Piperine as potential therapy of post-weaning porcine diarrheas: an in vitro study using a porcine duodenal enteroid model

Saravut Satitsri
Chakri Naruebodindra Medical Institute, Mahidol University
Nattaphong Akrimajirachoote
Kasetsart University
Kanokkan Nunta
Vet Products Research and Innovation Co., Ltd
Nitwarat Ruennarong
Vet Products Research and Innovation Co., Ltd
Orawan Amnucksoradej
Vet Products Research and Innovation Co., Ltd
Chatchai Muanprasat (chatchai.mua@mahidol.ac.th)
Chakri Naruebodindra Medical Institute, Mahidol University

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Abstract

Post-weaning diarrhea in piglets is a major problem resulting in significant loss in pig production. This study aimed to investigate effects of piperine, an alkaloid abundantly found in black peppers, on biological activities related to pathogenesis of post-weaning diarrheas using a porcine duodenal enteroid model, a newly established intestinal stem cell-derived *in vitro* model recapitulating physiology of porcine small intestinal epithelia. Porcine duodenal enteroid models were treated with disease-relevant pathological inducers with or without piperine (8 μg/mL and/or 20 μg/mL) before measurements of oxidative stress, mRNA expression of proinflammatory cytokines, nuclear factor-kappa B (NF-κB) nuclear translocation, barrier leakage, and fluid secretion. We found that piperine (20 μg/mL) inhibited H_2O_2-induced oxidative stress, TNF-α-induced mRNA expression of proinflammatory cytokines without affecting NF-κB nuclear translocation, and prevented TNF-α-induced barrier leakage in porcine duodenal enteroid monolayers. Importantly, piperine inhibited fluid secretion induced by both forskolin and heat-stable toxins (STa) in a three-dimensional model of porcine duodenal enteroids. Collectively, piperine possesses both anti-inflammatory and anti-secretory effects in porcine enteroid models. Further research and development of piperine may provide novel interventions for treatment of post-weaning porcine diarrheas.

Introduction

Post-weaning diarrheas (PWD) is a major problem in pig production causing economic loss, lower growth performance, and increased mortality rate (Amezcua et al., 2002; Fairbrother et al., 2005). The mortality rate caused by PWD may reach approximately 20–30% (Amezcua et al., 2002). Pathophysiology of PWD involves excessive intestinal fluid secretion, resulting in severe fluid loss (Heo et al., 2013). This pathological process is driven by transepithelial chloride secretion via cystic fibrosis transmembrane conductance regulators (CFTR), a cAMP-regulated chloride channel (Barrett and Keely, 2000; Leonhard-Marek et al., 2009). Moreover, weaning piglets are vulnerable to *Escherichia coli* infection causing more severe symptoms by promoting CFTR-mediated chloride secretion via effects of bacterial enterotoxins e.g. heat-stable toxins (STa) and intestinal barrier disruption via oxidative stress and proinflammatory responses (Moeser et al., 2017). Notably, tumor necrosis factor-α (TNF-α), a proinflammatory cytokine elevated in the intestine weaning piglet, plays important roles in eliciting inflammatory responses and associated pathogenesis in PWD (Pié et al., 2004). At present, antibiotics prescribed for medical conditions in humans including colistin have been used to treat PWD (Rhouma et al., 2017). with significant concern on promoting an emergence of antibiotics-resistant bacteria. Therefore, novel therapeutic options of PWD are urgently needed especially those targeting pathological processes in hosts including oxidative stress, inflammation-associated barrier dysfunction, and intestinal fluid secretion.

Black peppers (*Piper nigrum L.*) are generally used as spice and seasoning in household. Piperine, an alkaloid serving as a major chemical compounds in black peppers, has been demonstrated to possess several biological activities including antioxidative stress and anti-inflammation (Haq et al., 2021;

Mujumdar et al., 1990; Vijayakumar et al., 2004). Interestingly, we have recently reported that piperine has anti-secretory effects in human intestinal epithelial (T84) cell lines by inhibiting CFTR, calcium-activated chloride channels (CaCC), and calcium-activated potassium channels (Pongkorpsakol et al., 2015). Interestingly, piperine supplement promotes growth performance as well as meat quality in pigs (Sampath et al., 2020). However, effects of piperine on oxidative stress, inflammatory responses, and fluid secretion related to PWD pathogenesis in porcine intestinal epithelia are unexplored.

Enteroids or mini-guts are a powerful and physiologically relevant model of intestinal epithelia recently developed from intestinal stem cells residing in the intestinal crypts (Gehart and Clevers, 2019). Enteroids contain all types of cells in intestinal epithelia, which exhibit functional characteristics of each intestinal regions *in vivo* dependent on areas from which intestinal stem cells/crypts are isolated. Enteroids from mice, humans and pigs have recently been established for investigating both intestinal functions and pathogenesis using either two dimensional (2D; monolayers) or three dimensional (3D) models (Khalil et al., 2016; Sato et al., 2011; Sato et al., 2009). Interestingly, a swelling (3D) assay has been developed and proven useful to evaluate anti-secretory effects (Cil et al., 2017; Dekkers et al., 2013). Due to advantage of this model, this study aimed to investigate effects of piperine on oxidative stress, inflammation-associated barrier disruption, and fluid secretion related to PWD pathogenesis using 2D and 3D porcine enteroids derived from duodenal crypts isolated from weaning piglets.

Materials And Methods

Ethics statement

This study has been approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, Kasetsart University (permit number ACKU64-VET-064). Leftover samples from this study were collected in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, U.S.A.

Cell lines

L-WRN cells were from American Type Culture Collection (ATCC) (catalog number CRL-3276; Manassas, VA, USA). Cells were maintained by Dulbecco's Modified Eagle Medium (DMEM) high glucose supplemented with 0.5 mg/mL of G418 (catalog number 11811031, Gibco, Waltham, MA, USA) and 0.5 mg/mL of Hygromycin B (catalog number 10687010, Gibco, Waltham, MA, USA). Conditioned medium was prepared according to ATCC's guideline.

Materials

$CFTR_{inh}$-172 and 4,4´-diisothiocyanatostilbene-2,2 ´-disulfonic acid disodium salt hydrate (DIDS) were purchased from Merck (Darmstadt, Germany). GlyH-101 was obtained from R&D Systems (Minneapolis, MN, USA). Piperine (purity > 98%) was prepared from black peppers as previously described (Raman and Gaikar, 2002).

Crypt isolation for porcine enteroid culture

Three weeks after weaning, piglets were acquired from the Faculty of Veterinary Medicine, Kasetsart University. Porcine duodenums as leftover samples were collected and crypt isolation was performed with some modifications as previously described (Foulke-Abel et al., 2016). Briefly, porcine duodenal tissues were cut into small pieces. Porcine duodenal tissues were then washed by complete chelated solution containing 5.55 mM Na_2HPO_4, 7.9 mM KH_2PO_4, 95.82 mM NaCl, 1.6 mM KCl, 43.82 mM sucrose, 54.89 mM D-sorbitol, and 0.5 mM dithiothreitol. Crypts from porcine duodenal tissues were subsequently isolated by 0.5 M EDTA. Crypts were formed into 3D porcine duodenal enteroid in Matrigel (catalog number 356237, Corning, NY, USA) mixed with enteroid-growing media containing L-WRN-conditioned media, 1X B27, 1 mM N-acetyl cysteine, 100 µg/mL Primocin, 50 ng/mL EGF,

10 nM Gastrin, 10 µM SB202190, and 500 nM A83-01. Porcine duodenal enteroids after more than 10 passages were used for the experiments.

Oxidative stress measurement

Oxidative stress was measured using 2´,7´-dichlorofluorescin diacetate (DCFDA) assays (D6883, Sigma Aldrich, Burlington, MA, USA), which detected reactive oxygen species (ROS) inside the cells. The 2D porcine duodenal enteroids were seeded onto 24-well plates. Cells were incubated for 2 h in serum-free DMEM high glucose media containing 1 mM H_2O_2 with or without piperine (8 µg/mL or 20 µg/mL). Fluorescence intensity (485 nm/ 530 nm) was measured using the Synergy Neo2 Plate Reader (Biotek, Santa Clara, CA, USA.). Trolox at 2 mM (238813, Sigma Aldrich, Burlington, MA, USA) was used as a positive control.

Intestinal barrier integrity evaluation

Intestinal barrier integrity of 2D porcine duodenal enteroid monolayers was evaluated using fluorescein isothiocyanate (FITC)-dextran (4 kDa) flux assays. Cells were seeded onto 24-well inserts (catalog number 3470, Corning, NY, USA) with collagen type IV coating (C6725, Sigma Aldrich, Burlington, MA, USA). Cells were maintained for at least 14 days, when transepithelial electrical resistance was ~ 500 $\Omega.cm^2$. In this experiment, enteroid monolayers were treated for 24 h with TNFα (50 ng/mL) with or without piperine (8 µg/mL or 20 µg/mL) before an addition of 15 µL of 10 mg/mL FITC-dextran (4 kDa) (catalog number 46944, Sigma Aldrich, Burlington, MA, USA). An hour later, 100 µL of basolateral media was collected for measuring fluorescence intensity (495 nm/519 nm) using the Synergy Neo2 Plate Reader (Biotek, Santa Clara, CA, USA). Concentrations of FITC-dextran in the collected media were calculated using the standard curve of FITC-dextran with known concentrations.

Immunofluorescence staining of NF-κB nuclear translocation

2D porcine duodenal enteroids were seeded onto 24-well plates. Cells were then treated for 24 h with TNFα (50 ng/mL) with or without piperine (8 µg/mL or 20 µg/mL), followed by 1-h fixation with 4%

paraformaldehyde, washing with PBS, and 1-h blocking and permeabilization with 0.1% Triton X-100 and 1% bovine serum albumin in PBS. Fixed cells were incubated overnight with rabbit NF-κB p65 antibodies (catalog number D14E12, Cell signaling, Danvers, MA, USA), washed with PBS, incubated for an hour with Goat anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (catalog number A-11034, Thermo Fisher Scientific, Waltham, MA, USA), and counterstained for 10 min with Hoechst (catalog number H3570, Invitrogen, Waltham, MA, USA) for nuclear staining. Images were captured by fluorescence microscope (Nikon Eclipse Ts2R inverted fluorescence microscope, Tokyo, Japan). Localization of NF-κB p65 and nuclear staining was analyzed using Fiji ImageJ (Schindelin et al., 2012).

Quantitative real-time PCR

2D porcine duodenal enteroids were seeded onto 24-well plates. Cells were treated for 24 h with TNFα (50 ng/mL) with or without piperine (8 μg/mL or 20 μg/mL) and collected for RNA isolation using Monarch Total RNA Miniprep Kit (NEB, Ipswich, MA, USA). The quantity of isolated RNA was measured by nanophotometer (Implen NP80, Munich, Germany). Isolated RNA was converted into cDNA using iScript™ Reverse Transcription Supermix (Biorad, Hercules, CA, USA). Quantitative real-time PCR was performed using CFX96 Touch™ Real-Time PCR (Biorad, Hercules, CA, USA) with iTaq universal SYBR green supermix (Biorad, Hercules, CA, USA) for DNA amplicon measurement under the following conditions: 95°C 5 min; 40 cycles of 60°C for 30 s. Primers of target genes (Table 1) were synthesized by Bio Basic (Singapore). Target genes were normalized to GAPDH. The calculation of mRNA expression was performed using ddCT method.

Swelling assay

3D porcine duodenal enteroids were seeded onto 48-well plates with Matrigel. Cells were then treated with piperine (20 μg/mL), $CFTR_{inh}$-172 (20 μM), GlyH-101 (50 μM), or DIDS (200 μM) for 1 h and were followed by an addition of forskolin (5 μM) or STa toxin (100 nM). Images were captured every five min in the area that contained at least ten enteroids per area using Cytation 5 Cell Imaging Multi-Mode Reader (Biotek, Santa Clara, CA, USA). Time-lapse imaging was performed for monitoring enteroid swelling with 5 frames per second. Areas of each enteroid in the images were measured using Fiji ImageJ (Schindelin et al., 2012).

Data analysis

Data are expressed as means ± S.E.M. Each group was compared and analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. The *p*-value of < 0.05 was considered statistically significant. All data were analyzed in GraphPad Prism 5 (La Jolla, CA, USA)

Results

Effect of piperine on H2O2-induced oxidative stress in 2D porcine duodenal enteroids

To investigate the anti-oxidative effect of piperine in 2D porcine duodenal enteroid, DCFDA-based ROS assays were performed after H_2O_2 challenge with or without co-treatment with piperine. As shown in the Fig. 1, H_2O_2 treatment significantly increased DCFDA fluorescence intensity indicating an increase in ROS. Interestingly, co-treatment with piperine at low (8 µg/mL) and high (20 µg/mL) concentrations of piperine completely abolished H_2O_2-induced ROS generation, with the use of Trolox as a positive control. Piperine alone at both concentrations had no effect on ROS. This result indicates that piperine exerts an anti-oxidative effect in 2D porcine duodenal enteroid.

Effect of piperine on TNF-α-induced inflammatory responses in 2D porcine duodenal enteroid

Apart from oxidative stress, elevation of TNF-α levels and inflammatory responses was found in the intestinal tissues of weaning piglets (Pié et al., 2004). To investigate the effect of piperine on TNF-α-induced NF-κB activation, NF-κB nuclear translocation was analyzed using immunofluorescence staining after 30-min treatment with TNF-α (50 ng/mL) in the presence or absence of piperine in a 2D model of porcine duodenal enteroids. As shown in Fig. 2A and Fig. 2B, TNF-α induced NF-κB nuclear translocation, which was unaffected by co-treatment with piperine at 8 µg/mL and 20 µg/mL. This result indicates that piperine does not inhibit TNF-α-induced NF-κB activation.

We next investigated the effect of piperine on TNF-α-induced inflammatory responses by measuring mRNA expression of proinflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-8) after 24-h challenge with TNF-α with or without co-treatment with piperine in a 2D model of porcine duodenal enteroids. As depicted in the Fig. 3A and Fig. 3B, piperine at 8 µg/mL significantly suppressed mRNA expression of TNF-α and IL-1β. Interestingly, piperine at 20 µg/mL significantly inhibited expression of all four proinflammatory cytokines (Fig. 3C and Fig. 3D). These data indicate that piperine possesses an inflammatory effect against TNF-α in 2D porcine duodenal enteroid.

Effect of piperine on TNF-α-induced intestinal barrier leakage in 2D porcine duodenal enteroid monolayer

It is known that TNF-α triggers intestinal inflammation leading to intestinal barrier disruption in weaning piglets (Lechuga and Ivanov, 2017; Zhu et al., 2012). To investigate the effect of piperine on protecting against TNF-α-induced intestinal barrier dysfunction, flux of FITC-dextran (4 kDa), an indicator of intestinal barrier leakage, was measured in the 2D porcine enteroid monolayer exposed to TNF-α (50 ng/mL) for 24 h with or without co-treatment with piperine. It was found that TNF-α (50 ng/mL; 24 h) induced increased flux of FITC-dextran indicating intestinal barrier leakage (Fig. 4). The TNF-α-induced barrier leakage was suppressed by co-treatment with piperine in a concentration-dependent manner with a significant effect (~ 70% inhibition) being observed at 20 µg/mL (Fig. 4). Since piperine exerted all previous biological activities at 20 µg/mL, next experiments were done using this concentration.

Effect of piperine on fluid secretion in a 3D model of porcine swelling assay

It is known that secretory diarrheas in weaning piglets result from *Escherichia coli* infection via mechanisms involving cAMP/cGMP-dependent chloride and fluid secretion, which is induced by *E. coli*-derived enterotoxins especially heat-stable enterotoxin (STa) (Upadhaya and Kim, 2021). We next investigated whether piperine was able to suppress cAMP and STa-induced fluid secretion using a 3D model of porcine swelling assay. The 3D porcine enteroids were pretreated for an hour with piperine or other chloride channel inhibitors before 3-h incubation with forskolin (an adenylate cyclase activator) or STa toxin. As shown in Fig. 5 and supplemental video 1 to 6, forskolin (5 μM) induced fluid secretion, which was inhibited by piperine (20 μg/mL) and DIDS (200 μM; non-specific chloride channel blocker), and not by known CFTR inhibitors GlyH-101 (50 μM) and $CFTR_{inh}$-172 (20 μM). Interesting, the STa-induced fluid secretion was inhibited by piperine, GlyH-101 and DIDS, and not by $CFTR_{inh}$-172 (Fig. 6 and supplemental video 7–12).

Discussion

In this study, we successfully established a porcine duodenal enteroid model derived from intestinal crypts of weaning piglet. This newly established model was used to investigate effects of piperine on biological activities related to pathophysiology of post-weaning diarrheas (PWD). We demonstrated that piperine exerted anti-oxidative effect against H_2O_2 and anti-inflammatory effect in 2D porcine enteroid models. Piperine inhibited TNF-α-induced mRNA expression of proinflammatory cytokines without inhibiting NF-κB nuclear translocation and suppressed TNF-α-induced barrier disruption. Interestingly, piperine inhibited forskolin and STa-induced fluid secretion in a 3D model of porcine duodenal enteroid.

We found that piperine inhibited H_2O_2-induced oxidative stress in porcine enteroids. It is like that piperine directly scavenges ROS because its co-treatment with H_2O_2 produced near complete inhibition of ROS generation similar to Trolox, which is a direct scavenger of ROS. This notion is in agreement with several previous studies that have demonstrated that piperine acts as a direct scavenger of ROS (Boonyong et al., 2020; Liu et al., 2014; Ma et al., 2014). Likewise, it has been demonstrated that TNF-α-induced ROS was diminished by piperine in weaned Wuzhishan piglets (Shi et al., 2020). Results from our study provide the direct evidence of anti-oxidative effect of piperine in porcine intestinal epithelial cells.

Anti-inflammatory effect of piperine against TNF-α-induced inflammatory responses was evaluated in porcine enteroid models. We found that piperine did not inhibit TNF-α induced NF-κB nuclear translocation. However, piperine was found to inhibit NF-κB nuclear translocation in endothelial cells (Kumar et al., 2007). This opposite finding may be due to the fact that the effect of piperine on NF-κB nuclear translocation is cell-type specific. Nonetheless, we found that piperine at 20 μg/mL downregulated TNF-α-induced intestinal inflammation in 2D porcine duodenal enteroids by inhibiting mRNA expression of proinflammatory cytokines, suggesting that piperine affects the TNF-α signaling

downstream to NF-κB translocation. Additionally, it was demonstrated that piperine downregulated lipopolysaccharide (LPS)-induced TNF-α, IL-6, IL-1β, and prostaglandin E2 production in BV-2 microglia cells (Wang-Sheng et al., 2017). Several studies demonstrated that piperine decreased the level of IL-1β both *in vitro* and *in vivo* (Liang et al., 2016; Liu et al., 2020). In contrast, piperine did not inhibit TNF-α, IL-1β, and IL-6 in jejunal and ileal mucosa of weaned Wuzhishan piglets (Shi et al., 2020). These results suggest that there are some controversial findings regarding the anti-inflammatory effect of piperine in the weaning piglets that requires further investigation.

Of particular importance, we found that piperine at 20 μg/mL prevented the TNF-α-induced epithelial barrier disruption in the 2D model of porcine enteroids. Likewise, piperine treatment (20 mg/kg and 40 mg/kg) was found to upregulate protein expression of tight junction proteins including claudin-1, occludin, and ZO-1 in the large intestine of mice (Guo et al., 2020). Notably, different types of spices are known to differentially influence intestinal barrier integrity. For instance, capsaicin decreased transepithelial electrical resistance (TER), whereas piperine increased TER in HCT-8 cells (Jensen-Jarolim et al., 1998). Furthermore, it was found that piperine repaired intestinal barrier disruption in obese mice by downregulating TNF-α (Wang et al., 2021). These data suggest that piperine may have the beneficial effect on recovering intestinal barrier function provoked by inflammatory insults.

Anti-secretory effect of piperine was demonstrated in the 3D porcine duodenal enteroid. We found that piperine effectively blocked fluid secretion induced by both forskolin and STa. In the forskolin-induced swelling assay, $CFTR_{inh}$-172 and GlyH-101 did not inhibit fluid secretion, whereas piperine and DIDS (non-specific anion channel inhibitor) did. In the STa-induced swelling assay, piperine, GlyH-101 and DIDS inhibited fluid secretion, while $CFTR_{inh}$-172 had no effect. Lack of effect of $CFTR_{inh}$-172 may be because $CFTR_{inh}$-172 had no effect on porcine CFTR, which was reported in the previous studies (Rogers et al., 2008; Salinas et al., 2004; Stahl et al., 2012). It is noteworthy that fluid secretion induced by forskolin is more that STa at 3 h of incubation. This may be because there are additional mechanisms contributing to forskolin-induced enteroid swelling i.e. inhibition of Na^+/fluid absorption or stimulation of additional chloride secretion pathways e.g. calcium-dependent chloride secretion (Hoque et al., 2010; Ousingsawat et al., 2011; Reymann et al., 1986). This also explains the lack of effect of GlyH-101 CFTR inhibitor on forskolin-induced fluid secretion in this model. Furthermore, GlyH-101 completely inhibited STa-induced fluid secretion, indicating that STa-induced fluid secretion is mainly driven by CFTR-mediated chloride secretion. Piperine inhibited both forskolin and STa-induced fluid secretion indicating that efficacy of piperine was not dependent on secretagogues. This is consistent with the previous study reporting that piperine inhibit intestinal chloride secretion in human intestinal epithelial cells (T84 cells) by inhibiting several proteins involved in both cAMP and calcium-dependent chloride secretion including CFTR, calcium-activated chloride channels, and cAMP-dependent K^+ channels (Pongkorpsakol et al., 2015).

Conclusion

In conclusion, piperine exerts anti-oxidative, anti-inflammatory and anti-secretory effects in a porcine duodenal enteroids. The results from this study provide a rational basis for further research and development of piperine or piperine-containing black pepper extract as a functional feeds or pharmaceutical agents for prevention or treatment of PWD.

Declarations

Ethics approval and consent to participate

This study has been approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, Kasetsart University (permit number ACKU64-VET-064). Leftover samples from this study were collected in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, U.S.A. all methods are reported in accordance with ARRIVE guidelines (https://arriveguidelines.org) for the reporting of animal experiments.

Consent for publication

Not applicable.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author [C.M.] upon reasonable request

Competing interests

The authors declare that they have no conflicts of interest.

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Authors' contributions

S.S. – performed experiments, data collection, data evaluation, writing the final manuscript.; N.A. – performed experiments, data collection and data analysis; K.N, N.R, O.A. -data analysis; C.M. - conceptualisation, data collection, data evaluation, writing and reviewed the final manuscript. All authors reviewed the manuscript.

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Tables

Table 1 Primers of target genes analyzed by real-time PCR

Genes	Primer sequence (5′-3′)
TNF-α	FW: TGCCTACTGCACTTCGAGGTTATC
	RW: CAGATAAGCCCGTCGCCCAC
IL-1β	FW: AATTCGAGTCTGCCCTGTACCC
	RW: GCCAAGATATAACCGACTTCACCA
IL-6	FW: CAGAGATTTTGCCGAGGATG
	RW: TGGCTACTGCCTTCCCTACC
IL-8	FW: GACCCCAAGGAAAAGTGGGT
	RW: TGACCAGCACAGGAATGAGG
GAPDH	FW: ATGGTGAAGGTCGGAGTGAA
	RW: CGTGGGTGGAATCATACTGG

Figures

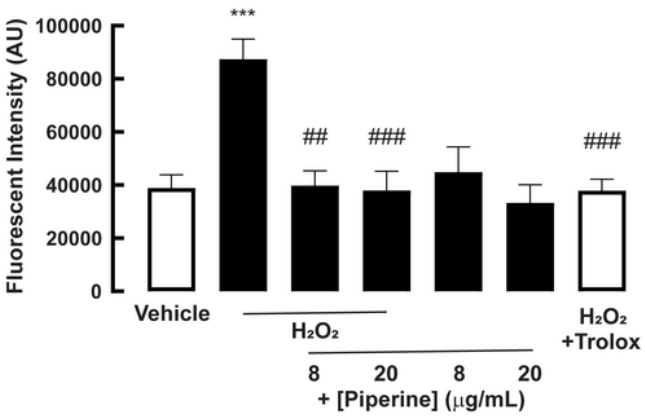

Fig. 1

Figure 1

The effect of piperine on H_2O_2-induced oxidative stress in 2D porcine duodenal enteroid. 2',7'-Dichlorofluorescin diacetate, (Reactive Oxygen Species (ROS)-sensitive dye, was incubated for 1 hour in 2D Porcine duodenal enteroid. After incubation, cells were treated with vehicle or 1 mM H_2O_2 with or without 8 or 20 µg/mL piperine. 2 mM Trolox was also used as positive control (n = 5) (***: *p*-value <

0.001 compared with vehicle, ##: *p*-value < 0.01 compared with 1 mM H_2O_2, ###: *p*-value < 0.001 compared with 1 mM H_2O_2)

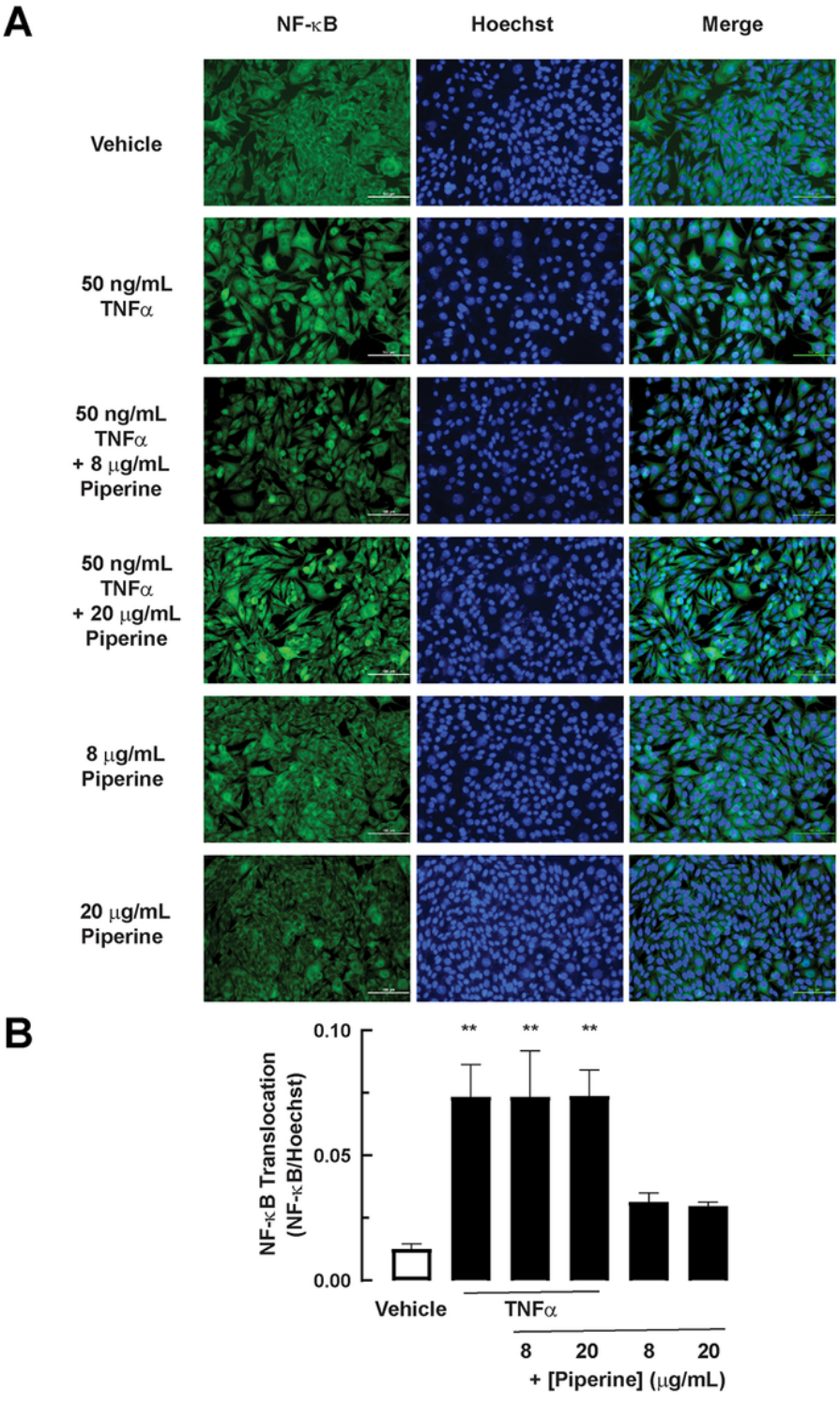

Fig. 2

Figure 2

The effect of piperine on TNF-α-induced NF-kB translocation in 2D porcine duodenal enteroid. (A) The representative image of 2D porcine duodenal enteroid. Cells were treated with 50 ng/mL TNF-α with or without 8 or 20 μg/mL piperine for 30 minutes. Cells were stained with rabbit NF-kB antibody, following with Alexa Fluor 488 anti-rabbit IgG and Hoechst as nuclear staining. (B) Positive localization of NF-kB staining inside nuclear staining was counted divided by the amount of nuclear staining. One sample was measured at least 5 areas (n = 3) (**: *p*-value < 0.01 compared with vehicle)

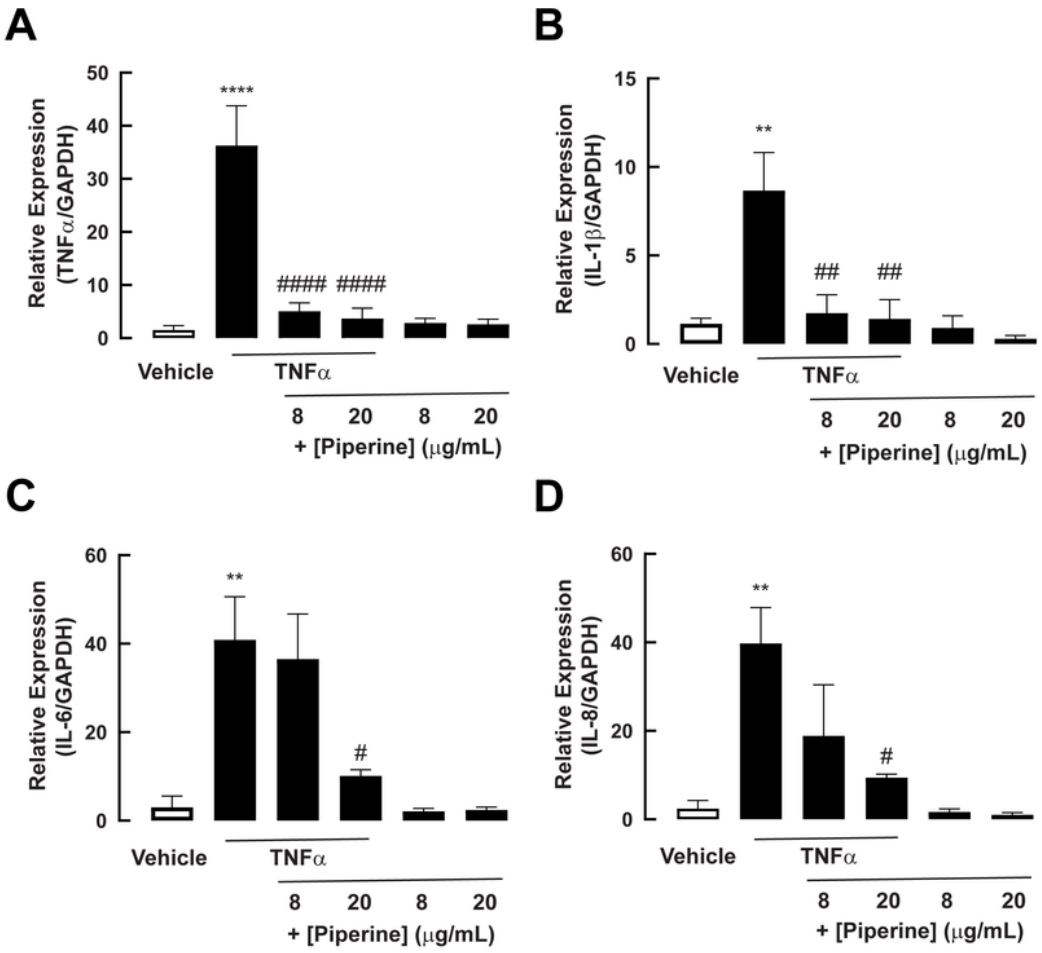

Fig. 3

Figure 3

The effect of piperine on mRNA of TNF-α-induced proinflammatory cytokines in 2D porcine duodenal enteroid. Cells were treated with 50 ng/mL TNF-α with or without 8 or 20 μg/mL piperine for 24 hours. Cell were harvested for mRNA expression. GAPDH was measured as housekeeping gene. (A) The mRNA expression of TNF-α in 2D porcine duodenal enteroid (n =4) (**** : *p*-value < 0.0001 compared with vehicle, #### : *p*-value < 0.0001 compared with 50 ng/mL TNF-α (B) The mRNA expression of IL-1β in 2D

porcine duodenal enteroid (n =4)(** : *p*-value < 0.01 compared with vehicle, ## : *p*-value < 0.01 compared with 50 ng/mL TNF-α) (C) The mRNA expression of IL-6 in 2D porcine duodenal enteroid (n =4) (** : *p*-value < 0.01 compared with vehicle, # : *p*-value < 0.05 compared with 50 ng/mL TNF-α) (D) The mRNA expression of IL-8 in 2D porcine duodenal enteroid (n =4) (** : *p*-value < 0.01 compared with vehicle, # : *p*-value < 0.05 compared with 50 ng/mL TNF-α)

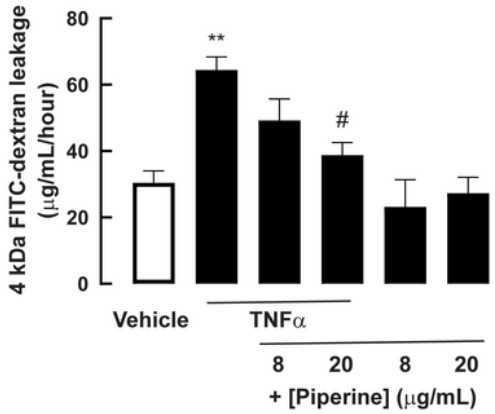

Fig. 4

Figure 4

Effect of piperine on TNF-α-induced intestinal barrier defects in 2D porcine duodenal enteroid monolayer. Cells were treated with 50 ng/mL TNF-α with or without 8 or 20 μg/mL piperine for 24 hours. Cells were then treated with 15 mL 4 kDa FITC-dextran for 1 hour. Basolateral medium was collected. The concentration of FITC-dextran in the cell culture medium at the basolateral side was calculated according to standard curve of FITC-dextran. (n =3) (** : *p*-value < 0.01 compared with vehicle, # : *p*-value < 0.05 compared with 50 ng/mL TNF-α)

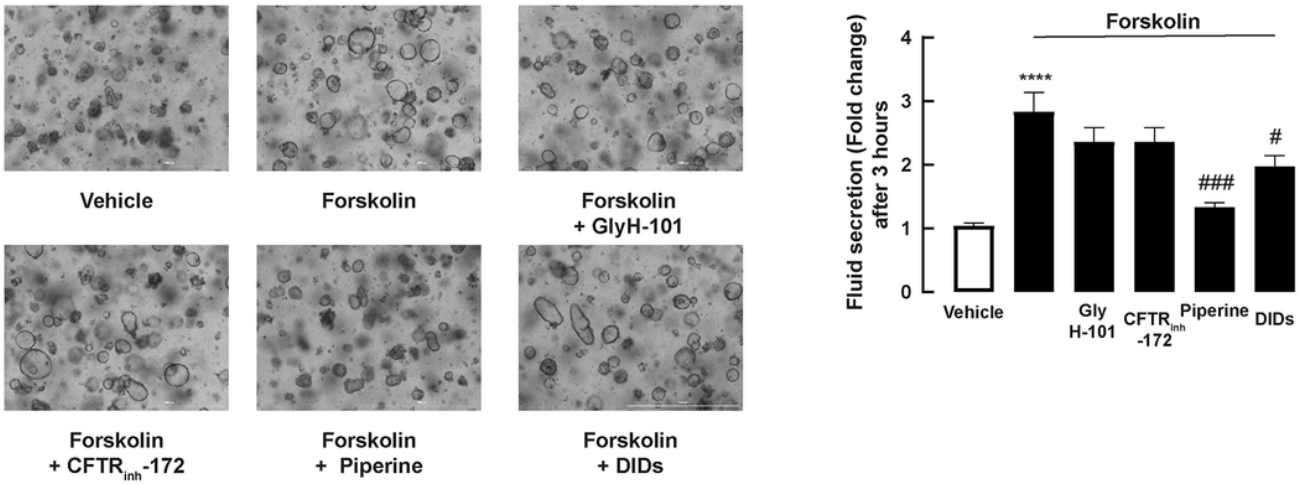

Fig. 5

Figure 5

Effect of piperine on CFTR-mediated fluid secretion in forskolin-induced swelling assay. 3D porcine duodenal enteroids were seeded on 48-well plate. Cells were then treated with piperine, $CFTR_{inh}$-172, GlyH-101, or DIDs for 1 hour prior to stimulate with 5 μM forskolin. (Left panel) Representative images of 3D porcine duodenal enteroids after 3 hours treated with 5 μM forskolin (Right panel) Quantitative data of enteroid area after 3 hours treated with 5 μM forskolin. Enteroids areas were measured at least 10 enteroids per 1 sample groups (n = 5) (****: *p*-value < 0.0001 compared with vehicle, #: *p*-value < 0.05, ###: *p*-value < 0.001 compared with 5 μM forskolin)

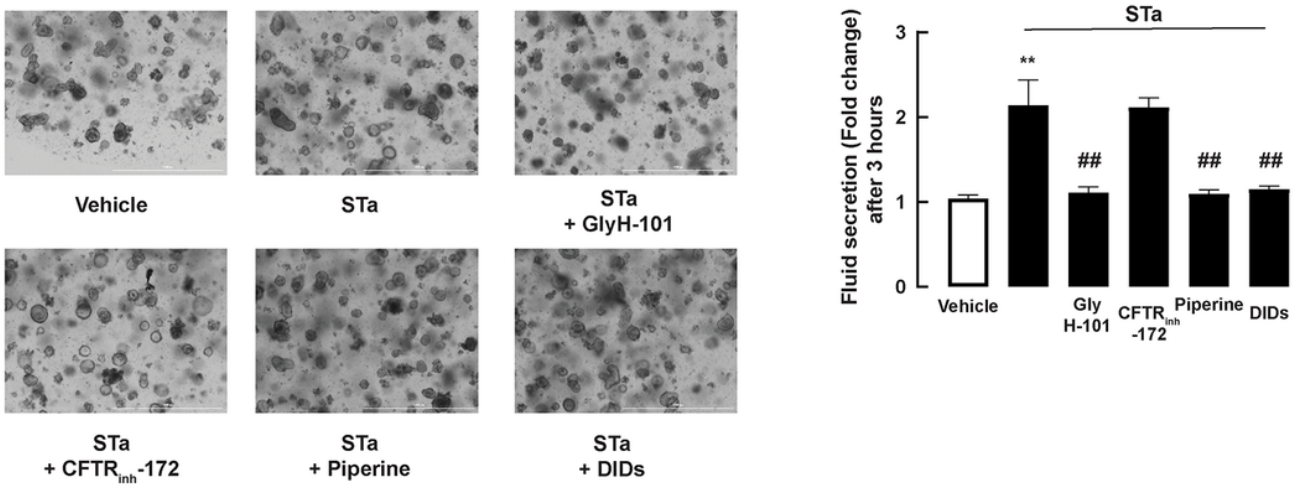

Fig. 6

Figure 6

Effect of piperine on CFTR-mediated fluid secretion in STa toxin-induced swelling assay. 3D porcine duodenal enteroids were seeded on 48-well plate. Cells were then treated with piperine, $CFTR_{inh}$-172, GlyH-101, or DIDs for 1 hour prior to stimulate with 100 nM STa toxin. (Left panel) Representative images of 3D porcine duodenal enteroids after 3 hours treated with 100 nM STa toxin (Right panel) Quantitative data of enteroid area after 3 hours treated with 100 nM STa toxin. Enteroids areas were measured at least 10 enteroids per 1 sample groups (n = 5) (**: *p*-value < 0.01 compared with vehicle, ##: *p*-value < 0.01 compared with 100 nM STa toxin)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementalvideo1vehicle.mp4
- Supplementalvideo25uMFSK.mp4
- Supplementalvideo35uMFSK50uMGlyH101.mp4

- Supplementalvideo45uMFSK20uMCFTRinh172.mp4
- Supplementalvideo55uMFSK20ugmLBPE.mp4
- Supplementalvideo65uMFSK200uMDIDs.mp4
- Supplementalvideo7vehicle.mp4
- Supplementalvideo8100nMSTa.mp4
- Supplementalvideo9100nMSTa50uMGlyH101.mp4
- Supplementalvideo10100nMSTaCFTRinh172.mp4
- Supplementalvideo11100nMSTa20ugmLBPE.mp4
- Supplementalvideo12100nMSTa200uMDIDs.mp4