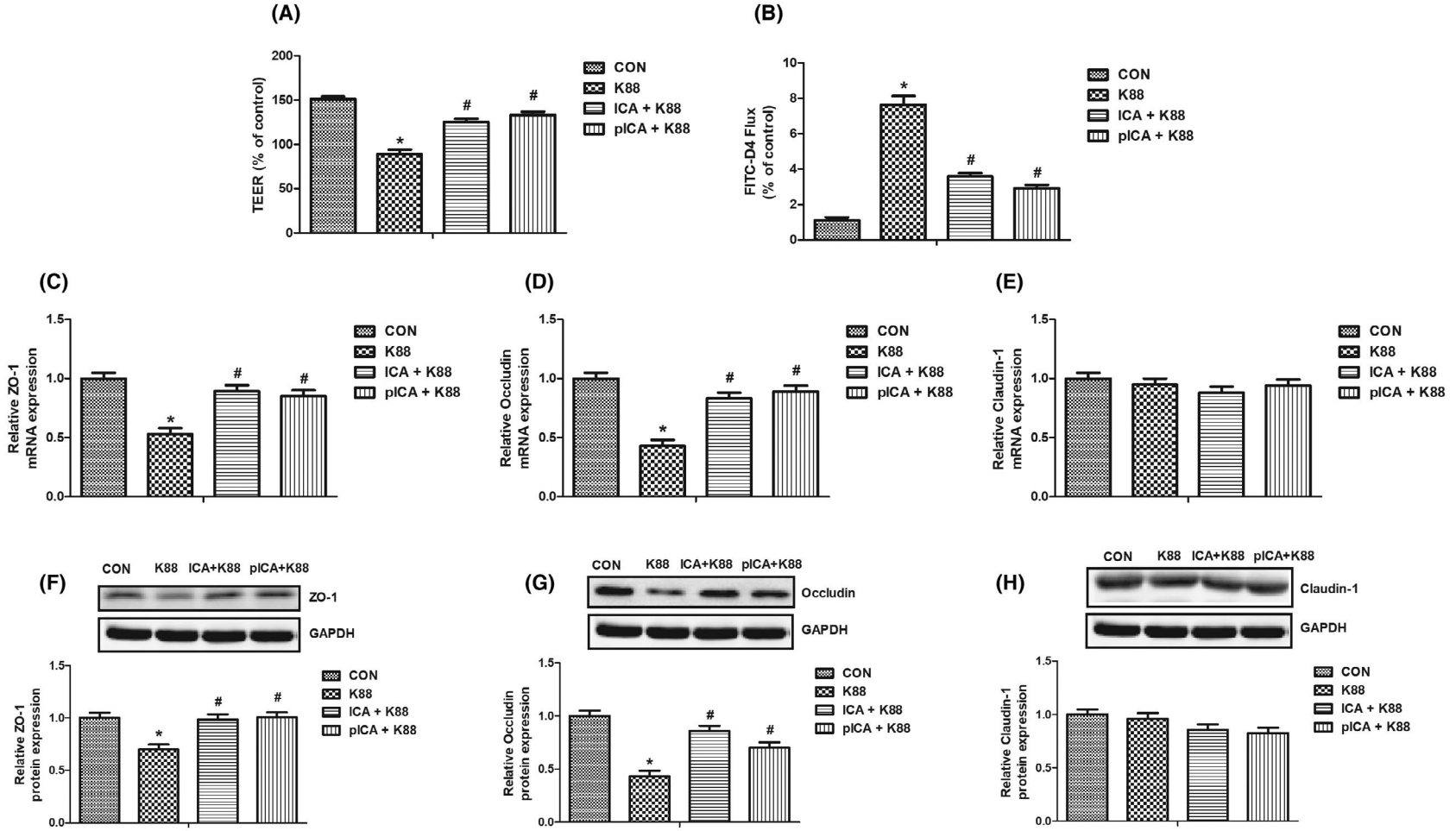

FIGURE 5 Effect of ICA and pICA on barrier function in IPEC-J2 cells with ETEC K88 challenge. (A) TEER and (B) FITC-D4 of IPEC-J2 cells. The gene expression of (C) ZO-1, (D) Occludin, and (E) Claudin-1 in IPEC-J2 cells. The protein expression of (F) ZO-1, (G) Occludin, and (H) Claudin-1 in IPEC-J2 cells. Data are represented as means ± SEM, n = 6, $^{*}P < .05$ vs CON group; $^{\#}P < .05$ vs ETEC K88 group. CON: cells were not any treated for 15 hours. K88: cells were not treated for the first 12 hours, then challenged with 6.5×10^7 cfu ETEC K88 for 3 hours. ICA + K88: ICA (40 μg/mL) pretreated cells for 12 hours, then challenged with 6.5×10^7 cfu ETEC K88 for 3 hours. pICA + K88: pICA (160 μg/mL) pretreated cells for 12 hours, then challenged with 6.5×10^7 cfu ETEC K88 for 3 hours

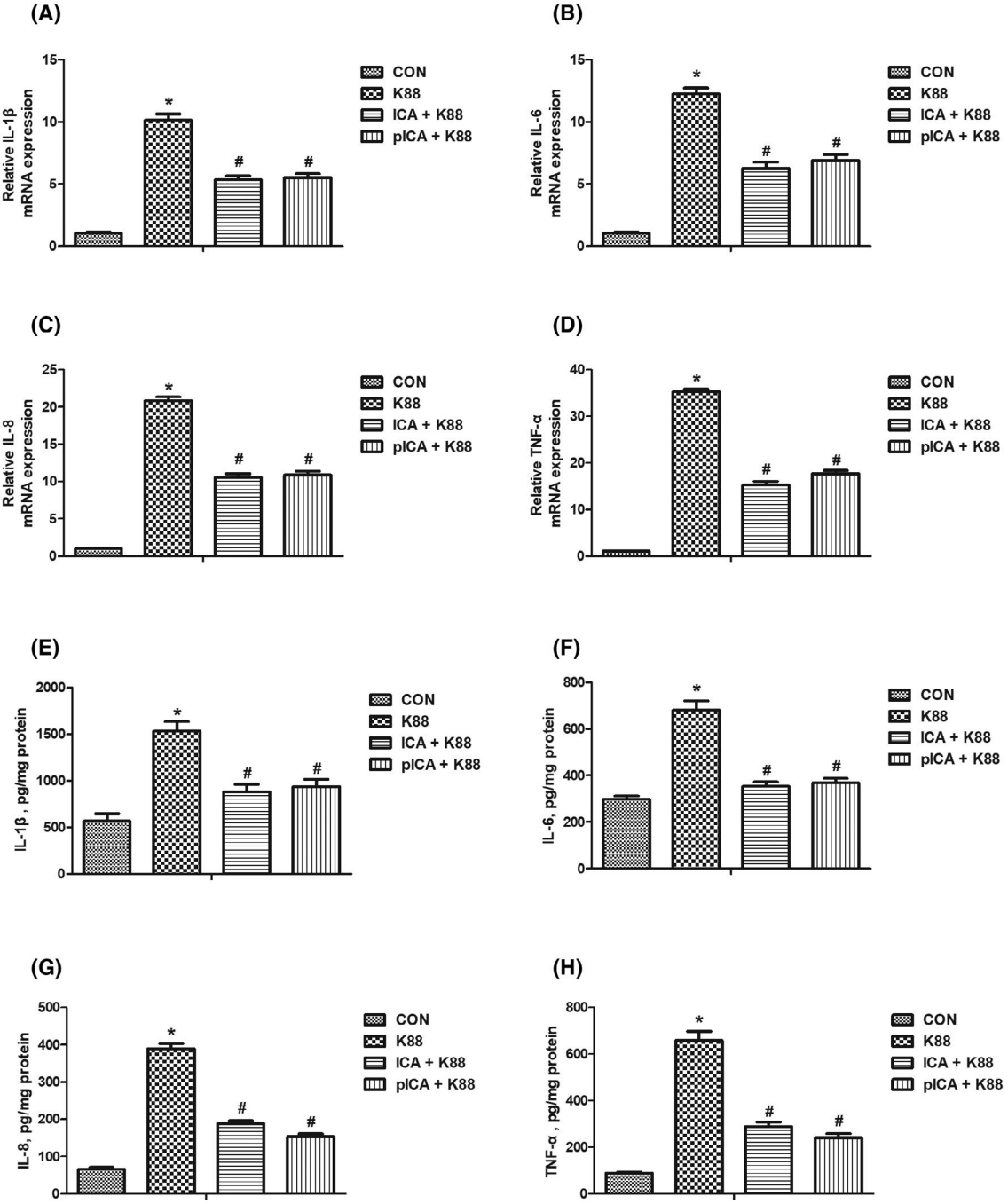

FIGURE 6 Effect of ICA and pICA on inflammatory status in IPEC-J2 cells with ETEC K88 challenge. The gene expression of (A) IL-1β, (B) IL-6, (C) IL-8, and (D) TNF-α in IPEC-J2 cells. The content of (E) IL-1β, (F) IL-6, (G) IL-8, and (H) TNF-α in IPEC-J2 cells. Data are represented as means ± SEM, n = 6, $^{*}P < .05$ vs CON group; $^{\#}P < .05$ vs ETEC K88 group. CON: cells were not any treated for 15 hours. K88: cells were not treated for the first 12 hours, then challenged with 6.5×10^7 cfu ETEC K88 for 3 hours. ICA + K88: ICA (40 μg/mL) pretreated cells for 12 hours, then challenged with 6.5×10^7 cfu ETEC K88 for 3 hours. pICA + K88: pICA (160 μg/mL) pretreated cells for 12 hours, then challenged with 6.5×10^7 cfu ETEC K88 for 3 hours

recognized as biological process involved in the ETEC K88-induced diarrhea.[28] Due to their high security and low cost, natural products and their derivatives are increasingly used in combating diseases. ICA and pICA have been shown to have excellent preventive effects against many diseases processes by exerting their potent anti-inflammatory and antioxidant properties.[29,30] In this study, ICA and pICA have an effective protective effect on ETEC-induced piglet diarrhea. The results of this study indicated that piglets fed the diet containing ICA and pICA had improved growth performance assessed by BW and ADG at postchallenge with ETEC K88. Besides, piglets fed ICA and pICA showed a lower diarrhea rate after suffering from ETEC K88 infection.

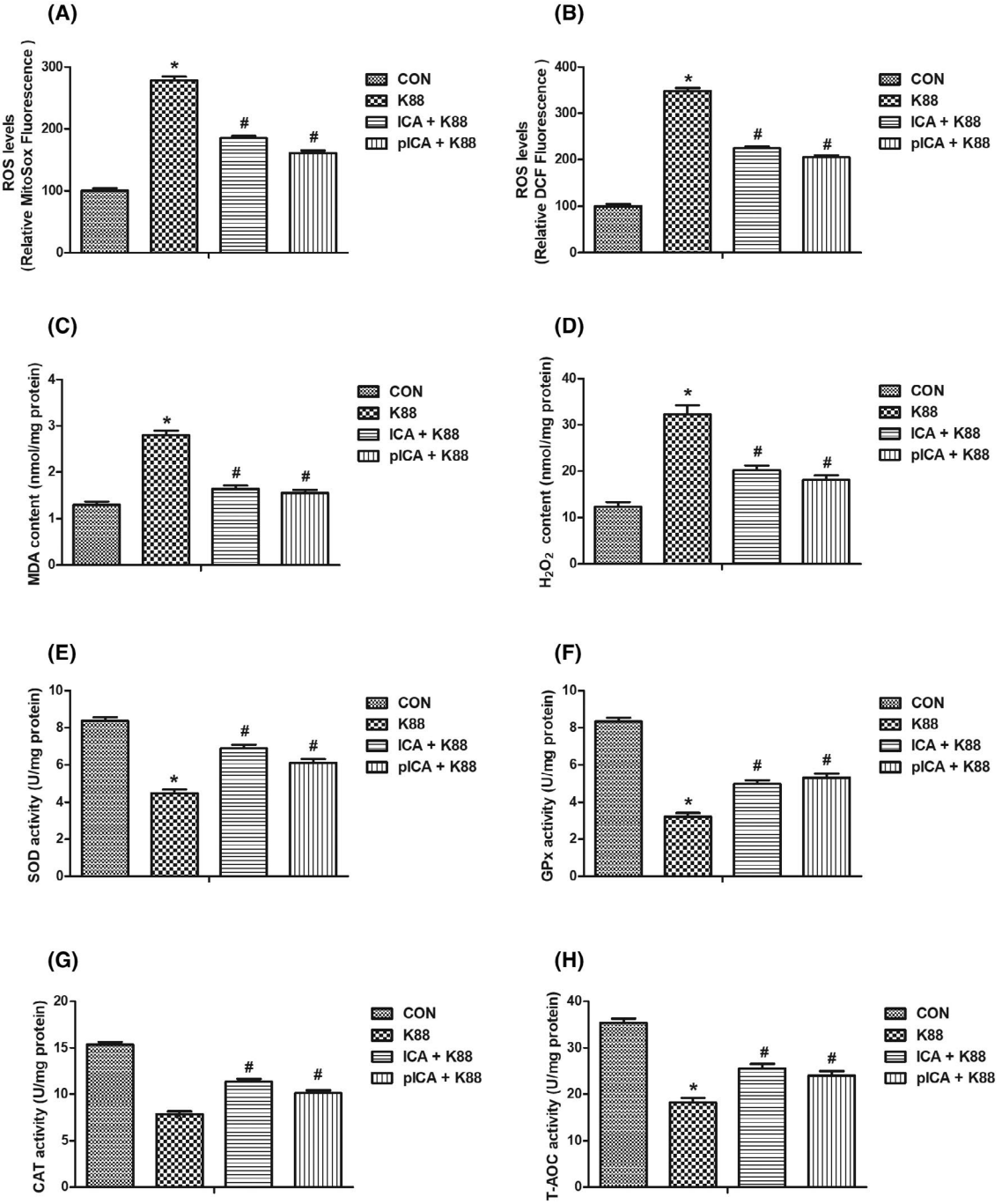

FIGURE 7 Effect of ICA and pICA on redox status in IPEC-J2 cells with ETEC K88 challenge. Changes in the levels of (A) mitochondrial ROS (MitoSox dye oxidation), (B) total intracellular ROS (H2DCF oxidation), (C) MDA, and (D) H_2O_2 in IPEC-J2 cells. The activity of (E) SOD, (F) GPx, (G) CAT, and (H) T-AOC in IPEC-J2 cells. Data are represented as means ± SEM, n = 6, $^*P < .05$ vs CON group; $^{\#}P < .05$ vs ETEC K88 group. CON: cells were not any treated for 15 hours. K88: cells were not treated for the first 12 hours, then challenged with 6.5×10^7 cfu ETEC K88 for 3 hours. ICA + K88: ICA (40 μg/mL) pretreated cells for 12 hours, then challenged with 6.5×10^7 cfu ETEC K88 for 3 hours. pICA + K88: pICA (160 μg/mL) pretreated cells for 12 hours, then challenged with 6.5×10^7 cfu ETEC K88 for 3 hours

Impaired of intestinal barrier function by ETEC infection is the direct cause of diarrhea in piglets. Previous studies have shown that ETEC challenge induces jejunal villus height and villus height/crypt depth in piglets, and the expression of TJs in intestinal epithelium is reduced, thereby destroying intestinal barrier function.[31] In addition, studies in vitro had shown that ETEC K88 reduces TJs protein abundance in porcine small intestinal epithelial cell line (IPEC)-J2.[32] In the current study, our data showed that ETEC K88 challenge significantly reduced the gene and protein expression of ZO-1 and Occludin in jejunum of piglets and IPEC-J2 cells, whereas the gene and protein abundance of Claudin-1 did not changed.

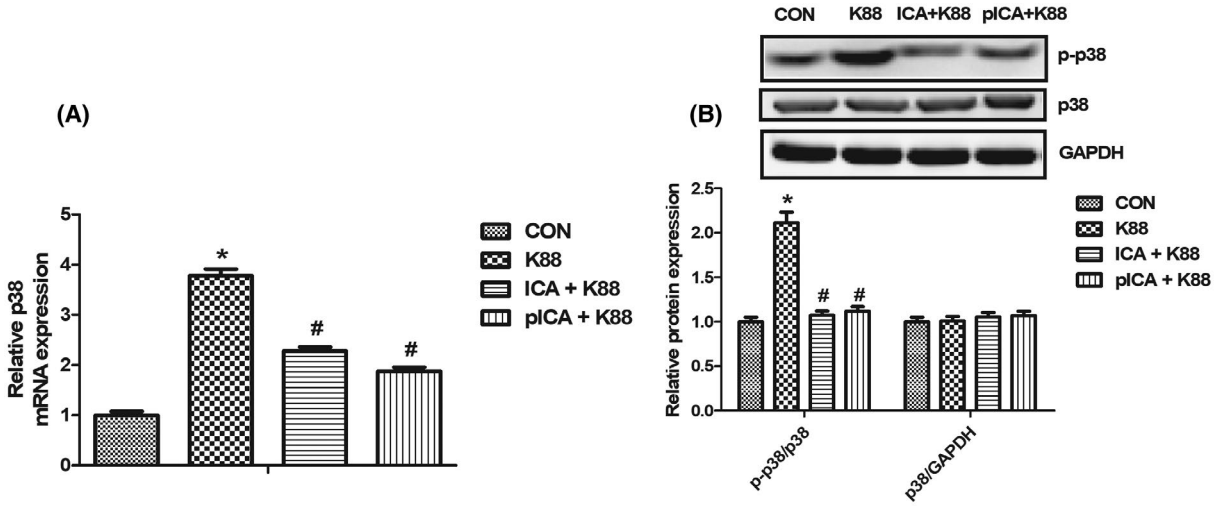

FIGURE 8 Effect of ICA and pICA on p38 MAPK expression in IPEC-J2 cells with ETEC K88 challenge. The gene (A) and protein (B) expression of p38 in IPEC-J2 cells. Data are represented as means ± SEM, n = 6, $^{*}P < .05$ vs CON group; $^{\#}P < .05$ vs ETEC K88 group. Data are represented as means ± SEM, n = 6, $^{*}P < .05$ vs CON group; $^{\#}P < .05$ vs ETEC K88 group. CON: cells were not any treated for 15 hours. K88: cells were not treated for the first 12 hours, then challenged with 6.5×10^7 cfu ETEC K88 for 3 hours. ICA + K88: ICA (40 μg/mL) pretreated cells for 12 hours, then challenged with 6.5×10^7 cfu ETEC K88 for 3 hours. pICA + K88: pICA (160 μg/mL) pretreated cells for 12 hours, then challenged with 6.5×10^7 cfu ETEC K88 for 3 hours

Among TJs, ZO-1 is a peripheral membrane protein that plays a very important role in the distribution and maintenance of TJs[33]; Occludin contributes to transfer of the macromolecular substances through the cellular bypass pathway by acting directly with claudins and actin.[34] Thus, occludin and ZO-1 play a vital role in maintaining the intestinal barrier. Claudins are composed of multiple gene families, and different claudins play different roles due to their host and cell types.[35] Studies have shown that claudin-1 has the ability to regulate the cell proliferation, suggesting it may not participate in barrier function.[36] The impaired intestinal barrier caused by ETEC infection will increase the permeability to harmful substrates in the intestinal lumen.[37] Our results revealed that the levels of endotoxin, DAO, D-lactate, and zonulin in plasma and jejunum were significantly increased after ETEC challenge. The chemical markers (endotoxin, DAO, D-lactate, and zonulin) are usually low in the circulation system in healthy individuals, will have a significant increase in the circulation system during the destruction of the intestinal barrier.[38] Besides, ETEC K88 infection reduced the transmembrane resistance of IPEC-J2 cells and increased FITC-D4 flux, which was consistent with previous study.[23] More importantly, ICA and pICA pretreatment significantly attenuated ETEC K88-induced damage to the barrier function and permeability of piglet jejunum and IPEC-J2 cells. These results indicated that ETEC K88-induced disruption of intestinal permeability and barrier function in vivo an in vitro, while ICA and pICA effectively alleviate these adverse effects.

To further elucidate the underlying mechanisms by which ICA and pICA protected the intestinal epithelial barrier function, we explored at the inflammatory and redox status in vivo and in vitro. Previous study has shown that ETEC induces high expression of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and TNF-α.[39] TNF-α has a significant destructive effect on the expression and distribution of TJs proteins. IL-1β, IL-6, and IL-8 not only regulate the reorganization of cytoskeletal proteins, but also directly lead to the rearrangement of TJs proteins and thus reduce the barrier function.[40,41] In the present study, our data showed that ETEC K88 challenge significantly increased the gene abundance and concentrations of IL-1β, IL-6, IL-8, and TNF-α in jejunum of piglets and IPEC-J2 cells. Pretreatment with ICA and pICA significantly attenuated ETEC K88-induced pro-inflammatory cytokines expression in jejunum of piglets and IPEC-J2 cells. Oxidative stress is a very important mechanism in many potential mechanisms by which toxic substances affect mammalian cells.[42] Under physiological conditions, some intermediates produced during oxidative phosphorylation, such as ROS, are important signaling molecules that regulate cellular biochemical processes. However, when the body is in oxidative stress, the ROS content is significantly increased, exceeding the body's ability to effectively scavenge free radicals, leading to DNA oxidative damage and abnormal protein expression, ultimately leading to physical damage.[43] Previous studies have demonstrated many potential mechanisms by which ETEC affects intestinal epithelial cells in animals. Among these mechanisms, the accumulation of oxidative stress intermediates has received great attention.[44] Besides, evidences suggested that oxidative stress is closely related to intestinal barrier dysfunction, mainly due to the ability of oxidative stress to disrupt TJ proteins.[45,46] Our data clearly showed that ETEC K88 challenge

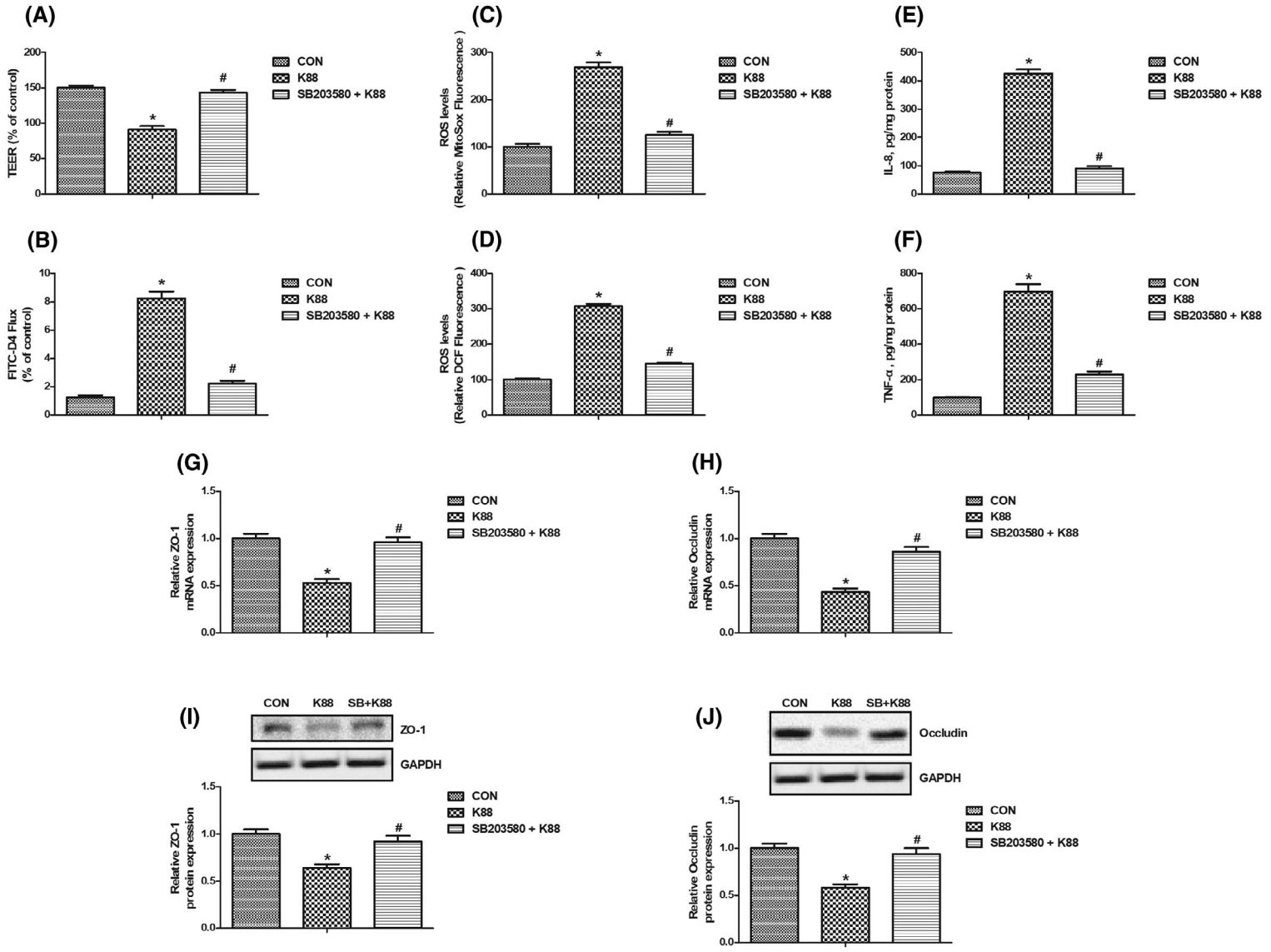

FIGURE 9 Effect of p38 MAPK inhibitor on barrier function, oxidative stress, and inflammatory status in IPEC-J2 cells with ETEC K88 challenge. (A) TEER and (B) FITC-D4 of IPEC-J2 cells. Changes in the levels of (C) mitochondrial ROS (MitoSox dye oxidation) and (D) total intracellular ROS (H2DCF oxidation) in IPEC-J2 cells. The content of (E) IL-8 and (F) TNF-α in IPEC-J2 cells. mRNA expression of (G) ZO-1 and (H) Occludin in IPEC-J2 cells. Protein expression of (I) ZO-1 and (J) Occludin in IPEC-J2 cells. Data are represented as means ± SEM, n = 6, $^{*}P < .05$ vs CON group; $^{\#}P < .05$ vs ETEC K88 group. CON: cells were not any treated for 15 hours. K88: cells were not treated for the first 12 hours, then challenged with 6.5×10^7 cfu ETEC K88 for 3 hours. SB203580: SB203580 (10 μM) pretreated cells for 12 hours, then challenged with 6.5×10^7 cfu ETEC K88 for 3 hours

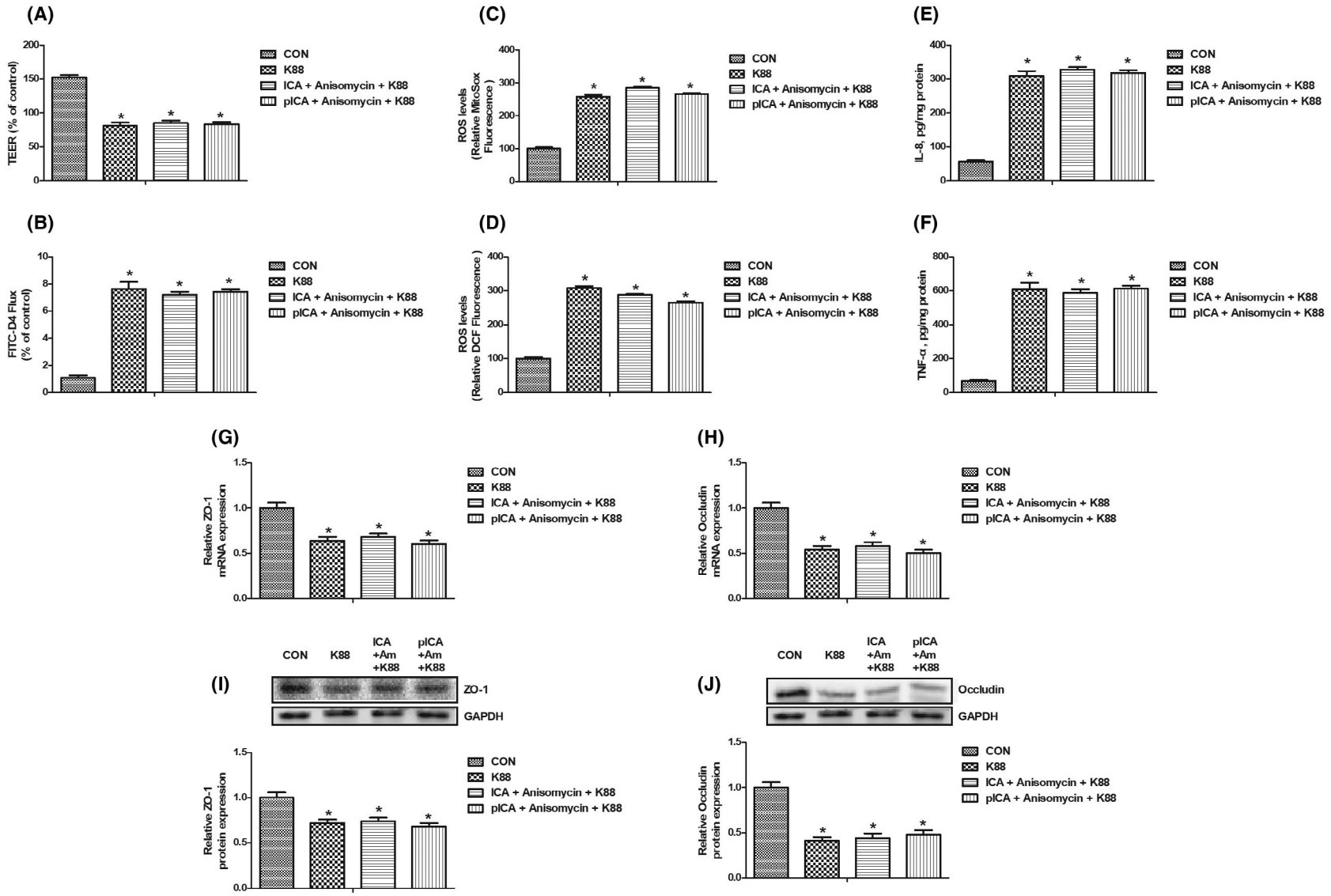

FIGURE 10 Effect of co-treatment with p38 MAPK activator and ICA/pICA on barrier function, oxidative stress, and inflammatory status in IPEC-J2 cells with ETEC K88 challenge. (A) TEER and (B) FITC-D4 of IPEC-J2 cells. Changes in the levels of (C) mitochondrial ROS (MitoSox dye oxidation) and (D) total intracellular ROS (H2DCF oxidation) in IPEC-J2 cells. The content of (E) IL-8 and (F) TNF-α in IPEC-J2 cells. mRNA expression of (G) ZO-1 and (H) Occludin in IPEC-J2 cells. Protein expression of (I) ZO-1 and (J) Occludin in IPEC-J2 cells. Data are represented as means ± SEM, n = 6, $*P < .05$ vs CON group. CON: cells were not any treated for 15 hours. K88: cells were not treated for the first 12 hours, then challenged with 6.5×10^7 cfu ETEC K88 for 3 hours. ICA + Anisomycin +K88: ICA (40 μg/mL) and Anisomycin pretreated cells for 12 hours, then challenged with 6.5×10^7 cfu ETEC K88 for 3 hours. pICA + Anisomycin + K88: pICA (160 μg/mL) and Anisomycin (25 μg/mL) pretreated cells for 12 hours, then challenged with 6.5×10^7 cfu ETEC K88 for 3 hours

induced oxidative stress and inhibited anti-oxidative capability in jejunum of piglets and IPEC-J2 cells. Pretreatment with ICA and pICA significantly attenuated ETEC K88-induced changed in oxidative-antioxidative status in jejunum of piglets and IPEC-J2 cells.

p38 MAPK regulates ETEC-induced intestinal barrier dysfunction primarily in two ways. On the one hand, the p38 signaling pathway is directly involved in the destruction of the intestinal epithelial TJ proteins ZO-1 and Occludin, while the expression of TJ protein returns to normal after inhibition of the p38 signaling pathway.[47] On the other hand, the p38 signaling pathway indirectly disrupts the intestinal epithelial barrier function by regulating the progression of inflammation and oxidative stress in intestinal epithelial cells.[48,49] In the current study, our data revealed that ETEC K88 challenge significantly increased p38 MAPK gene expression and phosphorylated p38 MAPK protein expression, while ICA and pICA pretreatment effectively reversed these changed. To further explore the underlying mechanisms by which ICA and pICA modulated the intestinal epithelial barrier function of IPEC-J2 cells, we employed specific inhibitor (SB203580) and activator (anisomycin) of p38 MAPK to perform subsequent experiments. Our results showed that specific blockade of p38 MAPK signaling can effectively alleviate ETEC K88-induced inflammatory response, oxidative stress, and intestinal permeability damage. The results indicated that ETEC K88 regulates inflammatory response and oxidative stress through p38 MAPK signaling and causes intestinal epithelial barrier dysfunction. Furthermore, when we pretreated cells with ICA or pICA using a specific activator of p38 MAPK, ICA, and pICA lost efficacy against ETEC K88-induced inflammatory response, oxidative stress, and intestinal permeability damage. The results indicated that ICA and pICA play a protective role against ETEC-induced intestinal barrier dysfunction by regulating p38 MAPK signaling.

In conclusion, our results demonstrated that ETEC K88 activates the inflammatory response and oxidative stress in the intestinal epithelial cells of piglets in a p38 MAPK-dependent manner, thereby disrupting the intestinal epithelial barrier and permeability, ultimately leading to piglet diarrhea. More importantly, our results highlight that ICA and pICA have excellent protective effects on the intestinal epithelial barrier function of ETEC-induced piglet diarrhea by regulating the expression of p38 MAPK. These results suggested that ICA and pICA may be effective natural products for the prevention of piglet diarrhea caused by ETEC K88.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

W. Xiong designed the research. W. Xiong, J.Huang and X. Li conducted the research. Z. Zhang, M. Jin, J. Wang, and Y. Xu analyzed the data. The manuscript was mainly written by W. Xiong and edited by Z. Wang. All the authors have read and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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