Quercetin Protects RIMVECs From Inflammatory Damage, Pyroptosis and Cell Migration by Inhibiting the TLR4/NF- κ B/NLRP3 Pathway

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Research

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Abstract

Background: Intestinal mucosal microvascular endothelial cells (MEC) have multiple functions and play an important role in intestinal bowel diseases (IBD). Quercetin is a flavonoid found in many plants and fruits. It was reported that quercetin can treat several gastrointestinal cancers, but its effect on bacterial enteritis and pyroptosis-related diseases has been rarely studied. This article aims to explore the effect and mechanism of quercetin on inflammatory injury and pyroptosis of RIMVECs.

Methods: The inflammatory damage and pyroptosis in RIMVECs were induced by LPS and ATP. Real-time quantitative polymerase chain reaction (RT-qPCR), western blot analysis, enzyme-linked immunosorbent assay (ELISA) and immunofluorescence methods were used to detect TLR4/NF- κ B/NLRP3 pathways, inflammatory factors (IL-1 β and IL-18) and pyroptosis-related proteins (Caspase-1 and GSDMD). The expression and distribution of ZO-1 were detected by western blot analysis and immunofluorescence method. The late apoptosis and necrosis of cells were measured by cell flow cytometry.

Results: The results showed that different concentrations (5, 10, 20 μ M) of quercetin not only significantly reduced the protein and mRNA levels of TLR4, NLRP3, Caspase-1 and GSDMD, but also down-regulated the protein expression, mRNA and secretion of IL-1 β and IL-18. Quercetin also inhibited the phosphorylation of NF- κ B p65 and the degradation of I κ B. At the same time, quercetin increased the cell migration rate and the expression level of ZO-1, and reduced the number of late apoptotic cells ($P < 0.05$).

Conclusions: Our data indicated that Quercetin reduced the inflammatory response and pyroptosis induced by LPS/ATP through the TLR4/NF- κ B/NLRP3 pathway, and protected the migration and tight junctions of RIMVECs.

1 Introduction

Intestinal mucosal capillaries are an important part of the intestinal tract, which can regulate the circulation of metabolic wastes in blood by nourishing the intestinal mucosa, and participate in intestinal barrier and secreting of cytokine^[1]. When the microvessels of the intestinal mucosa are damaged, it will increase vascular permeability and aggravate early endothelial damage^[2]. Relevant studies have shown that in some colitis, microvascular disease precedes epithelial disorders^[3]. Bacteria is one of the main reasons of infectious enteritis in newborn and juvenile animals^[4]. The common presenting sign in animals with infectious enteritis are diarrhea, and in the severe cause are endotoxemia or sepsis with death. Exogenous bacteria will recruit a large number of inflammatory cells and mediators after entering the intestinal blood circulatory system, resulting in a series of inflammatory reactions and tissue damage^[5].

Pyroptosis is a kind of programmed cell death between apoptosis and necrosis, which has been strongly linked to the development of bacterial enteritis^[6]. The expression of NOD-like receptor 3 (NLRP3) inflammasome is a typical feature of infectious diseases and the critical protein of pyroptosis^[7]. The

activation of NLRP3 inflammasomes requires two signals. Pathogen signals such as Gram-negative lipopolysaccharide (LPS) are recognized first by Toll-like receptor 4 (TLR4), activating the synthetic nuclear factor κ B (NF- κ B) and its downstream NLRP3, also cause inflammation and production of pro-forms of IL-1 β (interleukin-1) and IL-18(interleukin-18) [8–9]. The second signals are adenosine triphosphatase (ATP) [10–11], changes of ion concentration or pH value and other factors which activate Caspase-1 (cysteine aspartate proteolytic enzyme-1) to cut IL-1 β , IL-18 and GSDMD (succinic acid to make gasdermin D). In this way, more inflammatory factors are recruited to expand the inflammatory response, which can cause pyroptosis of microvascular endothelial cell [12].

Zonula Occluden 1 (ZO-1) is an important protein that constitutes tight junctions (TJ). Down-regulation or decreased activity of ZO-1 will affect the formation of TJ between cells [13], thereby affecting the normal function of the intestinal barrier. It also increases the risk of bacterial enteritis caused by harmful bacteria penetrating the intestinal mucosa and entering the bloodstream [14]. Bacterial toxins, inflammatory factors, tumor necrosis factor, etc. can cause direct damage to TJ and affect the protein activity and distribution of ZO-1 [15–16].

Based on the existing bacterial resistance problem, it is of great significance to study the application and mechanism of traditional Chinese medicine components in the treatment of bacterial enteritis. Quercetin is not only an effective ingredient of Chinese medicinal materials such as Bupleurum, mulberry leaves, Sophora japonicus and Inula, but also exists in the flowers, leaves and fruits of many plants in nature. Quercetin can impact the proliferation, metastasis, apoptosis, and metabolism of tumor cells through multiple signal pathways, and has inhibitory effects on colorectal cancer, gastric cancer, breast cancer, and leukemia [17–21]. Relevant studies have proved that quercetin can promote and protect the TJ barrier function of the intestine [22], and it can degrade some intestinal bacteria which increases in a time-dependent manner [23]. Regarding the regulation of NLRP3 inflammasome, quercetin can protect human retinal microvascular endothelial cells (HRMECs) from injury induced by high glucose and inhibit the expression of NLRP3, Caspase-1, IL-1 β , and IL-18 [24]. In addition, quercetin can also inhibit the protein and gene expression of NLRP3, Caspase-1, iNOS and inflammatory factors in mononuclear macrophages (RAW264.7) activated by LPS, and improve antioxidant and anti-inflammatory abilities [25]. Therefore, it can infer that quercetin can protect the integrity of the TJ barrier and has a regulatory effect on the NLRP3 inflammasome.

In this study, we established the model of inflammatory injury and pyroptosis on RIMVECs induced by LPS/ATP, and explored that quercetin regulates the inflammatory injury and pyroptosis of RIMVECs through the NLRP3 signaling pathway.

2 Material

2.1 Main drugs and reagents

Quercetin (Batch No. Z100081) was purchased from the National Institute for Food and Drug Control. The Dulbecco's Modified Eagle Medium (DEEM), fetal bovine serum (FBS) and trypsin were purchased from Biological Industries. LPS and ATP were obtained from Sigma.

2.2 Main instruments

High-speed centrifuge (Scilogex), real-time fluorescence quantitative RCR instrument (Beijing Institute of Animal Husbandry and Veterinary Medicine), microplate reader (Thermo Scientific), protein electrophoresis instrument and electrophoresis tank (BIO-RAD).

3 Method

3.1 RIMVECs cell culture

RIMVECs were obtained with isolating primary cells from rat intestinal mucosal tissue following by performing stable passages. Cells were cultured in the complete medium for normal culture of RIMVECs: 1% antibody was added to high-sugar DMEM containing 10% fetal bovine serum (FBS) (both from Gibco, Grand Island, NY, USA), and 1% penicillin–streptomycin mixed solution (Solarbio, Beijing, China) at 37°C in a humidified incubator with 5% CO₂ [26]. RIMVECs was frozen by the cryopreservation solution: Mix DMEM, FBS and DMSO in a ratio of 5:4:1 and mix well with the cells. The medium was refreshed every 2 days, and cells were passaged using 0.05% trypsin (Gibco).

3.2 Screening of the concentration of quercetin

RIMVECs were seeded on a 96-well cell culture plate (1×10^4 /well). When the cells grown to 80% and as monolayers, the original medium was aspirated and discard. Then different concentrations of quercetin were severally added and treated for 3, 6, 9, 12, 24 and 48 hours. After discarded the liquid, CCK-8 kit (Beyotime Biotechnology, China) which was applied by DMEM medium (incubate at 37°C for 40 min in the dark) was added, and measured the absorbance at 450nm with a microplate reader.

3.3 Test grouping

This experiment was divided into control group (complete medium), model group (10μg/mL LPS + 1mM ATP), LPS alone group (10μg/mL LPS), ATP alone group (1mM ATP) and drug treatment group (10μg/mL LPS + 1mM ATP and 5, 10, 20μM quercetin). Cells in each group were stimulated for 24 hours.

3.4 Western blot analysis,

RIMVECs were grouped and processed according to the above experiment. First, the supernatant was discard (washed three times with cold PBS), then added RAIP cell lysate for ten minutes, and collected the cells with a cell scraper to extract total protein. The protein concentration was determined with BCA protein detection kit (Beyotime Biotechnology, China), separated by SDS-PAGE, and transferred to PVDF (Bedford, MA, USA) membrane. Then the cell membrane was sealed with 5% bovine serum albumin (BSA) indoors for 1 hour at room temperature to prevent non-specific binding of the antibodies and incubated

overnight at 4°C with specific primary antibodies of TLR4 (1:800), NLRP3(1:1000), GSDMD(1:1000), Caspase-1(1:1000), NF-κB p65 (1:1000), p-NF-κB p65(1:400), p-IκB(1:1000), I-κB(1:1000), IL-18(1:1000), IL-1β(1:1000), β-tubulin (1:1000) and ZO-1(1:1000), followed by incubation with HRP-conjugated secondary antibodies(1:5000)(ABclonal Technology)for 1 hour at room temperature. Ultimately, the blots were performed a chemiluminescence test by using a Tanon 5200 chemiluminescence imaging system (Shanghai, China). The image was analyzed by Image J software (National Institutes of Mental Health, Bethesda, MD, USA).

3.5 Enzyme-linked immunosorbent assay to measure the secretion of IL-18 and IL-1β

RIMVECs were spread in a 12-well Transwell chambers (1.13 cm²; 0.4μm pore size, Corning Costar Crop, Cambridge, MA) at 1×10⁵/well and grown as monolayers, and washed with PBS when the cell density reached 90%. Different groups were stimulated for 24 hours, then the supernatant of cell was collected, and the secretion of IL-1β and IL-18 were measured according to the instructions of the ELISA kit (enzyme, China).

3.6 Determination of mRNA levels of TLR4, NLRP3, Caspase-1, GSDMD and inflammatory factors

RIMVECs were plated on a 6-well cell culture plate at 1×10⁵/well. Total RNA and reverse transcription were purchased from Aidlab (Beijing, China). GAPDH primers were obtained from Sangon (Shanghai, China). Reverse transcription was performed using the Prime Script RT reagent kit (TAKARA). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using CFX9600 apparatus (Bio-Rad, Hercules, CA, USA) and the RT quantitative reaction was performed as follows: 94°C pre-denaturation for 30s, 94°C denaturation for 5s, 60°C annealing for 15s, and repeat 40 reaction cycles) to expand and calculate the relative mRNA expression level. Rat monoclonal antibodies for TLR4, NLRP3, Caspase-1, IL-1β, and IL-18 were all obtained from Abcam (Shanghai, China). The reaction system was 20μL, including RCR Forward Primer (10μmol/L, 0.4μL), PCR Reverse Primer (10μmol/L, 0.4μL), cDNA 0.8μL, ddH₂O (8.4μL) and qPCR Supermix (10μL).

The gene sequences of rat-derived TLR4, GSDMD, Caspase-1, IL-1β and IL-18 were checked from GenBank. Primer Premier 5.0 design software was used to design the primers, which were synthesized by Sangon. Target gene expression was calculated with normalization to GAPDH values using the 2^{-ΔΔCt} method and the specific primer sequences of the target gene are shown in the Table 1.

Table 1
Primer sequence

Target gene	Primer sequence (5' to 3')	Number of bases
IL-18-1F	ACCGCAGTAATACGGAGCAT	20
IL-18-1R	TCTGGGATTCGTTGGCTGTT	20
Caspase-1-1F	TGGAGCTTCAGTCAGGTCCAT	21
Caspase-1-1R	ATGCGCCACCTTCTTTG TTC	20
TLR4-1F	ATGCCTCTCTTGCATCTGGC	20
TLR4-1R	TAGGAAGTACCTCTATGCAGGGAT	24
IL1-6F	CAGCTTTCGACAGTGAGGAGAA	22
IL1-6R	TCTTGTCGAGATGCTGCTGT	20
GSDMD-2F	AGATCGTGGATCATGCCGTC	20
GSDMD-2R	AGGGCTTGAAGCTGGTAGAAT	21

3.7 Confocal fluorescence microscope test

Cell slides were placed in a 24-well plate, and then spread the trypsinized RIMVECs cells on the plate. According to the experimental design, the culture medium of the treatment group was added for 24 hours and then the cell slide was taken out. After washing with pre-warmed PBS for three times, and then added 500μl paraformaldehyde (PFA) to each well for fixation for 40 minutes, followed by Permeabilized with 0.2%-0.5% Triton X-100 for 20 minutes, and washed three times with PBS before and after each step. The slides were incubated with the primary antibody was 4°C overnight, and the secondary antibody (diluted with 5% BSA) at room temperature for 1 hour, protected from light. After washing, 1ug/mL DAPI was added to each well, and fixed the film in the dark at room temperature for 20 minutes, and then washed with PBS. Followed by washed, the slides were dried on clean dust-free paper, and taken out them. The slides were sealed by nail polishes, and observed with a confocal fluorescence microscope.

3.8 Cell flow cytometry

Adherent cells were digested by the trypsin, which combined with the supernatant (centrifuge at 300g for 5 min in a centrifuge). The cells were mixed with 0.1% BSA into a 1×10^7 /mL cell suspension. The Fc receptor blockers were used to reduce non-specific staining during staining. Then the fluorescent-labeled antibody was incubated according to the recommended amount in the instructions. After mixing, cells were incubated for 30 min at 4°C in the dark, and the cell staining buffer was added to resuspend the cells to analysis by using the flow cytometry.

3.9 Cell scratch test

First, we drew several horizontal lines by a marker pen on the back of the 6-well plate (draw a line about every 0.5-1cm across the holes, and at least 5 lines for each hole). RIMVECs cells with a cell concentration of 5×10^5 cell/mL were added to each well, and the healing rate reached 100% after overnight. The next day, we used the pipette tip to scratch the vertical cell plane according to the marking line. After washed three times in PBS, the cells were cultivated in the fresh serum-free medium (cells were put in an incubator, 37°C, 5% concentration of CO₂). The cells were taken out after 0, 2, 4, 6, 12 and 24 hours respectively, and observed under a microscope and photographed with TS-View. Image J software was used to process the pictures (draw 6–8 horizontal lines randomly between the scratches to calculate the average distance between cells).

3.10 Data processing

The test results were analyzed and processed with SPSS 19.0 software, and the test data were expressed as mean $\pm$ standard error (mean $\pm$ SEM). The difference between the test groups was detected by One-way ANOVA. It was generally considered that $P < 0.05$ is considered to be a difference and statistically significant.

4 Results

4.1 The effect of quercetin on the survival rate of RIMVECs

The effect of quercetin on the survival rate of RIMVECs cells was first detected by the CCK-8 method. The results showed that quercetin had no effect on the survival rate of RIMVECs after 3, 6, 9, 12 hours. The effect of quercetin on RIMVECs for 24 hours on cell survival was shown in Fig. 1 below. Compared with the control group, the 320 μ M quercetin group had a significantly lower cell survival rate ($P < 0.01$). However, 80, 40, 20, 10, 5, 2.5 and 1.25 μ M quercetin had no significant effect on the survival rate of RIMVECs ($P < 0.01$) Fig. 1 (A, B). Therefore, referring to relevant literature and test results, 40, 20, 10 and 5 μ M quercetin were chosen for the next step.

4.2 Quercetin protects RIMVECs and reduces the effect of LPS and ATP on cell survival

Initially, to define whether quercetin has a protective role of survival rate on RIMVECs induced by LPS/ATP. The cell viability of RIMVECs was significantly decreased after being co-stimulated with 10 μ g/mL LPS and 1mM ATP for 24 hours. 5 μ M and 10 μ M quercetin significantly improved the survival rate of RIMVECs cells ($P < 0.05$) Fig. 1 (C).

4.3 The effect of quercetin on the mRNA levels of NLRP3 signaling pathway related proteins and inflammatory factors

4.3.1 The effect of quercetin on the mRNA levels of TLR4, Caspase-1 and GSDMD

Compared with the control group, the mRNA levels of TLR4 and GSDMD in the LPS/ATP group increased significantly ($P < 0.01$). 10 μ M quercetin significantly reduced mRNA levels of TLR4 and GSDMD in LPS/ATP group ($P < 0.01$), while 5 and 20 μ M quercetin had no obvious effect on mRNA levels of TLR4 and GSDMD Fig. 2 (A, B). 5 and 10 μ M quercetin prominently suppressed the level of Caspase-1 mRNA in RIMVECs stimulated by LPS/ATP ($P < 0.01$), while 20 μ M quercetin had no obvious effect on the mRNA level of Caspase-1 Fig. 2 (C).

4.3.2 Determination of quercetin on the mRNA levels of IL-1 β and IL-18

After LPS and ATP co-induced, IL-1 β and IL-18 mRNA increased distinctly ($P < 0.01$). Compared with the control group, 5, 10, 20 μ M quercetin visibly restrained the mRNA level of IL-1 β in RIMVECs ($P < 0.01$) Fig. 2 (D). 10 and 20 μ M quercetin efficiently decreased the mRNA level of IL-18 in RIMVECs ($P < 0.01$), while 5 μ M quercetin was far less effective to the mRNA level of IL-18 Fig. 2 (E).

4.4 The effect of quercetin on the expression levels of TLR4 and downstream pyroptosis-related proteins

The co-stimulation of LPS and ATP to RIMVECs aggrandized the protein expression levels of TLR4, NLRP3, pro-caspase-1, caspase-1 and GSDMD ($P < 0.01$) Fig. 3. Compared with the LPS/ATP group, 5, 10 and 20 μ M quercetin clearly reduced the protein expression levels of TLR4, NLRP3, pro-caspase-1, caspase-1 and GSDMD ($P < 0.01$).

The distribution and expression of NLRP3, GSDMD and Caspase-1 were detected by the immunofluorescence. 5, 10 and 20 μ M quercetin had taken a drop in the expression of NLRP3, GSDMD and Caspase-1. Among them, 5 and 10 μ M quercetin had an obvious down-regulation on NLRP3 and Caspase-1, while 20 μ M had a better effect on GSDMD Fig. 4.

4.5 The effect of quercetin on the expression level of NF- κ B pathway related proteins

The NF- κ B pathway is an important inflammatory signaling pathway that activates the NLRP3 inflammasome Fig. 5. The expressions of p-NF- κ B p65 and NF- κ B p65 in the LPS/ATP group were significantly increased, while the expression of I- κ B decreased ($P < 0.01$). On the contrary, the three concentrations of quercetin all reduced the expression of p-NF- κ B p65 and NF- κ B p65, and 20 μ M could increase the expression of I- κ B ($P < 0.01$) Fig. 5 (A, B, C).

4.6 The effect of quercetin on the protein expression and secretion of IL-1 β and IL-18

It can be seen from Fig. 5 (D, E, F, I, J) that the co-stimulation of LPS and ATP up-regulated the protein expression and secretion levels of IL-1 β and IL-18 in RIMVECs. Therefore, the total expression of IL-1 β and IL-18 was consistent with above ($P < 0.01$). Among them, the protein expression levels of pro-IL-18/IL-18 and pro-IL-1 β /IL-1 β in the LPS alone group were increased ($P < 0.05$). But ATP alone had a slight increase on the protein expression levels of pro-IL-18/IL-18 and pro-IL-1 β /IL-1 β . 5, 10 and 20 μ M quercetin sharply inhibited the protein expression levels of pro-IL-18/IL-18 and pro-IL-1 β /IL-1 β in RIMVECs cells ($P < 0.01$). 5 and 10 μ M quercetin exerted best effect on the IL-18 secretion of RIMVECs cells ($P < 0.05$) Fig. 5 (G, H).

4.7 The effect of quercetin on the migration of RIMVECs induced by LPS/ATP

As shown in Fig. 6 (A), the co-stimulation of LPS and ATP and the treatment of LPS alone for 24 hours both successfully inhibited the migration of RIMVECs ($P < 0.01$). 5, 10, 20 μ M quercetin observably promoted the migration of RIMVECs induced by LPS/ATP ($P < 0.01$). After 5, 10 and 20 μ M quercetin were added, the cell migration rates were 67%, 57%, and 59% respectively. 5 μ M quercetin most notably boosted the improvement of cell migration Fig. 6 (B).

4.8 Protein expression and distribution of ZO-1

ZO-1 is one of the important components of TJ. It not only participates in the transport of cell material, but also in the regulation of cell proliferation and differentiation, cell migration, gene transcription and other processes. The protein levels of ZO-1 in the LPS/ATP group were markedly reduced. 5 and 20 μ M quercetin significantly up-regulated the protein expression levels of ZO-1 ($P < 0.01$), while 10 μ M quercetin had an upward trend but no Significant Fig. 7 (B, C). The results of fluorescence immunoassay for ZO-1 showed that ZO-1 in normal cells was evenly distributed on the cell surface to form a compact structure, while ZO-1 in the LPS/ATP group was diffuse with its expression declined. 5, 10 and 20 μ M Quercetin up-regulated the expression of ZO-1 and improved its distribution on the cell surface. Among them, 5 and 20 μ M quercetin had a better effect, which was consistent with the results of protein level determination Fig. 7 (A).

4.9 Cell flow test results

In view of the current flow cytometry related methods for measuring scorch is not perfect. The degree of apoptosis and necrosis in cells were tested by the flow cytometry. The results were combined with key proteins above in the pyroptosis pathway to determine whether pyroptosis occurred. The proportion of late apoptotic cells and necrotic cells in the LPS/ATP group was much less, and the total apoptotic ratio had a sharp rise ($P < 0.01$). 5, 10 and 20 μ M quercetin all restrained the proportion of late apoptosis and necrosis in cells Fig. 8.

5 Discussion

Quercetin is one of the effective ingredients of many traditional Chinese medicines such as rutin and quercetin. In the current study, we confirmed that quercetin had a protective effect on the damage of intestinal microvascular endothelial cells, and played an anti-pyroptosis effect through the signal pathway mediated by the NLRP3 inflammasome. Based on previous studies, quercetin could protect RIMVECs from PGN-induced cell inflammatory damage. Quercetin inhibited the mRNA and protein levels of TLR2 and TLR4, and down-regulated the protein expression levels of MyD88 to reduce the release of TNF- α [27]. Pyroptosis is a kind of programmed cell death that is different from apoptosis and necrosis. Experiments have shown that quercetin could effectively protect the inflammatory damage of RIMVECs induced by LPS [28], but the effect of LPS-induced pyroptosis of RIMVECs is not ideal. The main reason is that LPS needs further stimulation of ATP after activating cells to induce inflammasome activation and pyroptosis.

Therefore, this experiment used LPS and ATP to induce RIMVECs to establish a pyroptosis model. In this way, the protective and regulatory effects of quercetin on the disease process of bacterial enteritis can be evaluated from the aspect of endothelial cell with pyroptosis. LPS is a component in the outer wall of gram-negative bacteria. It can stimulate inflammation-related cytokines by binding to the TLR4 protein on the cell membrane surface, and further induce pyroptosis under the stimulation of ATP and TNF- α [29]. Our results showed that the combined action of LPS and ATP for 24 hours posed a threat to the survival of RIMVECs. In addition, western blot analysis was used to determine important indicators related to pyroptosis. It was found that compared with the normal cell group, the protein expression levels of TLR4, NLRP3, Caspase-1, and GSDMD were markedly increased, and the protein expression of IL-18, IL-1 β also like this. These indicated that LPS and ATP work together successfully established a pyroptosis model of RIMVECs. On the contrary, quercetin (20, 10 and 5 μ M) improved the survival rate of cells, and attenuated the increase in the levels of pyroptosis-related indicators and inflammatory factor proteins, which were also consistent with the results of fluorescence immunoassay. Quercetin also effectively reduced the secretion of IL-1 β and IL-18 which were detected by the ELISA method. These proved that quercetin played an important role in protecting RIMVECs from reducing inflammatory and pyroptosis damage. The mRNA levels of key pyroptosis proteins and inflammatory factors were evaluated by RT-PCR technology. Quercetin reduced the mRNA expression of key pyroptosis proteins and inflammatory factors, and 5, 10 μ M quercetin down-regulated key pyroptosis proteins significantly. However, 20 μ M quercetin had failed to inhibit these indicators. Therefore, we considered that there may be a certain difference between protein expression and mRNA expression, but its specific regulatory mechanism needs further study.

Relevant studies have confirmed that NF- κ B can activate NLRP3 and up-regulate the expression of NLRP3 by specifically binding to nt-1303 to -1292 and nt-1238 to -1228 in the promoter region of NLRP3 [30]. Its inhibitory protein I- κ B is also reduced [31]. The results of Western blot had shown that the combined action of LPS and ATP promoted the activation of NF- κ B p65 and reduced the expression of I- κ B. On the contrary, quercetin inhibited the increase of NF- κ B p65 in protein levels and increased the protein expression levels of I- κ B induced by LPS/ATP. Combined with above results, quercetin could

restrain the activation of NLRP3 by inhibiting the TLR4/NF- κ B/NLRP3 pathway, and defended the damage of LPS/ATP on RIMVECs.

In addition, the activation of NF- κ B will also cause the transcription of TJ to be inhibited, which affects the normal structure, morphology, growth and migration of cells^[32–34]. Through the cell migration test, it could be seen that LPS/ATP significantly inhibited the growth and migration of cells, and physical scratches were difficult to heal within 24 hours. Quercetin effectively promoted the growth and migration of cells. 5 μ M quercetin made the cell migration rate reach 67%. TJ is closely related to cell growth and migration. If it is inhibited or missing, it will cause tremendous damage to cell scratch healing, in addition delay migration and decrease value increase^[35–37]. ZO-1 is one of the important members of TJ, and it has been proven to be widely used to judge the level of intestinal mucosal barrier damage^[38–39]. Our test also detected the expression and distribution of ZO-1. The results of fluorescence immunoassay showed that the spread of ZO-1 in LPS/ATP group was destroyed, and the structure of ZO-1 was damaged which was originally distributed around the cell membrane and nucleus, while quercetin could up-regulate the protein expression of ZO-1 and restore the distribution of ZO-1. Overall, quercetin had a protective effect on the TJ between RIMVECs. Finally, late apoptosis, necrosis and total apoptosis rate were examined by the cell flow cytometry. The results shown that quercetin significantly reduced the number of late apoptosis and necrosis cells. Relevant studies had confirmed that the membrane permeability of late apoptosis and necrotic cells will increase, and intestinal mucosal damage, apoptosis and necrosis in intestinal diseases are also related to the massive infiltration of inflammatory cells and release of inflammatory factors, which may aggravate reactions such as cell pyroptosis^[40].

In the organism's intestinal inflammatory response, MEC acts as an intestinal barrier, which can absorb inflammatory factors in tissues and inflammatory lesions and remove bacterial pathogenic products. When LPS enters the intestinal mucosal microvascular system, TLR4 on the surface of endothelial cells first recognizes and binds to pathogens, and then activates NLRP3 Inflammasome through the TLR4/NF- κ B signaling pathway. After that, it will activate Caspase-1 to cut GSDMD, causing a series of pyroptosis phenomena such as cell rupture and cell membrane pores^[41–43], and releasing inflammatory factors such as IL-1 β and IL-18 to expand the inflammatory response. At the same time, activation of NF- κ B also inhibits the expression of ZO-1, affecting the TJ, and the growth and migration of cells. In summary, this study proved that quercetin can protect RIMMVECs from pyroptosis caused by LPS/ATP, and enhance the ability of the TJ between cells, and cell growth and migration. However, its effects on non-classical pathways of pyroptosis and other aspects still need to be further studied.

6 Conclusions

We have proved through research that quercetin can down-regulate the inflammatory response and pyroptosis in RIMVEC caused by LPS/ATP through the TLR4/NF- κ B/NLRP3 signaling pathway. Quercetin also significantly inhibited the destruction of TJ between cells and ZO-1 protein by LPS/ATP. In addition, quercetin also contributes to promote the ability of cell migration and reduce cells in a state of late

apoptosis and necrosis. The above conclusions jointly support that quercetin can be used as a drug for the treatment of bacterial enteritis and pyroptosis-related diseases.

Abbreviations

RIMVECs=Rat intestinal mucosal endothelial cells

BCA=Bicinchoninic acid

SDS-PAGE=Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis

PVDF=Poly(1,1-difluoroethylene)

I- κ B=Inhibitor of NF- κ B

GAPDH=Glyceraldehyde-3-phosphate dehydrogenase

DAPI=4',6-diamidino-2-phenylindole

CCK-8=Cell Counting Kit-8

PGN=Peptidoglycan

TNF- α =Tumor necrosis factor- α

Declarations

Ethics approval and consent to participate

Not applicable.No animal and human test, only cell test is carried out in this project.

Consent to publish

Not applicable.

Availability of data and materials

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

There is no conflict of interest in this study.

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Authors' Contributions

Huixin Zhang: methodology, experimentation, data analysis, article writing; Yeye Li: conceptualization, article revision & comment; Zhongjie Liu: review, supervision, editing, and fundraising.

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Figures

Figure 1

(A) Chemical structure of quercetin. Chemical formula, $\text{C}_{15}\text{H}_{10}\text{O}_7$; molecular weight = 302.236 CAS number: 117-39-5. (B) Effect of quercetin on the survival rate of RIMVECs ($n = 6$). The results represent the means $\pm$ SEM of three independent experiments. *** $P < 0.01$ were compared with the control group. (C) Effect of quercetin on the cell survival rate of RIMVECs induced by LPS/ATP ($n = 6$). The results represent the means $\pm$ SEM of three independent experiments. ** $P < 0.01$ were compared with the LPS/ATP-induced group. # # $P < 0.01$ were compared with the control group.

Figure 2

Effect of quercetin on the mRNA levels of TLR4, Caspase-1, GSDMD and inflammatory factors (n= 3). (A) Changes in TLR4 mRNA levels (n = 3). (B) Changes in GSDMD mRNA levels (n = 3). (C) Changes in Caspase-1 mRNA levels (n = 3). The experimental data are expressed as mean $\pm$ SD. ** P < 0.01 and *** P < 0.01 were compared with the LPS/ATP-induced group. # # P < 0.01 were compared with the control group. The effect of quercetin on the mRNA levels of IL-1 β and IL-18(n= 3). (D) Changes in IL-1 β mRNA levels (n = 3). (E) Changes in IL-18 mRNA levels (n = 3). The experimental data are expressed as mean $\pm$ SD. *** P < 0.01 were compared with the LPS/ATP-induced group. # # P < 0.01 were compared with the control group.

Figure 3

The effect of quercetin on the expression levels of TLR4 and downstream pyroptosis-related proteins. (A) The protein levels of TLR4, NLRP3, pro-caspase-1, caspase-1 and GSDMD (n = 3) were measured by western blot analysis in RIMVEC incubated with LPS (10μg/mL) and ATP (1mM) in the presence or absence of quercetin for 24 hours. (B-G) Changes of TLR4, NLRP3, pro-caspase-1, caspase-1 and GSDMD at the protein level (n = 3). The experimental data are expressed as mean ± SD. * P < 0.05, ** P < 0.01 and *** P < 0.01 were compared with the LPS/ATP-induced group. # # P < 0.01 were compared with the control group.

Figure 4

The effect of quercetin on the protein distribution of NLRP3, GSDMD and Caspase-1. (A-C) Fluorescence immunoassay results of pyroptosis-related proteins (n = 3).

Figure 5

The effect of quercetin on the expression level of NF-κB pathway related proteins. (A) The protein levels of p-NF-κB p65, NF-κB p65 and I-κB (n = 3) were measured by western blot analysis in RIMVEC incubated with LPS (10μg/mL) and ATP (1mM) in the presence or absence of quercetin for 24 hours. (B, C) Changes of p-NF-κB p65, NF-κB p65 and I-κB at the protein level (n = 3). The experimental data are expressed as mean ± SD. *** P < 0.01 were compared with the LPS/ATP-induced group. # # P < 0.01 were compared with the control group. (d)The protein levels of pro-IL-18, IL-18, pro-IL-1β and IL-1βwere measured by western blot analysis in RIMVEC incubated with LPS (10μg/mL) and ATP (1mM) in the presence or absence of quercetin for 24 hours (n = 3). (E, F) Changes of pro-IL-18 and IL-18 at the protein levels (n = 3). (I, J) Changes of pro-IL-1β and IL-1β at the protein levels (n = 3). The experimental data are expressed as mean ± SD. * P < 0.05, ** P < 0.01 and *** P < 0.01 were compared with the LPS/ATP-induced group. # # P < 0.01 were compared with the control group. (G)The results of IL-18 measured by ELISA (n= 3). (H) The results of IL-1β measured by ELISA (n= 3). The experimental data are expressed as mean ± SD. ** P <

0.01 and *** $P < 0.01$ were compared with the LPS/ATP-induced group. # $P < 0.01$ were compared with the control group.

Figure 6

(A)Effect of quercetin on migration ability of RIMVECs induced by LPS+ ATP. (a)Control (b)LPS 10µg/mL +ATP 1µM.(c)LPS 10µg/mL (d)LPS 10µg/mL +ATP 1µM+Que 5µM (e)LPS 10µg/mL +ATP 1µM+Que 10µM(f)LPS 10µg/mL +ATP 1µM+Que 20Mm.(B) Effect of quercetin on the migration rate of LPS/ATP-induced RIMVECs cells. The scale bar was 250µm (Magnificationa:40×, n=3). The experimental data are expressed as mean ± SD. * $P < 0.05$ and *** $P < 0.01$ were compared with the LPS/ATP-induced group. # $P < 0.01$ were compared with the control group.

Figure 7

Protein expression and distribution of ZO-1. (A, B) The result of protein levels of ZO-1 was determined by Western blot analysis (n=3). (C) Fluorescence immunoassay results of ZO-1(n=3). The experimental data are expressed as mean $\pm$ SD. *** P < 0.01 were compared with the LPS/ATP-induced group. # #P < 0.01 were compared with the control group.

Figure 8

The results of flow cytometric test(n=3). # Compared with the blank control group, $P < 0.01$; *** compared with the LPS/ATP group, $P < 0.01$; the experimental data are expressed as mean $\pm$ SD (n=3) The experimental data are expressed as mean $\pm$ SD. *** $P < 0.01$ were compared with the LPS/ATP-induced group. # $P < 0.01$ were compared with the control group.

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