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Total Flavonoid Extract from Hawthorn (*Crataegus pinnatifida*) Improves Inflammatory Cytokines-Evoked Epithelial Barrier Deficit

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

AEF 1 **Feng Liu***
BCD 2 **Xuesong Zhang***
FG 3 **Yong Ji**

1 Department of General Anorectal Surgery, Jingjiang People's Hospital, Jingjiang, Jiangsu, P.R. China
2 Central Laboratory, Jingjiang People's Hospital, Jingjiang, Jiangsu, P.R. China
3 Department of General Surgery, Jingjiang People's Hospital, Jingjiang, Jiangsu, P.R. China

* Feng Liu and Xuesong Zhang contributed equally to this work

Corresponding Author: Yong Ji, e-mail: jyong0909@126.com
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Background: Intestinal epithelial barrier dysfunction is involved in the development and pathogenesis of intestinal diseases, such as irritable bowel syndrome, inflammatory bowel disease, and celiac disease. This study was performed to evaluate the ability of total flavonoid extract from hawthorn (TFH) to improve TNF-α-evoked intestinal epithelial barrier deficit.

Material/Methods: Caco-2 cells monolayers were exposed to TNF-α in different concentrations of TFH. Intestinal epithelial barrier function was evaluated using epithelial permeability and transepithelial electrical resistance (TER).

Results: Our findings showed that TFH alleviated the increase of paracellular permeability and the decline of transepithelial electrical resistance (TER) evoked by TNF-α. Additionally, 24-h pre-incubation with TFH inhibited TNF-α-evoked secretion of pro-inflammatory factors (IL-6, IL-8, MCP-1, and IL-1β). Furthermore, TFH inhibited TNF-α-evoked overexpression of pMLC and MLCK and alleviated breakdown of TJs protein (ZO-1 and occludin). The activations of Elk-1 and NFκBp65 were inhibited by TFH pre-incubation.

Conclusions: TFH can alleviate TNF-α-evoked intestinal epithelial barrier deficit via the NFκBp65-mediated MLCK-MLC signaling pathway.

MeSH Keywords: Flavonoids • Gastrointestinal Diseases • Tight Junction Proteins

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Background

The intestinal mucosal barrier plays a vital role in preventing antigenic molecules and luminal microbes from penetrating the intestinal mucosa [1]. In addition, destruction of the intestinal mucosal barrier can increase gut permeability and lead to the progression of intestinal mucosal inflammation [2]. Furthermore, intestinal barrier dysfunction is associated with the etiology of several gut diseases, including celiac disease, irritable bowel syndrome, and inflammatory bowel disease [3,4].

The structural integrity of the intestinal barrier depends on a complex network of cytoskeletal structures and intercellular tight junctions (TJs) [5]. TJs proteins are located at the top of intestinal epithelial cells, which are made up of cytoplasmic zonula occludens (ZO) proteins, junctional adhesion molecule, claudins, and occludin [6]. TJs proteins play a vital role in intestinal epithelial permeability [7]. The disruption of intestinal epithelial TJs also leads to increased gut permeability, resulting in acute or chronic inflammatory responses [1].

Previous studies demonstrated that numerous locally secreted inflammatory factors, including interleukin (IL)-1β, IL-6, IL-8, nitric oxide, and tumor necrosis factor alpha (TNF)-α, are associated with the pathogenesis of gut diseases, resulting in intestinal barrier dysfunction [8,9]. Additionally, clinical research has indicated that TNF-α is a primary factor in gut diseases [10] and anti-TNF-α antibody is widely used in treatment of Crohn's and ulcerative colitis disease [11,12]. Moreover, several studies have indicated that myosin light chain kinase (MLCK) is a primary factor in TNF-α-evoked intestinal barrier dysfunction [13,14]. Hence, suppression of TNF-α-evoked inflammatory response may be an alternative therapy to treat gut diseases. However, long-term treatment with chemical agents, including corticosteroids, immunomodulators, and aminosalicylates, can cause serious adverse effects, such as increased susceptibility to infections [15]. Therefore, there is an urgent need to develop effective and safe therapeutic strategies of inflammatory bowel disease, and flavonoids may be potential agents for the prevention and treatment of gut diseases [16].

Crataegus pinnatifida L., widely known as "hawthorn", is a Traditional Chinese Medicinal herb that belongs to the rose family, with a long history of use. The dried fruits of hawthorn have been used as traditional treatment for hernia, hemafecia, postpartum blood stasis, and dyspepsia in East Asia [17]. Modern pharmacological research has demonstrated that hawthorn possesses numerous pharmacological activities affecting digestion, pathogenic microorganisms, and the cardiovascular system [17]. Flavonoids, polyphenols, steroids, organic acids, and triterpenoids have been identified as the bioactive constituents from the fruits of hawthorn [17,18]. However, whether total flavonoid extract from hawthorn (TFH) can improve inflammation-evoked epithelial barrier deficit is still unknown.

In the present study, we evaluated the alleviation effect of TFH in the intestinal epithelial cells and investigated the underlying mechanisms of the intestinal barrier-protective effect of TFH.

Material and Methods

Materials and reagents

Human colonic cancer Caco-2 cells were obtained from the Chinese Academy of Sciences (Shanghai, China) and cell culture reagents were purchased from ScienCell Research Laboratories (San Diego, USA). ELISA kits for IL-6, IL-8, MCP-1, and IL-1β were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Other chemical reagents were purchased from Sigma Chemical. The fruits of *Crataegus pinnatifida* L. were collected in October 2018 from Qinghe (Hebei Province, China).

Preparation and analysis of TFH

The fruit of *Crataegus pinnatifida* L. was milled into powder and sieved through a 40-mesh sieve. The extraction method of TFH was performed according to a previous report [19]. Briefly, 400 g of dried powder was extracted twice with 4 L of 70% ethanol under reflux for 1 h. Then, the extract was filtered and concentrated in a vacuum. The concentrates were added into a D101 macroporous resin column for 12 h, and the resin column was eluted with water, and then eluted by 95% ethanol. The ethanol eluate was collected, concentrated, and then lyophilized using a freeze-dryer to obtain TFH.

HPLC analysis of TFH

The HPLC analysis was performed on a Shimadzu LC-20AT system (Tokyo, Japan) equipped with an Agilent Zorbax 300Extend C18 (150×4.6 mm, 5 μm) column. The mobile phase was set 0.8 mL/min at 30°C. The mobile phase consisted of acetonitrile (A) and 0.1% formic acid (B) was performed as follows: 5% (A) to 35% (A) for 20 min, and 35% (A) held for 10 min, and TFH was measured at 280 nm.

Caco-2 cell culture

Caco-2 cells were maintained in DMEM supplemented with 50 U/mL streptomycin, 50 U/mL penicillin, 1% nonessential amino acid, and 10% FBS and cultured in an atmosphere of 100% relative humidity and 5% CO_2 at 37°C. The culture medium was changed daily.

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Cell viability assay

The effect of TFH or TNF-α on Caco-2 cells viability was measured by MTT method according to a previous report with minor modification [20]. Briefly, 5×10^3 cells/well of Caco-2 cells was pre-incubated in a 96-well plate for 12 h. Then, cells were treated with TFH (0–400 μg/mL) or TNF-α (0–200 ng/mL) under a humidified atmosphere (5% CO_2) at 37°C for 24, 48, and 72 h. After indicated incubation times, 10 μl of MTT reagent was added into each well. After incubation for 4 h, the colonic epithelial cells were lysed with isopropyl alcohol (containing 0.04 N HCl) and quantified at 570 nm using a micro-plate reader (BioTek, Winooski, VT). All samples were assayed in quadruplicate and experiments were repeated 3 times.

Paracellular permeability measurement

The flux of fluorescein isothiocyanate-conjugated (FITC, 4 kDa, Aladdin) dextran across Caco-2 cells monolayers was used to evaluate paracellular permeability. Briefly, Caco-2 cells were gently cleaned with Hank's balanced salt solution. DMEM media in the top of chamber was lightly aspirated and then replaced with 1 mg/ml of FITC-dextran (100 μl) in Hank's balanced salt solution. After the monolayers were incubated for 24 h with 5% CO_2 atmosphere at 37°C, the lower medium (100 μl) was detected by assaying the fluorescence with the fluorescent plate reader at Em 520 nm and Ex 480 nm.

Measurement of transepithelial electrical resistance (TER)

In the TER experiment, colonic epithelial cells were pre-cultured into chambers for 1 to 2 weeks. The value of TER was measured by a Millicell-ERS volt-ohmmeter (Millipore, MA, USA). When the TER of the Caco-2 monolayers above 400 Ω•cm^2, the THF (0–200 μg/mL) was transferred to the basolateral side, then the 100 ng/mL of TNF-α was added to the wells for 24 h. TER value was normalized to the control.

Measurement of pro-inflammatory cytokine

Caco-2 cells monolayers were cultured with TFH (0–200 μg/mL) for 24 h and subsequently co-treated with TNF-α (100 ng/mL) for another 24 h. The media in the upper chamber was centrifuged at 10 000 g for 10 min. The supernatant was collected and the levels of IL-6, IL-8, MCP-1, and IL-1β were assayed with an ELISA kit according to the manufacturer's protocol (Jiancheng Bioengineering Institute, Nanjing, China).

Measurement of phosphorylation of myosin light chain kinase (MLCK), myosin light chain (pMLC) and TJs proteins

After the TFH treatment, Caco-2 cells monolayers were gently cleaned with Hank's balanced salt solution and subsequently lysed with RIPA buffer. The cell debris was removed through centrifugation for 10 min at 4°C (10 000×g). The supernatant was obtained for the assessment the expression of pMLC, MLCK, ZO-1, and occludin by an ELISA kit according to the manufacturer's protocol (Elisa Biotech Co., Shanghai, China). The protein content of the cell lysate was assayed by the bicinchoninic acid protein kit (Thermo Scientific, MA, USA). The expression levels of pMLC, MLCK, ZO-1, and occludin are represented as % of normal control.

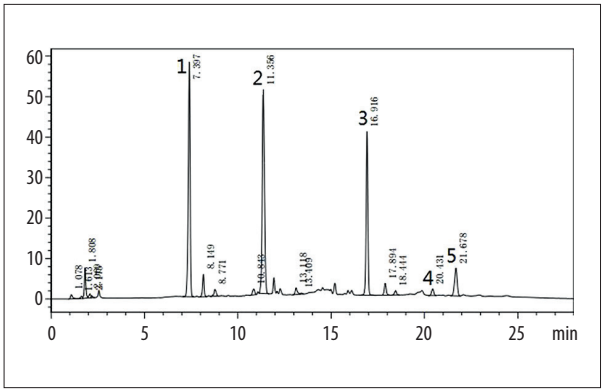

Figure 1. Chromatograms of active constituent occurring in hydroalcoholic extract of Hawthorn (*Crataegus pinnatifida*). (1 chlorogenic acid, 2 procyanidin B2, 3 epicatechin, 4 hyperoside, and 5 isoquercitrin).

Measurement of Elk-1 and NFκB p65 expression

The Nuclear Extraction Kit was purchased from Cayman Chemical Co. (MI, USA) and was used to extract the nuclear fraction of the Caco-2 cells lysate. The expressions of Elk-1 and NFκB p65 in nuclear extract were measured using the Elk-1 Kit and NFκB p65 transcription factor kits, respectively, according to the manufacturer's protocol (Active Motif, CA, USA; Cayman Chemical Co., MI, USA). The expression level of Elk-1 and NFκB p65 was represented as% of normal control.

Statistical analysis

Values are reported as the means±SD. Software (GraphPad software, Inc., La Jolla, USA) was used to analysis the experimental data. One-way ANOVA was performed to access the differences of all groups. $P<0.05$ was considered statistically significant.

Results

The constituent analysis of TFH

As shown in Figure 1, HPLC analysis showed the representative active ingredients in hawthorn extract. The major flavonoids were assigned as chlorogenic acid (1), procyanidin B2 (2), epicatechin (3), hyperoside (4), and isoquercitrin (5).

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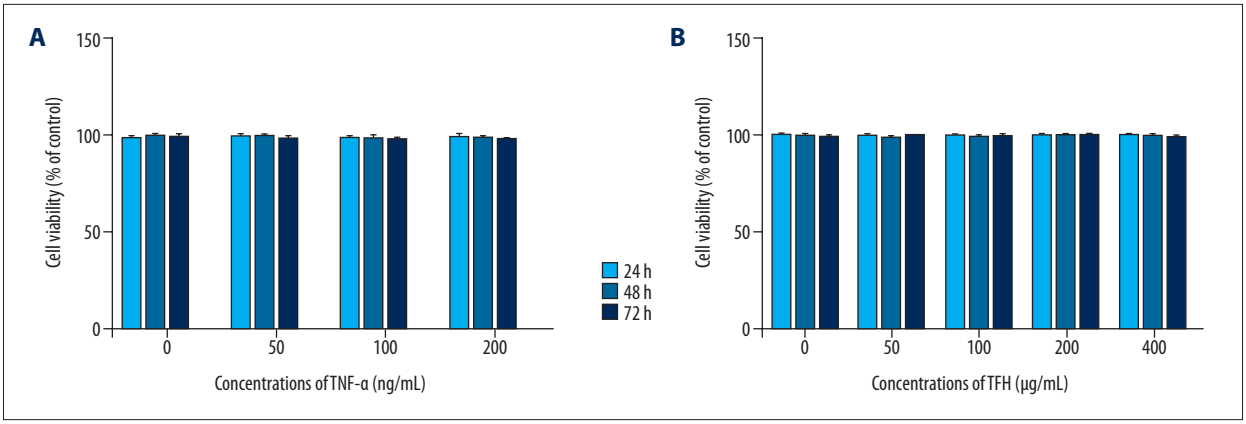

Figure 2. The toxicity effects of TFH and TNF-α on colonic epithelial cells. (**A**) Caco-2 cells were cultured with different concentrations of TNF-α (0-200 ng/mL). (**B**) Caco-2 cells were incubated with different concentrations of TFH (0-400 μg/mL). All results are showed as the mean ± SD (n=6).

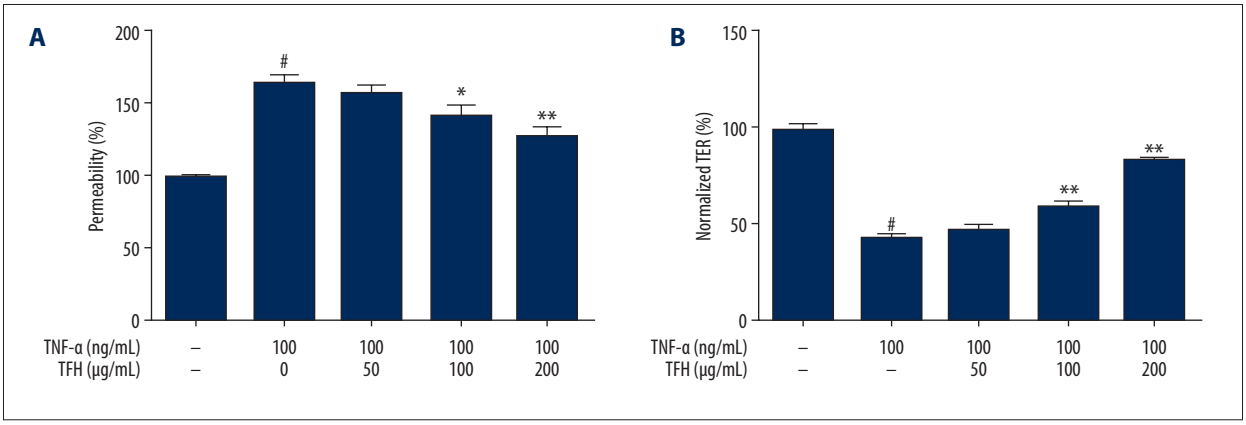

Figure 3. TFH improved inflammatory cytokines-induced intestinal epithelial barrier deficit. (**A**) The paracellular permeability of colonic epithelial cell monolayers. (**B**) The TER of colonic epithelial cell monolayers. Colonic epithelial cells were exposed to TNF-α (100 ng/mL) in the different concentrations of TFH (0-200 μg/mL) for 24 h. Values are expressed as mean±SD, n=6, # $P<0.01$, compared with normal control; * $P<0.05$, compared with TNF-α-challenged colonic epithelial cells; ** $P<0.01$ compared with TNF-α-challenged colonic epithelial cells.

The Coca-2 cells viability measurement

As shown in Figure 2, although the Caco-2 cells treated with the different concentrations of TNF-α (0–200 ng/mL) or TFH (0–400 μg/mL) for 72 h, the viability of colonic epithelial cells was not obviously changed. This finding suggests that the administered doses of TNF-α or TFH have no toxic effect on Caco-2 cells.

TFH alleviates the TNF-α-induced intestinal barrier deficit

As shown in Figure 3A, TNF-α treatment increased the paracellular permeability of cells monolayers, indicating that inflammatory factor could lead to intestinal barrier dysfunction. However, co-administration of TFH inhibited the increase of paracellular permeability in colonic epithelial cell monolayers. Consistent with the changes of paracellular permeability, the value of TER in TNF-α-challenged Caco-2 cell monolayers was obviously lower than that of control Caco-2 monolayers, indicating that TNF-α treatment increased the paracellular permeability of colonic epithelial cell monolayers. However, co-administration of TFH inhibited the decrease of TER in Caco-2 monolayers (Figure 3B). The findings show that TFH can alleviate the intestinal barrier deficit elicited by pro-inflammatory cytokines.

Inhibition effects of TFH on the TNF-α-evoked inflammatory cytokines release

As shown in Figure 4, we found that the stimulation of TNF-α (100 ng/mL) increased the secretion of IL-6, IL-1β, MCP-1, and IL-8 in Caco-2 cells. However, TFH (50-200 μg/mL) treatment inhibited the increase of inflammatory cytokines in a

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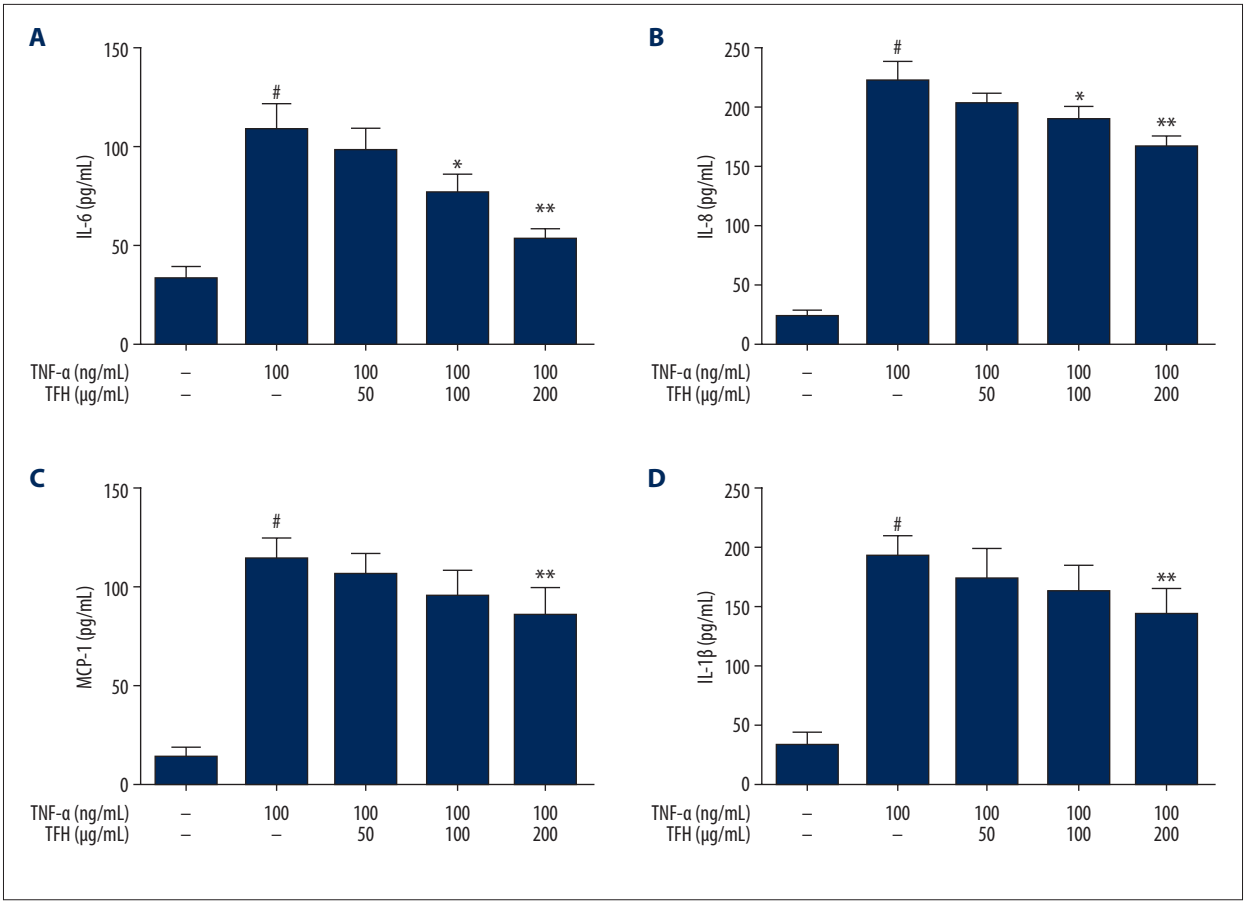

Figure 4. TFH suppressed the inflammatory cytokines-induced release of IL-6 (**A**), IL-8 (**B**), MCP-1 (**C**) and IL-1β (**D**) in colonic epithelial cells. Colonic epithelial cells were exposed to TNF-α (100 ng/mL) in the different concentrations of TFH (0-200 μg/mL). Values are expressed as mean±SD, n=6, # $P<0.01$, compared with normal control; * $P<0.05$, compared with TNF-α-challenged colonic epithelial cells; ** $P<0.01$ compared with TNF-α-challenged colonic epithelial cells.

dose-dependent manner. This shows that TFH can decrease the TNF-α-evoked release of inflammatory cytokines.

Effects of TFH on TNF-α-evoked pMLC and MLCK expression

As shown in Figure 5A, 5B, an obvious increase in pMLC and MLCK expression was observed in colonic epithelial cell monolayers exposed to TNF-α (100 ng/mL) for 24 h ($P<0.01$). However, TFH (200 μg/mL) pretreatment for 24 h inhibited the TNF-α-evoked pMLC and MLCK increase ($P<0.01$). Our findings show that TFH can improve the TNF-α-induced intestinal barrier deficit via suppressing the MLCK-mediated pMLC.

TFH improved TNF-α-induced epithelial barrier deficit via inhibiting Elk-1 and NFκB p65

As shown in Figure 5C, 5D, an obvious increase in Elk-1 and NFκB p65 expression was observed in Caco-2 monolayers exposed to TNF-α (100 ng/mL) for 24 h ($P<0.01$). However, TFH (200 μg/mL) pretreatment for 24 h inhibited the TNF-α-induced Elk-1 and NFκB p65 increase ($P<0.01$). Our findings show that TFH can improve TNF-α-induced intestinal barrier deficit via suppressing the activation of ERK1/2 and NFκB p65 pathways.

The protective effect of TFH on TNF-α-induced TJs proteins

As shown in Figure 6, compared with the normal control, TNF-α treatment decreased the expression of ZO-1 and occludin ($P<0.01$). Administration of TFH (200 μg/mL) restored the expressions of ZO-1 and occludin ($P<0.01$). Our findings indicate that TFH can prevent TNF-α-evoked dysfunction of TJs proteins in Caco-2 cells.

Discussion

Our findings indicate that TFH alleviated pro-inflammatory cytokine-evoked dysfunction of intestinal epithelial barrier in

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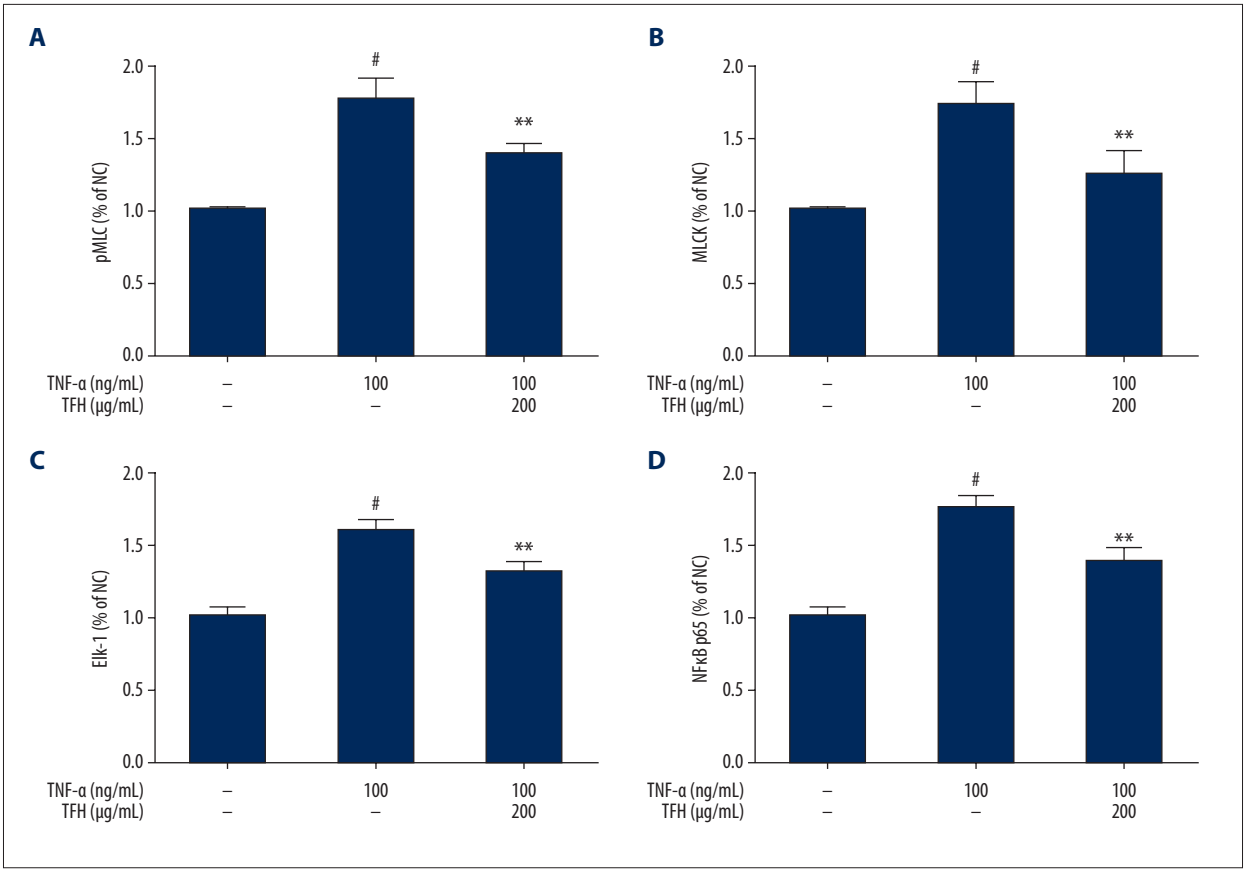

Figure 5. The effects of TFH on the expression of pMLC (**A**), MLCK (**B**), Elk-1 (**C**), and NF-κB p65 (**D**) in inflammatory cytokines-induced colonic epithelial cells. Values are expressed as mean±SD, n=6, # $P<0.01$, compared with normal control; **b $P<0.01$ compared with TNF-α-challenged colonic epithelial cells.

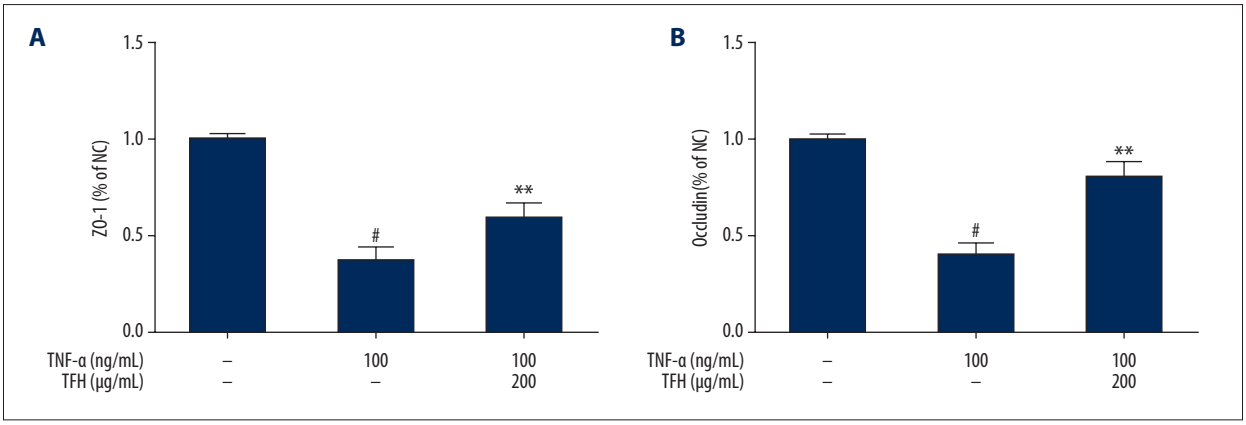

Figure 6. The effects of TFH on the expression of ZO-1 (**A**) and occludin (**B**) in inflammatory cytokines-induced colonic epithelial cells. Values are expressed as mean±SD, n=6, # $P<0.01$, compared with normal control; ** $P<0.01$ compared with TNF-α-challenged colonic epithelial cells.

colonic epithelial cell, as demonstrated by the fact that TFH attenuated both the increase of paracellular permeability and the reduction of TER, and inhibited the increase of pro-inflammatory cytokines in Caco-2 monolayers.

Human health is closely related to intestinal homeostasis. Ulcerative colitis and Crohn's disease are inflammatory bowel diseases characterized by recurring chronic inflammation. Although the etiology of gastrointestinal disorders is incompletely understood, and many theories have attempted to

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elucidate the pathogenesis of those diseases, such as infectious, metabolic, autoimmune, immune and allergic-mediated mechanisms [21]. It is accepted that inflammatory bowel diseases is characterized by epithelial barrier dysfunction and excessive secretion of inflammatory factors in the mucosa [22]. Anti-inflammatory drugs, including amino salicylate, biologics, and immunosuppressants, can cause serious adverse effects [23]. Therefore, the development of effective and safe therapeutic agents for inflammatory bowel diseases is urgently needed. A growing body of research suggests that natural produces derived from edible fruit and food, such as flavonoids and polysaccharides, are potential agents for inflammatory bowel diseases treatment [16,24,25].

The dried fruits of hawthorn have been used as a traditional treatment for dyspepsia. Scientific evidence has indicated the beneficial effects of hawthorn extracts on inflammation and dyspepsia [26,27]. In addition, a previous report has demonstrated that hawthorn fruit has pharmacological activity on murine colitis [28]. These studies are similar to our current findings.

An increasing number of studies indicate that excessive production of inflammatory cytokines induces intestinal epithelial barrier dysfunction and eventually results in impairment of TJs protein integrity [24,29]. Additionally, the imbalance of pro-inflammatory factors, including IL-6, IL-8, and IL-1β, is involved in the progression of the gut inflammatory cascade [24,30]. TJs proteins play an important role in preserving intestinal epithelial barrier integrity by effectively sealing the paracellular pathway of Caco-2 cells [31]. In accordance with these reports, our findings showed that the production of inflammatory cytokines was increased via induction of TNF-α. In addition, TFH effectively suppresses the inflammatory cytokines evoked by TNF-α. Similarly, a previous report has indicated that chlorogenic acid isomers inhibited release of IL-8 via increasing the integrity of Caco-2 cells [32].

However, the underlying mechanism by which TFH improves pro-inflammatory cytokines-induced dysfunction of the intestinal epithelial barrier is still unclear. The present study and previous studies indicate the overexpression of MLCK and upregulation of pMLC are required for induction of intestinal epithelial barrier deficit by TNF-α [30]. Our findings showed that TFH inhibited the upregulation of pMLC and MLCK expression of colonic epithelial cells exposed to TNF-α. Although detailed mechanisms need to be further illuminated, it is implied that TFH can improve TNF-α-evoked intestinal barrier deficit by inhibition of the pMLC pathway and MLCK activation.

Inflammatory factors are considered to activate NF-κB p65. A previous report found that NF-κB p65 activation was involved with intestinal barrier deficit and upregulation of MLCK in TNF-α-treated Caco-2 cells [33]. The MCLK-pMLC signaling pathway is mediated via ERK1/2 and NF-κB [34]. In addition, hyperoside, an active ingredient from hawthorn extracts, suppresses NF-κB p65 activation [35]. Based on these findings, we further investigated whether NF-κB p65 inactivation was involved with alleviating the effect of TFH on inflammatory cytokines-evoked intestinal barrier deficit, showing that inflammatory cytokines-induced Elk-1 and NF-κB p65 activation was suppressed by TFH treatment. Our findings indicate that the underlying mechanism by which TFH improved inflammatory cytokines-induced intestinal barrier deficit was mediated by the ERK1/2 and NF-κB pathways.

Conclusions

The present findings demonstrated that TFH alleviates inflammatory cytokines-induced intestinal barrier deficit and suppresses the production of inflammatory cytokines in Caco-2 cells, potentially via suppressing upregulation of MCLK-pMLC and inhibiting activation of ERK1/2 and NF-κB. Therefore, TFH might be a promising therapeutic agent for the treatment of inflammatory bowel disease.

Conflict of interest

None.

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