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Geniposide, a component of *Gardenia jasminoides* Ellis, inhibits NF- κ B to attenuate LPS-induced injury in intestinal epithelial cells

Qiuju Wang ^{1,†}, Xinyue Qiao ^{1,†}, Mengzu Wang ¹, Junfeng Jia ¹, and Yizhe Cui ^{1,2,*}

¹ College of Animal Science and Technology, Heilongjiang Bayi Agricultural University, Daqing, Heilongjiang 163319, China; wqj_9@126.com (Q.W.); 2362943901@qq.com (X.Q.); 380688962@qq.com (M.W.); 779289667@qq.com (J.J.)

² Heilongjiang Provincial Key Laboratory of Prevention and Control of Bovine Diseases, Heilongjiang Bayi Agricultural University, Daqing, Heilongjiang 163319, China

* Correspondence: cuiyizhe1979@126.com (Y.C.); Tel.: +86-13936887981 (Y.C.)

† These authors contributed equally to this work.

Simple Summary: Gardenia decoction markedly inhibited inflammatory cytokine production and reduced the expression of NF- κ B following LPS stimulation *in vivo*. Geniposide has certain anti-inflammatory and therapeutic effects on soft tissue injury. We investigated the role of Geniposide in protection against LPS using *in vitro* cultured IEC-6 cells. Our results showed that GE exerts an anti-inflammatory effect and that Geniposide blocks the activation of NF- κ B signaling by targeting I κ B in IEC-6 cells *in vitro*.

Abstract: The nuclear factor- κ B (NF- κ B) transcriptional system is a major effector pathway involved in inflammatory responses. Previous studies found that a Gardenia decoction (GD) inhibited the expression of NF- κ B in a lipopolysaccharide (LPS)-stimulated mouse intestinal injury model. Herein, we hypothesized that geniposide (GE), a component of *Gardenia jasminoides* Ellis, also exerts anti-inflammatory effects and inhibits NF- κ B activity in LPS-induced intestinal epithelial cells (IEC-6). IEC-6 cells were stimulated with LPS, following which the effects of GE on NF- κ B signaling in the IEC-6 cells were examined by western blotting to detect I κ B phosphorylation/degradation. The expression of NF- κ B was determined by immunofluorescence assay (IFA). Enzyme-linked immunosorbent assay (ELISA) was used to detect the inhibitory effect of GE on the release of tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6) and interleukin-1 β (IL-1 β) activated by LPS in IEC-6 cells. In addition, the migration ability of IEC-6 cells was observed by the scratch method. These results showed that GE dose-dependently downregulated levels of the proinflammatory cytokines TNF- α , IL-6 and IL-1 β that had been upregulated by LPS and suppressed the phosphorylation of I κ B and NF- κ B induced by LPS. Our findings indicated that GE could reduce LPS-induced NF- κ B signaling and proinflammatory expression in IEC-6 cells and significantly enhance the migration of IEC-6 cells. Moreover, GE inhibited the expression of NF- κ B, nuclear transfer, and transcriptional activity in IEC-6 cells. GE could block the synthesis of inflammatory factors of IEC-6 cells by inhibiting activation of the I κ B/NF- κ B signaling pathway induced by LPS.

Keywords: Geniposide; NF- κ B; IEC-6; Signaling pathway; Cell migration

1. Introduction

The nuclear factor- κ B (NF- κ B) family is a group of nuclear protein factors that regulate the expression of a wide range of genes. These nuclear protein factors play an important role in regulating the transcription of genes related to inflammation; cell proliferation, differentiation and apoptosis; the immune response; and tumorigenesis. In most cells, NF- κ B proteins can form homodimers or heterodimers that bind I κ Bs in an inactive state in the cytoplasm. When stimulated by stimulants such as viruses, radiation, oxygen

free radicals, bacterial lipopolysaccharide (LPS) and tumor necrosis factor- α (TNF- α), the NF- κ B-I κ Bs complex is activated, NF- κ B is released and transferred to the nucleus, and its binding at specific sites promotes transcription of the corresponding genes, thus initiating and regulating the immune response, cell adhesion, differentiation, proliferation, apoptosis and inflammation.

Expression of the NF- κ B promoter gene involves the activation of IKK, phosphorylation and ubiquitination of I κ B, cleavage of I κ B, migration of NF- κ B to the nucleus, and binding of NF- κ B to DNA. Overactivation of NF- κ B can lead to a series of diseases, including asthma, rheumatoid arthritis, enteritis and so on. Inhibiting signal transduction pathways with a core of NF- κ B through drugs or gene transformation may be an effective way to prevent and treat some diseases. Some traditional Chinese medicine extracts, such as flavonoids [1] and steroid-like compounds [2], can strongly block IKK activity, thus inhibiting the phosphorylation and degradation of I κ B and blocking the activity and protein-binding ability of NF- κ B. These compounds have advantages in the treatment of some inflammatory diseases.

In previous studies, we found that Gardenia decoction (GD) markedly inhibited inflammatory cytokine production and reduced the expression of NF- κ B following LPS stimulation in vivo [3]. Geniposide (GE), the main active ingredient of Gardenia jasminoides, is a kind of iridoid terpenoid glucoside [4]. Modern studies have shown that GE has substantial effects on digestive system, cardiovascular system and central nervous system diseases. In addition, GE has certain anti-inflammatory and therapeutic effects on soft tissue injury. However, the mechanism through which GE mediates its anti-inflammatory effects is not completely understood.

In this study, we investigated the role of GE in protection against LPS using in vitro cultured IEC-6 cells. Our results showed that GE exerts an anti-inflammatory effect and that GE blocks the activation of NF- κ B signaling by targeting I κ B in IEC-6 cells in vitro.

2. Materials and Methods

2.1. Reagents

GE (purity >98% SG8100) was purchased from Solarbio Co. (Beijing, China). LPS (Escherichia coli 055: B5) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). *G. jasminoides* Ellis extract was obtained according to a previous method [3] with slight modifications. In the previous experiment, we determined the composition and content of *G. jasminoides* Ellis extract by LC/MS Analysis. Briefly, the mixture was filtered through a 100-mesh metal sieve. The filtrate (crude decoction) was sterilized using a 0.22- μm syringe filter for cell processing. The extract of GD was identified by Qingdao Kechuang Quality Inspection Co., Ltd. (Qingdao, China). (Application No. PDFD-16-01 D/0). The specimen has been disposed in the laboratory of animal pharmacy, Heilongjiang Bayi Agricultural University

2.2. Cell culture and viability assay

Intestinal epithelial cells (IEC-6 cells) were obtained from the Institute of Basic Medicine, Chinese Academy of Medical Sciences (Beijing, China) and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS), antibiotics (0.1 mg•mL⁻¹ streptomycin and 100 U•mL⁻¹ penicillin) and 10 $\mu\text{g}\cdot\text{mL}^{-1}$ bovine insulin. Cells were maintained at 37 °C in a 5% CO₂ atmosphere. After 24 h, the cells were harvested, plated on 96-well plates at a density of 5,000 cells/well and then treated with LPS (4, 6, 10, 25, 50, 80, 100 $\mu\text{g}/\text{mL}$), GE (25, 50, 100, 200, 400, 800 μM) and GD (25, 50, 100, 200, 400, 800 $\mu\text{g}/\text{mL}$) at different concentrations. After 24 h of incubation, 20 μL of MTT (5 mg/mL) was added to each well, and the cells were incubated for 4 h at 37°C. The medium was replaced with 100 μL of dimethyl sulfoxide (DMSO), and the cells were incubated for 5 min while shaking. Cellular viability was determined by measuring the optical density (OD) at 490 nm with a spectrophotometer.

2.3. Cell treatment

IEC-6 cells were cultured in 96-well plates at a density of 5×10^3 cells/well. The cells were divided into a normal control group, LPS model group, GD treatment group (treated with different concentrations of GD) and GE treatment group (treated with different concentrations of GE). After pretreatment with GD and GE for 24 h, LPS was added to the cell culture for 12 h. Cells were then collected for subsequent testing.

2.4. Cell migration assays

The IEC-6 cells were seeded into 12-well cell culture plates for 24 h at 37 °C in a humidified atmosphere containing 5% CO₂, at a concentration of 5×10^3 cells/mL, and cultured in a medium containing 10% FBS to produce a nearly confluent cell monolayer. A linear wound was subsequently generated in the monolayer using a cutting knife in the monolayer, a scratch was made and the cell scraped off the other side. Any cellular debris was removed by washing the wells with PBS. Place the dish in a tissue culture incubator at 37 °C for 12 h. After incubation, the dish was placed under a phase-contrast microscope, the photographed region was aligned, and images were acquired. The acquired images of each sample were further quantitatively analyzed with computational software. The number of cells across each scratch was then determined using Image-Pro Plus software.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Levels of the proinflammatory cytokines TNF- α , interleukin-6 (IL-6) and interleukin-1 β (IL-1 β) were tested using specific ELISA kits (eBioscience) following the operation manual of the manufacturer.

2.6. Western blot analysis

In 10% sodium dodecyl sulfate polyacrylamide gels a total of 40 μg of protein was electrophoresed. After electrophoresis, the proteins were transferred to a PVDF membrane. The membrane was soaked in a solution containing 5% bovine serum albumin and primary antibodies against NF- κB p65 (Abcam, ab32536, 1:5000), p-NF- κB p65 (#3033, 1:1000), I κB - α (#2859, 1:1000), p-I κB - α (#4812, 1:1000) (CST, Beverly, MA, USA) and β -actin (1:1,000) (Proteintech Group, Inc., Beverly, Chicago, USA, 60008-1-ig) overnight at

4 °C. The membrane was immersed in the corresponding secondary antibody diluted 1:5000 for 1 h in the dark at 37 °C to enhance chemiluminescence detection of the protein. Protein density was quantified by means of imaging software.

2.7. Immunofluorescence assay (IFA)

Cells (1×10^4) were seeded on coverslips and treated with both digests then IEC-6 cells were washed twice with PBS, fixed with 4% formaldehyde in PBS for 10 min, and permeabilized with 0.2% Triton X-100 in PBS for 5 min at 20 °C. The IEC-6 cells were blocked with 5% BSA in PBS for 1 h at 20°C and then immunostained with anti-NF- κ B p65 primary antibody (Abcam, ab32536, 1:1000) and secondary antibody bound to FITC (A0568, 1:500, Beyotime) overnight at 4 °C. Then, the nucleus subjected to DAPI counterstaining. The NF- κ B p65 proteins were observed under an Olympus IX71 fluorescence microscope.

2.8. Statistical analysis

Data are expressed as the means \pm SDs and were analyzed by one-way ANOVA. The mean separation was assessed by Tukey's multiple comparison tests (GraphPad Prism version 5 software). Differences with $P < 0.05$ were considered statistically significant.

3. Results

3.1. MTT assays

The chemical structure of GE is shown in Figure 1A. The results of MTT assays showed that the activity of IEC-6 cells treated with GD and GE alone was not significantly different with than of IEC-6 cells in the normal control group ($P > 0.05$) (Figure 1B, C); however, 25 μ g/mL LPS significantly inhibited the activity of IEC-6 cells ($P < 0.01$) (Figure 1D). Therefore, LPS with concentration of 10 μ g/mL was selected to prepare IEC-6 cells induce model.

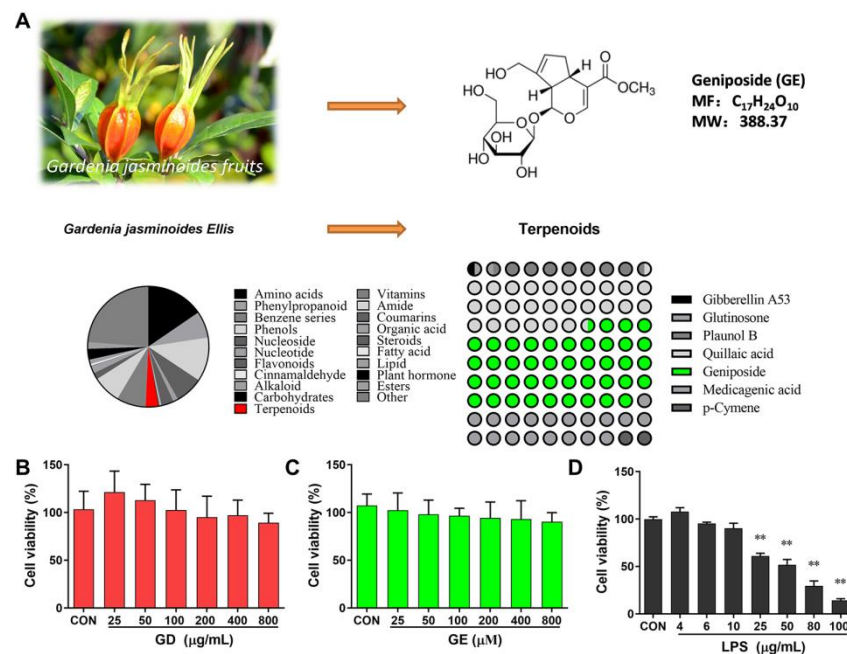


Figure 1. Effect of Ge on IEC-6 cell viability. (A) Ge content and structure in GD. (B) An MTT assay was performed to determine the effects of GD on IEC-6 cell viability. (C) An MTT assay was performed to determine the effects of GE on IEC-6 cell viability. (D) An MTT assay was performed to determine the effects of LPS on IEC-6 cell viability. Treatment with GE at concentrations up to 800 μ M did not significantly decrease the number

of viable cells. * $P < 0.05$, ** $P < 0.01$ compared with CON. MW: molecular weight; GD: gardenia decoction; GE: geniposide; CON: control; LPS: lipopolysaccharide.

3.2. GE promoted the migration of IEC-6 cell in vitro

The effect of GE on cell migration after injury is shown in Figure 2. Treatment with GD (100 $\mu\text{g}/\text{mL}$) or GE (100 μM) remarkably promoted cell migration compared to that in the normal control group ($P < 0.01$, Figure 2A, B). The LPS-induced injury of IEC-6 cells significantly inhibited cell migration, and the migration rate in the LPS group at 12 h decreased to 64% of that of the control group (Figure 2).

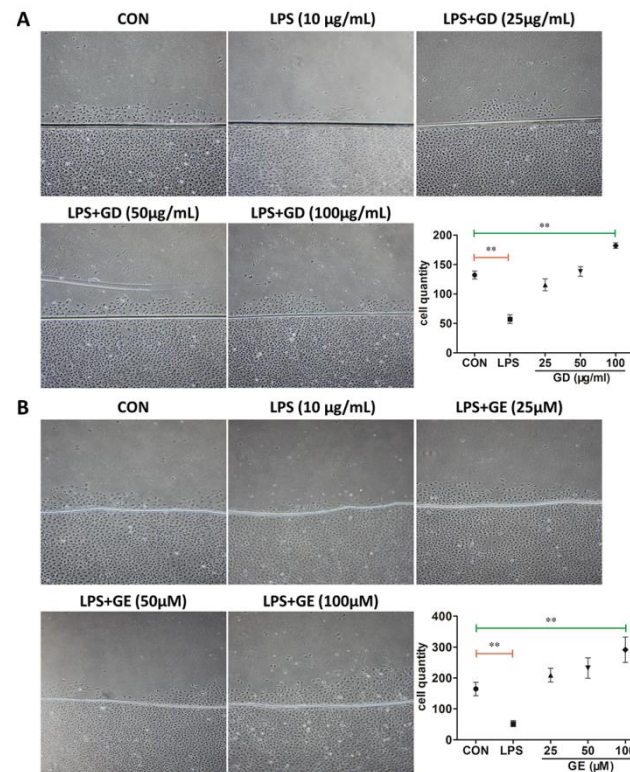


Figure 2. GE promoted IEC-6 migration in vitro. (A) Images showing the migration of IEC-6 cells treated with GD (25, 50, 100 $\mu\text{g}/\text{mL}$) at different concentration in plates. (B) Images showing the migration of IEC-6 cells treated with GE (25, 50, 100 μM) at different concentrations in plates. The IEC-6 cells were stimulated with 10 $\mu\text{g}/\text{mL}$ LPS. Migration was quantified as the number of cells in the covered wound area relative to the initial scratched area. Values are presented as the means \pm SDs ($n=8$). ** $P < 0.01$ compared with CON. Original magnification, 100 \times .

3.3. GE inhibited inflammatory cytokine production in IEC-6 cells stimulated by LPS

We measured the effect of GD and GE on levels of the inflammatory cytokines TNF- α , IL-6 and IL-1 β using ELISAs. A significant increase in the levels of TNF- α , IL-6 and IL-1 β in IEC-6 cells induced by LPS treatment was detected compared with their levels in the control group ($P < 0.01$). Compared with LPS model group, the expression of inflammatory cytokines in GD group was significantly lower (Figure 3A). Furthermore a significant dose-dependent decrease in inflammatory cytokine production in the GE treatment group compared with the LPS model group (Figure 3B) ($P < 0.01$).

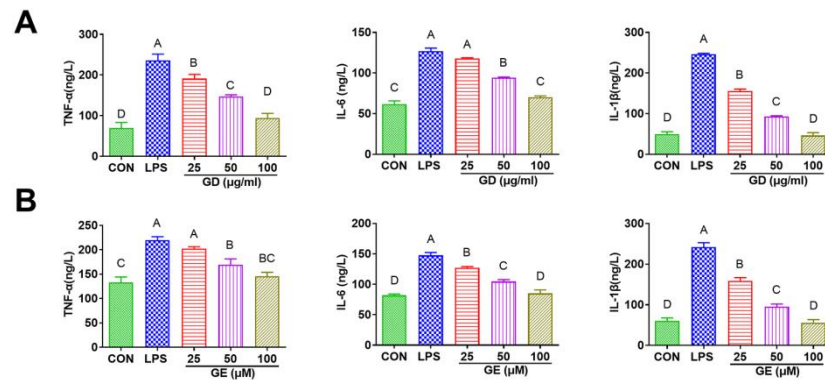


Figure 3. GE inhibited LPS-activated inflammatory cytokines in IEC-6 cells. (A) The effect of GD on the levels of TNF- α , IL-6, and IL-1 β in IEC-6 cells activated by LPS (10 $\mu\text{g}/\text{mL}$). A significant decrease in TNF- α , IL-6, and IL-1 β expression was detected in the GD treatment group compared with the LPS model group. (B) The effect of GE on the levels of TNF- α , IL-6, and IL-1 β in IEC-6 cells activated by LPS (10 $\mu\text{g}/\text{mL}$). A significant decrease in TNF- α , IL-6, and IL-1 β production in the GE treatment group compared with the LPS model group was detected by ELISA. ELISA: enzyme-linked immunosorbent assay; IL: interleukin; LPS: lipopolysaccharide; GD: gardenia decoction; GE: geniposide; TNF- α : tumor necrosis factor- α . Values from different groups marked with the same letters are not significantly different ($P > 0.05$), and those marked with different letters are significantly different ($P < 0.05$).

3.4. GE inhibited the NF- κB / I κB - α proteins pathway IEC-6 cells

As can be seen from Figure 4, Compared with the normal control group, LPS significantly activated the phosphorylation of p-NF- κB p65 and p-I κB - α protein ($P < 0.01$), while GD (Figure 4A) inhibited the phosphorylation of p-NF- κB p65 and p-I κB - α protein induced by LPS. The effect of GE on p-NF- κB p65 and p-I κB - α protein was consistent with the trend of GD (Figure 4B).

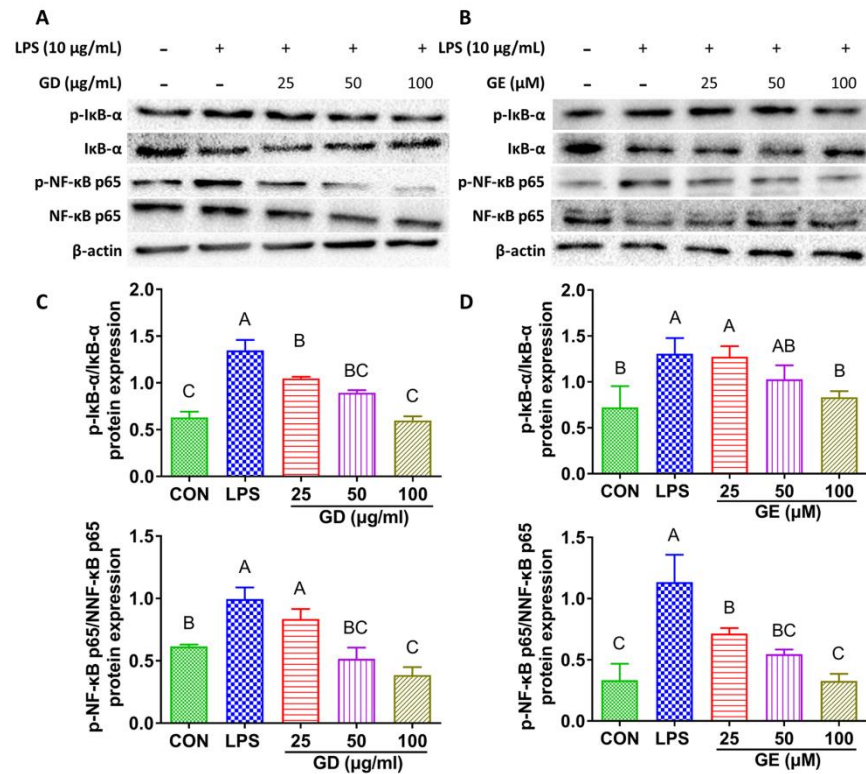


Figure 4. GE inhibited phosphorylation of the NF- κ B and I κ B- α proteins in IEC-6 cells activated by LPS. (A, C) Effect of GD on phosphorylation of the NF- κ B and I κ B- α proteins in IEC-6 cells, as shown by western blot analysis. (B, D) Expression of LPS activated phosphorylated proteins of NF- κ B and I κ B- α in IEC-6 cells, as shown by western blot analysis. LPS significantly increased p-NF- κ B p65 protein phosphorylation, which was suppressed by GE. Similarly, LPS significantly increased p-I κ B- α protein phosphorylation, which was suppressed by GE. LPS: lipopolysaccharide; GD: gardenia decoction; GE: geniposide. Values from different groups marked with the same superscript letters are not significantly different ($P > 0.05$), while those marked with the same letters are significantly different ($P < 0.05$).

3.5. GE inhibited the LPS-induced translocation of NF- κ B in IEC-6 cells

As shown in Figure 5, NF- κ B was obviously retained in the cytoplasm of IEC-6 cells in the normal control group. The translocation of NF- κ B to the nucleus was increased, and the immunostaining signal in the cytoplasm after LPS stimulation was weaker than that in the normal control group (Figure 5). However, treatment with GE (100 μ M) prevented the effects of LPS on nuclear NF- κ B translocation.

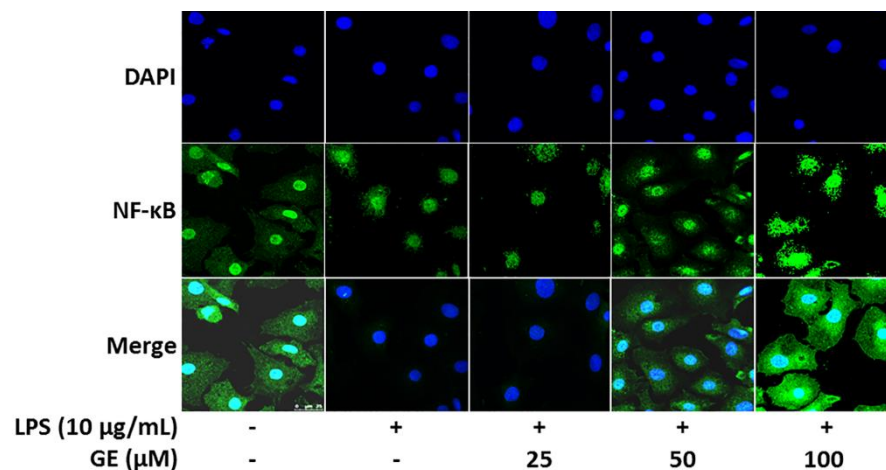


Figure 5. GE inhibited the LPS-induced translocation of NF- κ B. Subcellular localization of NF- κ B (green) in IEC-6 cells treated with GE at different concentrations. Nuclei were stained with DAPI (blue). Images (original magnification, 630 \times) were taken by laser confocal microscopy.

4. Discussion

GE, a bioactive iridoid glucoside derived from *G. jasminoides*, has anti-inflammatory effects [5]. In sepsis model mice, GE directly bound LPS and neutralized LPS in vitro, and significantly protecting against sepsis [6]. Gardenia, a plant belonging to the Rubiaceae family, contains various iridoid glycosides, which are unstable, non-volatile, thermally labile, polar natural compounds [7]. The fruit of *G. jasminoides* Ellis (gardenia fruit) is an Asian folk medicine that has been widely used for centuries for the treatment of inflammation, jaundice, headache [8], edema, fever, hepatic disorders [9] and hypertension [10]. The iridoid compounds GE and genipin were found to be the anti-inflammatory components of gardenia fruit. Both GE and genipin have anti-inflammatory activities, as shown via their inhibition of exudate and nitric oxide (NO) production in a carrageenan-induced rat paw edema model. Genipin suppressed both NO production and cyclooxygenase expression at an early stage and exhibited stronger anti-inflammatory activity than GE [11]. Genipin-mediated inhibition of NO production has been associated with the inhibition of NF- κ B activation [12]. However, the material basis of the anti-inflammatory activity gardenia fruit is not completely clear.

In this study, we investigated the impact of GE on LPS signaling in IEC-6 cells and elucidated its mechanism of action.

Activation of the NF- κ B signaling pathway can induce expression of the proinflammatory cytokines IL-6 [13], TNF- α , and IL-1 β [14], and these proinflammatory factors can further activate NF- κ B, thus aggravating inflammation [15]. IL-6, TNF- α and IL-1 β are important cytokines secreted by cells used to evaluate the inflammatory response. The secretion of IL-6, TNF- α and IL-1 β is significantly increased in inflammatory cells [16,17]. Much evidence indicates that LPS-induced inflammation is closely related to increased secretion of the inflammatory factors IL-6, TNF- α [18] and IL-1 β [19]. Therefore, we detected the levels of IL-6, TNF- α and IL-1 β in IEC-6 cells treated with LPS to observe the effects of LPS-induced inflammation.

In this study, LPS induction was used to establish a model of inflammation in IEC-6 cells. LPS treatment increased IL-6, TNF- α and IL-1 β secretion by IEC-6 cells and thus upregulated inflammation in IEC-6 cells. We found that GE at a certain concentration could downregulated the expression levels of IL-6, TNF- α and L-1 β in LPS-damaged IEC-6 cells. Therefore, we speculated that GE at this concentration can effectively regulate the expression levels of IL-6, IL-1 β and TNF- α through the I κ B/NF- κ B signaling pathway to further control the occurrence of inflammatory reactions.

NF- κ B is an important transcription factor that regulates the inflammatory response. At rest, NF- κ B binds its inhibitor, I κ B, and has no biological activity [20]. When cells are stimulated by LPS, I κ B-mediated inhibition of NF- κ B is relieved, and the NF- κ B complex is dissociated and transferred to cells. After binding the corresponding site on specific DNA, it can induce the transcription of related genes and promote the release of cytokines, thus generating a systemic inflammatory response [21]. The research shows that GD could reduce the level of inflammatory cytokines in the injury model and had anti-inflammatory effects on tissues. However, the treatment of LPS-induced IEC-6 cells with GE has not been investigated. Western blot analysis showed that the protein expression of p-I κ B- α was increased, and the distribution of p-NF- κ B p65 was significantly increased in IEC-6 cells stimulated by LPS. GE prevented activation of the NF- κ B pathway and decreased the nuclear transfer of p-NF- κ B p65 in cells induced by LPS (Figure 6).

Cell migration plays an important role in the repair of intestinal mucosal injury. IEC-6 cells have been widely used in the study of intestinal mucosal repair [22]. Scholars have conducted in-depth and systematic research on the physiological regulation [23], signaling pathways [24] and pathology [25] of intestinal mucosal injury, but there have been few reports of drugs to repair the gastrointestinal mucosa. This study demonstrated that GE can promote the migration of IEC-6 cells and reduce LPS-induced damage and has a protective effect on IEC-6 cells.

5. Conclusions

GE, a component of *G. jasminoides* Ellis, can block the synthesis of inflammatory factors by inhibiting activation of the I κ B/NF- κ B signaling pathway induced by LPS, thus effectively inhibiting inflammatory damage to IEC-6 cells induced by LPS.

Author Contributions: YZC and XYQ conducted most of the experiments and the data analysis; QJW MAW, FJJ and YZC contributed to the discussion; YZC supervised the project; and YZC and XYQ wrote the manuscript. All authors read and approved the final manuscript.

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