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Original Research Article

Quercetin inhibits porcine intestinal inflammation *in vitro*

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Abstract

Purpose: *To investigate the effect of Quercetin (Que) on inflammatory reaction in intestinal porcine enterocyte cells, IPEC-J2, induced by lipopolysaccharide (LPS).*
Methods: *IPEC-J2 cells were pretreated with Que and then incubated with LPS. Cell viability, cell morphology, nitric oxide (NO) content, gene expression levels of interleukin-6 (IL-6) and interleukin-8 (IL-8), were then evaluated.*
Results: *LPS incubation at 10 μg/mL for 24 h showed no effect on the viability and cell morphology of IPEC-J2, while Que pre-incubation significantly enhanced the viability of IPEC-J2 and improved cell morphology ($p < 0.05$). Que decreased the expression levels of pro-inflammatory cytokines IL-6 mRNA and IL-8 mRNA raised by LPS ($p < 0.05$). Interestingly, both LPS and Que demonstrated no influence on the release of NO.*
Conclusion: *Pre-treatment of Que shows a positive effect on intestinal porcine enterocyte cells and inhibited porcine intestinal inflammation in vitro induced by LPS, but the mechanism may be not associated with NO-related signaling pathway. Therefore, Que might have a potential effect as a veterinary drug or feed additive for the treatment of enteritis in pigs.*

Keywords: *Quercetin, Inflammatory cytokines, Intestinal porcine enterocyte cells, Lipopolysaccharide*

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INTRODUCTION

Severe inflammation of animal intestines has several disadvantages on the livestock and poultry breeding industry, and research on the intestinal inflammation of animals has gradually become the focus of academic field researchers. The inflammatory response of porcine intestinal tract not only affects the absorption of nutrients and reduces the growth performance, but also decreases the immunity, leading to the infections due to the pathogenic microorganisms, and even death of pigs [1]. Intestinal porcine enterocyte cells-jejunum2 (IPEC-J2) are normal cells with good biological characteristics of intestinal epithelial cells. These are derived from the jejunal epithelial cells of the piglet and often used as an experimental model in sifting of the functional substances, research of toxicological effects and mechanisms of toxicants, as well as utilization of nutrients [2,3].

Lipopolysaccharide (LPS) an endotoxin, can trigger inflammatory response in IPEC-J2 by

stimulating the expression of inflammatory cytokines such as IL-6 and IL-8 [4]. Moreover, LPS at a high concentration of 100 μg/mL, induced a remarkable effect on the death of IPEC-J2, as well as loss of intercellular tight junctions [5]. Therefore, it is greatly indispensable to screen the natural products that are effective in the suppression of the occurrence and development of animal intestinal inflammation. In addition, quercetin (Que), a typical flavone, mainly exists in many vegetables and fruits, and can inhibit several types of inflammatory responses [6,7]. However, till now, there are no reports regarding the inhibitory activity of Que on intestinal inflammation in pigs. Thus, in this study, IPEC-J2 were initially pretreated with Que, and then treated with LPS, to explore whether or not Que has the potential to inhibit the inflammation of small intestine in pigs.

EXPERIMENTAL

Materials

Dulbecco's Modified Eagle Medium (DMEM), trypsin, phosphate buffered saline (PBS), 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and penicillin-streptomycin were got from Procell Life Science Co., Ltd (Hubei, China). Fetal bovine serum (FBS) was obtained from Hangzhou Sijiqing Bioengineering Material Co., Ltd (Zhejiang, China). LPS and Que were purchased from Sigma-Aldrich Co., Ltd (Shanghai, China) and Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China), respectively. Sterile LPS stock solution of 1 mg/mL was prepared in PBS and Que stock solution of 20 mg/mL was prepared in DMSO. The LPS and Que stock solutions were stored at -20 ℃ and Que stock solution was used within 3 weeks. Nitric oxide (NO) assay kit was bought from Beyotime Biotechnology Co., Ltd (Shanghai, China).

Cell culture

The IPEC-J2 cells were purchased from Guangzhou Jennio Biotechnology Co. Ltd (Guangdong, China), which were incubated with DMEM supplemented with 10 % FBS, 1 % of penicillin and streptomycin. The IPEC-J2 were maintained in a humid atmosphere of 5 % CO_2 at 37 ^{o}C.

Cell viability assay

Cell viability was measured using MTT assay. The IPEC-J2 were incubated in a 96-well plate. To observe the effect of LPS on viability of IPEC-J2, cells were exposed to 0, 1, 5, and 10 μg/mL LPS for 24 h. To explore the effect of Que at non-toxic concentrations of 1.25, 2.5, and 5 μg/mL on the activity of IPEC-J2 exposed to LPS, IPEC-J2 were pre-incubated with Que for 2 h, before exposed to LPS for 22 h. After the treatments mentioned above, 10 μL MTT was added to the cell-cultured wells containing 100 μL serum-free medium. The IPEC-J2 were incubated for 4 h in an incubator at 37 ℃. Then 150 μL DMSO was added into each well after discarding the culture medium. After 10 min, the absorbance was quantified on a microplate reader (Thermo Fisher Scientific Inc., Shanghai, China) at 490 nm. Cell viability (V) was computed as in Eq 1.

$$V\ (\%) = \{(At - Ab)/(Ac - Ab)\}100 \quad \ldots\ldots\ldots (1)$$

where At, Ab and Ac are the absorbance of treated, blank and control samples, respectively.

Assessment of cell general morphology

Cells were pre-incubated with 1.25, 2.5, and 5 μg/mL of Que for 2 h, then exposed to LPS for 22 h. After the indicated treatments, the morphology of IPEC-J2 was observed via an inverted microscope.

Measurement of NO production

The IPEC-J2 cells were incubated in 96-well plates at a density of 1.5×10^3 cells/well. After pre-incubation with Que, IPEC-J2 were exposed to LPS. Then the cell culture supernatant was collected. The content of NO in the supernatant was determined by Griss assay kit, according to the manufacturer's protocol, at a detection wavelength of 540 nm using a microplate reader.

RNA extraction and real-time PCR

The IPEC-J2 cells were treated with Que, and then cultured with DMEM containing 10 μg/mL LPS. Total RNA was extracted using Trizol reagent referring to the manufacturer's instructions. Then the RNA was reverse transcribed to cDNA by using 5 × TransScript® All-in-One SuperMix and gDNA remover in the following conditions: 42 ℃ for 15 min, 85 ℃ for 5 s and 4 ℃ forever. Real-time PCR was applied by TransScript® Tip Green qPCR Super Mix according to the manufacturer's instructions. Cycling contained two steps: pre-denaturation at 94 ℃ for 30 s, and 40 cycles of reaction at 94 ℃ for 5 s and 60 ℃ for 30 s. The primers of target genes, IL-6 and IL-8, and reference gene GAPDH are shown in Table 1. The $2^{-\Delta\Delta Ct}$

method was used to calculate the relative expression levels of the target genes.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Differences between the groups were tested by one-way ANOVA and independent-sample *t* test. All data analyses were conducted with SPSS 17.0. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of LPS on viability of IPEC-J2

The cell viability was measured by MTT assay method, and the results showed that 1 to 10 µg/mL of LPS showed no obvious effects on cell viability ($p > 0.05$) (Figure 1).

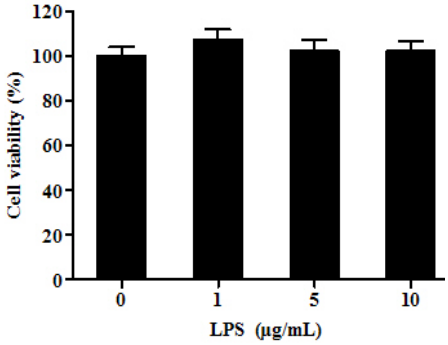

Figure 1: Effect of LPS on cell viability of IPEC-J2. Viability of IPEC-J2 was determined by MTT method. The IPEC-J2 were treated with indicated concentrations of LPS (1 to 10 µg/mL) for 24 h. Values are presented as mean ± SD

Effects of Que on viability and morphology of IPEC-J2 incubated with LPS

The IPEC-J2 were treated with 10 µg/mL of LPS for 22 h and the results showed no significant changes on cell viability ($p > 0.05$), but Que treatment increased the viability of IPEC-J2 exposed to 10 µg/mL of LPS ($p < 0.05$) (Figure 2). Moreover, the cell morphology was not changed post LPS treatment. And Que treatment increased the cell number, and greatly improved the cell status (Figure 3).

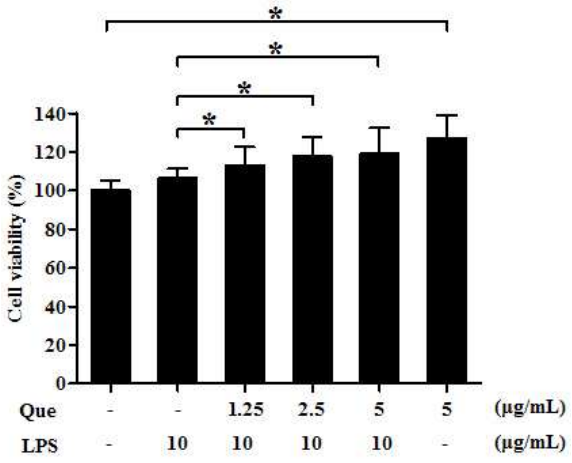

Figure 2: Effect of Que on cell viability of IPEC-J2 exposed to LPS. Viability of IPEC-J2 was determined by MTT method. The IPEC-J2 were pre-treated with Que (1.25 to 5 µg/mL), before treatment with LPS. Values are presented as mean ± SD; *$p < 0.05$

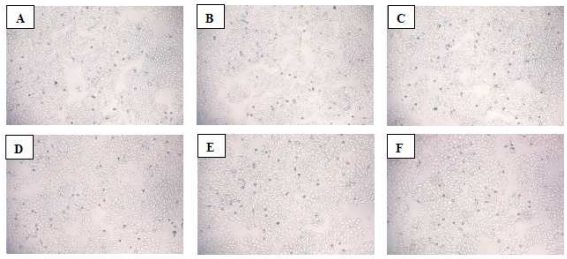

Figure 3: Effect of Que on cell morphology of IPEC-J2 exposed to LPS. Morphology of IPEC-J2 was observed under a microscope (40×). The IPEC-J2 were pre-treated with Que (1.25 to 5 µg/mL), and subsequently with LPS. 0 µg/mL Que + 0 µg/mL LPS (A); 0 µg/mL Que + 10 µg/mL LPS (B); 1.25 µg/mL Que + 10 µg/mL LPS (C); 2.5 µg/mL Que + 10 µg/mL LPS (D); 5 µg/mL Que + 10 µg/mL LPS (E) ; 5 µg/mL Que + 0 µg/mL LPS (F)

Effect of Que on NO release IPEC-J2 exposed to LPS

The content of NO was tested via Griess method. The NO content showed no significant differences in the absence or presence of Que and/or LPS ($p > 0.05$). In addition, the NO contents of all cells treated with different concentrations of drugs were below the lower limit of detection (Figure 4).

Table 1: Primer sequences of genes for Real-time PCR

Gene	Forward sequence	Reverse sequence
GAPDH	GGGCATGAACCATGAGAAGT	TGTGGTCATGAGTCCTTCCA
IL-6	TCACCACCGGTCTTGTGGAGT	AGGCCGGCATTTGTGGTGGG
IL-8	AGAGGTCTGCCTGGACCCCA	GGGAGCCACGGAGAATGGGT

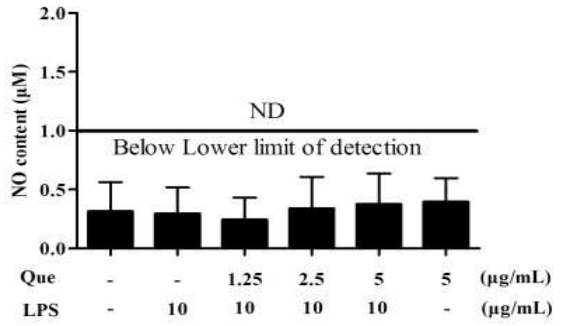

Figure 4: Effect of Que on NO content in cell culture medium of IPEC-J2 exposed to LPS. ND means "not detected". Values are presented as mean ± SD

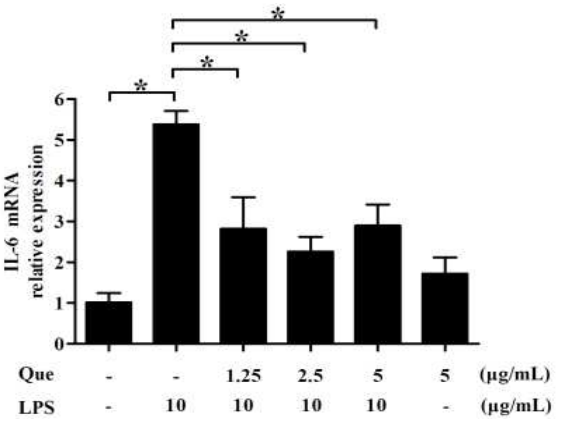

Figure 5: Effect of Que on IL-6 mRNA expression level in IPEC-J2 exposed to LPS. IL-6 mRNA expression was detected by real-time PCR. The IPEC-J2 were pre-cultured with Que, followed by incubation with or without 10 μg/mL LPS. Values were expressed as mean ± SD; *$p < 0.05$

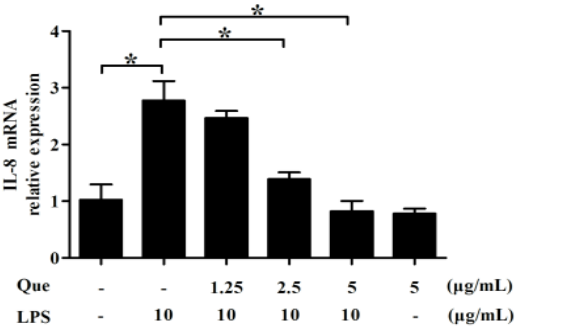

Figure 6: Effect of Que on IL-8 mRNA expression level in IPEC-J2 exposed to LPS. IL-8 mRNA expression was determined by real-time PCR. The IPEC-J2 were pre-cultured with Que, followed by incubation with or without 10 μg/mL LPS. Values were expressed as mean ± SD; *$p < 0.05$

Effect of Que on gene expression levels of IL-6 and IL-8 in IPEC-J2 exposed to LPS

Real-time PCR was applied to detect the gene expression levels of IL-6 and IL-8. The data showed, that the expression of IL-6 and IL-8 at the mRNA level was significantly up-regulated post exposure to LPS ($p < 0.05$). However, 1.25 to 5 μg/mL of Que pre-treatment reduced IL-6 along with IL-8 mRNA expression levels, which were increased by 10 μg/mL LPS treatment ($p < 0.05$) (Figure 5 and Figure 6).

DISCUSSION

At present, porcine intestinal inflammation is a very important risk factor for pig industry. The symptom of porcine intestinal inflammation is often manifested as diarrhea, which seriously affects the growth performance of pigs and pork quality, and even causes great economic losses to the piggery industry. Therefore, it is of great significance to explore drugs to prevent or treat porcine enteritis. IPEC-J2, an intestinal porcine epithelial cell line, can be used as a great model to investigate the immuno-modulatory and anti-inflammatory activities of drugs *in vitro* [8-10]. Importantly, porcine intestinal cells have high similarity to human intestinal cells, and hence are helpful to perform research on human medicine and feed science. LPS is an important component of the cell wall of Gram-negative bacteria, and can result in the production of pro-inflammatory cytokines and expression of inflammatory genes and proteins [11]. In addition, Que possesses a property of anti-inflammation. Thus, in this study, IPEC-J2 were selected as a model cell line to investigate the anti-inflammatory activity and the associated molecular mechanism of Que.

In previous studies, controversies still existed on whether LPS affect the viability and morphology of various cells. Different from Tang's results [12], in present study, the relative activity and morphology of cells exposed to 1, 5, and 10 μg/mL of LPS was not remarkably changed in comparison with the control group. However, Lee's results were similar to that of this research, where after continuous treatment with 1 μg/mL LPS for 24 h, the viability of HT29 and RAW264.7 cells showed no obvious changes, despite the different cell lines used [13]. In addition, the results obtained from MTT assay and photomicrographs showed that the IPEC-J2 incubated with 1.25, 2.5, and 5 μg/mL of Que can increase the viability and improve the status of IPEC-J2.

As a signaling molecule, NO, mainly generated by immune cells, acts as an inflammatory mediator, and excessive production of NO may aggravate the inflammatory response [14]. However, the results suggested that the NO levels of Que and/or LPS treatment groups were lower than the minimum detection limit of the kit

in this study. It could be inferred that the IPEC-J2 might not produce NO, or the amount of NO might be extremely low. Therefore, NO might not be involved in the inflammatory process of IPEC-J2.

IL-6 plays an important role in inflammation, and provokes a broad range of physiological responses. IL-6 is a vital mediator in chronic and acute inflammation, and the increase of IL-6 concentration may cause some inflammatory reactions and functional injuries to the tissues and organs. In the mice model of acute liver injury [15] and human peripheral blood mononuclear cells (PBMCs) of inflammatory reaction [16], Que treatment significantly diminished the production of IL-6 and other inflammatory markers. Similarly, these results also showed that Que remarkably attenuated IL-6 mRNA expression in IPEC-J2 induced by LPS. Besides, IL-8 is a chemokine that can attract and activate neutrophils then to release a series of active products, resulting in local inflammatory reactions. Hyaluronic acid anohydrogel of Que significantly reduced the production of IL-8 and IL-6 in glioblastoma cells under inflammatory conditions [17]. In the present study, Que markedly down-regulated the expression of IL-8 in LPS-treated IPEC-J2. Nuclear factor-kappa B-p65 (NF-κBp65) is an important nuclear transcription factor in the regulation of pro-inflammatory genes including IL-6 and IL-8. Under a resting condition, NF-κBp65 is combined with IκB-α in the cytoplasm. During inflammation, IκB-α is degraded, and the NF-κBp65 is activated to induce the expression of cytokines [18]. Wu *et al* proved that after IPEC-J2 were treated with *Escherichia coli* K88, the expression of IκB-α was decreased [19]. Similarly in RAW264.7 cells, Que also could suppress the degradation of IκB-α caused by LPS [18]. Thus, the inhibition of inflammation of IPEC-J2 cells by Que might be associated with NF-κBp65 pathway.

CONCLUSION

In summary, Que pre-treatment showed positive effects on the viability and cell morphology of IPEC-J2. However, LPS treatment showed no effects on IPEC-J2 viability and morphology but induced inflammatory response *in vitro*. Moreover, Que treatment inhibited porcine intestinal inflammation *in vitro* induced by LPS via inhibiting IL-6 and IL-8 gene expressions. However, the mechanism of inflammatory inhibition of Que might not be associated with NO-related signaling pathway. Thus, these findings suggest that Que may have a potential effect for treating enteritis in porcine.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this study.

Contribution of authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Zhi-Gang Chen and Guang-Ren Xu, as co-first authors, contributed equally to the work.

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