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Original Article

Esculin alleviates palmitic acid-induced intestinal barrier damage via Nrf2/HO-1 signaling

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Abstract: Objective: To investigate the effects and mechanisms of esculin in protecting against palmitic acid-induced intestinal barrier disruption in mice. Methods: Thirty-two male BALB/c mice were randomly assigned to four groups: control, model, esculin, and esculin+ML385 groups. A model of intestinal barrier injury was induced by daily gavage of 10 mg/kg palmitic acid for 3 days in all groups except for the control group. The esculin group received 100 mg/kg esculin orally for the same duration, while the esculin+ML385 group was additionally treated with 30 mg/kg ML385 intraperitoneally before each gavage. Disease severity was assessed using the disease activity index (DAI). TNF-α, IL-1β, IL-6, MDA, and T-AOC levels were measured using biochemical assays. mRNA expression of inflammatory and protective markers was determined using qPCR, and the protein levels of occludin, ZO-1, Nrf2, and HO-1 were detected using Western blot. Results: The DAI was significantly lower in the esculin group compared to the model group ($P < 0.001$). Serum TNF-α, IL-6, and IL-1β levels were significantly reduced in the esculin group ($P < 0.001$), while T-AOC increased and MDA decreased ($P < 0.001$). Intestinal mucosa showed elevated levels of TNF-α, IL-1β, ZO-1, Nrf2, HO-1, and occludin in the esculin group ($P < 0.05$), while ML385 reversed these protective effects. Conclusion: Esculin alleviates palmitic acid-induced intestinal barrier damage by activating the Nrf2/HO-1 signaling pathway and inhibiting inflammation, indicating its potential therapeutic role in managing intestinal barrier dysfunction.

Keywords: Esculin, intestinal barrier damage, oxidative stress, inflammatory factor, Nrf2/HO-1

Introduction

The digestive system is essential for nutrient absorption and digestion, serving as a critical barrier to hazardous substances, including bacteria and toxins in the intestinal lumen [1]. The intestinal barrier, composed of intestinal epithelial cells, intercellular tight junctions, and the mucus layer, acts as the primary defense against harmful stimuli [2]. Located at the interface between the body and the intestinal lumen, intestinal epithelial tissue is highly vulnerable to external stimuli such as diet, drugs, or harmful substances, which can trigger the production of reactive oxygen species (ROS). Excessive ROS leads to oxidative stress, compromising intestinal barrier integrity, resulting in epithelial cell apoptosis and structural damage to tight junction proteins [3, 4]. Timely repair of the intestinal barrier is crucial, as its damage can rapidly result in several intestinal illnesses [5]. While chemically synthesized drugs are commonly used to treat intestinal diseases, they often irritate the intestinal tract and cause secondary damage [6]. Thus, exploring the effects of safe, non-toxic natural substances on intestinal damage has become a key research focus. Notably, nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) are recognized for their significant roles in regulating oxidative stress and inflammation. For instance, taurine has been shown to protect the intestinal mucosal barrier in methotrexate-induced injury by modulating the Nrf2/HO-1 pathway [7].

Esculin, also known as hepcidin, is a coumarin-like substance rich in Qinpi and has been shown to possess diverse pharmacological activities [8-12]. Chen et al. [13] demonstrated its efficacy in ameliorating ulcerative colitis in mice, showing that esculin significantly improved clin-

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ical conditions, inhibited cyclooxygenase-2 and inducible nitric oxide synthase, and upregulated Nrf2, thereby exerting notable anti-inflammatory effects. Considering the therapeutic effect of esculin on enteritis, this study preliminarily investigated role of esculin in alleviating palmitic acid-induced intestinal barrier damage and the underlying mechanism.

Material and methods

Animals

Thirty-two male BALB/c (7-8 weeks old, 18-22 g) mice were obtained from Shanghai Sierpikai Laboratory (Production License No. SCXK (Shanghai) 2018-0006). The experimental mice were housed at 25°C±2°C with ad libitum access to food for one week of acclimatization. All animal experiments were conducted following ethical guidelines and were approved by the Institutional Animal Care and Use Committee of Beijing Life River Animal Experiment Co., Ltd. (SMH-149007).

Drugs and reagents

Esculin (purity ≥98%, Article No. ZL09032) was purchased from Chengdu Manstar Biotechnology Co. (Chengdu, China). The Nrf2 inhibitor ML385 (No. S8790) was purchased from Selleck Chemicals (Houston, TX, USA). Assay kits for interleukin (IL)-1β (No. H002-1-1), IL-6 (No. H007-1-1), tumor necrosis factor-α (TNF-α) (No. H052-1-1), hematoxylin-eosin (HE) (No.D006-1-1), malondialdehyde (MDA) (No. A003-1-1), total antioxidant capacity (T-AOC; No. A015-2-1), and ROS (No. E004-1-1) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Primary antibodies, including occludin (No. ab216327), zonula occludens protein 1 (ZO-1; No. ab276131), Nrf2 (No. ab62352), HO-1 (No. ab305290), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (No. 263962), and secondary antibodies (HRP anti-mouse serum albumin antibody; No. ab19195) were purchased from Abcam (Cambridge, MA, USA). SuperSignal™ West Pico PLUS ECL kit (No. 34579) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The reverse transcription kit (No. RR037Q) and reverse transcription PCR (RT-PCR) kit (No. RR014A) were supplied by TaKaRa (Tokyo, Japan).

Animal model and grouping

Mice were randomly divided into four groups (control, model, esculin, and esculin+ML385 groups), with eight mice in each group. Three groups, excluding the control group, were gavaged with 10 mg/kg palmitic acid for 3 days to establish a model of intestinal barrier injury [14], whereas the control group received saline. The esculin group was gavaged with 100 mg/kg esculin [15] daily for 3 days, and the esculin+ML385 group received the same treatment with an additional intraperitoneal injection of 30 mg/kg ML385 [16] before each gavage.

General observations

Daily observations were conducted for each group of mice. The disease activity index (DAI) was calculated using the formula: DAI = (weight loss score + fecal consistency score + bloody stool score)/3 (**Table 1**) [17]. Following euthanasia using (1.0% (w/v) sodium pentobarbital at a dose of 50 mg/kg, the length of the colon of each mouse was measured.

Serum index test

At 24 h post-treatment, 0.5-1.0 mL of blood was collected from the apex of the heart, allowed to clot for 30 min, and centrifuged at 3000 rpm for 10 min to obtain the serum. Serum levels of IL-1β, IL-6, TNF-α, MDA, and T-AOC were measured using assay kits according to the manufacturer's instructions. ELISA kits were employed to quantify IL-1β, IL-6, and TNF-α levels. MDA levels were determined using a TBA assay kit, while T-AOC was evaluated via the ABTS method.

HE staining

The mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Blood samples were obtained via eyeball extirpation for serum preparation by centrifugation, followed by storage at -80°C. After euthanasia, suitable amounts of ileal tissues were collected, fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. HE staining was performed on the pathological sections using a staining kit. Pathological changes in the intestinal mucosal were observed under a microscope (Olympus BX51, Tokyo, Japan).

Table 1. DAI scoring scale

Score	Weight Loss (%)	Fecal Consistency	Blood in Stool
0	0	Normal	Negative
1	1-5	Loose	Occult blood positive (+)
2	6-10	Loose	Occult blood positive (++)
3	11-15	Watery	Occult blood positive (+++)
4	> 15	Watery diarrhea	Grossly bloody stool

DAI, disease activity index.

Table 2. Primer sequences

Gene	Accession Number (NCBI)	5'-3'
IL-1β	NM_008361	F: TGGACCTTCCAGGATGAGGACA R: GTTCATCTCGGAGCCTGTAGTG
TNF-α	NM_013693	F: GGTGCCTATGTCTCAGCCTCTT R: GCCATAGAACTGATGAGAGGGAG
IL-6	NM_031168	F: TACCACTTCACAAGTCGGAGGC R: CTGCAAGTGCATCATCGTTGTTC
Occludin	NM_008756.1	F: TGGCAAGCGATCATACCCAGAG R: CTGCCTGAAGTCATCCACACTC
Zo-1	NM_009386	F: GTTGGTACGGTGCCCTGAAAGA R: GCTGACAGGTAGGACAGACGAT
Nrf2	NM_006164	F: CACATCCAGTCAGAAACCAGTGG R: GGAATGTCTGCGCCAAAAGCTG
HO-1	NM_002133	F: CCAGGCAGAGAATGCTGAGTTC R: AAGACTGGGCTCTCCTTGTTGC
NQO-1	BC000906	F: CCTGCCATTCTGAAAGGCTGGT R: GTGGTGATGGAAAGCACTGCCT
GAPDH	NM_002046	F: GTCTCCTCTGACTTCAACAGCG R: ACCACCCTGTTGCTGTAGCCAA

Note: *IL-1β*, interleukin-1β; *TNF-α*, tumor necrosis factor-α; *IL-6*, interleukin-6; *Zo-1*, Zonula Occludens Protein 1; *Nrf2*, Nuclear factor erythroid 2-related factor 2; *HO-1*, heme oxygenase 1; *NQO-1*, NADH Quinone Oxidoreductase 1; *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase.

Western blot (WB)

Suitable amounts of intestinal mucosal tissue from the ileum were collected, washed with physiological saline, dried, and homogenized in tissue lysis buffer for protein extraction. After determination of protein concentration, 30 μg of each sample was loaded onto polyacrylamide gels for electrophoresis. The proteins were then transferred to nitrocellulose membranes, blocked with 5% skim milk for 1 h, and washed. The membranes were incubated overnight at 4°C with specific primary antibodies against occludin, ZO-1, Nrf2, and HO-1 (diluted at 1:1000) or specific GAPDH antibodies (diluted at 1:5000). The following day, the membranes were incubated with a secondary antibody (diluted at 1:5000) for 1 h. Finally, protein bands were visualized using the ECL method with the Molecular Immer ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA). Band intensities were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The expression levels of occludin, ZO-1, Nrf2, and HO-1 were normalized to GAPDH bands, which served as internal controls.

Fluorescence quantitative PCR (qPCR)

Total RNA was extracted from intestinal mucosal tissues of each group using the Trizol method, and cDNA was synthesized using a reverse transcription kit. The mRNA levels of *IL-1β*, *TNF-α*, *IL-6*, *occludin*, *ZO-1*, *Nrf2*, *HO-1*, and *NQO-1* were amplified using a PCR instrument (Applied Biosystems, Foster City, CA, USA). The reaction system (20 μL) included 10 μL of 2×SYBR Premix Ex Taq™ II Probe qPCR Mix, 8 μL of ddH_2O, 1 μL of cDNA (50 mg/L), and 0.5 μL each of upstream and downstream primers (10 μmol/L). The reaction conditions were as follows: 95°C for 5 min, followed by 40 cycles of 94°C for 15 s and 59°C for 30 s. Relative quantitative analysis was performed using the $2^{-\Delta\Delta Ct}$ method. The sequences of the primers are provided in **Table 2**.

Statistical analysis

Data were analyzed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). In statistical reporting, for data that follow a normal distribution, the collected data were presented as mean ± standard deviation (x±s). For data that do not follow a normal distribution, it is more appropriate to report the median (interquartile range, IQR). For comparisons between multiple groups, one-way ANOVA followed by Tukey's post-hoc test was applied. Normality was assessed using the Shapiro-Wilk test, and

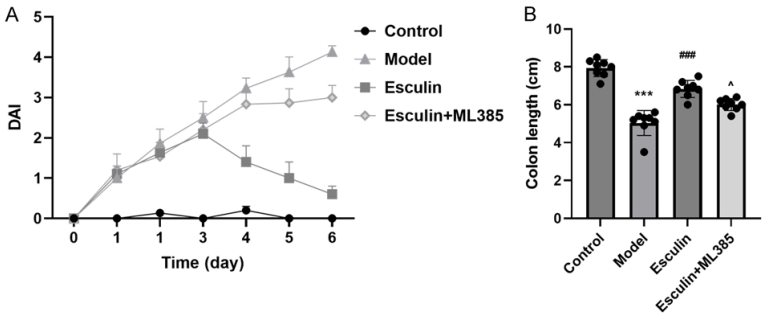

Figure 1. Comparison of DAI (A) and colon length (B) among the four groups of mice (n = 8). $^{***}P < 0.001$, compared with control group; $^{\#\#\#}P < 0.001$, compared with model group; $^{\wedge}P < 0.05$, compared with Esculin group. DAI, disease activity index.

homogeneity of variance was evaluated using Levene's test. Statistical significance was set at $P < 0.05$.

Results

Effects of esculin on DAI and colon length in mice

Compared with the model group, the esculin group showed a significantly lower DAI ($P < 0.001$), indicating reduced disease severity (**Figure 1A**). Conversely, DAI in the esculin+ML385 group was significantly higher than in the esculin group ($P < 0.05$), suggesting a worsening of disease activity. The esculin group demonstrated a notable increase in colon length compared to the model group ($P < 0.01$), signifying a protective effect on intestinal structure (**Figure 1B**). In contrast, colon length in the esculin+ML385 group was significantly shorter than in the esculin group ($P < 0.05$), indicating partial attenuation of esculin's protective effect.

Regulatory effects of esculin on inflammatory factors in murine intestines

Compared with the control group, the serum levels of IL-1β, IL-6, and TNF-α significantly increased in the model group ($P < 0.001$). Treatment with esculin significantly reduced these serum levels compared to the model group ($P < 0.001$). However, the esculin+ML385 group showed higher serum levels of IL-1β, IL-6, and TNF-α compared to the esculin group ($P < 0.001$). The details are demonstrated in **Figure 2A**. Correspondingly, the mRNA expression levels of these cytokines in the intestinal mucosal tissue showed similar expression patterns, as illustrated in **Figure 2B**.

Effects of esculin on oxidative stress markers

As shown in **Figure 3**, the model group showed significantly elevated levels of MDA and ROS compared to the control group ($P < 0.001$), indicating increased oxidative stress. Treatment with esculin significantly reduced MDA and ROS levels compared to the model group ($P < 0.01$). However, in the esculin+ML385 group, MDA and ROS levels were significantly higher than in the esculin group ($P < 0.05$), suggesting that ML385 mitigated the antioxidative effects of esculin.

The model group demonstrated a significant decrease in T-AOC compared to the control group ($P < 0.05$), reflecting impaired total antioxidant capacity. Esculin treatment significantly increased T-AOC levels compared to the model group ($P < 0.001$). Conversely, T-AOC levels in the esculin+ML385 group were significantly lower than in the esculin group ($P < 0.001$).

Pathological changes of the intestinal mucosal barrier in mice affected by esculin

As shown in **Figure 4**, The colonic mucosa of control mice displayed a regular epithelial structure with normal goblet cell distribution and no inflammatory infiltration. By contrast, the model group exhibited significant mucosal damage, characterized by a marked reduction in goblet cells and inflammatory cell infiltration. Esculin treatment significantly improved the intestinal tissue structure and goblet cell arrangement. However, the esculin+ML385 group showed worse histomorphology, with fewer goblet cells and increased inflammatory cells, compared with the controls.

Tight junction proteins in the intestinal mucosa affected by esculin

In the model group, both protein (**Figure 5A**) and mRNA (**Figure 5B**) levels of occludin and ZO-1 were lower than those in the control (both $P < 0.001$), indicating compromised intestinal

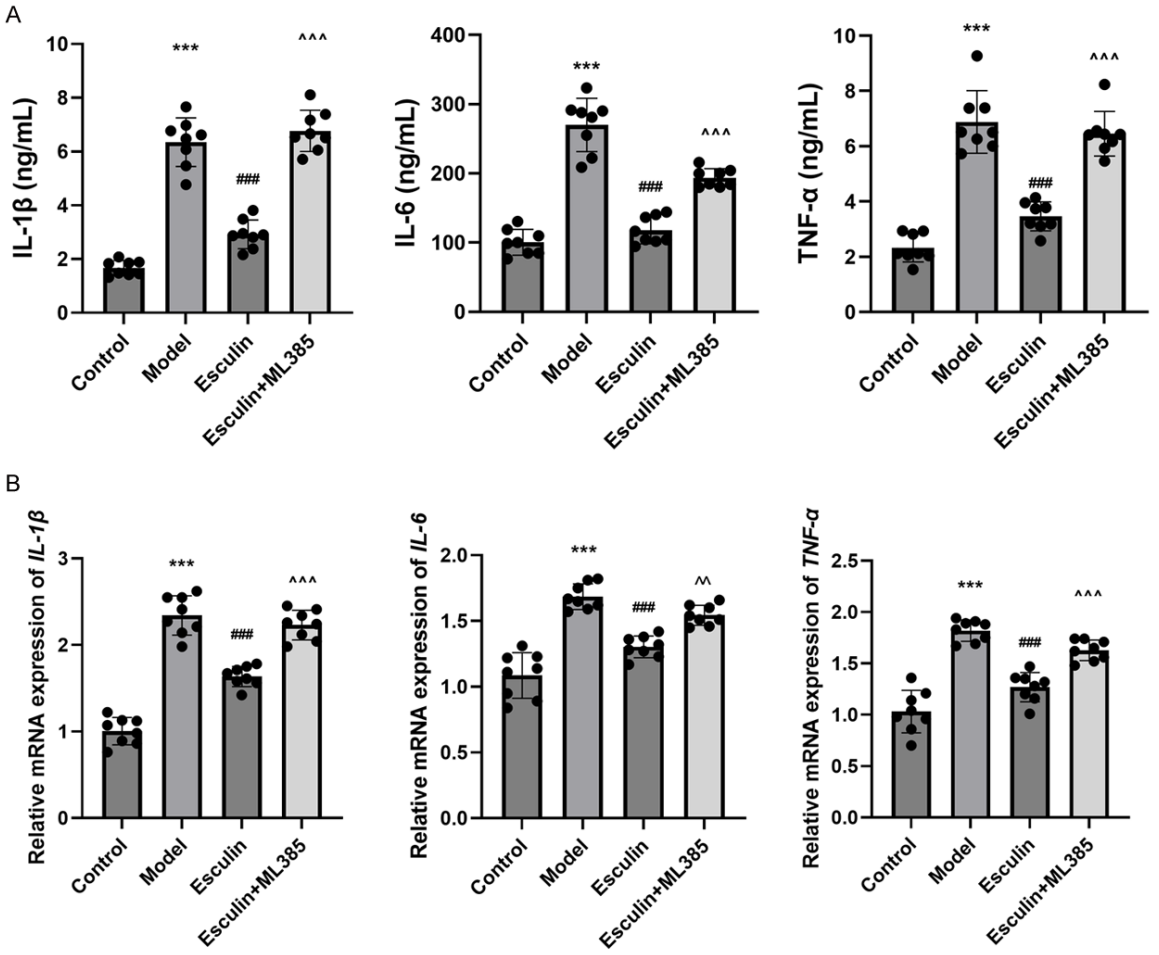

Figure 2. Comparison of serum (A) and mRNA levels (B) of interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α) in intestinal mucosal tissues among the four groups (n = 8). $^{***}P < 0.001$, compared with control group; $^{\#\#\#}P < 0.001$, compared with model group; $^{\wedge\wedge}P < 0.01$, $^{\wedge\wedge\wedge}P < 0.001$, compared with esculin group.

tight junction integrity. In the esculin group, the expression levels of occludin and ZO-1, both at the protein and mRNA levels, were significantly higher compared to the model group ($P < 0.001$), suggesting that esculin enhances the integrity of the intestinal mucosal barrier. However, in the esculin+ML385 group, the expression levels of occludin and ZO-1 were significantly reduced compared to the esculin group ($P < 0.05$), indicating that ML385 antagonized the beneficial effects of esculin on tight junction protein expression.

Effect of esculin on Nrf2 pathway in the intestinal mucosa

In the model group, both the protein (**Figure 6A**) and mRNA (**Figure 6B**) expression levels of the Nrf2 pathway related molecules were lower than those in the control group ($P < 0.001$), indicating impaired activation of the Nrf2-mediated antioxidant defense pathway. Those molecules in the esculin group, both at mRNA and protein levels, were significantly higher than those of the model group ($P < 0.05$). However, in the esculin+ML385 group, the levels of Nrf2 were significantly reduced compared to the esculin group ($P < 0.05$), indicating that ML385 inhibited the Nrf2 pathway activation induced by esculin ($P < 0.05$).

Discussion

The gut serves as both a digestive and absorptive organ and a major immune organ, with a larger lymphoid presence than the reticuloendothelial system, liver, and spleen [18]. Damage to the intestinal barrier can result in severe out-

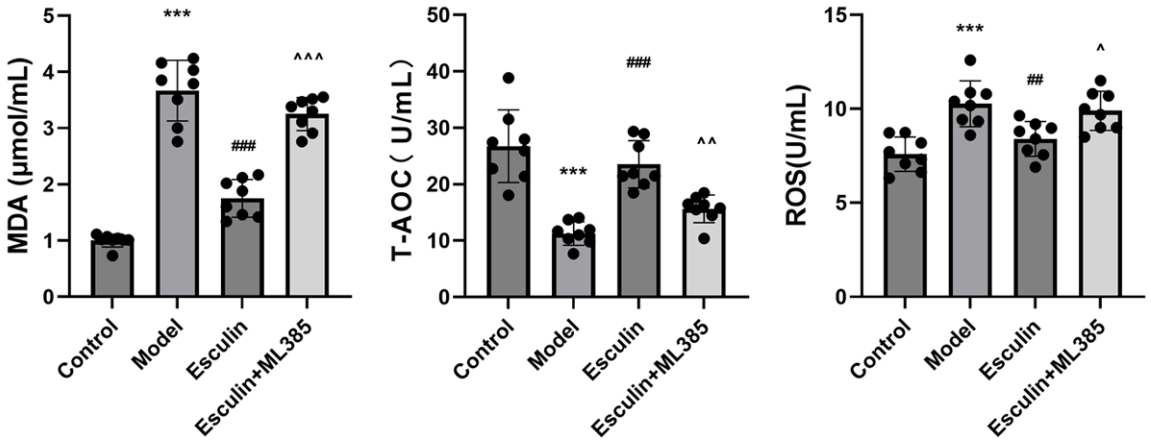

Figure 3. Comparison of serum malondialdehyde (MDA), total antioxidant capacity (T-AOC), and reactive oxygen species (ROS) levels among the four groups of mice (n = 8). $^{***}P < 0.001$, compared with control group; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with model group; $^{\wedge}P < 0.05$, ^^P < 0.05, $^{\wedge\wedge\wedge}P < 0.001$, compared with esculin group.

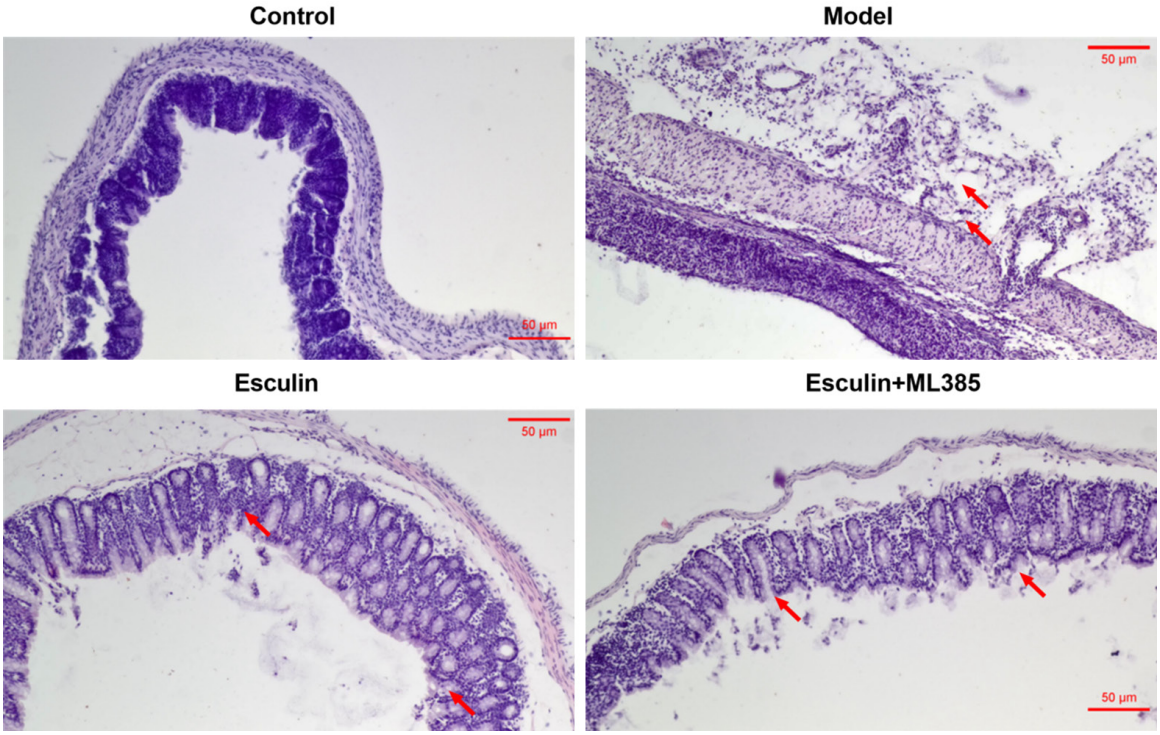

Figure 4. HE staining of the intestinal mucosa in the four groups of mice (200×).

comes such as intestinal failure and bacterial displacement, potentially leading to life-threatening multi-organ failure [19]. This study established a palmitic acid-induced mouse model of intestinal barrier injury and investigated the role of esculin in enhancing intestinal mucosal barrier function via the Nrf2/HO-1 pathway.

Previous studies have highlighted that the imbalance between anti-inflammatory and inflammatory factors is closely linked to the onset and progression of ulcerative colitis. The disease progression can be assessed by measuring inflammatory factors, such as IL-1β, IL-6, and TNF-α levels in serum [20, 21]. In this study, esculin reduced the inflammatory cytokines IL-1β, IL-6, and TNF-α in mice with intestinal barrier injury, suggesting that esculin could alleviate the inflammatory response to such injury. Intestinal epithelial cells and tight junc-

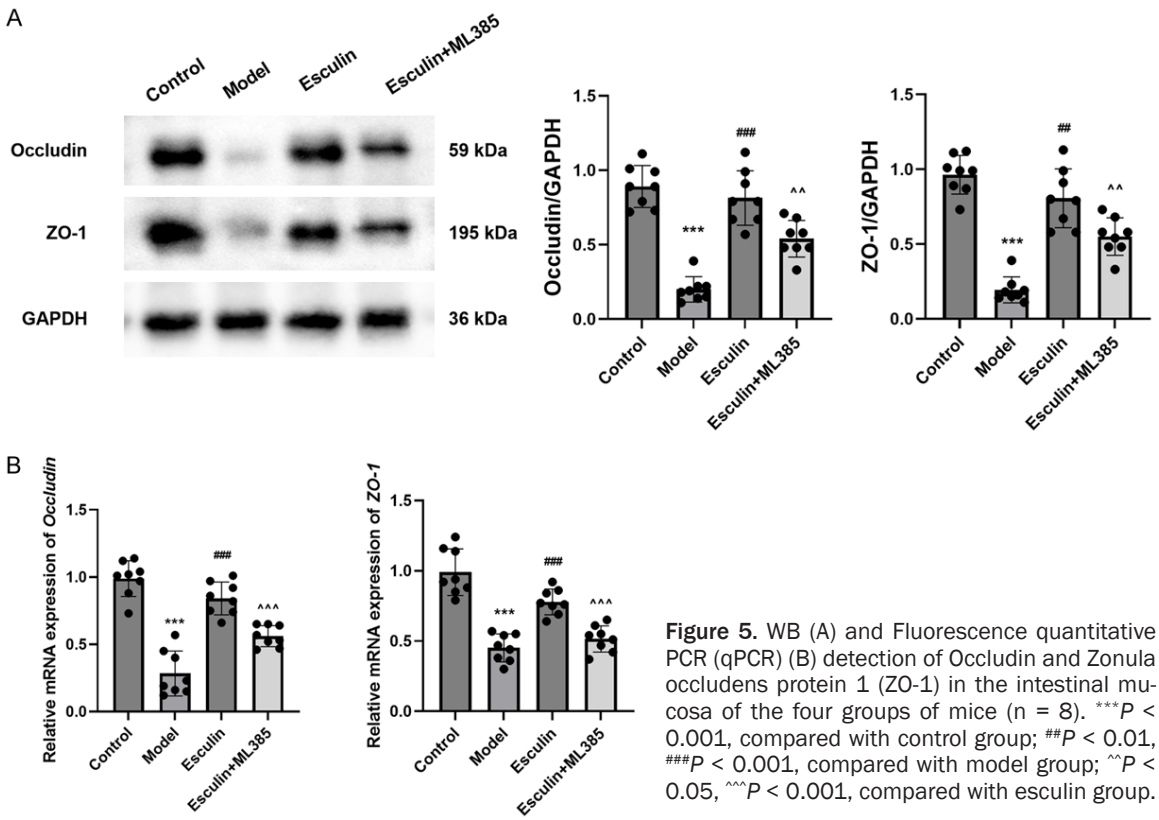

Figure 5. WB (A) and Fluorescence quantitative PCR (qPCR) (B) detection of Occludin and Zonula occludens protein 1 (ZO-1) in the intestinal mucosa of the four groups of mice (n = 8). $^{***}P < 0.001$, compared with control group; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with model group; $^{\wedge\wedge}P < 0.05$, $^{\wedge\wedge\wedge}P < 0.001$, compared with esculin group.

tion proteins, including occludin and ZO-1/2/3, are crucial for maintaining mucosal integrity. Reduced expression of Occludin has been observed in animal models of inflammatory bowel disease, highlighting its importance in preserving the mucosal barrier [22, 23]. In this study, esculin increased the expression levels of occludin and ZO-1 in the intestinal mucosa of mice with intestinal barrier injury, indicating its potential to restore mucosal barrier function. Oxidative stress adversely impacts the intestinal barrier by generating excess free radicals, which can degrade key functional proteins and disrupt tight junctions, leading to increased mucosal permeability [24, 25]. Furthermore, oxidative stress stimulates the immune system, leading to immunosuppression and dysfunction [26]. In this study, esculin decreased the serum MDA content and increased the T-AOC content in mice with intestinal barrier injury, suggesting that esculin could alleviate the oxidative stress response to intestinal barrier injury.

The Nrf2/HO-1 pathway is an important antioxidant response pathway regulating the expression of a variety of antioxidant enzymes and enzymes involved in phase II detoxification [27]. Nrf2/HO-1-mediated anti-oxidative stress mechanisms have been shown to be effective in attenuating intestinal barrier damage [20, 28]. In this study, the level of Nrf2/HO-1 pathway associated molecules in the intestinal mucosa increased in the esculin group, which aligns with the study conducted by Chen et al. [13]. These results suggest that esculin activates the Nrf2/HO-1 pathway, contributing to its therapeutic effects. To further investigate this hypothesis, we established an esculin+ML385 group, in which the Nrf2 inhibitor ML-385 was administered intraperitoneally prior to esculin gavage. This intervention resulted in significantly increased inflammatory responses and oxidative stress in the intestinal barrier-injured mice, confirming that the activation of the Nrf2/HO-1 pathway is integral to the therapeutic effects of esculin in intestinal barrier damage.

Conclusion

In this study, our findings lay the groundwork for the potential use of esculin in the treatment of intestinal barrier disorders. The results suggest

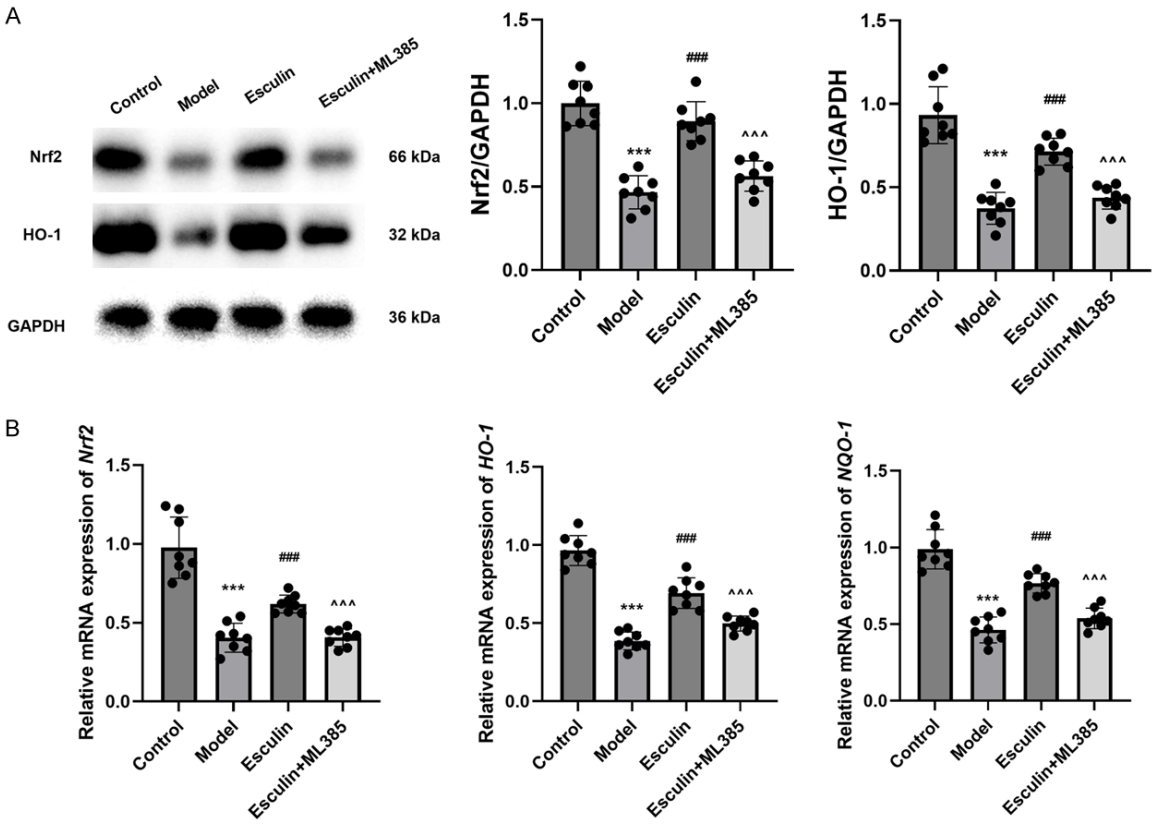

Figure 6. WB (A) and qPCR (B) detection of nuclear factor erythroid 2 (Nrf2) pathway related molecules in the four groups of mice (n = 8). $^{***}P < 0.001$, compared with control group; $^{\#\#\#}P < 0.001$, compared with model group; $^{\wedge\wedge\wedge}P < 0.001$, compared with esculin group.

that esculin can alleviate oxidative stress and inflammation associated with intestinal barrier injury, enhance the levels of occludin and ZO-1 in mice with compromised intestinal barriers, and improve intestinal barrier function. The underlying mechanism appears to involve the activation of the Nrf2/HO-1 pathway.

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Disclosure of conflict of interest

None.

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