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# *Allomyrina dichotoma* larval extract attenuates intestinal barrier disruption by altering inflammatory response and tight junction proteins in lipopolysaccharide-induced Caco-2 cells

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ABSTRACT

Intestinal epithelial cells form a barrier between the intestinal lumen and host connective tissues and play an important role in maintaining intestinal nutrient homeostasis. This study investigated effects of *Allomyrina dichotoma* (rhinoceros beetle) larval extract (ADLE) on the intestinal barrier damage and explored mechanisms for reversing intestinal barrier dysfunction in lipopolysaccharide (LPS)-stimulated Caco-2, human intestinal epithelial cells. LPS reduced intestinal epithelial barrier function by increasing transepithelial electrical resistance, and this effect was significantly attenuated by ADLE treatment. ADLE also significantly countered the inhibition of tight junction-related protein expression in both LPS-induced Caco-2 cells and intestine from HFD-induced mice. Moreover, ADLE significantly decreased expression and production of inflammatory factors, such as iNOS, cox-2, nitric oxide, and cytokines induced by LPS stimulus. Reduction in phosphorylation of adenosine monophosphate-activated protein kinase was averted by ADLE treatment in LPS treated INS-1 cells. Finally, reactive oxygen stress level was decreased and ATP production was increased by ADLE treatment. ADLE appears to display gut health-promoting effects by reducing inflammation and inducing tight junction proteins in Caco-2 cells. Therefore, ADLE might be useful for preventing or treating intestine cell damage in inflammatory bowel disease.



## 1. Introduction

The incidence and prevalence of inflammatory bowel diseases (IBDs) are increasing worldwide [1]. The pathogenesis of IBDs are still unclear, though immune dysfunction, infection, and gut microbiota are likely involved [2]. Treatment of IBDs is limited and includes corticosteroids, immunosuppressive agents and some biological agents [3]. All are associated with limitations and side effects. New drugs and alternative treatment strategies encompassing natural sources of medicines are therefore needed.

The intestinal mucosal barrier binds to the surface of epithelial cells and blocks entry of microbial toxins and other substances into the bloodstream [4,5]. The epithelial barrier is maintained mainly by apical multi-protein complexes termed tight junction (TJ) proteins [6,7]. TJs are composed of three crucial proteins, zonula occludens-1 (ZO-1), occludin, and claudins. Impairment of the TJ barrier by pathogens contributes to the progression of IBD via increased intestinal permeability [8]. Moreover, impaired intestinal permeability is an important factor for the pathophysiology of inflammation associated with chronic diseases, such as diabetes and obesity [9,10].

Insects are proposed as a promising alternative source of proteins, and their value as food is garnering more attention. Traditionally, *Allomyrina dichotoma* (Korean rhinoceros beetle) larvae (ADL) are an accepted edible insect in South Korea. The larvae are also widely used for their anti-hepatofibrotic, anti-neoplastic, anti-inflammatory and anti-obesity properties [11]. Based on our

*Abbreviations:* HFD, High-fat diet; IBD, Inflammatory bowel disease; NFD, Normal fat diet; ROS, Reactive oxygen species; TJ, Tight junction.

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previous studies, an ADL extract (ADLE) may ameliorate hepatic insulin resistance through inhibition of hepatic lipogenesis in high-fat diet (HFD) fed mice [12]. In this study, we investigate the effect of ADLE on intestinal permeability and elucidate mechanisms in LPS treated Caco-2 cells.

## 2. Materials and methods

### 2.1. Preparation of A. dichotoma larva extract (ADLE)

*A. dichotoma* larvae (ADL) were purchased from Yechun Bugs land (Yecheon-gun, Gyungsangbuk-do, Korea). ADL was extracted as previously described [12].

### 2.2. Cell culture and cytotoxicity

Caco-2 cells were grown at 37 °C and 5% $CO_2$ in MEM medium containing 25 mM HEPES (Welgene, Daegu, Korea), 10% FBS (Gibco, Paisley, UK), and 1% penicillin-streptomycin (Welgene). For cytotoxicity, MTT (thiazolyl blue) was dissolved in 2-propanol (Duchefa Biochemie BV, Haarlem, Netherlands) at a concentration of 0.5 mg/mL and added to Caco-2 cells. After incubation for 2 h, absorbance was recorded at 540 nm (TECAN Group Ltd, Shanghai, China).

### 2.3. Transepithelial resistance

Transepithelial resistance (TEER) was measured to assess barrier integrity of Caco-2 cells. Cells were seeded in 12-well plates at a density of $1.0 \times 10^5$ cells/well. Cells were incubated for 0, 2, 4, 6, and 8 h with or without LPS (1 μg $mL^{-1}$) in the medium. TEER values were measured using an ohmmeter with chopstick electrodes (EVOM2, World Precision Instruments, Sarasota, FL, USA).

### 2.4. Nitrite, ROS, and ATP assay

Cells at a density of $2.5 \times 10^4$ cell/well were cultured in LPS (1 μg $mL^{-1}$) with or without ADLE in opaque black 96-well plates for 48 h. Nitric oxide (NO) in media was measured as previously described [12]. Intracellular reactive oxygen species (ROS) were measured using 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Invitrogen). ATP levels in the cells were measured with the PerkinElmer ATPLite system according to the manufacturer's instructions by luminometer (TECAN).

### 2.5. Western blotting

Immunoblotting was performed as previously described [12]. Briefly, 20–40 mg of proteins were separated by 6% or 10% SDS–PAGE, transferred onto nitrocellulose membranes (Amersharm, GE Healthcare Life science, Germany) and incubated with primary antibodies, washed with TBST, and incubated with secondary antibodies. Protein bands were detected using an ELC kit (Millipore, USA). Bands were normalized to β-actin and quantified by Quantity 1 version 4.6.7 software (Bio-Rad).

### 2.6. Quantitative RT-PCR

Total RNA extracted by Trizol reagent (Invitrogen, Grand Island, NY, USA) was reverse transcribed with a cDNA synthesis kit (Takara Bio Inc., Shiga, Japan). Reactions were performed on an ABI real-time PCR system from Applied Biosystem Inc. (Forster City, CA) using SYBR Premix Ex Taq II, ROX plus (Takara Bio Inc., Shiga, Japan) as directed by the manufacturer. Amplification was performed as follows: 10 min at 90 °C, 15 s at 95 °C, and 1 min at 60 °C for a total of 40 cycles. Gene-specific primers are listed in Table 1.

### 2.7. Animal studies

Four-week-old C57BL/6J male mice were obtained from the Korea Research Institute Bioscience & Biotechnology (Daejeon, Korea). After one week of adaptation, HFD (60% fat, D12492; Research Diets, New Brunswick, NJ, USA, n = 16) and the normal fat diet (NFD, 4.5% fat, Purina, n = 6) were provided to mice for six weeks. Mice were then treated with ADLE orally (100 mg/kg/day, n = 8) or vehicle (distilled water, n = 8) once a day for six weeks. At the end of the experimental period, intestines were removed for morphological analysis and TJ-related protein analysis. All animal procedures were approved by the Institution Animal Care and Use Committee at Eulji University (EUIACUC-18-7).

### 2.8. Immunohistochemistry

Paraffin-embedded small intestine sections (4 μm) were fixed in acetone, deparaffinized, and stained in hematoxylin and eosin. For the immunohistochemistry, sections were incubated with specific primary antibodies, anti-occludin (1:1000; Invitrogen, USA), anti-claudin-1 (1:1000; Invitrogen), and anti-ZO-1 (1:1000; Invitrogen). Slides were imaged with an Olympus DP70 digital camera (Olympus Co., Tokyo, Japan) and Olympus BX61 microscope (Olympus Co., Tokyo, Japan).

### 2.9. Statistical analysis

Statistical analysis was performed using SPSS 20.0 software (IBM SPSS ver. 20.0.0 for Windows; IBM Co., Armonk, NY, USA). Results are presented as mean ± standard deviation. Significance of differences among groups was analyzed by LSD comparisons tests. Statistical significance was set at $p < 0.05$.

## 3. Results

### 3.1. ADLE protects against permeabilization of LPS-induced Caco-2 cells

Optimal concentrations of ADLE from the treatment of Caco-2 cells were determined by examining cell viability after exposure of cells to various concentrations (0.1–4 mg/mL) of ADLE. No significant reduction in cell viability was observed at any concentration (Fig. 1A). Based on this result and our previous studies [12], 0.5 mg/mL ADLE was used in Caco-2 cells. Next, the effects of ADLE on intestinal epithelial barrier function impaired by LPS treatment were examined with TEER measured in Caco-2 cell monolayers. Time zero TEER (0 h) values were measured before ADLE treatment, and time-dependent TEER values at 2, 4, 6 and 8 h was measured after LPS exposure with or without ADLE. As a result, LPS treated cells showed a significant increase in permeabilization of Caco-2 cells, evidenced by TEER values significantly decreased to 78% of controls after 8 h of treatment. ADLE treated cells protected the Caco-2 cell monolayer, indicated by significantly increased TEER values compared with LPS treated cells (Fig. 1B). Finally, expression of TJ-related proteins, ZO-1, occludin and claudin-1, altered by LPS and ADLE treatments were measured by Western blotting. Levels of all three proteins were significantly reduced by LPS treatment, but ADLE treatment significantly ameliorated decreases of TJ-related proteins (Fig. 1C).

### 3.2. ADLE attenuated inflammatory response in LPS-induced Caco-2 cells

Anti-inflammatory effects of ADLE were assessed by examining the production of inflammatory cytokines and NO after ADLE

**Table 1**
Primer sequences for real-time PCR.

| Gene | Forward (5′–3′) | Reverse (5′–3′) |
|---|---|---|
| TNF-α | 5′- TGCTCCTCACCCACACCAT-3′ | 5′- GGAGGTTGACCTTGGTCTGGTA-3′ |
| IL-6 | 5′- GCTGCAGGCACAGAACCA-3′ | 5′-TAAAG TGCGCAGAATGAGATG -3′ |
| IL-1β | 5′-ACGATGCACCTGTACGATCACT-3′ | 5′-CACCAAGCTTTTTTGCTGTGAGT-3′ |
| IFNγ | 5′-ACTCATCCAAGTGATGGCTGAA-3′ | 5′-TCCTTTTTCGCTTCCCTGTTT-3′ |
| Cyclophilin | 5′-TGCCATCGCCAAGGAGTAG-3′ | 5′-TGCACAGACGGTCACTCAAA-3′ |

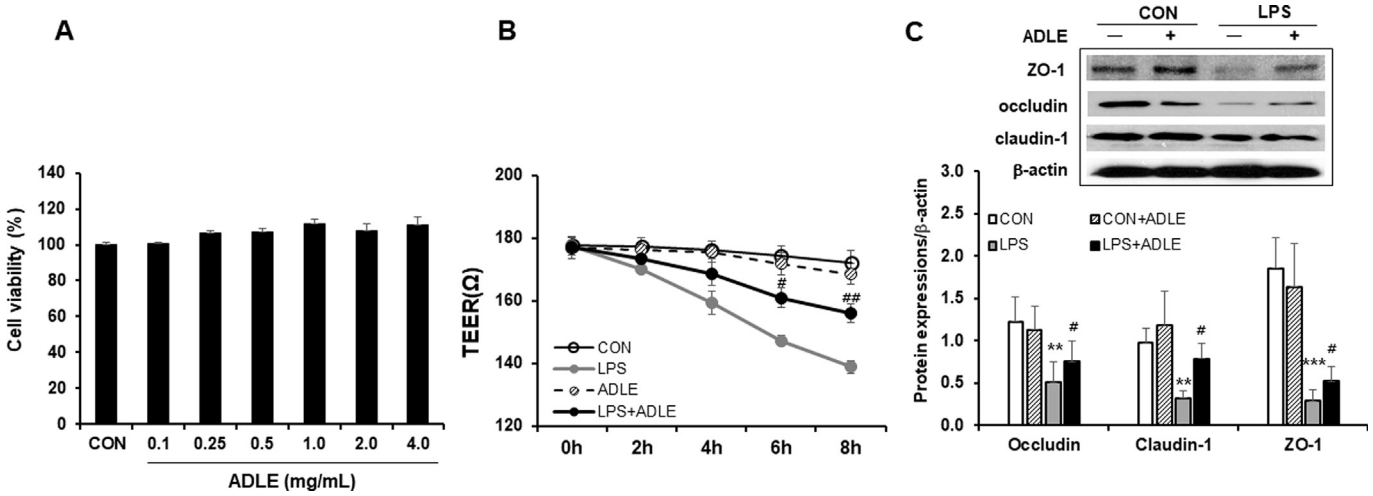


**Fig. 1.** ADLE prevents reduction of transepithelial electrical resistance and regulates tight junction proteins in LPS-induced Caco-2 cells. (A) The viability of Caco-2 cells was evaluated by MTT. (B) Representative immunoblot and quantification of TJ protein expression in Caco-2 cells. (C) TEER values for Caco-2 monolayers treated with LPS up to 8 h in 2 h intervals. Data represent mean ± SD (n = 3–5). $^{**}p < 0.01$, $^{***}p < 0.001$ versus the CON group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus the LPS group.

treatment of Caco-2 cells. Protein expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (Cox-2) were increased by LPS treatment, and these levels were significantly reduced by ADLE cotreatment (Fig. 2A). NO production induced by LPS was also attenuated (Fig. 2B). LPS stimulation increased mRNA expression of pro-inflammatory cytokines, such as TNFα, IL-6, IFNγ and IL-1β in Caco-2 cells compared with controls. This increased expression was significantly attenuated by ADLE treatment (Fig. 2C).

### *3.3. ADLE prevented a decrease of phosphorylation of AMPK and rescued ROS production and ATP levels in LPS-induced Caco-2 cells*

AMPK plays a key role in maintaining homeostasis of the intestinal barrier during proliferation and differentiation [13], and we investigated possible mechanisms for ADLE actions on intestinal barrier function. Cotreatment with ADLE significantly blocked the decrease in phosphorylation of AMPK caused by LPS (Fig. 3A). AMPK activation by ADLE affects ROS production and cellular ATP levels in LPS-induced Caco-2 cells. Treatment with LPS increased ROS production, but the level was significantly reduced by ADLE cotreatment (Fig. 3B). Moreover, ADLE significantly countered the decrease in ATP levels induced by LPS treatment (Fig. 3C).

### *3.4. ADLE promoted tight junction-related protein expression in HFD induced mice*

HFD induces low-grade intestinal inflammation in mice [14,15], and we investigated ADLE effects on the expression of TJ-related proteins in intestine of HFD fed mice. Mice fed NFD showed strong TJ protein staining, with homogeneous distribution in enterocytes. Stained cells were observed at a low level in HFD animals. However, ADLE treatment significantly increased the expression of ZO-1, occludin and claudin-1 compared with HFD mice (Fig. 4A and B). Western blotting showed that an HFD reduced TJ protein expression by 33% for ZO-1, 44% for occludin, and, significantly, by 79% for claudin. Levels of these proteins recovered to normal after ADLE treatment.

## 4. Discussion

Impairment of intestitnal epithelial TJ formation by pathogens is an important factor in determining gut permeability [8]. Penetration of luminal noxious agents via disrupted TJs induces perturbation of the mucosal immune system and inflammation that contributes to the progression of intestinal disease. ADLE significantly improved gut integrity via increased expression of TJ-related proteins in LPS-induced Caco-2 cells and confirmed these results in mice fed an HFD.

Chronic inflammation upregulates signaling proteins and enzymes of iNOS and COX-2, as well as NO and prostaglandins (PG). This expression leads to various disease associated with inflammation [16,17]. LPS-induced NO production was significantly inhibited in Caco-2 cells by treatment with ADLE, suggesting a reduction in inflammation. Moreover, patients with IBDs appear to have significantly higher levels of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 [18] and blocking the production of these signals may be an effective treatment for IBDs. Cytokines regulate the expression of TJ proteins. In particular, TNF-α causes downregulation of ZO-1, occludin and claudin-1 protein expression in Caco-2 cells [19]. We confirmed that ADLE reduces inflammation in LPS-induced Caco-2 cells through regulation of pro-inflammatory mediators, such as COX-2, and iNOS. Therefore, our findings of ADLE inhibition of NO signaling and expression of

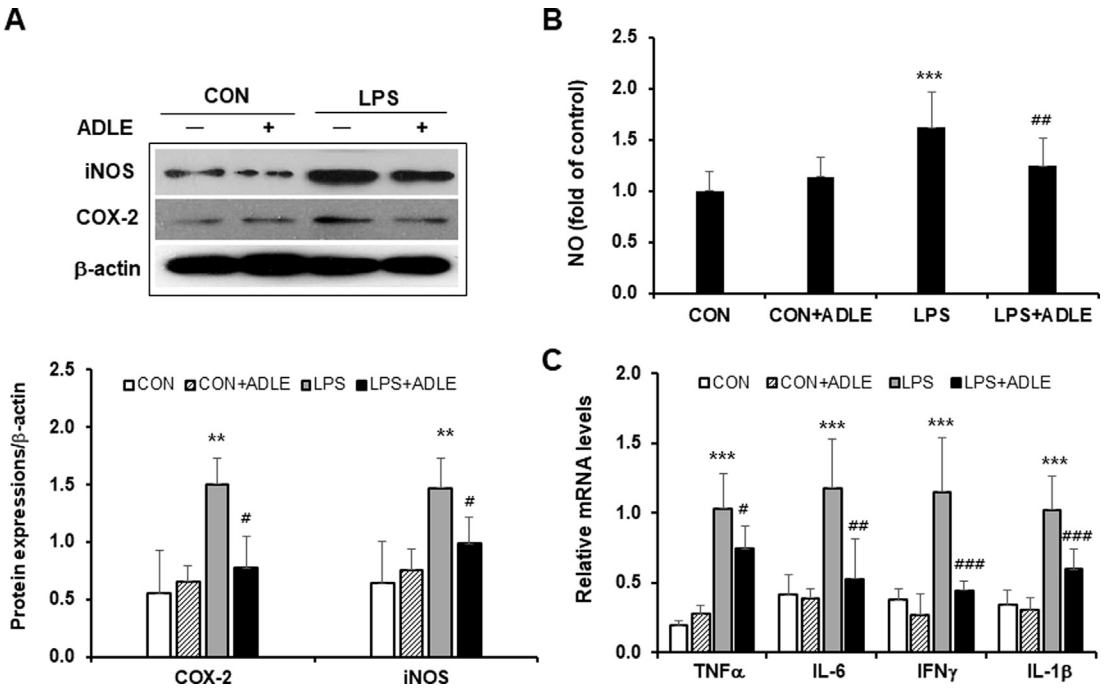


**Fig. 2.** ADLE suppresses inflammation-related factors in LPS-induced Caco-2 cells. (A) Representative immunoblot and quantification of Cox-2, and iNOS expression in Caco-2 cells. (B) NO content was assayed using griess reagent. (C) Cytokine expression was measured by qRT-PCR. Data represent mean ± SD (n = 3–5). $^{**}p < 0.01$ and $^{***}p < 0.001$ versus the CON group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ versus the LPS group.

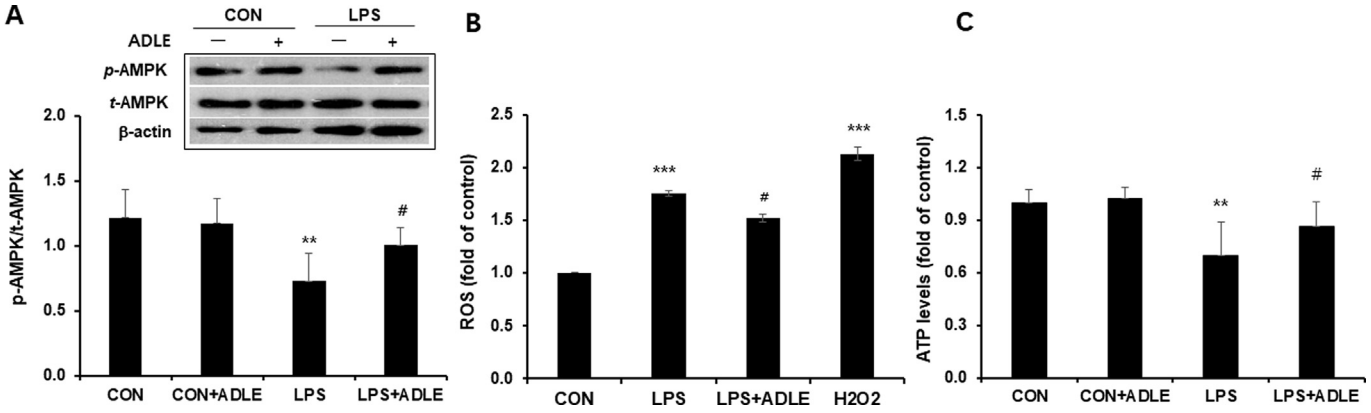


**Fig. 3.** ADLE activates AMPK phosphorylation, increases ATP levels, and reduces oxidative stress in LPS-induced Caco-2 cells. (A) Representative immunoblot and quantification of AMPK in Caco-2 cells. (B) Intracellular ROS was measured by FACS. (C) Intracellular ATP level was measured with an ATP determination kit. Data represent mean ± SD (n = 3–5). $^{**}p < 0.01$ and $^{***}p < 0.001$ versus the CON group; $^{\#}p < 0.05$ versus the LPS group.

cytokines could support beneficial impacts on gut health.

The critical role of AMPK activation in strengthening epithelial TJs are reported. AMPK is crucial for assembly of TJ proteins in cell-cell junctions, and initiation of TJ assembly is impaired in cells lacking AMPK activation [20,21]. Some short chain fatty acids are reported to enhance intestinal barrier function through activation of AMPK. The underlying mechanism is still not clear, but we obtained results consistent with previous reports on AMPK activation by ADLE treatment in LPS-induced Caco-2 cells [22,23]. Reduction of AMPK expression also causes abnormal oxidative stress [24]. Our results suggest that ADLE can regulate AMPK activation and ATP levels for intracellular anabolic metabolism, to support intestinal epithelial barrier tight junction protein synthesis. Biologically active constituents of ADLE are now characterized, and we are currently working to isolate antioxidants among these chemicals.

Long-term consumption of a HFD generates chronic inflammation that interferes with intestinal barrier function, leading to metabolic diseases, such as obesity and diabetes [9,10]. We confirmed that ADLE strengthens weakened cellular barriers caused by hyperglycemia *in vivo* by augmenting TJs and adherent junction protein abundance in HFD mice. Thus, supplementation with ADLE may protect the intestine from damaging effects caused by enteropathogenic bacterial toxins.

In conclusion, ADLE ameliorates intestinal barrier dysfunction by improving pro-inflammatory factors and regulating TJ proteins. Our results suggest a possible benefit of ADLE as a supplement for

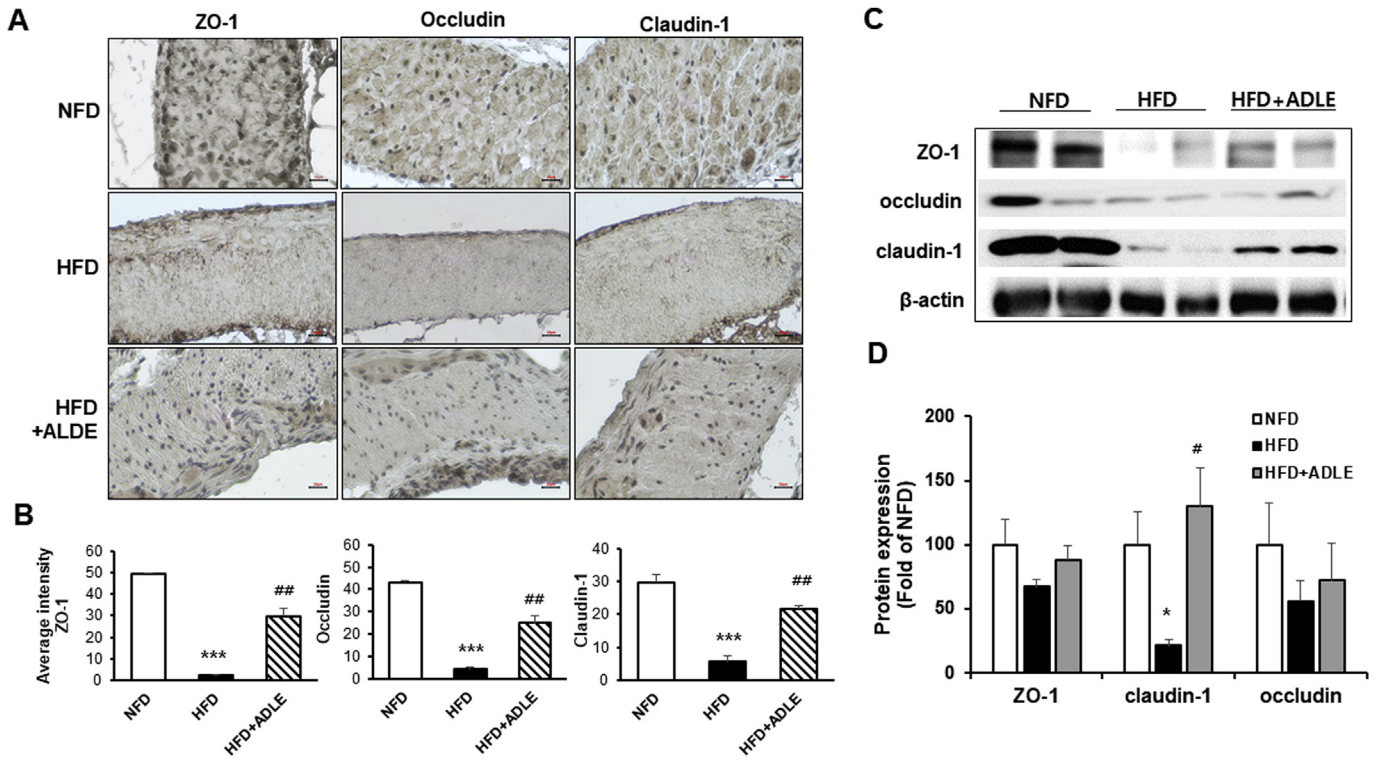


**Fig. 4.** ADLE increased expression of tight junction proteins in intestine from HFD-fed mice. (A) Immunohistochemistry staining (400×) for ZO-1, occludin and claudin-1 in small intestine of HFD-induced mice (bar = 10 μm). (B) Quantification of TJ proteins used image J and was calculated as staining site/total area X 100%. (C) Representative immunoblot and (D) quantification of TJ proteins were detected by Western blotting. Data represent mean ± SD (n = 5–8). *$p < 0.05$, ***$p < 0.001$ versus the NFD group; ##$p < 0.01$ versus the HFD group. NFD, normal fat diet; HFD, high-fat diet; HFD + ADLE, high-fat diet + ADLE.

the treatment of IBD.

## Declaration of competing interest

The authors declare no conflict of interest.

## Acknowledgments

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