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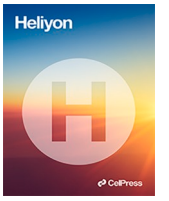

Research article

Baicalin-aluminum complex on the regulation of IPEC-1 infected with enterotoxigenic *Escherichia coli*

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ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) is the main bacterial cause of diarrhea in weaned piglets. Baicalin-aluminum (BA) complex is the main active ingredient of *Scutellaria baicalensis* Georgi extracted-aluminum complex, which has been used to treat diarrhea in weaning piglets, however the underlying mechanism remains unclear. To investigate the effects of the BA complex on the regulation of porcine intestinal epithelial (IPEC-1) cells infected with ETEC, IPEC-1 cells were incubated with an ETEC bacterial strain at a multiplicity of infection of 1 for 6 h and then treated with different concentrations of the BA complex for 6 h. ETEC infection increased the levels of cAMP and cGMP, upregulated *CFTR* (cystic fibrosis transmembrane conductance regulator) mRNA, and downregulated *NHE4* mRNA in IPEC-1 cells. Treatment with the BA complex inhibited ETEC adhesion and the production of cAMP and cGMP, reduced *CFTR* mRNA expression, and increased *NHE4* mRNA expression. Overall, the BA complex weakened the adhesion of ETEC to IPEC-1 cells, and inhibited cAMP/cGMP-CFTR signaling in IPEC-1 cells.

1. Introduction

Diarrhea in piglets is a challenging disease that causes huge economic losses to the swine industry [1]. There are many factors related to piglet diarrhea, enterotoxigenic *Escherichia coli* (ETEC) is generally considered to be one of the most common causative agents of diarrhea in post-weaned piglets [2]. ETEC is mainly spread via the fecal–oral route, where it attaches to the intestinal epithelial cells of piglets through fimbrial adhesins and produces enterotoxins that lead to diarrhea [3]. The enterotoxins belong to two major classes: heat-labile (LT) and heat-stable toxins (ST), which bind specific enterocyte receptors to increase intracellular cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) levels, respectively [4], and disrupt fluid secretion across the epithelium by alteration of ion transport via faulting cystic fibrosis transmembrane conductance regulator (CFTR), calcium activated chloride channels, and ion exchanger functions [5].

Using antibiotics is the traditional way to control bacterial infections [6]. However, the prevalence of bacteria that are resistant to multiple antibiotics is steadily increasing worldwide, leading to difficulties in preventing and controlling clinical infections [7]. Thus, it has become increasingly important to find alternatives to antibiotics. The baicalin-aluminum (BA) complex is synthesized from

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baicalin and aluminum salt, which are the main active ingredients of the Huangqinsulv capsule, which has been used clinically for more than 30 years and demonstrates a range of pharmacological activities, including temperature reducing, toxicity decreasing, dampness eliminating, and diarrhea relieving properties. Huangqinsulv capsule is also useful for treatment of acute or chronic enteritis and dysentery without any adverse effects, with recovery rates of 97.27 % and 93.00 %, respectively, especially in patients in whom antibiotics and sulfa are ineffective [8,9]. In our previous study, we obtained a dry *Scutellaria baicalensis* Georgi extract-aluminum complex suspension using the extraction method used to produce the Huangqinsulv capsule, which was used to treat diarrhea in weaning piglets, with a recovery rate of ~90 % [10].

The therapeutic effect of BA complex in treating bacterial diarrhea in piglets is definite. The main molecular mechanisms underlying piglet diarrhea caused by ETEC have been clarified, but the exact mechanism of BA complex in the treatment of ETEC-induced diarrhea in piglets is unclear. Therefore, we hypothesized that BA complex could attenuate damage of porcine intestinal epithelial (IPEC-1) cells via reducing the adhesion of ETEC to IPEC-1 cells and inhibiting cAMP/cGMP-CFTR signaling pathways. The purpose of this study was to investigate the effects of the BA complex on the regulation of IPEC-1 cells infected with ETEC and further explore molecular mechanisms.

2. Materials and methods

2.1. BA complex, bacterial strains and cells

The BA complex was homogeneously synthesized referring to previous methods [11]. Briefly, an equimolar amount of baicalin was added to a 1 % sodium bicarbonate aqueous solution and stirred to completely dissolve it. Aluminum salt was added according to the molar ratio of baicalin to copper sulfate of 1:2, and after stirred for 30 min at 60 °C. A orange precipitate was obtained via filtration. This was then washed with 75 % ethanol and ddH_2O, respectively, then a drying these precipitations at 50 °C, further confirmed by UV-spectra, FTIR and MS analysis. The products were achieved with high pureness (>90 %) detected by HPLC-UV [12]. The BA complex was dissolved and diluted in DMEM-F12 medium (Gibco, Grand Island, NY, USA).

ETEC K88 (LT-I+ and STb+) and ETEC C83715 (STa+) was identified and maintained by the college of veterinary medicine, Huazhong Agricultural University (Wuhan, China). The ETEC strains were grown in Luria-Bertani broth (Solarbio Life Sciences, Beijing, China) at 37 °C.

IPEC-1 were cultured in the presence of DMEM-F12 medium with 10 % fetal bovine serum and 1 % antibiotic mixture (100 μg/mL streptomycin and 100 U/mL penicillin) at 37 °C under a humidified atmosphere containing 5 % CO_2 as previously described [13,14].

2.2. Cell viability assay

The viability of IPEC-1 cells was measured using a Cell Counting Kit-8 (CCK-8) assay [15]. Briefly, IPEC-1 cells were seeded in 96-well plates (Costar, Coppell, TX, USA) at 5×10^4 cells/well and treated with the BA complex at 0, 12.5, 25, 50, 100, or 200 μg/mL for 6, 12, or 20 h at 37 °C under 5 % CO_2. CCK-8 solution (10 μL) was added to each well and incubated for 90 min at 37 °C and the optical density was measured at 450 nm. Cell viability was calculated according to the following formula: cell viability (%) = (experimental well − blank well/control well − blank well) × 100 %. The data were expressed as mean ± standard deviation of triplicate samples from at least three independent experiments.

2.3. Infection of IPEC-1 cells

The multiplicity of infection (MOI) of ETEC with the IPEC-1 cells was first explored. IPEC-1 cells (2.5×10^5 cells/well) were seeded in 6-well plates (Corning, NY, USA) and washed with antibiotic-free DMEM-F12 medium after reaching 50 % confluence. Then, ETEC C83715 or K88 (2×10^5, 2×10^6, 2×10^7, and 2×10^8 CFU) was added to each well of the infective groups and incubated under 5 % CO_2 at 37 °C for 0, 1, 3, and 6 h, respectively. Cell morphology was observed by inverted microscope (OLMPUS, BX53, Japan) to determine the MOI and optimal interaction time.

2.4. Adherence assay

IPEC-1 cells (100 μl) were seeded in 96-well plates and cultivated at 37 °C in CO_2 for 11–12 h until covering the bottom of wells. Following treatment with the MOI value of bacterial strains, different concentrations of the BA complex (0, 12.5, 25, 50, or 100 μg/mL) were added and the plates were incubated for 1 h at 37 °C. The cells were then washes three times with sterile phosphate buffered saline (PBS) to remove non-adhering material. After treating the IPEC-1 cells with a 0.5%–1.0 % Tritonx-100 solution, the cells were resuspended. The suspension, containing bacterial cells, was diluted 10-fold in PBS and inoculated in trypcasein soy agar overnight at 37 °C to determine the mean number of bacteria attached per cell. Bacterial cells incubated with PBS were used as controls. All tests were performed in triplicate.

2.5. Determination of cAMP and cGMP

IPEC-1 cells were cultured in 96-well plates, treated with the MOI value of 1 and incubated for 6 h with culture medium containing different concentrations of BA complex (0, 12.5, 25, 50, or 100 μg/mL). Then, cell supernatants were collected and centrifuged at

400×*g* for 15 min at 4 °C. cAMP and cGMP levels were measured by enzyme immunoassay (cAMP or cGMP ELISA Kit, Jining Shiye, Shanghai, China) according to the manufacturer's instructions.

2.6. RNA extraction and quantitative RT-PCR

To determine the expression levels of *CFTR* and *NHE4* in infected IPEC-1 cells, following co-incubation with the BA complex (0, 12.5, 25, 50, or 100 μg/mL) for 6 h, the cells were collected and total cellular RNA was extracted using the TRISOL reagent (Invitrogen, USA). The RNA was reverse-transcribed to cDNA using reverse transcriptase (TaKaRa, Dalian, China) and cDNA amplification was assessed using the SYBER Green PCR Kit (ABI, USA). Individual transcripts in each sample were assessed three times. *HMBS* was chosen as an internal control, based on both expression stability and expression level [16]. Nucleotide sequences of the primers used for qPCR are listed in Table 1.

2.7. Statistical analysis

Effects of treatment were assessed by one-way ANOVA using SPSS 17.0 software. Data were expressed as mean ± SD. $2^{-\triangle\triangle Ct}$ values were used to analyze qPCR data.

3. Results

3.1. Cytotoxicity of the BA complex in IPEC-1 cells

To optimize the concentration and co-incubation of the BA complex, an IPEC-1 cell viability assay was performed to evaluate cytotoxicity of the BA complex. At concentrations of 200 μg/mL or less, the BA complex had no cytotoxic effect on IPEC-1 cells after 6 h (Fig. 1). Following stimulation with 200 μg/mL BA complex for 12 h, or 200 μg/mL and 100 μg/mL BA complex for 20 h, cell viabilities were significantly reduced ($P < 0.01$). To investigate the effects of the BA complex on the regulation of IPEC-1 cells, the maximum safety concentrations were selected for further research. Thus, 100 μg/mL BA complex for 6 h was used for further experiments.

3.2. Establishing an infection model of ETEC in IPEC-1 cells

An infection model was established to determine the optimal MOI for ETEC in IPEC-1 cells. Morphological changes were observed at 6 h when IPEC-1 cells were infected with ETEC at MOIs of 1, 10, 100, and 1000. Compared with control cells, more cells were unhealthy and rounded up when infected at an MOI of 10 (Fig. 2). The cells infected with ETEC at an MOI of 100 started to detach from the surface of culture dishes and float into the medium (Fig. 2). At an MOI of 1000, most of the cells underwent cell death and detached from the surface of the plate (Fig. 2a and b, panels B). At an MOI of 1, the viability of cells did not significantly decrease, but significantly reduced to 55 % in cells infected at MOI 10 (Fig. 2a and b, panels E). The viability of cells infected with ETEC at an MOI of 100 or 1000 decreased 10%–20 % and most of the cells were dead (Fig. 2c). Therefore, we chose ETEC at an MOI of 1 to infect IPEC-1 cells and co-culture of 6 h as the infection model.

3.3. Effect of the BA complex on ETEC adherence to IPEC-1 cells

As shown in Fig. 3, in the presence of the BA complex (25, 50, or 100 μg/mL), the number of adherent ETEC K88 and C83715 cells was significantly lower than in the absence of the BA complex in a dose-dependent manner ($P < 0.01$). Thus, the BA complex inhibited the ability of ETEC K88 and C83715 to adhere to IPEC-1 cells.

3.4. Effect of the BA complex on cAMP and cGMP production

In the absence of the BA complex, production of cAMP and cGMP from IPEC-1 cells was significantly increased following stimulation with ETEC K88 or C83715 compared to that in control cells (Fig. 4, $P < 0.01$). When 50 or 100 μg/mL BA complex was added, production of cAMP and cGMP was significantly lower than that in cells stimulated with ETEC C83715 alone (Fig. 4a, $P < 0.01$). When 25, 50, or 100 μg/mL BA complex was added, production of cAMP was significantly reduced ($P < 0.01$), treatment with 100 μg/mL BA complex significantly reduced cGMP concentration compared with cells stimulated with ETEC K88 alone (Fig. 4b, $P < 0.01$). These data indicated that the BA complex inhibits the production of cAMP and cGMP during infection of IPEC-1 cells with ETEC.

Table 1
Summary table of IPEC-1 cell gene primer sequences.

Gene symbol	Forward primer	Reverse primer	Amplicon size (bp)	Primer efficiency ±SD	R
HMBS	AGGATGGGGCAACTCTACCTG	GATGGTGGCCTGCATAGTCT	83	2.01 ± 0.067	0.9949
CFTR	CCTCTGGAAAAGGCCAGCAT	CTGCTTTGGTGACTTCCCCT	277	1.98 ± 0.032	0.9927
NHE4	GATGGTCATCTGAGCACGGT	AGACGGGCCCTGGAAAATC	128	1.95 ± 0.085	0.9945

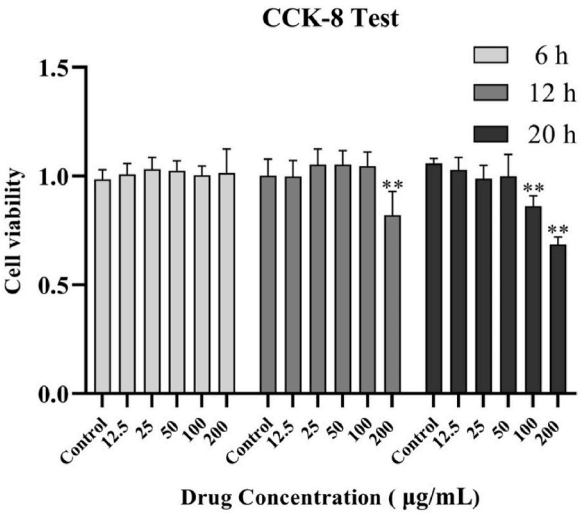

Fig. 1. Effect of BA complex on IPEC-1 viability *in vitro*. ** indicates significance at $p < 0.01$.

3.5. Effect of the BA complex on mRNA expression of CFTR and NHE4

Expression of *CFTR* at the mRNA level in IPEC-1 cells was significantly upregulated upon ETEC infection induction at 6 h compared with the control (Fig. 5, $P < 0.01$). Treatment with the BA complex at concentrations of 50 and 100 μg/mL downregulated the expression of *CFTR* following stimulation with C83715 (Fig. 5a, $P < 0.01$), while treatment with 12.5, 25, 50, and 100 μg/mL BA complex downregulated the expression of *CFTR* following stimulation with K88 (Fig. 5b, $P < 0.01$). Conversely, infection with ETEC reduced mRNA expression of *NHE4* ($P < 0.05$). In addition, treatment of IPEC-1 cells with the BA complex significantly affected the mRNA expression of *NHE4* ($P < 0.01$).

4. Discussion

Medicinal herbs, which are characterized as being safe, purely natural, and not prone to drug resistance, are now widely used in animal husbandry [17]. Baicalin, the main active compound of *S. baicalensis*, has high medicinal value. We previously reported that baicalin reduced the inflammatory response and protected peritoneal tight junctions of piglets from *Glaesserella parasuis* (*G. parasuis*) [18,19]. Baicalin has also been used to treat diarrheic piglets by inhibiting bacterial adhesion and inflammatory responses due to ETEC infection [20,21]. With electron-rich carbonyl and carboxyl groups, baicalin can complex with metal ions to result in higher bioactivity [22]. It has previously been shown that baicalin-zinc and baicalin-copper can enhance the antioxidant capacity and nutrient absorption of pigs [23,24]. Baicalin-copper can also alleviate the impairment in growth, and modulate the gut microbiota, inflammatory responses, and hormone secretion in deoxynivalenol challenged piglets [24,25]. Our previous studies reported that the BA complex protected piglets from diarrhea; however, the underlying mechanism is still unclear [10]. Therefore, this research is consistent with our previous reports.

IPEC-1 cells are a biologically relevant model system for studying porcine ETEC-host intestinal epithelial cell interactions *in vitro* [26]. ETEC adherence is the first step of the invasion process, and is followed by the production of enterotoxins [3]. ETEC strains cause diarrhea through the action of a LT and ST [27]. LT can increase intracellular cAMP levels by binding the ganglioside GM1, activating the CFTR. Increased cAMP levels inhibits the NHEs. STa and STb are two heat-stable enterotoxins responsible for diarrhea in pigs. STa binds guanylyl cyclase C (GC-C), activating the intracellular domain of GC-C, resulting in intracellular accumulation of cGMP. The increased levels of cGMP activate cGMP-dependent protein kinase II (cGMPKII), which in turn phosphorylates the CFTR. The elevated levels of cGMP also inhibit phosphodiesterase 3 (PDE3), leading to an increased level of cAMP. The elevated cAMP activates protein kinase A (PKA), which phosphorylates CFTR. Furthermore, PKA inhibits NHEs. STb activates calmodulin-dependent protein kinase II (cAMPKII), though a GTP-binding regulatory protein and triggers the activation of protein kinase C (PKC), which enzyme phosphorylates CFTR and inhibits NHEs. Moreover, cAMPKII opens a calcium-activated chloride channel (CaCC) and may also be involved in the phosphorylation of CFTR [3.28,29].

NHE3 plays an important role in the regulation of Na+/H+ exchange in the small intestine; increased cAMP and cGMP levels inhibit NHE3 in piglets challenged with ETEC [27,29].

In this study, we used IPEC-1 as a cell model for ETEC infection. Our study showed that few bacteria adhered to the surface of IPEC-1 cells following treatment with the BA complex. Levels of cAMP and cGMP were significantly increased after ETEC infection. Compared with the ETEC infection group, treatment with the BA complex significantly decreased the production of cAMP and cGMP. When cells were infected with ETEC, mRNA levels of *CFTR* were significantly increased compared with those of control cells as previously reported [3,27]. Treatment with the BA complex significantly decreased the expression of *CFTR* mRNA. NHE4, which plays

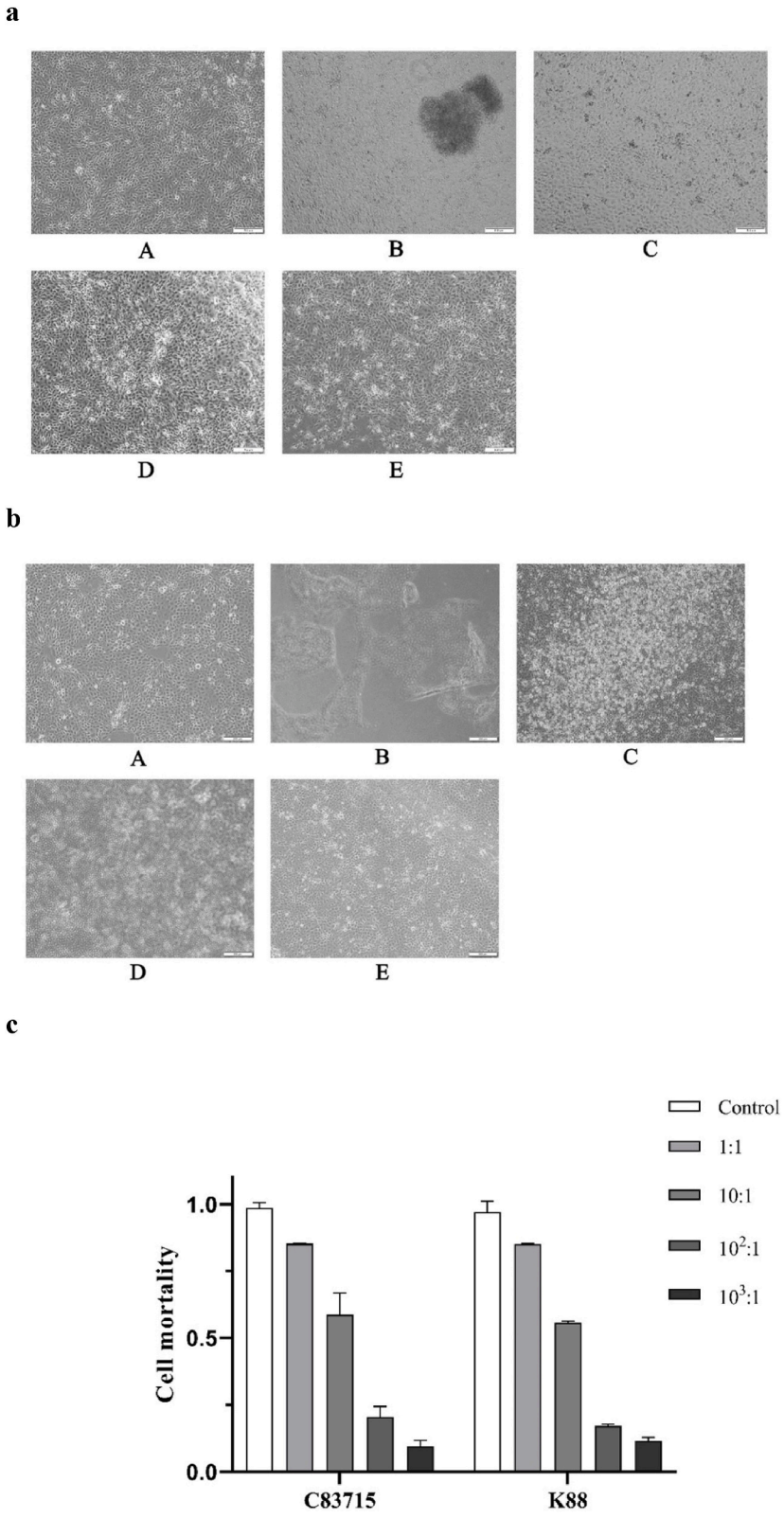

Fig. 2. Establishing a model of infection of IPEC-1 cells by ETEC (**a.** C83715; **b.** K88; **c.** cell survival after co-incubation of IPEC-1 with C83715 and K88). (**A.** blank control group; **B.** MOI = 1000: 1; **C.** MOI = 100: 1; **D.** MOI = 10: 1; **E.** MOI = 1: 1).

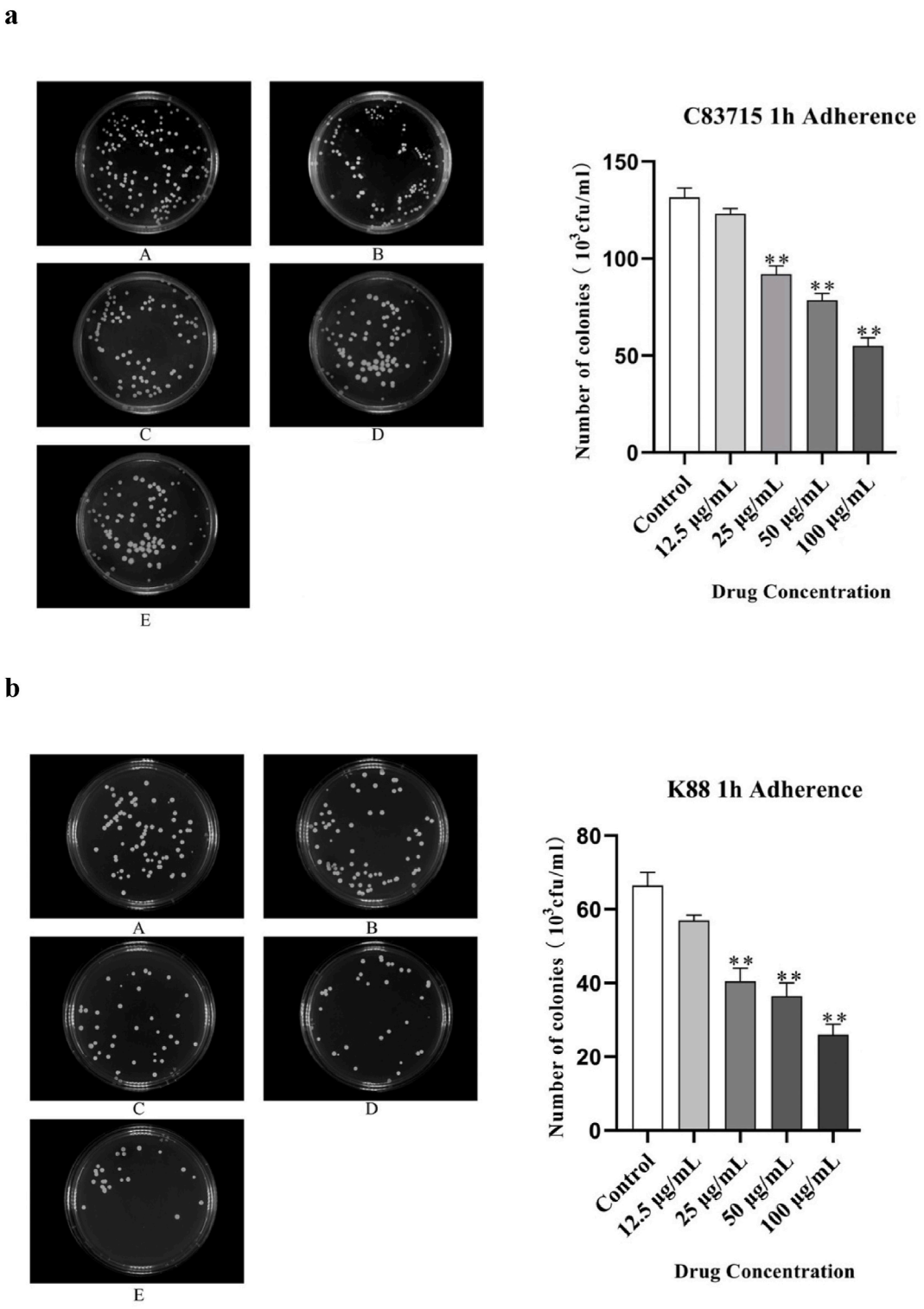

Fig. 3. Inhibitory effect of BA complex against the adherence of ETEC (**a.** C83715 and **b.** K88) to IPEC-1 cells. (**A-E** in the left panels represent blank control group, 12.5, 25, 50 and 100 μg/mL BA complex treatment groups.) ** indicates significance at $p < 0.01$.

a large role in controlling pH, was identified in the human colon carcinoma cell line T84 and human colonic crypts, and in the stomachs of guinea pig [28,30]. Nothing is known, however, about the regulation of NHE4 activity in IPEC-1 cells following ETEC infection. Our findings suggest that BA complex treatment increases the expression of *NHE4* mRNA in IPEC-1 cells which was inhibited by ETEC.

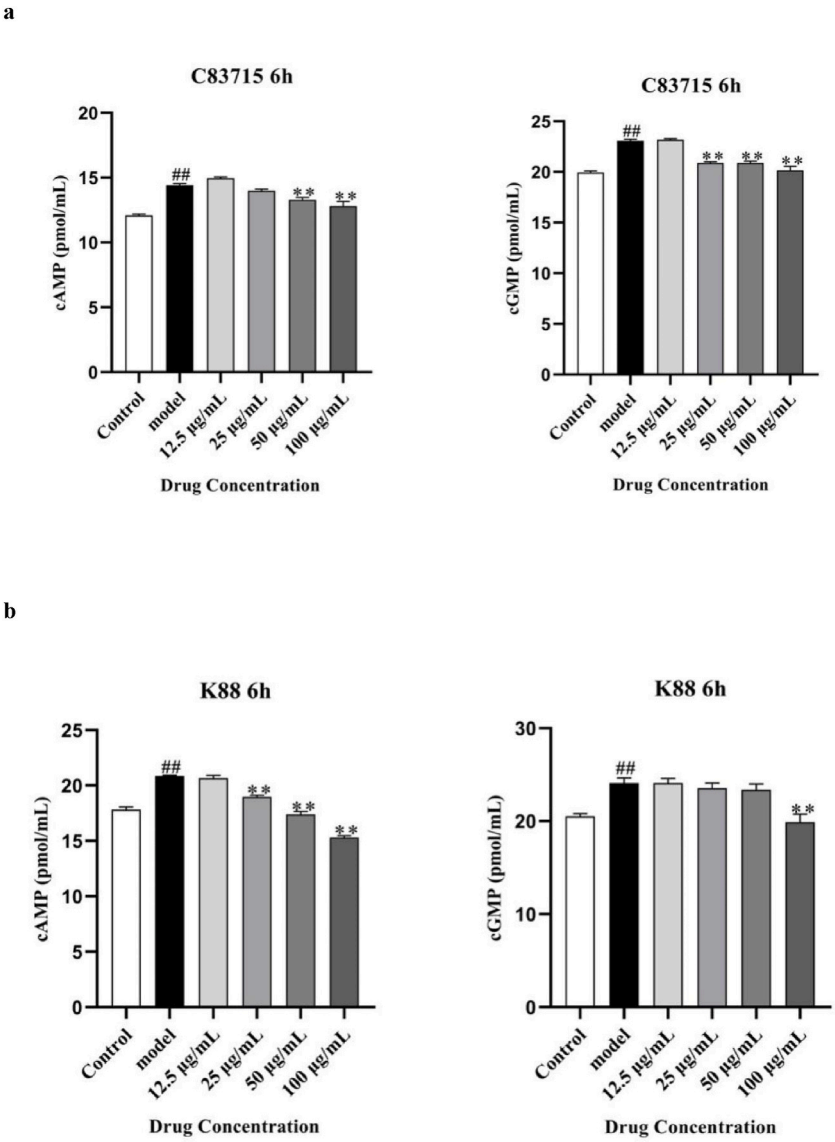

Fig. 4. Effect of BA complex on production of cAMP and cGMP induced by ETEC in IPEC-1 cells (**a.** C83715 and **b.** K88). $^{\#\#}p < 0.01$ vs. control; **indicates significance at $p < 0.01$.

However, treatment with the BA complex alone has little influence on IPEC-1 cells (Fig. 6a and b). Thus, the BA complex can interrupt attachment of ETEC to IPEC-1 cells and inhibit cAMP/cGMP-CFTR signaling.

Pig diarrhea caused ETEC is often associated with the innate immune response and impaired intestinal barrier function [31–33]. Consistent with our previous studies, the results have shown that baicalin could inhibit activation of nuclear factor-kappa B and NLRP3 inflammasome signaling pathways in piglet mononuclear phagocytes and porcine aortic vascular endothelial cells of piglets during *G. parasuis* infection or LPS stimulation [15,34,35], and protect peritoneal/vascular tight junctions damaged with inhibition of the activation of protein kinase C and myosin light chain kinase/myosin light chain signaling pathway induced by *G. parasuis* [19,36]. This effect may be attributed to protection of the BA complex against pig diarrhea, which promotes protection of porcine intestinal cells and maintains the epithelial barrier and immunity function. Future studies will explore the role of the BA complex on the inflammatory response and tight junction changes in ETEC infection.

In summary, the present study explored the effects of the BA complex on the regulation of IPEC-1 cells against ETEC. We also established an ETEC infection model with IPEC-1 cells. Our findings revealed that the BA complex exhibited minimal cytotoxicity in the IPEC-1 cell line, but significantly reduced the adhesion of ETEC to IPEC-1 cells and interrupted cAMP/cGMP-CFTR signaling. Our results will improve our understanding of the possible reaction mechanism of the BA complex and may facilitate the development of promising antibacterial candidates for treatment of weaned piglets infected by ETEC.

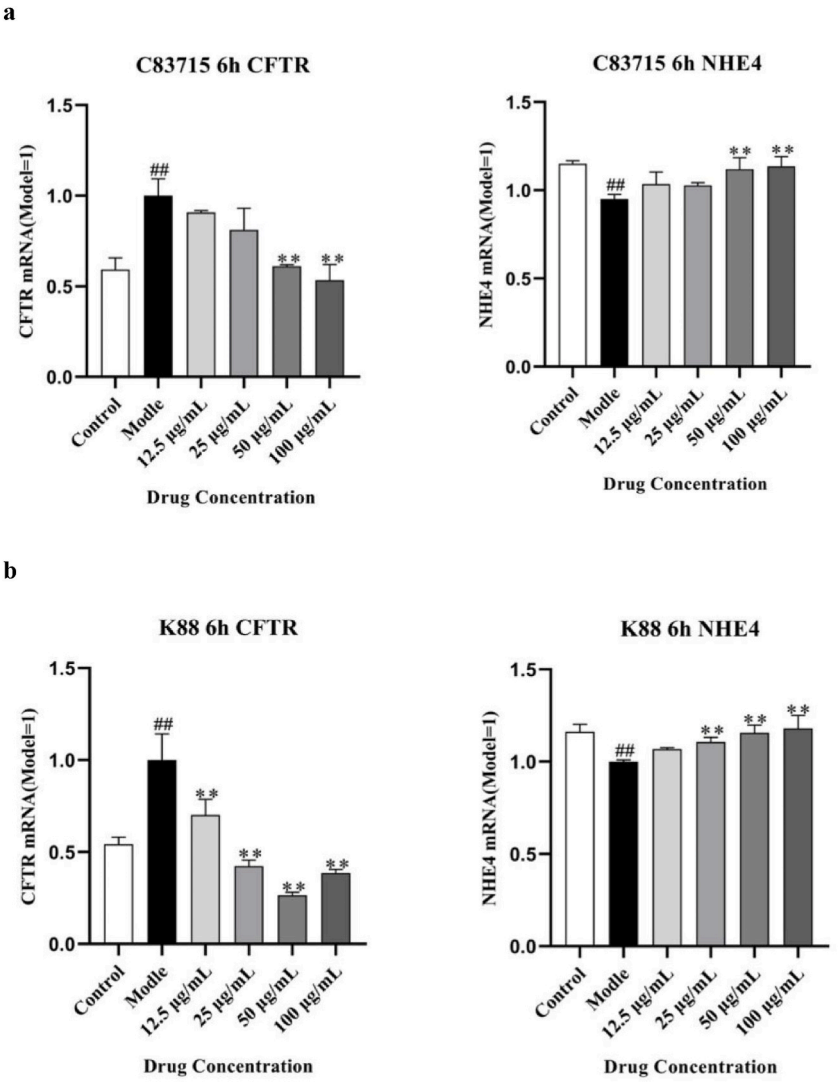

Fig. 5. Effect of BA complex on mRNA expression of *CFTR* and *NHE4* induced by ETEC in IPEC-1 cells (**a.** C83715 and **b.** K88). $^{\#\#}p < 0.01$ vs. control; **indicates significance at $p < 0.01$.

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Chun Ye: Writing – review & editing, Writing – original draft, Project administration, Funding acquisition, Conceptualization. **Yuqian Chen:** Writing – review & editing. **Ruixue Yu:** Visualization, Investigation. **Ming Zhao:** Writing – original draft, Investigation, Data curation. **Ronghua Yin:** Writing – original draft, Visualization, Methodology, Formal analysis. **Yinsheng Qiu:** Resources, Funding acquisition, Conceptualization. **Shulin Fu:** Validation. **Yu Liu:** Supervision. **Zhongyuan Wu:** Software.

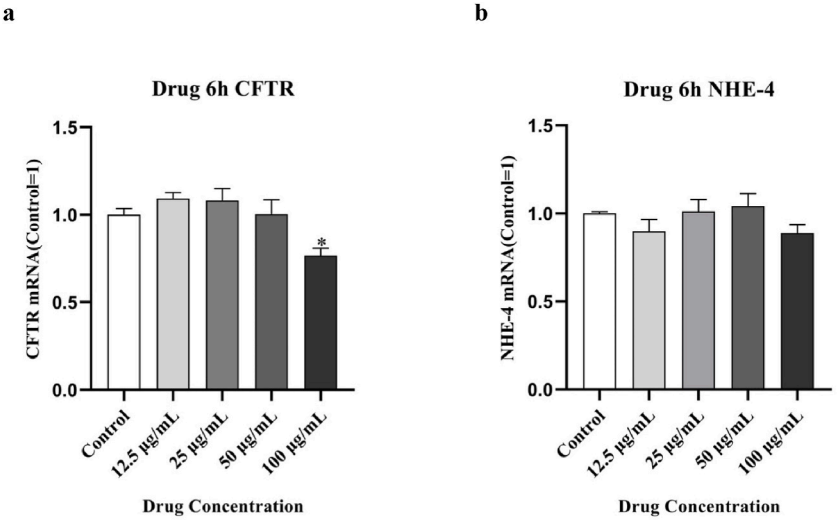

Fig. 6. Effect of BA complex on mRNA expression of *CFTR* and *NHE4* in IPEC-1 cells (**a.** *CFTR* and **b.** *NHE4*).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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