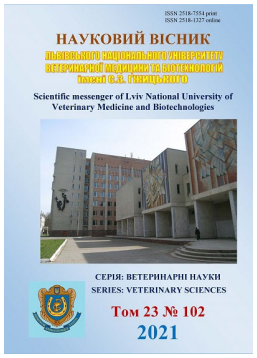

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The function of antimicrobial peptide MPX on the apoptosis and barrier of IPEC-J2 cells

Xueqin Zhao

Faculty of Veterinary Medicine, Sumy National Agrarian University, Sumy, Ukraine
College of Animal Science and Veterinary Medicine, Henan Institute of Science and Technology, Xinxiang, China

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Faculty of Veterinary Medicine, Sumy National Agrarian University, Herasima Kondratieva Str., 160, Sumy, 40021, Ukraine.

College of Animal Science and Veterinary Medicine, Henan Institute of Science and Technology, Xinxiang, 453003, China.
E-mail: 1124227899@qq.com

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The porcine intestinal epithelial cell line IPEC-J2 cells, which were isolated from neonatal piglet mid-jejunum, the main components of the intestinal epithelium and play an important role in the intestine. Escherichia coli is an important cause of diarrhea in human and animal. E.coli infections are the leading cause of travelers' diarrhea and a major cause of diarrhea in developing nations, where it can be life-threatening among children. The aim of this study is to explore MPX against E. coli to inhibit IPEC-J2 cells apoptosis and enhance cell barrier. In this study, scanning electron microscopy results found that E. coli infection caused cell apoptosis and destroyed cell membranes of IPEC-J2 cell. MPX effectively alleviated apoptosis of IPEC-J2 cells. The laser confocal results further found that MPX prevented cell apoptosis by inhibiting caspase-3 and caspase-9 activation. In addition, it was found that MPX regulated the expression of tight junction proteins ZO-1, Occludin, and Claudin-1 in IPEC-J2 cells and is closely related to Rac1 by adding a Rac1 inhibitor. The results of adhesion and invasion experiments further found that MPX inhibited bacterial adhesion and invasion through Rac1. The above results indicated that MPX has better function in inhibiting IPEC-J2 cells apoptosis and enhancing cell barrier.

Key words: *antimicrobial peptide MPX, Escherichia coli, IPEC-J2 cells.*

Introduction

The gut is composed of a single layer of intestinal epithelial cells and plays important roles in the digestion and absorption of nutrients, immune and barrier functions and amino acid metabolism. IPEC-J2 cells have differentiated characteristics and exhibit strong similarities to primary intestinal epithelial cells, which in turn might serve as a good model for humans, demonstrating that IPEC-J2 cells represent a better model of normal intestinal epithelial cells than transformed cell lines (Schierack et al., 2006). Pigs as a good gastrointestinal (GI) model for humans which have the high similarity between pigs and humans, the advantages of using IPEC-J2 cells as *in vitro* model of the GI tract are the high resemblance between humans and pigs (Geens et al., 2011). Previous studies have found that MPX can alleviate the inflammatory response caused by *E. coli* infection. However, the mechanism of MPX on IPEC-J2 cells apoptosis and barrier function is still unknown.

Antimicrobial Peptides (AMPs) are a class of small molecular peptides produced by the body to resist the invasion of pathogenic microorganisms when pathogenic microorganisms invade which have good inhibitory effect and are the first line of defense of the body's innate immune system and are resistant to most drug-resistant bacteria, viruses, fungi, etc (Payganova et al., 2021; Gumienna et al., 2021; Sahoo et al., 2021). Antimicrobial peptides have a wide range of sources, exist in most organisms, and can be isolated from insects, invertebrates, mammals, and even humans. Antimicrobial peptides are small molecule peptides with short amino acid sequence and generally no more than 100 amino acids. They have hydrophilic and most antimicrobial peptides are positively charged and therefore have stronger antibacterial activity (Ajingi et al., 2021). Antimicrobial peptides have small molecular weight, easily soluble in water, high temperature resistance, resistance to protease hydrolysis, broad antibacterial spectrum, and antibacterial mechanism is different from traditional antibiotics. They have good antibacterial activity against super-resistant strains produced by antibiotics and have no drug residues (Yang et al., 2021). There are many types of antimicrobial peptides, and the types have more than 2,000 (Guani-

Guerra et al., 2010). MPX was extracted from wasp venom and has good antibacterial and anti-inflammatory functions. The purpose of this study is to explore the effect of MPX on the apoptosis and barrier function of IPEC-J2 cells induced by *E.coli.*

Materials and methods

Antimicrobial peptide and cell. Antimicrobial peptide MPX (H-INWKGIAAMAKKLL-NH2) was synthesized and purified by Ji er sheng hua (Shanghai, China) at purity greater than 98 % and antimicrobial peptide MPX was very soluble in ddH_2O. The porcine intestinal epithelial cell line IPEC-J2 cells is intestinal columnar epithelial cells, which were isolated from neonatal piglet mid-jejunum, and donated by professor Liancheng Lei of Jilin University.

Scanning electron microscope. Place an autoclaved 6mm round glass slide on the bottom of the 12-well plate. When the IPEC-J2 cells grow into a single layer on the 12-well plate, infect the cells with *E. coli* at MOI=10, and add MPX (10 ug/mL) for 2 h, after aspirating DMEM, add 500 μL 2.5 % glutaraldehyde to each well, fixed overnight at 4 °C. After the 2.5 % glutaraldehyde is aspirated, 30 %, 50 %, 70 %, 80 %, 90 %, 95 %, 100 %, and 100 % alcohol gradient dehydration, with an interval of 15 minutes each time. After the dehydration is completed, the sample is sprayed with gold and observed with a scanning electron microscope SU8010 FASEM (HITACHI, Japan).

Laser confocal detection. After the IPEC-J2 cells grew into a single layer, they are spread on a laser confocal dish with $2x10^5$ cells per well. After overnight culture, the DMEM1640 cell culture medium is changed to DMEM 1640 without dual-antibody serum and the MOI = 10 and MPX action for 2.5 h, preheat PBS and wash 3 times. Add 1 mL 2.5 % glutaraldehyde to each well and fixed overnight at 4 °C. Room temperature for 30 min, discard the fixative solution and washed with PBS at 3 times for 5 min every time, 5 % PBS skim milk, block for 1 h at room temperature, and acted on the primary antibody for 1 h. It can be gently shaken on a shaker and washed 3 times with PBS for 5 min every time. Goat anti-rabbit fluorescent secondary antibody 594 (Zhongshan Janqiao) for 1 h in dark, and shaken gently on a shaker. Washed with PBS at 3 times for 5 min every time in dark. DAPI was stained for 15 min in the dark, washed with PBS at 3 times for 5 min every time, and the results were observed with a laser confocal microscope.

qRT-PCR. After IPEC cells were cultured into a monolayer in a 6-well plate, the group were IPEC+*E.coli*, IPEC+*E.coli*+MPX, IPEC+*E.coli*+MPX+NSC 23766, IPEC+*E.coli*+ NSC 23766. Infection was carried out at MOI=10. IPEC-J2 cells were collected for the detection of tight junction protein ZO-1, Occludin, Claudin-1 mRNA expression after 12 h, and 1 mL RNAio plus was added to each well to extract total cell RNA. 200 μL of chloroform to each well, centrifuged at 12000 rpm, 4 ℃ for 10 min, slowly aspirated the supernatant, added 500 μL of isopropanol to mix centrifuged at 12000 rpm at 4 °C for 10 min. Added 1 mL of 75 % ethanol to each tube centrifuged at 12000 rpm at 4 °C for 5 min. Added 20~30 μL DEPC water. Reversed transcription adopts Takala kit (Cat. No. DRR047A), the reaction conditions are: 95 ℃ 5 min, 95 ℃ 30 s, 55 ℃ 30 s, 72 ℃ 20 s, total 40 cycles. The relative expression levels were determined using the $2^{-\Delta\Delta CT}$ method and overexpression efficiency was also calculated using the $2^{-\Delta\Delta CT}$ method (Guani-Guerra et al., 2010). The primer sequences as shown in Table 1.

Table 1
The primer sequences for qRT-PCR

Genes	Sequence
Occludin	F:5'- GACAGACTACACAACTGGCGG-3' R:5'-TGTACTCCTGCAGGCCACTG-3'
Claudin-1	F: 5'-CCATCGTCAGCACCGCACTG-3' R: 5'-CGACACGCAGGACATCCACAG-3'
ZO-1	F: 5'-ATGAGCAGGTCCCGTCCCAAG-3' R: 5'-GGCGGAGGCAGCGGTTTG-3'
GAPDH	F: 5'-ACTCACTCTTCCACTTTTGATGCT-3' R: 5'-TGTTGCTGTAGCCAAATTCA-3'

IPEC-J2 cells adhesion and invasion. IPEC-J2 cells were cultured in a 6-well plate, $1x10^6$ cells were added to each well, after 24 hours of culture, replaced with fresh DMEM:F 12 blank medium, added 50 uM Rac1 inhibitor NSC 23766 or the same volume of 0.1 % DMSO, and then added 10 ug/mL MPX, or sterile water, put it in the cell incubator and continue culturing for 12 h, washed it with PBS at 3 times , and then added it at MOI = 10. Incubated *E. coli* suspension at 37 °C for 1 h, washed away unadhered and invaded bacteria with PBS; added 200 uL 0.5 % Triton X-100 to each well for 5 min, added 800 uL of pre-cooled PBS, collected the cells and proceed multiply dilution and LB medium coating, inverted culture in a biochemical incubator at 37 °C for 16 h.

Statistical Analysis. GraphPad Prism 5 data processing software to carry out and difference analysis of experimental results (One-Way ANOVA), $P < 0.05$ means significant difference (marked in the text *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Results

MPX reduces IPEC-J2 cells apoptosis. After *E. coli* infected IPEC-J2 cells with MOI = 10, the morphology of the infected cells was observed by scanning electron microscope, as shown in Figure 1. The results showed that the surface of IPEC-J2 cells alone was smooth and round, and the cell morphology was intact. Phenomenon such as swelling and collapse of the cytoskeleton. After *E. coli* infection with IPEC-J2 cells, the cells appear obvious apoptosis, the cells swell, there are apoptotic vesicles around, and the cytoskeleton collapses and collapses. IPEC-J2 cells did not appear to collapse, or apoptotic vesicles after the action of MPX.

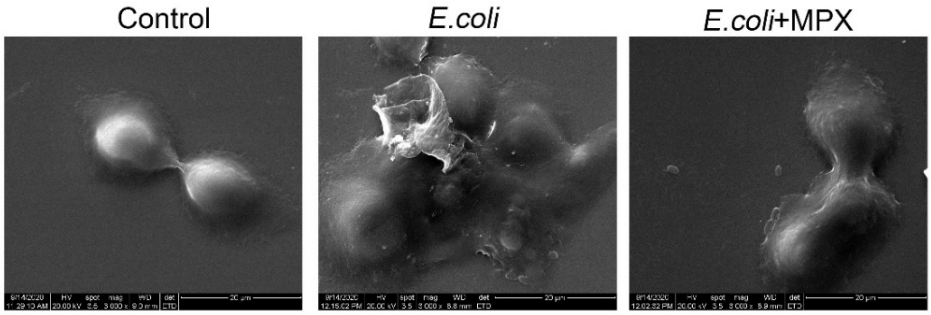

Fig. 1. The results of IPEC-J2 cells infected with *E. coli* observed by scanning electron microscope

MPX inhibits Caspase-3 and Caspase-9 activation. Scanning electron microscopy results showed that MPX could alleviate the apoptosis of IPEC-J2 cells caused by *E.coli.* The effect of MPX on the activation of Caspase-3 and Caspase-9 was further detected by laser confocal microscopy. As shown in Figure 2, the results showed that IPEC-J2 cells activated Caspase-3 and Caspase-9 after *E. coli* infection, while MPX significantly decrease the activation of Caspase-3 and Caspase-9, thereby inhibiting the apoptosis of IPEC-J2 cells.

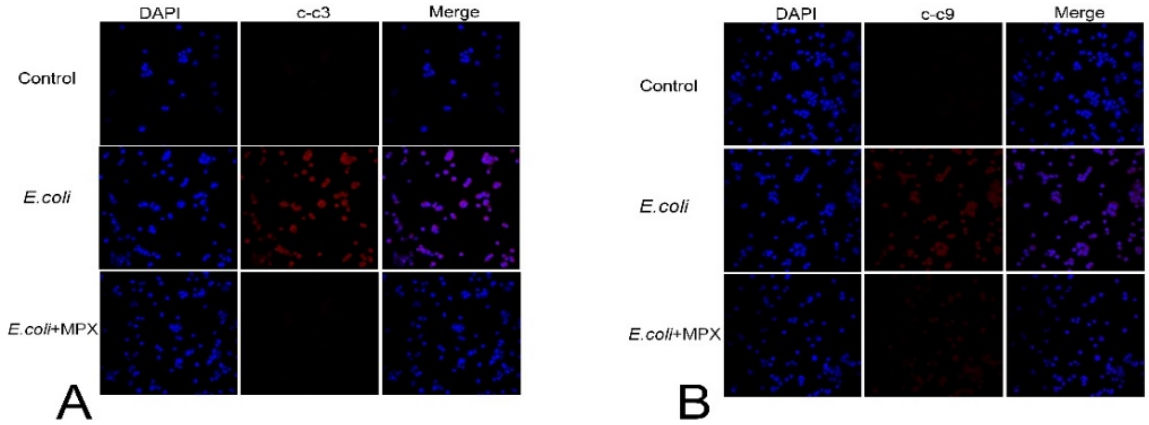

Fig. 2: The results of IPEC-J2 cells infected with *E.coli* observated by Laser confocal. A: The activation result of Caspase-3 of IPEC-J2 cells after *E.coli* infection; B: The activation result of Caspase-9 of IPEC-J2 cells after *E. coli* infection

MPX regulates tight junction protein expression by Rac1. Rac 1 inhibitor NSC 23766 was used to study the regulation mechanism of MPX on tight junction proteins (Fig. 3 A, B, C). The results showed that compared with IPEC-J2+*E.coli* group, MPX pretreatment could increase the mRNA expression of ZO-1 and Occludin in IPEC-J2 cells, While the mRNA expression of Claudin-1 was not significant. However, the effect of MPX on ZO-1 and Occludin was inhibited after adding inhibitor NSC 23766. The above results suggest that MPX may regulate the tight junction protein of intestinal epithelial cells and enhance the barrier function through the Racl pathway.

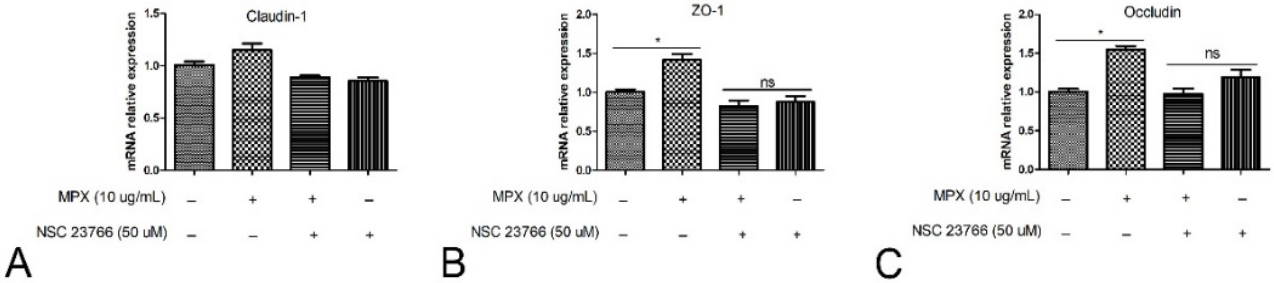

Fig. 3. The mRNA expression of tight junction protein after *E. coli* infection. A: The mRNA expression of Claudin-1 after *E. coli* infection; B: The mRNA expression of ZO-1 after *E. coli* infection; C: The mRNA expression of Occludin after *E. coli* infection

MPX inhibits *E. coli* adhesion and invasion. Then, we tested the effect of MPX on the adhesion and invasion of IPEC-J2 cells by adding Rac1 inhibitor. As shown in Figure 4, the results found that *E. coli* adhered and invaded more in the IPEC-J2 + *E. coli* group. While pretreatment with MPX could significantly alleviated the

adhesion and invasion of *E. coli* in IPEC-J2 cells. In the presence of NSC 23766, MPX failed to reduce *E. coli* adhesion and invasion in IPEC-J2 cells. The above results indicate that Rac1 is related to the cell barrier of IPEC-J2 cells, and MPX enhances the cell barrier function of IPEC-J2 cells by regulating Rac1, thereby reducing *E. coli* adhesion and invasion in IPEC-J2 cells.

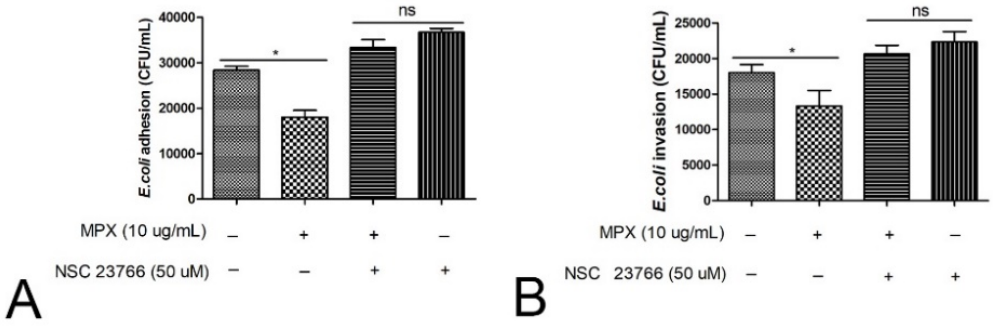

Fig. 4. The results of *E. coli* adhesion and invasion in IPEC-J2 cells. A: The results of *E.coli* adhesion in IPEC-J2 cells; B: The results of *E.coli* invasion in IPEC-J2 cells

Discussion

The intestinal barrier is composed of four parts: intestinal epithelial barrier, immune barrier, chemical barrier and biological barrier. Intestinal epithelial barrier is the first barrier for animals to resist the invasion of pathogens. It is particularly important in protecting the body from pathogenic bacteria and other microorganisms. The tight junction structure is the most critical part of the intestinal epithelial barrier (Xie et al., 2021). ZO-1, Occludin and Claudin-1 are important proteins in the tight junction structure (Karada et al., 2017). Yi et al found that antimicrobial peptide CWA increased the expression of tight junction proteins ZO-1 and Occludin in the intestinal tract of weaned piglets with diarrhea, and enhanced the intestinal barrier function (Yi et al., 2016). LL-37 could also increase the expression of tight junction proteins Claudins and Occludin, increase cell transmembrane resistance (TER), and enhance cell barrier function (Akiyama et al., 2014). Han et al. found that defensin pBD2 could increase the gene expression of ZO-1 and Occludin in mouse colon, and relieve the colon inflammation induced by DSS in mice (Han et al., 2015). Lin et al. found that *E.coli* K88 lead to a decrease in the expression of tight junction protein ZO-1 in IPEC-J2 cells, and the antimicrobial peptide orcine NK-Lysine (PNKL) could significantly increase the expression of tight junction protein ZO-1 (Lin et al., 2021). The above results suggest that MPX can increase the expression of intestinal tight junction proteins and enhance the intestinal epithelial barrier.

The regulatory mechanism of tight junction proteins is very complicated, and the signal pathways involved include: Rho, MAPK, PI3K/Akt, protein kinase C (PKC), MLCK and other pathways (Khan et al., 2021). In recent years, studies have found that Rho signaling pathway may play an important role in the regulation of tight junction proteins by antimicrobial peptides (Hemshekhar et al., 2018). At present, more than 20 Rho family members have been discovered and mainly included RhoA. Rac1, Cdc42. In addition, many studies have shown that activation of Rac 1 enhanced cell barrier function (Yi et al., 2017). Studies have shown that the antimicrobial peptide hBD3 improves the barrier function of tight junctions of epithelial cells by increasing the activation level of Rac I (Kiatsurayanon et al., 2014). This study found that the effect of MPX on tight junction proteins ZO-1 and Occludin was inhibited after adding inhibitor NSC 23766, and inhibited the function of MPX against *E. coli* adhesion and invasion in IPEC-J2 cells, suggesting that MPX may regulate IPEC-J2 cells tight junction proteins through the Racl pathway, and enhance the intestinal epithelial barrier function.

In conclusion, intestinal epithelial barrier as the first line of defense for animals against the invasion of pathogens plays an important role in maintaining the health of the body. Thereby, how to protect the intestinal epithelial barrier function and improve the self-repair ability of damaged intestinal epithelial tissue is particularly important. In this study, the results found that MPX reducing the apoptosis of IPEC-J2 cells caused by *E. coli* infection by inhibiting the activation of Caspase-3 and Caspase-9. Further studies found that MPX regulates tight junction protein gene expression through the Rac1 pathway by adding Rac1 inhibitors, and adhesion and invasion studies further prove that MPX enhances the barrier function of IPEC-J2 cells by Rac1. This study is the first to evaluate the effect of MPX on the apoptosis and barrier function of IPEC-J2 cells, laying a foundation for the development and research of new antimicrobials.

Author's contributions

Xueqin Zhao participated in the study design, carried out data analyses, participated and performed measurements, laboratory testing's and wrote this manuscript.

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Conflict of interest

Author does not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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