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Protective Effects of Intestinal Trefoil Factor against Endotoxin-Induced Injury of Intestinal Mucosal Epithelial Cells in Piglets

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Authors' Contributions
ZC, ZL and DZ conceived and designed the experiments. HZ, MUR, MK, FN, XW, XT, XL, JX and XY contributed reagents, materials and analysis tools. ZL, HZ and ZC wrote the manuscript.

Key words
Intestinal trefoil factor, Porcine intestinal epithelial cells, Endotoxin, TFF3, rhTFF3.

ABSTRACT

Individual intestinal epithelial cells can survive and maintain their activity for several hours in tissue samples. For this study piglets without colostrum feeding were selected. Primary cell culture and subculture was performed from the intestinal epithelial cells of piglets. Then the effects of intestinal trefoil factor (TFF3) in intestinal disease was explored. Porcine intestinal epithelial cells were treated with lipopolysaccharide (LPS) and recombinant human (rh) TFF3. Cell proliferation and TFF3 mRNA expression were examined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays and real-time quantitative PCR, respectively. The results showed that rhTFF3 effectively prevented the cell injury induced by LPS ($P<0.05$). A low concentration of LPS (10 μg/mL) down-regulated the expression of TFF3 mRNA, whereas a high concentration of LPS (100 μg/mL) up-regulated TFF3 mRNA expression. Furthermore, TFF3 mRNA expression was decreased by rhTFF3 treatment. These findings indicate that TFF3 attenuates LPS-induced cell damage, and TFF3 mRNA expression is influenced by LPS and rhTFF3 in intestinal mucous epithelial cells in piglets.

INTRODUCTION

Individual intestinal epithelial cells can survive and maintain their activity for several hours in tissue samples. Intestinal cells cultured in serum-free medium can be used to study endocrinology, energy metabolism, and apoptosis for a short time (16-48 h). However, long-term primary culture of intestinal cells (up to 10 d) is still difficult, despite progress in isolation methodologies and manipulation of the microenvironment (Kaeffer, 2002). Intestinal epithelial cells are an essential component of the intestinal barrier, and play an important role in maintaining the integrity of the mucosal barrier, absorption of nutrients, and prevent intrusion of harmful substances (Iizuka and Konno, 2011).

Trefoil peptides (TFFs) are regulatory peptides for mucosal protection and repair in the gastrointestinal tract (Thim, 1989). TFF1 is expressed in surface epithelial cells of the stomach. TFF2 is expressed in mucous neck cells, pyloric glands, and Brunner's glands, while intestinal trefoil factor (TFF3) is mainly expressed in goblet cells of the small and large intestines (Hoffmann *et al*., 2001). Many in vitro studies have shown that TFF3 plays an important role in intestinal diseases. For example, TFF3 participates in epithelial cell repair, induces intestinal epithelial cell migration via a transforming growth factor β (TGFβ)-independent pathway (Dignass *et al*., 1994), and induces nitric oxide production in intestinal epithelial cells (Tan *et al*., 1999). These TFF-related results can provide a more profound analysis of the TFF functional background to better explore the effects of TFFs.

Bacterial endotoxin is a major component of the outer membrane of all gram-negative bacteria. Endotoxin has a major stimulating role in the immune system, which causes fever and intestinal diseases when it enters in blood circulation (Abutaweel *et al*., 2013). In addition, endotoxin influences the expression of mucin and cytokines. This study was conducted to determine whether TFF3 prevents

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cell damage caused by endotoxin, lipopolysaccharide (LPS). Additionally, recombinant human (rh) TFF3 were applied to intestinal mucosa epithelial cells to observe the effect of TFF3 on cell repair and TFF3 expression.

MATERIALS AND METHODS

Animals and materials

Piglets raised without colostrum were provided by Hubei Academy of Agricultural Sciences China. All experimental procedures were reviewed and approved by the appropriate Animal Use Committee of Huazhong Agricultural University. The piglets were anaesthetized with sodium pentobarbital (45mg/kg), followed by rapid collection of small intestinal segments under sterile conditions. DMEM-F12 was obtained from Hyclone, fetal bovine serum was purchased from Gibco (Gibco Company, USA), and epidermal growth factor (EGF) was purchased from Sigma (Sigma-Aldrich, USA).

Primary culture of intestinal epithelial cells and subculture

The small intestines were rinsed with phosphate buffered saline (PBS) several times under aseptic conditions, transferred to a sterile Petri dish after removal of the mesentery, and cut into 8-10 cm segments. The intestinal cavities were then thoroughly flushed with PBS. After longitudinal cuts on intestine with scissors, the small intestines were repeatedly washed with PBS containing antibiotics (Penicillin-Streptomycin, Cat#: GA3502, Wuhan), then washed with DMEM-F12 twice, and transferred to a new a Petri dish. The small intestines were cut into small pieces (< 1 mm^3), transferred to a sterile 50 mL centrifuge tube, and then washed several times with DMEM-F12 until the supernatant was clear. The tissue was transferred to a sterile 200 mL Erlenmeyer flask containing collagenase (0.1%) and shaken at 10 g for 2 h at 37 °C. Cells were isolated from the digested tissue by density gradient centrifugation using Percoll. The cells were then incubated at 37 °C with 5% CO_2. The culture medium was replaced after 24 h. The residual tissue was washed with PBS, digested with collagenase again at 4 °C overnight, and then processed as described above. At 80% confluence, the cells were subcultured at 1:2 ratios by incubation with 1 mL pancreatic enzymes at 37 °C for 2 min.

Identification of intestinal epithelial cells

Immunohistochemistry was performed using a monoclonal antibody against cytokeratin 18 (CK18), an epithelial cell marker. At 100% confluence, cells were transferred to glass coverslips in six-well plates and cultured to confluency. The cells were washed and then fixed in ice-cold acetone at room temperature for 10 min. Endogenous peroxidase activity was blocked by hydrogen peroxide, and then non-specific antibody binding was blocked by incubation with 5% bovine serum albumin for 20 min at room temperature. Then, the cells were incubated with a rabbit anti-human CK18 antibody at 37 °C for 1 h, followed by a goat anti-rabbit IgG at 37 °C for 20 min. The negative control omitted the primary antibody. Coloration was performed with a DAB Kit and images of the cells were obtained under microscope.

Prevention and treatment of injury

Cells cultured in 96-well plates were designated into three TFF3 treatment groups (A, B and C), three TFF3 prevention groups (D, E and F), and the control group (five wells each group). Cell in the treatment groups were incubated for 30 min with LPS @ 1 μg/mL (group A), 10 μg/mL (group B), or 100 μg/mL (group C), and then 50 μg/mL rhTFF3 for 30 min. Cells in the prevention groups were incubated for 30 min with 50 μg/mL rhTFF3, and then LPS @ 1 μg/mL (group D), 10 μg/mL (group E), or 100 μg/mL (group F) for 30 min. Cells in the control group were incubated with the same volumes of PBS.

Cell proliferation was detected by a MTT assay. MTT (20 μl) was added to each well, followed by incubation for 4 h. The cells were washed and 150 μL Dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. Absorbance values were read at 570 nm wavelength using an automated microplate reader (Stockert *et al*., 2012).

Effect of bacterial endotoxin on TFF expression

At 80% confluence, cells were washed and treated with 1, 10, or 100 μg/mL LPS, and 50 μg/mL rhTFF3. Saline was used for the control. The cells were incubated at 37 °C with 5% CO_2 for 24 h. Total RNA was then extracted with Trizol following the manufacturer's instructions, and immediately reverse transcribed to cDNA for PCR analysis.

Table I.- Primers for real-time quantitative PCR.

Genes	Primer (5'-3')	Band (bp)	Gen Bank ID
TFF3	F: GGGAGTATGTGGGCCTGTC	174	F14493
	R: AGGTGCATTCTGTTTCCTG		
18S rRNA	F: AATCGGTAGTAGCGACGG	275	AY265350
	R: AGAGGGACAAGTGGCGTTC		

Real-time quantitative PCR

Primers for the TFF3 gene (based on the TFF3 gene sequence in GenBank) and 18s RNA was designed from Primer Premier 5.0 (Table I) and synthesized by YINGJUN (Wuhan, China).

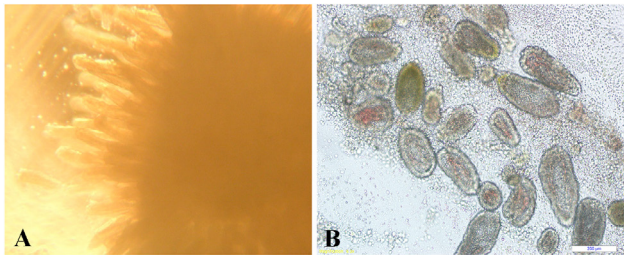

Fig. 1. Intestinal villus, crypt units and lots of cells were detected by microscope, scale bars represent 100 μm.

RESULTS

Cultivation of intestinal mucosa epithelial cells from porcine

During cell extraction, crypts and intestinal villi were observed in the intestinal tissue pieces under a microscope (Fig. 1A). After the tissue fragments were digested with collagenase for 2 h, a large number of intestinal epithelial cells were observed (Fig. 1B).

After 12 h of culture, a small number of cells were adherent among primary cultured cells. At 24 h of culture, there was a large number of adherent cells. After 48 h of culture, radial growth was observed around the tissues (Fig. 2A). Cells grew as clumps and the cell morphology was triangle and polygon shapes and some rounded cells with two nuclei were also seen. At 72 h of culture, there was a clear increase in the number of cells. Cells were mostly distributed evenly and showed a typical triangular shape (Fig. 2B). After 96 hr of culture, a monolayer of cells began to form, exhibiting a typical cobblestone appearance with tightly packed cells (Fig. 2C). At confluency, cells were tightly packed in a monolayer at high density (Fig. 2D).

Cell identification

We used CK18 immunohistochemical identification of intestinal mucosa epithelial cells. The results showed that cells in the test group were positive, whereas cells in the control group were negative (Fig. 3).

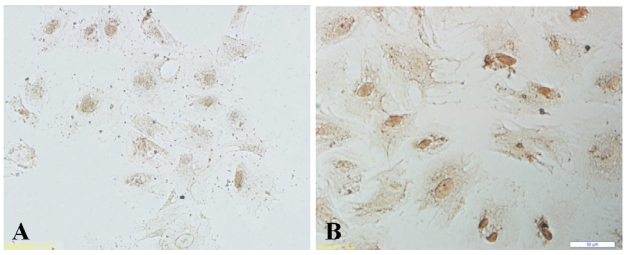

Fig. 3. Immunodetection of cytokeratin in neonatal swine intestinal epithelia cells. Left: negative control, Right: epithelial cells assayed by Immunodetection of cytokeratin, scale bars represent 50 μm.

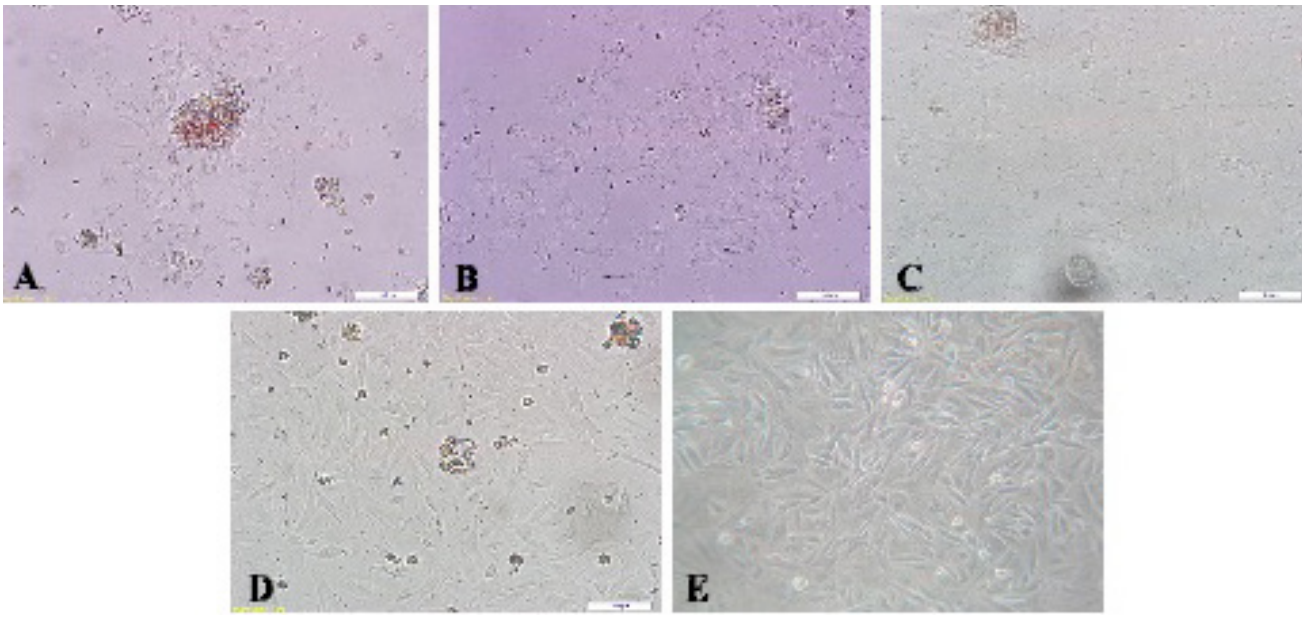

Fig. 2. The culture of neonatal swine intestinal epithelial cell (a, after 48 h; b, after 72 h; c, after 96 h; d and e, overgrow; scale bars represent 100 μm).

Table II.- MTT assay results showing prevention and treatment of cell damage by rhTFF3.

Groups	CG	Treatment groups			Prevention groups		
		TA	TB	TC	PD	PE	PF
OD	0.63±0.04[a]	0.67±0.03[a]	0.62±0.06[a]	0.66±0.06[a]	0.59±0.04[ac]	0.69±0.01[b]	0.69±0.07[ad]

Note: a, b, c, and d lacking a common superscript within a row indicates a significant difference at P<0.05.

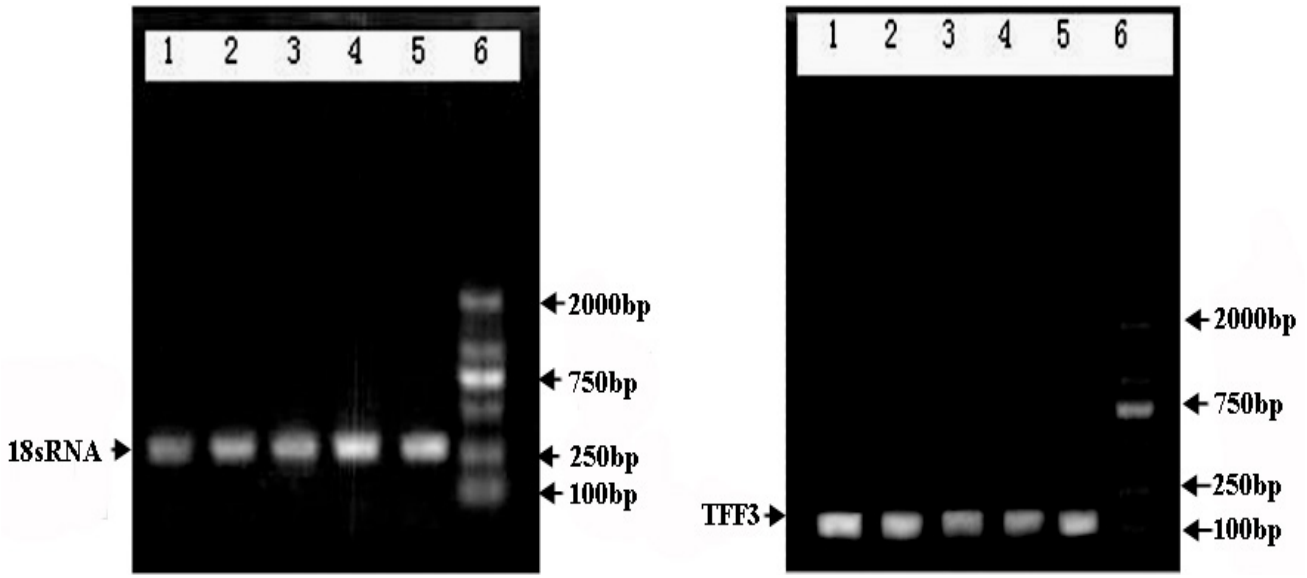

Fig. 4. The results of PCR product 1% Agarose Gel Electrophoresis. Left, The PCR results of 18S rRNA. Right, The PCR results of TFF3. After total RNA reverse transcription and PCR, PCR products by 1% agarose gel electrophoresis, after the completion of the electrophoresis observe in the gel imaging system. The pictures shows the stripes clarity after PCR products by 1% agarose gel electrophoresis, reference gene 18S rRNA amplification, amplified fragment length of 275 bp, consistent with design. TFF3 target gene amplified fragment length of 174 bp, matches the designed length of the primers. Lane 1~4 respectively endotoxin final concentration of 0, 1, 10, 100 μg/mL group, lane 5 is TFF3 induced group, lane 6 is DL-2000 Marker. The results show that the internal gene and TFF3 gene PCR amplification effect is good, achieve the desired effect.

Prevention and control of cell damage

The MTT assay is a colorimetric assay for measuring the activity of NAD (P)H-dependent oxidoreductase to reflect the number of viable cells. Compared with the control group, the MTT assay results (Table II) showed that addition of 50 μg/mL rhTFF3 before 10 μg/mL LPS (group E) had a preventive effect on cell damage (P<0.05). There were also significantly different preventive effects between groups D and E (P<0.05). Cell proliferation in experimental groups was higher than that in control groups, except for groups B and D that showed an increase of cell proliferation by addition of rhTFF3 to neutralize the damage by LPS. These result indicate that prevention of endotoxin-induced cellular damage by rhTFF3 is more effective than treatment with rhTFF3 following such damage.

Effect of LPS and rhTFF3 on TFF3 gene expression

After various treatments of the cells with LPS and rhTFF3 for 24 h, quantitative PCR was used to detect TFF3 gene expression. At a high concentration of LPS (100 μg/mL), we found a 1.48-fold increase of TFF3 gene expression compared with that in the control group, suggesting that cell damage induced by LPS caused an increase of TFF3 gene expression (Fig. 5). Low concentrations of LPS (1 and 10 μg/mL) inhibited TFF3 gene expression. Interestingly, treatment with rhTFF resulted in a decrease of TFF3 gene expression to 0.71-fold when compared with the control group (Fig. 5).

In summary, during this study primary porcine intestinal epithelial cell culture was done successfully as shown by immunohistochemistry of CK18. In addition, rhTFF3 effectively prevented LPS-induced damages in cells and LPS affected by TFF3.

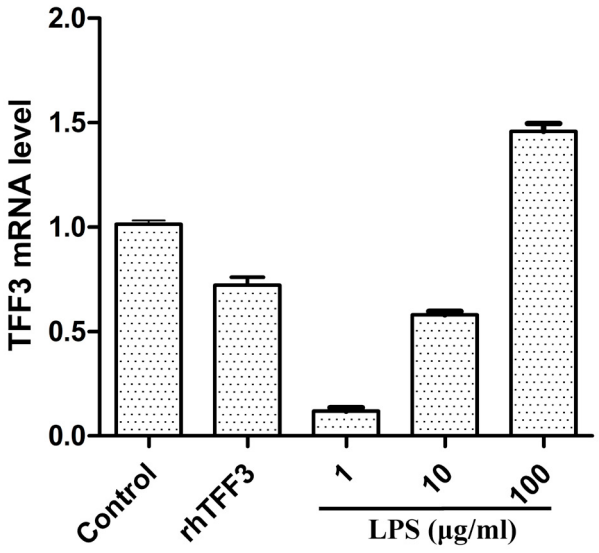

Fig. 5. The expression of TFF3 genes in cells effect by LPS and rhTFF3. Cells were treated with a final concentration of, respectively; 1, 10, 100 μg/mL of endotoxin and 50 μg/mL of rhTFF3, the amount of TFF3 gene expression respectively 0.11, 0.58, 1.48, and 0.71 times of the control group, shows that concentration of 100 μg/mL of endotoxin plays an enhanced role in TFF3 gene expression in cells. With the concentration of 1 μg/mL, 10 μg/mL of endotoxin and 50 μg/mL rhTFF3, TFF3 gene expression in cells with different degree of inhibition.

DISCUSSION

The recovery of epithelial cells with a defined phenotype and growth potential has been investigated by four methodologies as follows. Tissue explantation, mechanical isolation by dissection and trituration of tissue samples, chelation to obtain isolated crypts or cells, and proteolytic enzyme digestion to prepare cellular aggregates and retain direct interactions of epithelial cells with stromal cells and the extracellular matrix (Kaeffer, 2002). In this study, we used proteolytic enzyme digestion of the intestinal tissue. The small intestines of newborn piglets without colostrum were used in this study. The intestines of piglets are relatively sterile, which avoids contamination. In addition, compared with adult pigs, the intestinal mucosa of newborn piglets has a greater growth and differentiation potential. Collagenase was used to digest the intestinal tissue because collagenase hydrolyses proline polypeptides, which has no effect on the cell membrane or the growth potential attached cells. Previously, we successfully isolated and cultured cells to determine the optimal concentration and digestion duration of collagenase (Kaeffer, 2002). In this study, we also used tissue explantation and proteolytic enzyme digestion at 4 °C to isolate the epithelial cells. However, tissue explantation is very difficult with a high risk of contamination. Furthermore, the isolated cells include a large number of fibroblasts. Cells are able to grow normally after digestion at 4 °C overnight, which results in more cells with a higher purity compared with those obtained by digestion at 37 °C. However, the cells obtained by digestion at 4 °C overnight do not have sufficient cell viability. Therefore, we performed the initial digestion at 37 °C and used the residual tissue for digestion at 4 °C overnight to achieve higher cell viability and improve the yield.

In the present study, we used 5% fetal bovine serum to reduce the fibroblast growth rate and obtain more epithelial cells. Immunohistochemical staining of CK18 showed a significant positive rate to identify the epithelial cells. In addition, morphological characteristics demonstrated the presence of epithelial cells, such as a cobblestone appearance of triangle- or polygon-shaped cells.

Numerous studies have shown that there are important relationships between TFF3 and intestinal diseases. Inadequate secretion of TFF3 is one of the reasons that intestinal mucosa damage is more likely to occur in premature infants and during asphyxia (Lin *et al*., 2003). TFF3 may be associated with gastric cancer invasion and metastasis because an increase of TFF3 expression indicates an adverse prognosis (Feng *et al*., 2009). Moreover, TFF3 may be involved in the early recovery of gastric mucosa (Alison *et al*., 1995). TFF3 decreases diamine oxidase activity in plasma, inhibits mRNA and protein expression of tumor necrosis factor alpha in intestinal tissue, and plays a protective role by relieving intestinal injury induced by LPS in young rats (Li *et al*., 2006). Intraperitoneal and subcutaneous injection of TFF3 reduces oxygen free radical damage and intestinal inflammation (Chen *et al*., 2003). TFF3 alleviates TNBS-induced murine inflammatory bowel disease and inflammation of the intestinal mucosa and promotes repair (Büschenfelde *et al*., 2006; Kjellev *et al*., 2007). Protection and therapeutic effects of TFF3 are primarily dependent on its ability to promote mucosal repair including promotion of cell migration to the site of injury. Repair of the epithelial cells plays an important role in complete recovery after injury to the intestinal barrier and the cell repair can begin within a few minutes after injury. Stomach repair mainly relies on gastric mucosal cells and intestinal repair mainly depends on intestinal epithelial cells (Masuda *et al*., 2003). The repair of these organs involves cells-cell interactions as well as cell migration and apoptosis. An in vitro study has shown that TFF3 is associated with all of these processes simultaneously (Hoffmann, 2005). TFF3 also

down-regulates the expression of calcium binding proteins involved in cell-cell interactions (Kjellev *et al*., 2007). In this study, intestinal mucosal epithelial cells treated with both rhTFF3 and LPS were subjected to MTT assays to detect cell viability. We found that rhTFF3 effectively prevented cell damage and played a protective role in the cells. In the treatment group, TFF3 acted on cells after endotoxin damage. Therefore, TFF3 mainly promoted cell recovery. In the prevention groups, TFF3 acted on cells before the endotoxin to protect the cells from injury. Therefore, the effect of TFF3 on prevention of cell injury is stronger than its therapeutic effect, indicating that TFF3 effectively prevents cell death caused by endotoxin.

In a rat gastric ulcer model, TFF3 and TFF2 are expressed faster after injury than classic repair peptides such as EGF and TGFα (Alison *et al*., 1995), which is also observed in human gastrointestinal cells (Taupin *et al*., 1999). TFF3 plays a key role in the immediate repair of mucosal injury. In the acute stage of colitis caused by acetic acid in mice, TFF3 expression is down-regulated and may be associated with acute reduction of the number of goblet cells (Tomita *et al*., 1995). In mouse intestinal mucositis caused by methotrexate, TFF3 mRNA expression increases slightly, but protein expression in the acute stage is reduced significantly. TFF3 mRNA levels in the small and large intestines are significantly reduced when exposed to methotrexate and irradiation (Xian *et al*., 1999). However, TFF3 protein expression increases in acute dextran sulfate sodium-induced colitis in rats and mice, which may be caused by a difference in mucosal surface damage in the different models. Endotoxin is a major factor in sepsis and septic shock. It can directly damage intestinal mucosal epithelial cells, stimulate mucosal secretion of cytokines, and affect the permeability of cells. After LPS stimulation of endothelial cells, there is a remarkable increase of p38 MAPK phosphorylation that is LPS concentration-dependent (Lee and Young, 1996). TFF3 inhibits some of the channel capacity of MAPK (Alison *et al*., 1995), stimulates proliferation, and induces phosphorylation of the EGF receptor (Kosriwong *et al*., 2011). Upon endotoxin damage in epithelial cells, the TFF3 signaling pathway is activated and generates TFF3 protein to protect against mucosal epithelial cell injury and promote mucosal repair. Furthermore, there is a concentration-dependent relationship between TFF3 and endotoxin. Real-time quantitative PCR was used in this study to analyze the relationship between TFF3 and LPS. We found that high concentrations of LPS (100 μg/mL) promoted TFF3 gene expression, which is consistent with a previous report (Cook *et al*., 1999). At low concentrations of LPS (1 and 10 μg/mL), TFF3 gene expression in intestinal epithelial cells was inhibited because the activation threshold of TFF3 signaling the pathway may not have been reached and endotoxin inhibits the expression of mucosal protective factors. Treatment of cells witrhTFF3 decreased the TFF3 gene expression level to 0.71-fold of that in the control group, which may be a self-regulating mechanism.

CONCLUSIONS

From this study it is concluded that LPS and rhTFF3 both affect the expression of TFF3 in porcine intestinal epithelial cells. However, the signaling pathways regulating TFF3 gene expression are unclear and require further study.

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Conflict of interest statement

None of the authors have any conflict of interest.

REFERENCES

Abutaweel, G., Ajarem, J.S., Ebaid, H. and Rady, A.M., 2013. Behavioral changes induced by prenatal acute endotoxemia in mice offspring. *Pakistan J. Zool.,* **45**: 359-369.

Alison, M.R., Chinery, R., Poulsom, R., Ashwood, P., Longcroft, J.M. and Wright, N.A., 1995. Experimental ulceration leads to sequential expression of spasmolytic polypeptide, intestinal trefoil factor, epidermal growth factor and transforming growth factor alpha mRNAs in rat stomach. *J. Pathol.*, **175**: 405-414. https://doi.org/10.1002/path.1711750408

Büschenfelde, D.M.Z., Tauber, R. and Huber, O., 2006. TFF3-peptide increases transepithelial resistance in epithelial cells by modulating claudin-1 and -2 expression. *Peptides*, **27**: 3383-3390. https://doi.org/10.1016/j.peptides.2006.08.020

Chen, L.P., Zhang, B.H. and Li, Y., 2003. The effect and significance of intestinal trefoil factor IL-8 and MDA for neonatal rat model for hypoxia-induced intestinal injury. *Chinese J. Perin. Med.*, **5**: 306-309.

Cook, G.A., Familari, M., Thim, L. and Giraud, A.S.,

1999. The trefoil peptides TFF2 and TFF3 are expressed in rat lymphoid tissues and participate in the immune response. *FEBS Lett.*, **456**: 155-159. https://doi.org/10.1016/S0014-5793(99)00940-0

Dignass, A., Lynch-Devaney, K., Kindon, H., Thim, L. and Podolsky, D.K., 1994. Trefoil peptides promote epithelial migration through a transforming growth factor beta-independent pathway. *J. clin. Invest.*, **94**: 376-383. https://doi.org/10.1172/JCI117332

Feng, L.Y., Qin, Y.C. and Yin, X., 2009. Expression of trefoil factor family members in gastric cancer, precancerous lesion and their significance. *Chinese Gen. Pract.*, **24**: 2197-2203.

Hoffmann, W., Jagla, W. and Wiede, A., 2001. Molecular medicine of TFF-peptides: from gut to brain. *Histol. Histopathol.,* **16**: 319-334.

Hoffmann, W., 2005. TFF-triggered signals promoting restitution of mucous epithelia. *Cmls-Cell Mol. Life Sci.*, **62**: 2932-2938. https://doi.org/10.1007/s00018-005-5481-9

Kaeffer B., 2002. Mammalian intestinal epithelial cells in primary culture: A mini-review. *In Vitro Cell Dev. Biol. Anim*., **38**: 123-34.

Kjellev, S., Thim, L., Pyke, C. and Poulsen, S.S., 2007. Cellular localization, binding sites, and pharmacologic effects of TFF3 in experimental colitis in mice. *Digest. Dis. Sci.*, **52**: 1050-1059. https://doi.org/10.1007/s10620-006-9256-4

Kosriwong, K., Menheniott, T.R., Giraud, A.S., Jearanaikoon, P., Sripa, B. and Limpaiboon, T., 2011. Trefoil factors: Tumor progression markers and mitogens via EGFR/MAPK activation in cholangiocarcinoma. *World J. Gastroenterol*., **17**: 1631-1641. https://doi.org/10.3748/wjg.v17.i12.1631

Masuda, K., Ikeda, H., Kasai, K., Fukuzawa, Y., Nishimaki, H., Takeo, T. and Itoh, G., 2003. Diversity of restitution after deoxycholic acid-induced small intestinal mucosal injury in the rat. *Digest. Dis. Sci.*, **48**: 2108-2115. https://doi.org/10.1023/A:1026295014525

Lee, J.C. and Young, P.R., 1996. Role of CSB/p38/RK stress response kinase in LPS and cytokine signaling mechanisms. *J. Leukoc. Biol.*, **59**: 152-157.

Li, J., Xu, L.F., Sun, M., Li, Q., Gao, H. and Jiang, W.G., 2006. Protective effects of recombinant intestinal trefoil factor against intestinal injuries induced by endotoxin in young rats. *Zhongguo Dang Dai Er Ke Za Zhi*, **8**: 425-428.

Lin, J., Nadroo, A.M., Chen, W., Holzman, I.R., Fan Q.X. and Babyatsky, M.W., 2003. Ontogeny and prenatal expression of trefoil factor 3/ITF in the human intestine. *Early Hum Devel.*, **71**: 103-109. https://doi.org/10.1016/S0378-3782(02)00113-5

Iizuka, M. and Konno, S., 2011. Wound healing of intestinal epithelial cells. *World J. Gastroenterol.*, **17**: 2161-2171. https://doi.org/10.3748/wjg.v17.i17.2161

Stockert, J.C., Blázquez-Castro, A., Cañete, M., Horobin, R.W. and Villanueva. A., 2012. MTT assay for cell viability: intracellular localization of the formazan product is in lipid droplets. *Acta Histochem.*, **1148**: 785-796. https://doi.org/10.1016/j.acthis.2012.01.006

Tan, X.D., Liu, Q.P., Hsueh, W., Chen, Y.H., Chang, H. and Gonzalez-Crussi, F., 1999. Intestinal trefoil factor binds to intestinal epithelial cells and induces nitric oxide production: priming and enhancing effects of mucin. *Biochem. J.*, **338**: 45-751. https://doi.org/10.1042/0264-6021:3380745

Taupin, D., Wu, D.C., Jeon, W.K., Devaney, K. and Wang, T.C., 1999. Podolsky D.K. The trefoil gene family are coordinately expressed immediate-early genes: EGF receptor-and MAP kinase-dependent interregulation. *J. clin. Invest.*, **103**: 31-38. https://doi.org/10.1172/JCI3304

Thim, L., 1989. A new family of growth-factor-like peptides. 'Trefoil' disulphide loop structures as a common feature in breast cancer associated peptide (pS2) pancreatic spasmolytic polypeptide (PSP), and frog skin peptides (spasmolysins). *FEBS Lett.*, **250**: 85-90. https://doi.org/10.1016/0014-5793(89)80690-8

Tomita, M., Itoh, H., Ishikawa, N., Higa, A., Ide, H., Murakumo, Y., Maruyama, H., Koga, Y. and Nawa, Y., 1995. Molecular cloning of mouse intestinal trefoil factor and its expression during goblet cell changes. *Biochem. J.*, **311**: 293-297. https://doi.org/10.1042/bj3110293

Xian, C.J., Howarth, G.S., Mardell, C.E., Cool, J.C., Familari, M., Read, L.C. and Giraud, A.S., 1999. Temporal changes in TFF3 expression and jejunal morphology during methotrexate-induced damage and repair. *Am. J. Physiol.*, **277**: 785-795.