Combined administration of anisodamine and neostigmine reduced Dextran Sulfate Sodium-Induced Colitis through inducing autophagy and inhibiting inflammation

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Research Article

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Abstract

Background: Our Previous work demonstrated that combined treatment of ANI (anisodamine) and NEO (neostigmine) produced anti-shock effect and rescued acute lethal crush syndrome via activating α7 nicotinic acetylcholine receptor (α7nAChR). In this study, we attemptted to reveal the therapeutic effect and underlying mechanisms of ANI/NEO combination in dextran sulfate sodium (DSS)-induced colitis.

Methods: The expression of autophagy related proteins was detected by Western blot and the autophagy flux was evaluated by confocal microscopy transfected with tandem fluorescent AdPlus-mCherry-GFP-LC3B plasmid. The inflammatory cytokines were measured by RT-PCR and ELISA. Intestinal inflammation was reflected by HE staining.

Results: Combining treatment of ANI and NEO at a ratio of 500:1 alleviated the symptoms of DSS-induced mice colitis, reduced body weight loss, improved the disease activity index, enhanced colon length and alleviated colon inflammation. Treatment with ANI/NEO combination also enhanced autophagy both in colon from DSS-induced colitis mice and Caco-2 cells stimulated with LPS/DSS. In addition, ANI/NEO significantly inhibited INF-γ, TNF-α, IL-6, and IL-22 in colon tissues and decreased TNF-α, IL-1β and IL-6 mRNA levels in Caco-2 cells. These effects were attenuated by autophagy inhibitor 3-methyladenine (3-MA) and ATG5 siRNA. Further more, autophagy inhibitor 3-MA and α7nAChR antagonist methyllycaconitine (MLA) weakened the protective effect of ANI/NEO on colitis in DSS-induced mice.

Conclusion: Collectively, these results demonstrate that ANI/NEO combination could produce therapeutic effects in DSS-induced mice colitis and this effect is mediated by autophagy and a7nAChR.

Background

Although the exact etiology of inflammatory bowel diseases (IBD), which mainly consist of ulcerative colitis (UC) and Crohn's disease (CD) have not yet been clarified, it is well established that genetic susceptibility, commensal microbiota, epithelial barrier function and immune response are the main causes leading to the pathogenesis and progression of IBD [1, 2]. Disturbance of microbial homeostasis and disturbed intestinal epithelial barrier function largely trigger the innate and adaptive immune responses to stabilize intestinal environment. However if the self-protective immune responses are over-triggered, it can lead to the elevated secretion of pro-inflammatory cytokine and the damage of intestine itself as well as the intestinal mucosal barrier, which contributes to the pathogenesis and progression of IBD [3, 4]. Consequently, inhibiting the over-activation of innate and adaptive immune responses may serve as a potential and effective strategy for the treatment of IBD.

Autophagy is a self-protecting cellular catabolic pathway through which some long-lived proteins, damaged organelles and misfolded proteins are degraded and recycle [5, 6]. It is widely acknowledged that autophagy plays a vital role in intestinal homeostasis, as well as in regulating the interactions between host defense and commensal microbiota or intestinal pathogens [7, 8]. A dysfunction of autophagy is associated with several inflammatory diseases including UC and CD [5]. Our previous works

have demonstrated that enhancement of autophagy could ameliorate the pathogenesis of EAE and DSS-induced colitis through the inhibiting of inflammation [9, 10]. In addition, we also found combining anisodmine (ANI), a muscarinic cholinergic receptor antagonist, with neostigmine (NEO), a cholinesterase inhibitor, significantly rescued acute lethal crush syndrome and collagen-induced arthritis through reducing inflammation [11, 12]. Based on these studies, we hypothesize that combined ANI and NEO may promote autophagy which corrects the balance of immune response to produce therapeutic benefits in DSS-induced mice colitis models.

The α7 nicotinic acetylcholine receptor (α7nAChR) is a subtype of N-acetylcholine receptor, which belongs to ligand gated ion channel [13]. It has been showed that α7nAChR plays an immunomodulatory role in a variety of diseases. Works from our laboratory have also confirmed the protective effect of α7nAChR in many models such as ischemic stroke and atherosclerosis [14, 15]. Moreover, we found that ANI and NEO combination rescued acute lethal crush syndrome through activating α7nAChR [11]. Accordingly, in this study, we try to assume that combined ANI and NEO can protect mice from DSS-induced colitis by activating α7nAChR.

Materials And Methods

Animals and agents

C57BL/6 mice were purchased from Shanghai Super-B&K Laboratory Animal Corp., Ltd. (Shanghai, China) and kept at 22°C under a 12-h light/dark cycle with unlimited access to water and standard rodent diet. All experiments were approved and conducted in accordance with the guidelines of the Animal Care Committee of Second Military Medical University. Anisodamine (ANI) was obtained from Shanghai First Biochemical Phamaceutical Company (Shanghai, China). Neostigmine (NEO) was obtained from San-Wei Pharmaceutical Company (Shanghai, China). 3-methyladenine (3-MA, Cat.No. M9281) and methyllycaconitine (MLA, Cat.No. M168) were obtained from Sigma-Aldrich (Saint Louis, MO, USA).

Cell culture

Caco-2 cells were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured with DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) at 37°C in a humidified incubator with 5% CO_2. Caco-2 cells were primed with 10 ng/ml LPS (Sigma, Louis, MO, USA) for 1 h, and then stimulated with 3% DSS in the presence or absence of 10, 30, 100 μM ANI/NEO compound for 24 h.

Induction of Colitis

Colitis was induced with 3% (w/v) DSS (mol.wt. 36,000 to 50,000 kDa, MP Biomedicals, Solon, OH, USA) dissolved in drinking water for 7 days as previously described. Briefly, C57BL/6 mice were provided with a solution of filtered water containing 3% DSS ad libitum. Every other day, the DSS solution was replenished. Control mice received only normal drinking water.

Clinical score and histological analysis

Body weight, the disease activity index (DAI) were determined by two investigators blinded to the treatment groups. The DAI combines scores for diarrhoea and the presence of occult or overt blood in the stool. A scoring system was used to assess diarrhea and the presence of occult or overt blood in the stool [16]. Changes of body weight are indicated as loss of baseline body weight as a percentage. Postmortem, the colon was removed and pieces of colonic tissue were used for *ex vivo* analysis. For histology, rings of the transverse part of the colon were fixed in 4% buffered formalin and embedded in paraffin. Sections were stained with H&E according to standard protocols. Histological scoring was performed in a blinded way by a pathologist as previously described [16]. Briefly, no evidence of inflammation was scored as 0, low level of inflammation with scattered infiltrating mononuclear cells as 1, moderate inflammation with multiple focim as 2, high level of inflammation with increased vascular density and marked wall thickening as 3, maximal severity of inflammation with transmural leukocyte infiltration and loss of goblet cells as 4.

Reverse Transcription and Real-time PCR

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used for the extraction of total RNA from colon tissuess and Caco-2 cells. Reverse transcription was conducted for the extracted RNA to obtain the cDNA with PrimeScript™ RT Master Mix (Takara, Otsu, Shiga, Japan). Real-time PCR was then conducted in the LightCycler quantitative PCR apparatus (Stratagene, Santa Clara, CA, USA) using the FastStart Unitversal SYBR Green Master (Roche, Konzern-Hauptsitz, Grenzacherstrasse, Switzerland). In order to eliminate the effect of DSS on PCR, 1.25 mM spermine was added into PCR reactions. The expression values of INF-γ, TNF-α, IL-6 and IL-22 were normalized to GAPDH in the same sample and then normalized to the control. After transfection with control siRNA or ATG5 siRNA, Caco-2 cells were primed with 10 ng/ml LPS for 1 h, and then stimulated with 3% DSS in the presence or absence of 100 μM ANI/NEO compound for 24 h. The expression values of TNF-α, IL-1β and IL-6 were were normalized to β-actin in the same sample and then normalized to the control. The primer sequences are listed in Table 1.

Enzyme-linked Immunosorbent Assay

Levels of INF-γ and TNF-α in colonic tissue were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (Westang Biotechnology, Shanghai, China).

Western blotting

Proteins were extracted from colonic tissue and Caco-2 cells using a standard extraction reagent supplemented with protease inhibitors (Kangchen, Shanghai, China). Protein concentration was determined using a BCA protein assay kit (Beyotime Biotechnology, Shanghai, China). The proteins were separated using SDS-PAGE and electro-transferred to nitrocellulose membranes and then incubated with the rabbit anti-Beclin-1 monoclonal antibody (1:500; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-LC3 polyclonal antibody (1:500; Novus Biologicals, Littleton, CO, USA), rabbit anti-p62 antibody

(1:500; Cell Signaling Technology, Danvers, MA, USA) and mouse anti-beta-actin antibody (1:1000, Beyotime Biotechnology, Shanghai, China). After that, the membranes were incubated with a Donkey anti-rabbit or Donkey anti-mouse secondary antibody (1:5000, LI-COR Biosciences, Lincoln, NE, USA) accordingly. The image was acquired with an Odyssey infrared imaging system (Li-Cor Bioscience, Lincoln, NE, USA).

Autophagy Flux Assessment

Caco-2 cells were seeded on the cultural slides and transfected with tandem fluorescent AdPlus-mCherry-GFP-LC3B plasmid (Beyotime Biotechnology, Shanghai, China) when the confluence reached to 50–70% [10]. In brief, cultured in DMEM supplemented with 10% FBS for 24 h, cells were incubated with plasmids for 8 h and then changed into DMEM supplemented with 10% FBS for another 48 h to ensure the expression of the genes. After transfection, cells were primed with 10 ng/ml LPS for 1 h, and then stimulated with 3% DSS in the presence or absence of 10, 30, 100 μM ANI/NEO compound for 24 h. Cellular autophagosomes (G^+C^+) and autolysosomes (G^-C^+) were detected by confocal microscopy (Leica TCS SP8, Leica, Biberach, Germany). Total number of puncta (>1 μm) per cell was counted.

Transient transfection and siRNA

The following siRNAs against ATG5 (Gene ID: 11793) were synthesized by Genepharm Biotech (Shanghai, China): siRNA1, 5’-GCCUGUAUGUACUGCUUUATT-3’, 5’-UAAAGCAGUACAUACAGGCTT-3’; siRNA2, 5’-GAACCAUACUAUGCAUUAUTT-3’, 5’-AUAAUGCAUAGUAUGGUUCTT-3’; siRNA3, 5’-GGGAAGAAGAGAUUGUUUATT-3’, 5’-UAAACAAUCUCUUCUUCCCTT-3’. All siRNAs consisted of 21 nucleotides and contained symmetric 3’ overhangs of two deoxythymidines. Caco-2 cells were transfected with siRNAs as previously reported [17].

Animals and treatment

For the detection of ANI/NEO compound effection on symptoms of DSS-induced colitis, C57BL/6 mice were received 3% DSS for 7 days and treated with vehicle, ANI (20 mg/kg, i.p.), NEO (40 μg/kg, i.p.) or ANI and NEO compound (ANI/NEO, 5 mg/kg and 10 μg/kg, 10 mg/kg and 20 μg/kg, 20 mg/kg and 40 μg/kg, i.p.) twice a day from day 3 to day 7. Body weight, the disease activity index, colon length and histologic score were examined as mentioned above. Protein levels of LC3-II/LC3-I ratio, Beclin-1, P62 and mRNA levels of INF-γ, TNF-α, IL-6, IL-22 in colonic tissue were measured as mentioned above. To test the influence of autophagy and 7nAChR on the protective effect of ANI/NEO combination in DSS-induced colitis, C57BL/6 mice were received 3% DSS for 7 days and treated with vehicle, 3-MA (10 mg/kg, i.p.), MLA (10 mg/kg, i.p.), ANI/NEO compound (20 mg/kg and 40 μg/kg, i.p.), 3-MA+ANI/NEO compound or MLA+ANI/NEO compound respectively. 3-MA or MLA was given daily and ANI/NEO compound twice a day from day 3 to day 7. Body weight, the disease activity index, colon length and histologic score were examined and the expression of INF-γ, TNF-α, IL-6, IL-22 in colonic tissue were tested with RT-PCR as mentioned above.

Statistical analysis

Data are expressed as means ± SEM. For nonparametric data, a Kruskal-Wallis test followed by a Dunn's post-test was used. For continuous variables, the statistical differences between groups were determined by one-way analysis of variance followed by a Student-Newman-Keuls test. Statistical analyses were performed using GraphPad Prism 4 software (GraphPad Software, San Diego, CA). $P < 0.05$ was considered statistically significant.

Results

ANI and NEO combination alleviates the symptoms and severity of dextran sulphate sodium (DSS)-induced colitis

C57BL/6 mice were received 3% DSS for 7 days, the control mice were given tap water. The mice in the DSS group developed a severe illness characterized by the presence of sustained weight loss, bloody diarrhea, and severe colon inflammation, as well as hyperemia, ulceration and bowel wall thickening. The body weight in the ANI alone group or the NEO alone group was higher compared with the DSS group. However, in the ANI/NEO compound group , the body weight was evidently higher than ANI alone group or the NEO alone group (Figure 1A). As shown in the Figure 1B-C, the disease activity index were lower and the colon length longer in the ANI alone group or the NEO alone group than the DSS group. Furthermore, in the ANI/NEO compound group, the disease activity index was evidently lower and the colon length significantly longer than ANI alone group or the NEO alone group. And the ANI/NEO compound treatment relatively alleviative inflammatory infiltration in mucosa and submucosa in DSS-induced colitis mice (Figure 1D).

Dose-dependent effects of ANI/NEO combination at 500:1 ratio on the symptoms and severity of DSS-induced colitis

To determine the best therapeutic effect of ANI/NEO on DSS-induced colitis, three different doses (5 mg/kg and 10 μg/kg, 10 mg/kg and 20 μg/kg, 20 mg/kg and 40 μg/kg, i.p.) of ANI/NEO compound at 500:1 ratio were used twice a day from day 3 to day 7. ANI/NEO at two higher doses (10 mg/kg and 20 μg/kg, 20 mg/kg and 40 μg/kg) significantly reduced body weight loss (Figure 2A), improved the disease activity index (Figure 2B) and enhanced colon length (Figure 2C-D). Moreover, at these two doses, ANI/NEO reduced colon inflammation remarkably and the highest dose (20 mg/kg and 40 μg/kg, i.p.) of ANI/NEO was more effective (Figure 2E). For this reason, a dose of 20 mg/kg and 40 μg/kg (i.p.) of ANI/NEO was used in subsequent experiments.

ANI/NEO combination increases autophagy and alleviates colon inflammation of DSS-induced colitis mice

Since autophagy could mediate the homeostasis of intestinal function and inflammatory cytokines expression, we detected the autophagy related proteins LC3, Beclin-1, P62 and pro-inflammatory

cytokines. Results showed that ANI/NEO compound significantly increased the LC3-II/LC3-I ratio and Beclin-1 and decreased P62 in colon from DSS-induced colitis mice (Figure 3A). Besides, treatment with ANI/NEO compound decreased the mRNA levels of INF-γ, TNF-α, IL-6, IL-22 and the concentration of TNF-α, INF-γ in colon tissues induced by DSS apparently (Figure 3B).

ANI/NEO combination increases autophagy in Caco-2 cells challenged with LPS/DSS

Caco-2 cells were primed with 10 ng/ml LPS for 1 h, and then stimulated with 3% DSS in the presence or absence of 10, 30, 100 μM ANI/NEO compound for 24 h. Incubation with ANI/NEO dose-dependently enhanced LC3-II/LC3-I ratio, Beclin-1 and reduced P62 in LPS/DSS stimulated Caco-2 cells (Figure 4A). Since autophagosomes are basic functional units of autophagy, here we further detected the number of autophagosomes in Caco-2 cells. Under the stimulation of LPS/DSS, the number of autophagosomes was greatly increased. Treatment with ANI/NEO (100 μM) further enhanced the effect of LPS/DSS on autophagosomes (Figure 4B).

Autophagy mediates the inhibition of ANI/NEO combination on the expression of inflammatory cytokines in Caco-2 cells challenged with LPS/DSS

Intestinal tissue inflammation caused by intestinal immune dysfunction plays an important role in the pathogenesis of UC. The level and proportion of inflammatory cytokines in the intestinal tissue are closely related to the pathogenesis of UC. LPS/DSS stimulation induced the high levels of TNF-α, IL-1β and IL-6 in Caco-2 cells, which was significantly reduced by pre-incubation with ANI/NEO. Blocking autophagy by ATG5 siRNA attenuated the inhibitory effect of ANI/NEO on the expression of TNF-α, IL-1β and IL-6 (Figure 5A-D).

Blockade of autophagy attenuates the protective effect of ANI/NEO combination on DSS-induced colitis and inflammation

In DSS-induced colitis mice, treatment with 3-MA (10mg/kg, i.p.) attenuated the beneficial effects of ANI/NEO on weight loss, disease activity index and colon length (Figure 6A-D). We further tested the changes in intestinal inflammation after the autophagy blocker 3-MA was administered. Compared with the DSS group, ANI/NEO combination significantly reduced the expression of pro-inflammatory factors (TNF-α, IL-6, IFN-γ, IL-22) in colon tissue after treatment. After simultaneous administration of the autophagy blocker 3-MA, compared with the DSS+ANI/NEO group, the pro-inflammatory factors (TNF-α, IL-6, IFN-γ, IL-22) in DSS+3-MA+ANI/NEO group increased. 3-MA partially weakened the inhibitory effect of ANI/NEO combination on inflammation (Figure 7). The above results indicate that autophagy mediates the protective effect of ANI/NEO combination on the intestinal inflammation caused by DSS.

Blockade of α7nAChR partly inhibits the protective effect of ANI/NEO combination on DSS-induced colitis

Treatment with MLA (10mg/kg, i.p.), a selective inhibitor of α7nAChR, abolished the inhibitory effects of ANI/NEO on weight loss, disease activity index and colon length shorten in DSS-induced colitis mice

(Figure 8), suggesting that activating α7nAChR contributed to the protective effect of ANI/NEO combination on DSS-induced colitis.

Discussion

In this study, we for the first time found that combined treatment with ANI and NEO could significantly ameliorate DSS-induced colitis by reducing weight loss, disease activity index and colon length shorten, leading to remarkably improved histopathology scores. And we found the best combination of anisodamine and neostigmine was 500:1. ANI/NEO combination could not only elevate the protein levels of LC3-II/LC3-I ratio, Beclin-1 and decrease P62, but also attenuate several inflammatory cytokines including INF-γ, TNF-α, IL-6, and IL-22 in colon tissue from DSS-induced colitis mice. Similar results were observed in Caco-2 cells stimulated with LPS/DSS in vivo. The ANI/NEO preincubation can increase LC3-II/LC3-I ratio, Beclin-1 and decrease P62, and promote autophagy flux, as well as reducing TNF-α, IL-1β and IL-6 mRNA levels. These findings suggest that ANI/NEO could augment autophagy and inhibit inflammatory response both in vivo and in vitro.

Recent studies have reported the role of ANI on regulating inflammatory response in inflammatory and autoimmune diseases [18, 19, 20]. In our previous works, we found that ANI significantly reduced the levels of TNF-α and IL-1β in LPS-induced shock rats [21]. Afterwards, we combined ANI with NEO, an inhibitor of acetylcholinesterase, and found the anti-inflammatory effect greatly increased compared with ANI alone. Combined treatment with ANI and NEO produced the significant increase in therapeutic efficiency in shock mice, acute lethal crush syndrome mice and collagen-induced arthritis mice [22, 11, 12]. In accordance with those studies, our current study showed that combination of ANI/NEO significantly reduced body weight loss, improved the disease activity index, enhanced colon length and alleviated colon inflammation in DSS-induced mice colitis. Furthermore, the data show that the combining ANI and NEO is superior than ANI or NEO alone to protect DSS-induced colitis.

During the process of ulcerative colitis, the intestinal lamina propria (LP) macrophages, derived from circulating monocytes, increase and produce excessive inflammatory cytokines, which stimulates neutrophil infiltration into the colon and induce intestinal epithelial tissue damage [1]. Yan found that Artemisinin analogue SM934 could ameliorate DSS-induced mice ulcerative colitis by inhibiting neutrophils and macrophages infiltration and IL-1β, IL-6, TNF-α production [23]. Moreover, cytokines have been demonstrated as potential new target for the treatment of IBD patients and IBD animal [24]. Anti-TNF-α agents like infliximab ameliorate disease activity index and histological scores of DSS-induced murine colitis and induces clinical remission and mucosal healing UC patients [25]. In this work, we found that administration of ANI/NEO combination (500:1) at the dose of 20 mg/kg and 40 μg/kg significantly decreased the mRNA levels of INF-γ, TNF-α, IL-6, and IL-22 in colon tissues raised by DSS. ANI/NEO (100μM) also reduced TNF-α, IL-1β and IL-6 mRNA levels in Caco-2 cells stimulated by LPS/DSS. These results suggest that combining application of ANI and NEO could alleviate the symptoms of DSS-induced colitis through inhibiting inflammatory reaction.

Previous studies and our works demonstrated that autophagy played an important role in ulcerative colitis through the modulation of inflammatory response [26]. Intestinal epithelial cell (IEC)-specific deletion of ATG16 developed severely exacerbated pathology of colitis, and exhibited elevated pro-inflammatory cytokine secretion and increased IEC apoptosis, indicating that autophagy in the epithelium could ease intestinal damage through regulating inflammation and apoptosis [27]. Our previous studies demonstrated that inducing autophagy via activation of cannabinoid receptor 2 could inhibit NLRP3 inflammasome activation in mice colitis and EAE models [9, 28]. Here, in our work, we revealed that treatment with ANI/NEO combination contributed to the enhancement of autophagy both in colon from DSS-induced colitis mice and Caco-2 cells stimulated with LPS/DSS. In addition, blocking autophagy by ATG5 siRNA in Caco-2 cells attenuated the effect of ANI/NEO on TNF-α, IL-1β and IL-6. 3-MA, an autophagy inhibitor, significantly attenuated the protective effect of ANI/NEO combination on DSS-induced colitis and the inhibitory effect of ANI/NEO combination on cytokines production. Taken together, these data suggest that inducing autophagy at least partly contributed to ANI/NEO mediated suppression of inflammatory response in Caco-2 cells as well as DSS-induced colitis.

A clinical investigate reported that smoking can inhibit the progression and severity of UC, while stopping smoking can increase the recurrence rate. The mechanism is that nicotine in tobacco activates α7nAChR to produce anti-inflammatory effect [13, 29, 30]. Nicotine can also activate α7nAChR on CD4T cells in colonic mucosa to inhibit IL-6, STAT3 and TNF-α resulting in the reduction of UC [31]. In addition, Alsharari et al. demonstrated that colitis severity and level of TNF-α were significantly elevated in α7nAChR knockout mice. In contrast, pretreatment with selective agonists of α7nAChR, PHA-543613 and PNU120596, alleviated the symptoms of colitis in mice [32]. Similarly, selective agonists of α7nAChR, PNU282987 and Enenicline, reduced enteritis immune inflammation and ulcerative colitis [33, 34]. In this work, we found that MLA, a selective inhibitor of α7nAChR, significantly inhibited the protective effect of ANI/NEO combination on DSS-induced mice colitis, indicating that α7nAChR mediates the therapeutic effect of ANI/NEO combination on DSS-induced colitis. Interestingly, some studies reported that AR-R17779 and GSK1345038A, two selective agonists of α7nAChR, could aggravate UC and increased the level of colonic proinflammatory cytokines [35]. This discrepancy may be related to a variety of experimental factors, such as α7nAChR agonist selectivity, the dosage, the mode of administration, disease severity and disease model.

Conclusion

Taken together, our study demonstrate that the combining treatment of ANI and NEO at 500:1 could produce the therapeutic effects on DSS-induced colitis mice, which is at least partly through the induction of autophagy and inhibition of inflammation. The protective effect of ANI/NEO is related with α7nAChR. This study may provide a new therapeutic strategy for UC, but there is a long way to go from bench to bedside before the side-effects, stability of the preparation, pharmacokinetics, efficacy and toxicology should be determined.

Abbreviations

ANI
anisodamine
NEO
neostigmine
α7nAChR
α7 nicotinic acetylcholine receptor
DSS
dextran sulfate sodium
RT-PCR
real-time quantitative polymerase chain reaction
ELISA
enzyme-linked immunosorbent assay
LPS
lipopolysaccharide
3-MA
3-methyladenine
ATG5
autophagy related gene 5
MLA
methyllycaconitine
IBD
inflammatory bowel diseases
UC
ulcerative colitis
CD
Crohn’s disease
EAE
Experimental autoimmune encephalomyelitis
DMEM
Dulbecco's modification of Eagle's medium
FBS
fetal bovine serum
DAI
disease activity index
H&E
hematoxylin-eosin.

Declarations

Ethics approval and consent to participate

All animal experiments were approved and conducted in accordance with the guidelines of the Animal Care Committee of Second Military Medical University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets generated for this study are available on request to the corresponding author.

Authors' Contributions

NK performed the experiments. ZD interpreted the results of experiments. ML, GZ, QX and DZ helped to performe the experiments. PK performed the experiments, analyzed the data and drafted the manuscript. CL designed research and revised the manuscript.

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Table 1

Table 1 is available in the Supplementary Files section.

Figures

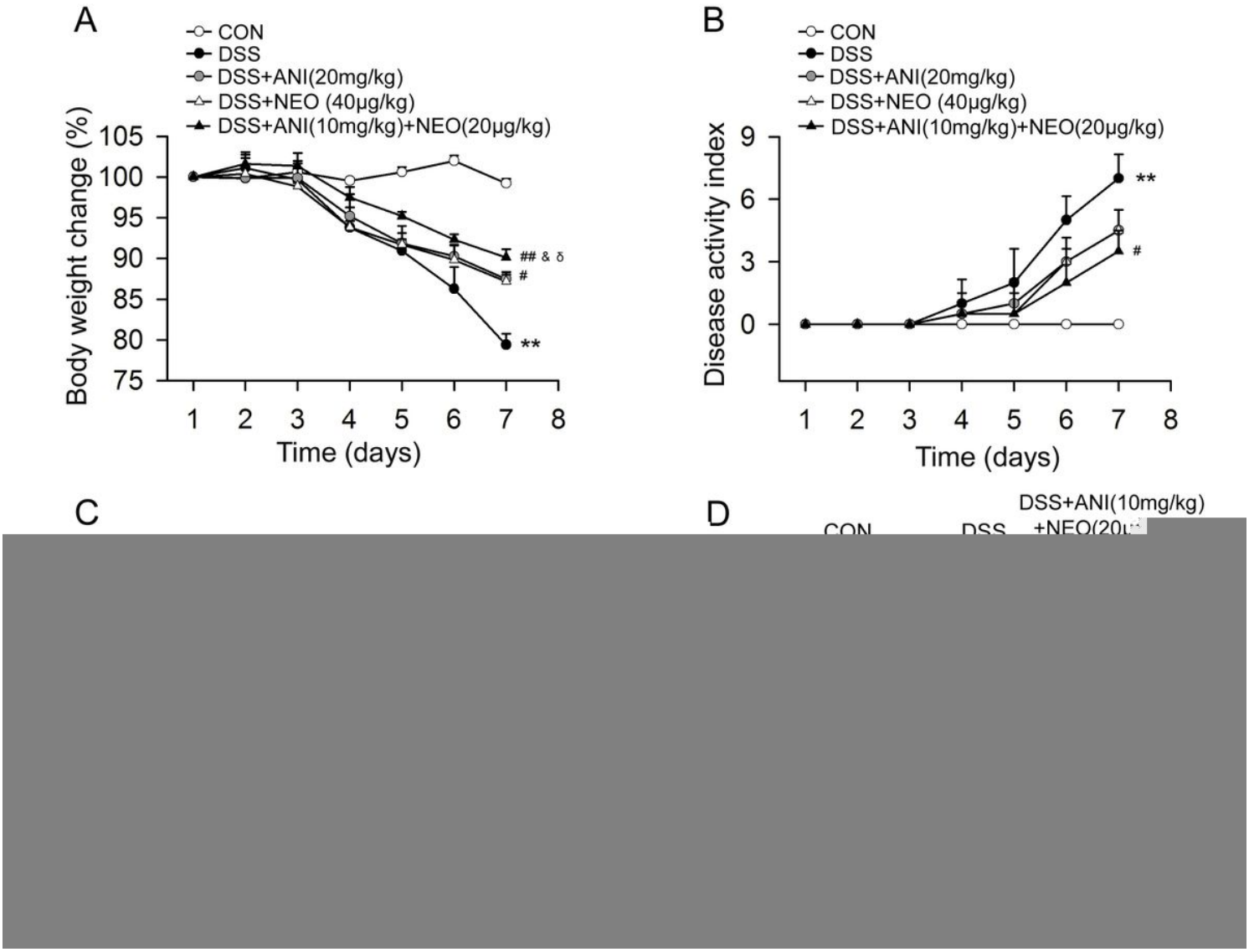

Figure 1

Effects of ANI and NEO on the symptoms and colon inflammation in DSS-induced colitis mice. C57BL/6 mice were received 3% DSS for 7 days, the control mice were given tap water. Animals were treated with vehicle, ANI (20 mg/kg, i.p.), NEO (40 μg/kg, i.p.) or ANI/NEO compound (10 mg/kg and 20 μg/kg, i.p.)

twice a day from day 3 to day 7. (A) The body weight loss was tested in the mice. n = 3 per group. **p < 0.01 vs. control groups; #P<0.05 vs. DSS group; ##P<0.01 vs. DSS group; &P<0.05 vs. ANI group; $\bar{Q}$P<0.05 vs. NEO group; (B) The disease activity index was tested in the mice. n = 4 per group. **p < 0.01 vs. control groups; #P<0.05 vs. DSS group; (C) The colon length was tested in the mice. n = 4 per group. **P<0.01 vs. control groups; #P<0.05 vs. DSS group; ##P<0.01 vs. DSS group. &&P<0.05 vs. ANI group; $\bar{Q}\bar{Q}$P<0.05 vs. NEO group; (D) The colon inflammation in the DSS-induced colitis mice. n = 4 per group. **P<0.01 vs. control groups; #P<0.05 vs. DSS group.

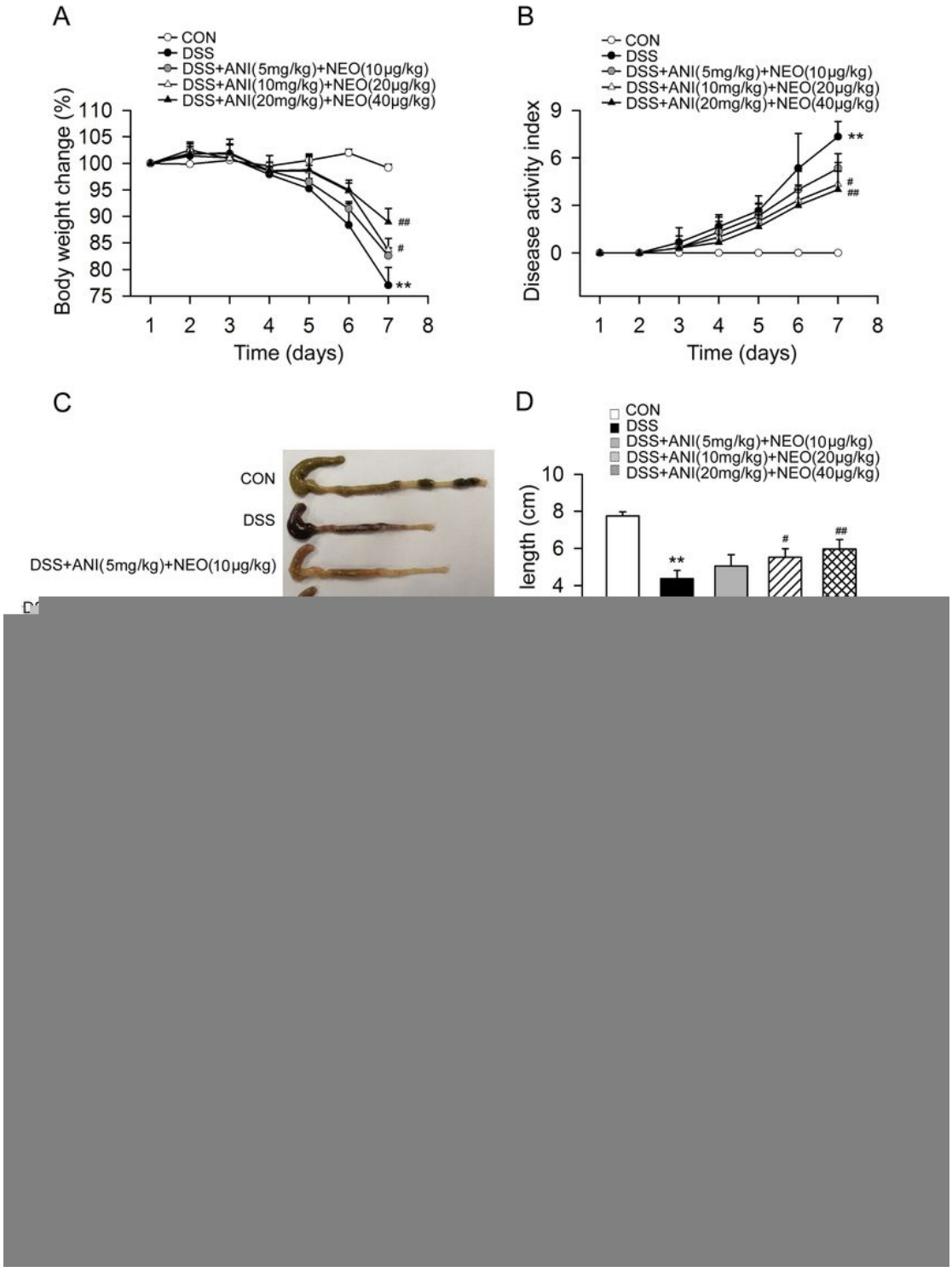

Figure 2

Effects of different dose of ANI/NEO combination on the symptoms and colon inflammation in DSS-induced colitis mice. C57BL/6 mice were received 3% DSS for 7 days, the control mice were given tap water. Animals were treated with vehicle or ANI/NEO compound at three different doses (5 mg/kg and 10 μg/kg, 10 mg/kg and 20 μg/kg, 20 mg/kg and 40 μg/kg, i.p.) twice a day from day 3 to day 7. (A) n = 3 or 4 per group. The body weight loss was tested in the mice. **P<0.01 vs. the control group; #P<0.05 vs. the

DSS group, ##P<0.01 vs. the DSS group; (B) The disease activity index was tested in the mice. n = 6 per group. **P<0.01 vs. the control group; #P<0.05 vs. the DSS group, ##P<0.01 vs. the DSS group; (C-D) The colon length was tested in the mice. n = 4 per group. **P<0.01 vs. the control group; #P<0.05 vs. the DSS group, ##P<0.01 vs. the DSS group. (E) The colon inflammation was tested in the mice. n = 4 per group. **P<0.01 vs. the control group; #P<0.05 vs. the DSS group.

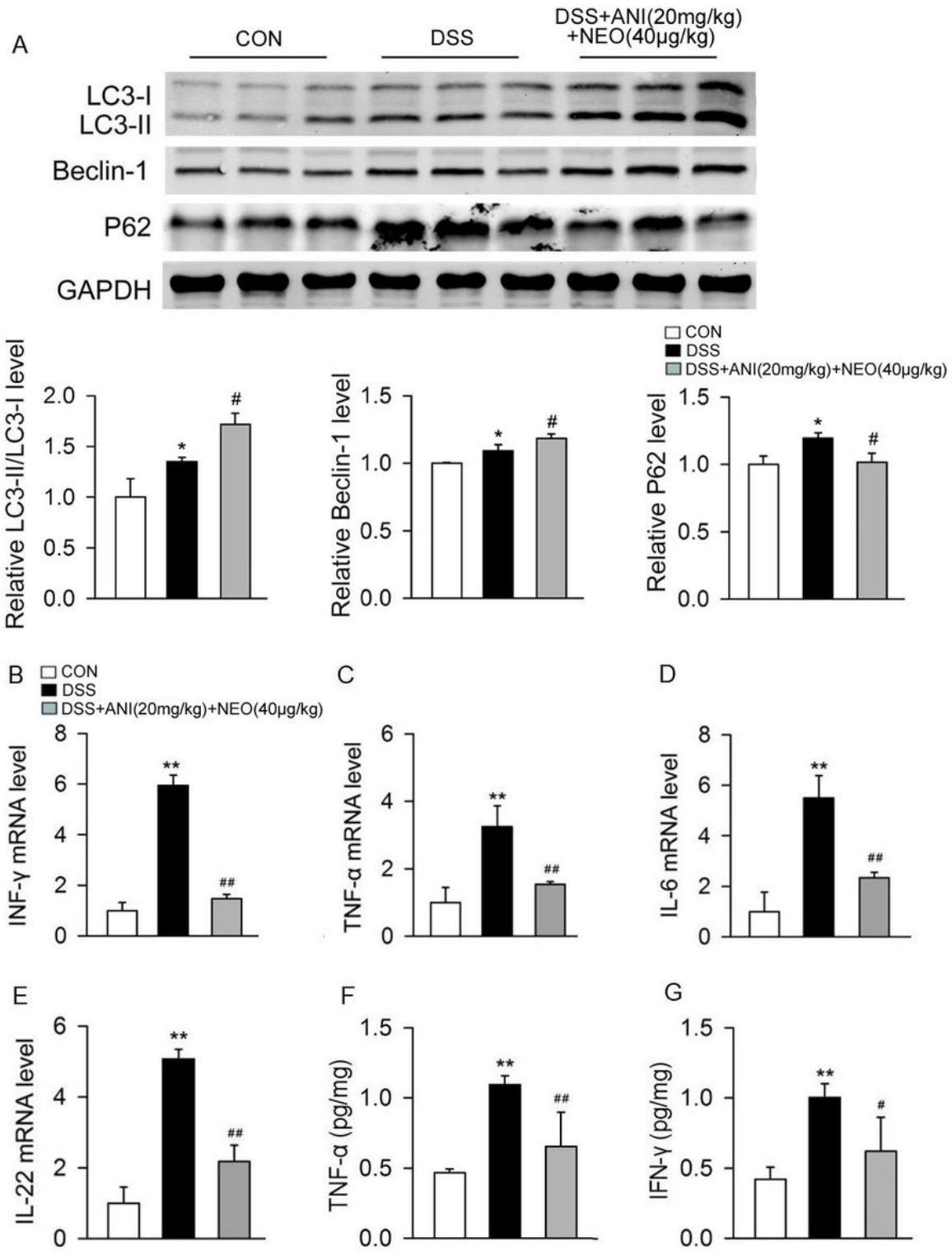

Figure 3

Effects of ANI/NEO combination on autophagy and colonic inflammatory cytokines in DSS-induced colitis mice. C57BL/6 mice were received 3% DSS for 7 days, the control mice were given tap water. Animals were treated with vehicle or ANI/NEO compound (20 mg/kg and 40 μg/kg, i.p.) twice a day from day 3 to day 7. The protein levels of LC3-II/LC3-I ratio, Beclin-1, P62, the concentration of INF-γ, TNF-α and the mRNA levels of INF-γ, TNF-α, IL-6, IL-22 in colonic tissue were detected. (A) Representative western blot and quantification data for LC3-II/LC3-I, Beclin-1 and P62 level in the intestinal tissues. n = 3 per group. *P<0.05 vs. the control group; $^{\#}$P<0.05 vs. the DSS group. (B-E) The INF-γ, TNF-α, IL-6, and IL-22 mRNA levels were detected in the mice. n = 3 per group. **P<0.01 vs. the control group; $^{\#\#}$P<0.01 vs. the DSS group. (F-G) The TNF-α and INF-γ protein levels were detected in the mice. n = 4 per group. **P<0.01 vs. the control group; $^{\#}$P<0.05 vs. the DSS group, $^{\#\#}$P<0.01 vs. the DSS group.

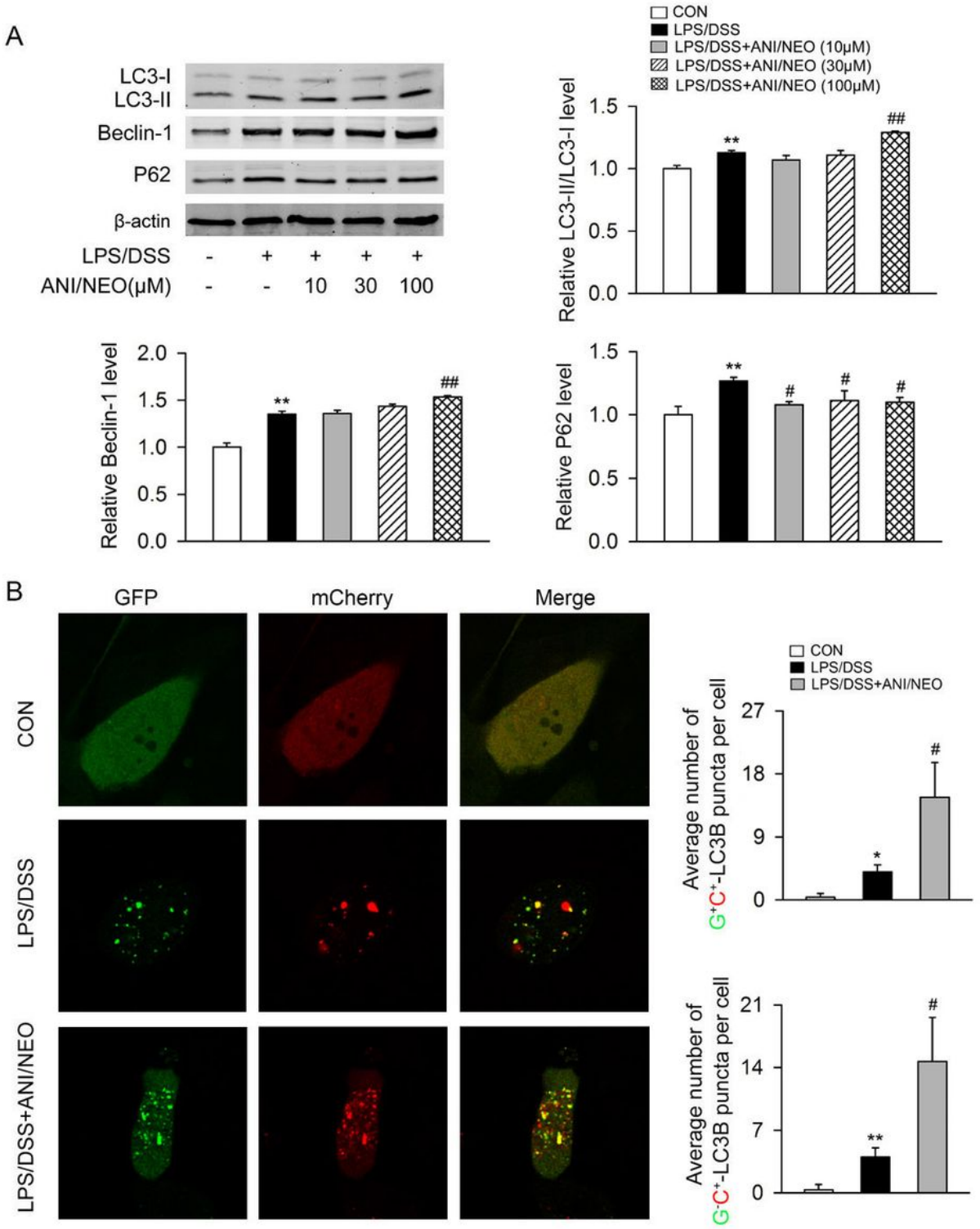

Figure 4

Effects of ANI/NEO combination on autophagy in LPS/DSS simulated Caco-2 cells. (A) Caco-2 cells were primed with 10 ng/ml LPS for 1 h, and then stimulated with 3% DSS in the presence or absence of 10, 30, 100 μM ANI/NEO compound for 24 h. The LC3-II/LC3-I ratio, Beclin-1 and P62 in colonic tissue and Caco-2 cells were analyzed. n = 3 per group. **P<0.01 vs. the control group; ##P<0.01 vs. the DSS group. (B) Caco-2 cells were transfected with tandem fluorescent AdPlus-mCherry-GFP-LC3B plasmid for

8 h and then cultured in fresh DMEM supplemented with 10% (vol/vol) FBS for another 48 h. After primed with 10 ng/ml LPS for 1 h, cells were stimulated with 3% DSS in the presence or absence of 100 μM ANI/NEO compound for 24 h. Cellular autophagosomes (G^+C^+) and autolysosomes (G^-C^+) were detected. ANI/NEO significantly increased the number of autophagosomes (G^+C^+) and autolysosomes (G^-C^+). n = 3 per group. *P<0.05 vs. the control group, **P<0.01 vs. the control group; #P<0.05 vs. the DSS group.

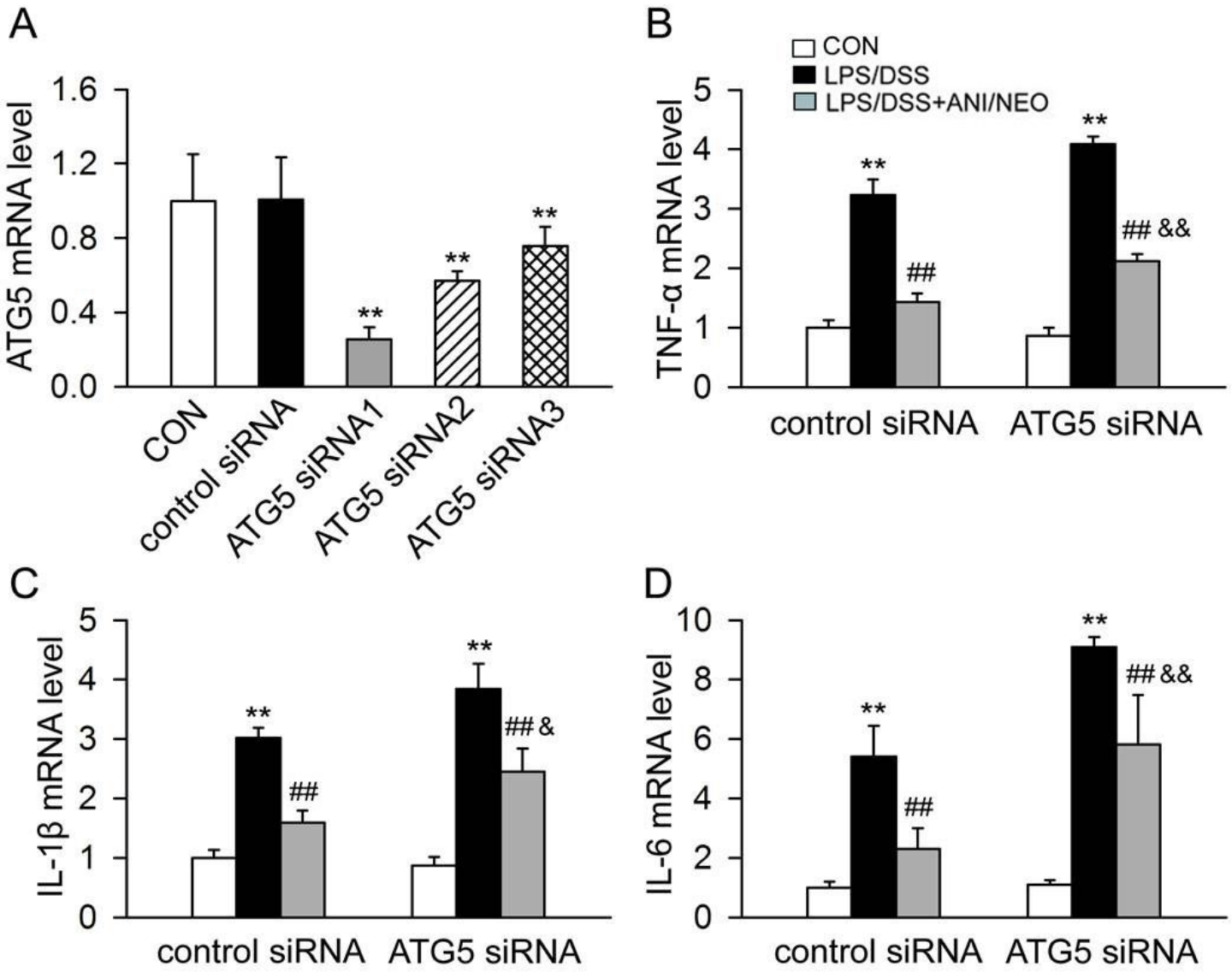

Figure 5

Autophagy mediates the inhibition of ANI/NEO combination on the expression of inflammatory cytokines in Caco-2 cells challenged with LPS/DSS. (A) The quantitative expression of Atg5 mRNA in Caco-2 cells after transfection with control siRNA or Atg5 siRNA. n = 3 per group. **P<0.01 vs. the control siRNA. (B-D) After transfection with control siRNA or Atg5 siRNA for 24h, Caco-2 cells were primed with 10ng/ml LPS for 1 h, and then stimulated with 3% DSS in the presence or absence of 10, 30, 100 μM ANI/NEO compound for 24 h. The mRNA levels of TNF-α, IL-1β and IL-6 were analyzed with RT-PCR. n = 3

per group. **P<0.01 vs. the corresponding control group; ##P<0.01 vs. the corresponding LPS/DSS group; &P<0.05 vs. the control siRNA LPS/DSS+ANI/NEO group, &&P<0.01 vs. the control siRNA LPS/DSS +ANI/NEO group.

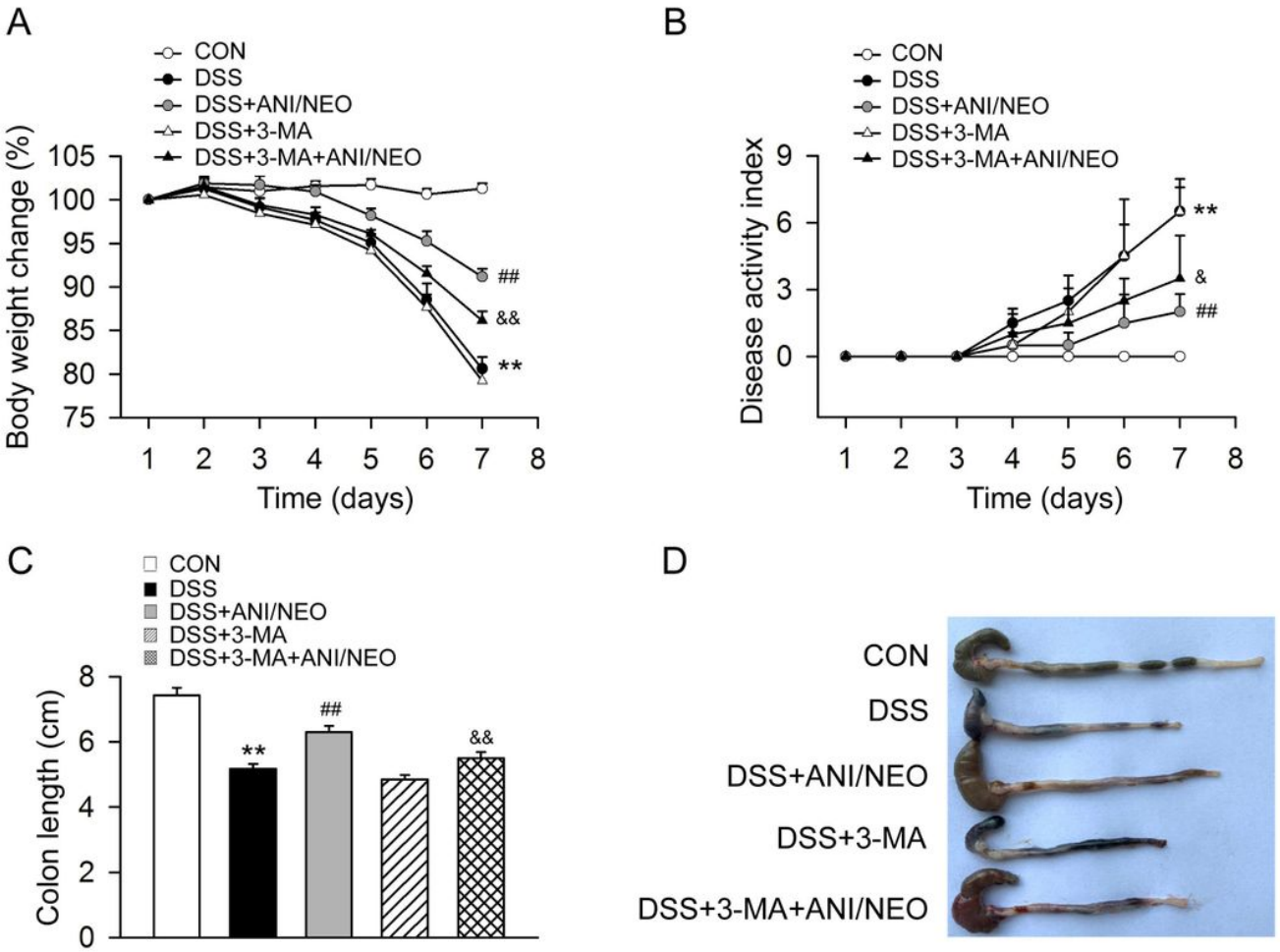

Figure 6

3-MA attenuates the protective effect of ANI/NEO combination on DSS-induced colitis in mice and the inhibitory effect of ANI/NEO combination on inflammatory cytokines in colon from DSS-induced colitis mice. C57BL/6 mice were received 3% DSS for 7 days, the control mice were given tap water. The DSS mice were treated with vehicle, 3-methyladenine (3-MA, 10 mg/kg, i.p.), ANI/NEO compound (20 mg/kg and 40 μg/kg, i.p.) or 3-MA+ ANI/NEO compound. 3-MA was given daily and ANI/NEO compound twice a day from day 3 to day 7. (A) The body weight loss was tested in the mice. n = 4 per group. **P<0.01 vs. the control group; ##P<0.01 vs. the DSS group. &&P<0.01 vs. the DSS+ANI/NEO group; (B) The disease activity index was tested in the mice. n = 4 per group. **P<0.01 vs. the control group; ##P<0.01 vs. the DSS group. &P<0.05 vs. the DSS+ANI/NEO group; (C-D) The colon length was tested in the mice. n = 4 per group. **P<0.01 vs. the control group; ##P<0.01 vs. the DSS group. &&P<0.01 vs. the DSS+ANI/NEO group.

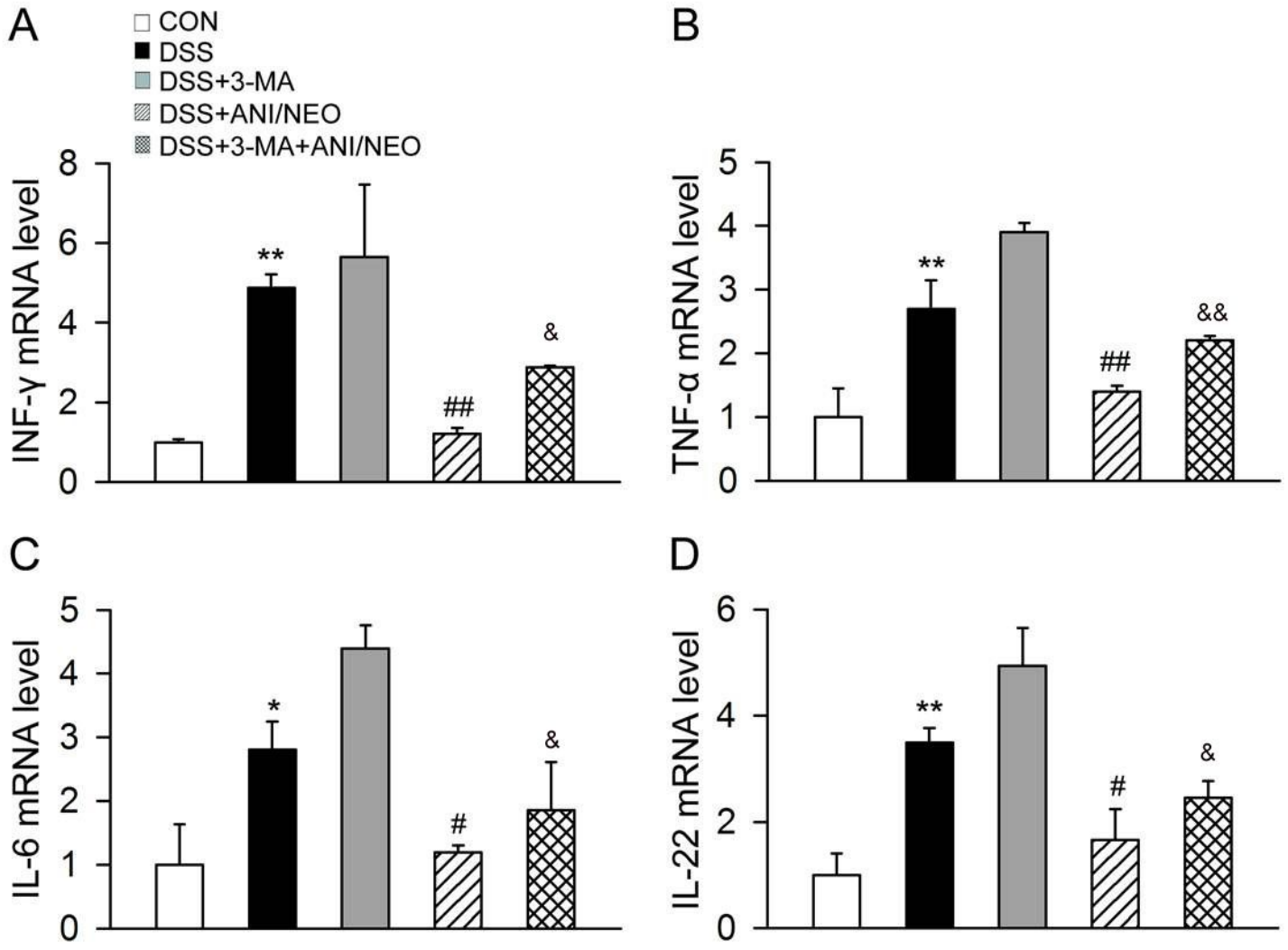

Figure 7

3-MA attenuates the inhibitory effect of ANI/NEO combination on inflammatory cytokines in colon from DSS-induced colitis mice. C57BL/6 mice were received 3% DSS for 7 days, the control mice were given tap water. The DSS mice were treated with vehicle, 3-MA (10 mg/kg, i.p.), ANI/NEO compound (20 mg/kg and 40 μg/kg, i.p.) or 3-MA+ ANI/NEO compound. 3-MA was given daily and ANI/NEO compound twice a day from day 3 to day 7. The expression of INF-γ, TNF-α, IL-6 and IL-22 in colonic tissue were detected with RT-PCR. n = 3 per group. *P<0.05 vs. the control group, **P<0.01 vs. the control group; $^{\#}$P<0.05 vs. the DSS group, $^{\#}$P<0.01 vs. the DSS group; $^{\&}$P<0.05 vs. the DSS+ANI/NEO group, $^{\&\&}$P<0.01 vs. the DSS+ANI/NEO group.

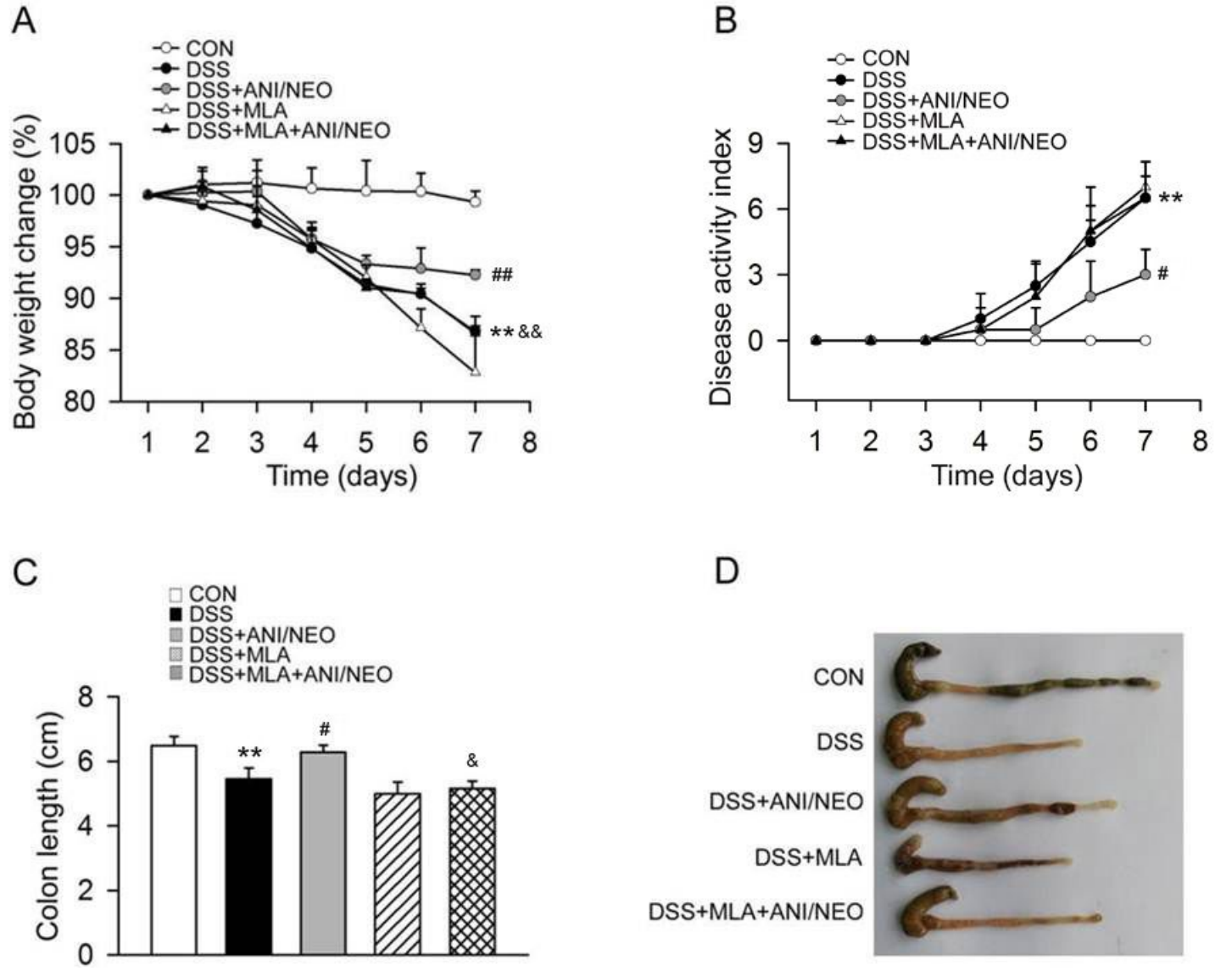

Figure 8

Methyllycaconitine (MLA) significantly inhibits the protective effect of ANI/NEO combination on DSS-induced colitis. C57BL/6 mice were received 3% DSS for 7 days, the control mice were given tap water. The DSS mice were treated with vehicle, MLA (10 mg/kg, i.p.), ANI/NEO compound (20 mg/kg and 40 μg/kg, i.p.) or MLA+ ANI/NEO compound. MLA was given daily and ANI/NEO compound twice a day from day 3 to day 7. (A) The body weight loss was tested in the mice. n = 3 per group. **P<0.01 vs. the control group; $^{\#\#}$P<0.01 vs. the DSS group; $^{\&\&}$P<0.01 vs. the DSS+ANI/NEO group. (B) The disease activity index was tested in the mice. n = 4 per group. **P<0.01 vs. the control group; $^{\#}$P<0.05 vs. the DSS group. (C-D) The colon length was tested in the mice. n = 4 per group. **P<0.01 vs. the control group; $^{\#}$P<0.05 vs. the DSS group; $^{\&}$P<0.05 vs. the DSS+ANI/NEO group.

Supplementary Files

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- table1.jpg