

# CD73 Blockade Aggravate Dextran-Sodium-Sulfate-Induced Colitis via Down Regulating Inosine

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**Background:** CD73, a pivotal enzyme in the purinergic signaling cascade, modulates the concentrations of adenosine and inosine. These metabolites are involved in immune responses and inflammatory processes. This study aims to investigate the function of CD73 in the pathogenesis of inflammatory bowel disease (IBD) and explore the potential mechanism.

**Methods:** Dextran-Sodium-Sulfate (DSS)-induced colitis mice models were established by orally administering 3% DSS. CD73 was blocked by intraperitoneal injection of Adenosine 5'- ( $\alpha$ ,  $\beta$ -methylene) diphosphate (APCP). Inosine was supplemented by intraperitoneal injection. Hematoxylin-eosin (H&E), PAS and Alcian blue staining were used to evaluate the inflammation infiltration and colon damage. Serum IL-6 levels were detected by ELISA assay. High-performance liquid chromatography-mass spectrometry (LC-MS) was used to investigate the level of inosine.

**Results:** Blockade of CD73 by APCP aggravated disease severity in DSS-induced colitis mice models, characterized by increased weight loss, colon shortening and pathological damage, increased disease activity and IL-6 production. Blocking CD73 impairs intestinal barrier function and integrity by reducing the expression of tight junction proteins (claudin-1, occludin and ZO-1), both in colon tissues and intestinal epithelial cell-MODE-K. In addition, APCP increased oxidative stress in colon tissue and MODE-K (increased MDA level, decreased SOD and GSH activities). Moreover, blocking CD73 reduced inosine levels in vivo and in vitro. We found that inosine treatment significantly ameliorated DSS-induced colitis in mice, as demonstrated by decreased weight loss, less colon shortening and histological injury, reduced disease activity and IL-6 production. Notably, the effects of inosine on MODE-K cells were opposite to those of APCP, including the effects on the expression of oxidative stress molecules and tight junction proteins.

**Conclusion:** This study indicates that CD73 exerts a protective effect in the progress of DSS-induced colitis. Inosine supplementation might be a potential therapeutic strategy for colitis.

**Keywords:** CD73, inosine, adenosine, inflammatory bowel disease, oxidative stress

## Introduction

Inflammatory bowel disease (IBD) is a chronic intestinal inflammation with systemic manifestations, including ulcerative colitis (UC) and Crohn's disease (CD).<sup>1</sup> Recurrent diarrhea, rectal bleeding, abdominal pain and bowel obstruction are the main clinical manifestation.<sup>2,3</sup> Periodic of symptomatic relapse and remission resulting in epithelial barrier disruption, ulceration formation and causing damage of epithelial structure.<sup>4</sup> IBD has emerged as a public health challenge worldwide with rapidly increasing incidence.<sup>5,6</sup> The pathogenesis of IBD were complex and remains unclear. Environmental factors, genetics, immune dysregulation, mucous barrier and epithelial barrier defects are believed to be association with IBD progress.<sup>7</sup>

CD73 is a key molecule in the purine signaling pathway, influencing the levels of adenosine and inosine, which play important roles in inflammation regulation. Wang R's study showed that inhibition of CD73 alleviated intestinal inflammation by regulating macrophage differentiation in ulcerative colitis and significantly alleviated DSS-induced colitis in mice,<sup>8</sup> which

indicated the facilitating role of CD73 in colitis progression. However, there were other study showed the protective role of CD73 in the progression of colitis. Bynoe et al reported that CD73 deficient mice showed high susceptibility to DSS-induced colitis, along with significant weight loss, slower weight recovery, an increased intestinal permeability.<sup>9</sup> Thus, the function of CD73 in colitis pathogenesis remains to be fully elucidated. Elevated oxidative stress levels and reduced antioxidant levels contribute to the pathogenesis of IBD.<sup>10</sup> In addition, intestinal barrier dysfunction is a key factor in the initiation and progression of IBD. Tight junction proteins expressed by intestinal epithelial cells play a crucial role in maintaining intestinal barrier integrity.<sup>11,12</sup> However, the effects of CD73 on oxidative stress and tight junction proteins has not been clearly defined.

In this study, to investigate the function, potential mechanism and therapeutic potency of CD73 in IBD, we systematically evaluated the effects of CD73 blockade on colitis severity, oxidative stress and tight junction proteins expression in DSS-induced colitis mice models. Given that CD73 regulates inosine levels, we also investigated the effect of inosine administration on DSS-induced colitis progression. In addition, we investigated the influences of CD73 inhibition and inosine treatment on intestinal epithelial cells. These findings will provide new insights into the role of CD73 and inosine in IBD development.

## Materials and Methods

### Materials

Dextran-Sodium-Sulfate (DSS) was purchased from GLPBIO (GC19829, USA). CD73 inhibitor-APCP was purchased from sigma (M3763, USA) and inosine was purchased from GLPBIO (GC17902, USA). ELISA kit for serum IL-6 detection was purchased from Novus (VAL604G, USA). Lipid Peroxidation MDA Assay Kit was purchased from Beyotime (S0131S, China). Total Superoxide Dismutase Assay Kit was purchased from Beyotime (S0101S, China). Total Glutathione Peroxidase Assay Kit was purchased from Beyotime (S0059S, China).

### DSS-Induced Colitis Mice Models

DSS-induced colitis mice models were used in this study, which was a classical colitis animal model. Male C57BL/6J mice (6–8weeks) were purchased from experimental animal center, Fourth Military Medical University. All mice were housed in a standard environment (56–65% humidity,  $24 \pm 2$  °C, 12 h light/dark cycle). The mice were randomly divided to four groups: control group (n = 8), DSS group (n = 8), APCP group (4mg/kg, n=8), and inosine group (300mg/kg, n = 8). Control group were provided with normal distilled water. DSS group were provided with distilled water containing 3% DSS (w/v) from day 1 to day 7. APCP group: APCP (4mg/kg) was administered via intraperitoneal (i.p.) injected into DSS mice once a day from day 3 to day 7. Inosine group: inosine (300 mg/kg i.p.) were injected into DSS mice once a day from day 3 to day 7. On day 8, mice were sacrificed with an overdose of isoflurane (RWD, R510-22-10), and then, blood and colon tissues were isolated according to the American Veterinary Medical Association. Colon length, body weight and the disease activity index (DAI) of mice were measured.

This animal study protocol was reviewed and approved by Fourth Military Medical University's Institutional Animal Care and Use Committee (No.20250036). All the experiments were performed in accordance with ARRIVE guidelines and guidelines for the ethical review of laboratory animal welfare (GB/T35892-2018) issued by China.

### Assessment of Disease Activity Index (DAI)

DAI score was used to evaluate the severity of colitis, including weight loss, diarrhea, and hematochezia. The DAI scores were monitored and recorded according to the previous study.<sup>13,14</sup> Briefly, weight loss was scored as follows: score 0: none; score 1: 1–5%; score 2: 5–10%; score 3: 10–20%; score 4: >20%. Diarrhea was scored as follows: score 0: normal; score 2: loose stools; score 4: watery diarrhea. Hematochezia was scored as follows: score 0: normal; score 2: slight bleeding; score 4: gross bleeding. DAI score is the sum of the scores of weight loss, diarrhea, and hematochezia.

### Histopathology Analysis

The colons were collected followed by washing with PBS and then fixed with 4% paraformaldehyde at room temperature overnight. Then, the paraffin-embedded sections of colon tissues were prepared (5µm thick). The H&E staining was

performed to evaluate the inflammatory infiltration. Alcian blue and PAS staining were used to assess the number and structure integrity of goblet cell. Panoramic and Case Viewer 2.4 software (3DHISTECH, Hungary) were used for image acquisition and analysis. Subjective assessment was used in this study.

## Immunohistochemical (IHC) Analysis

IHC analysis for paraffin-embedded sections of colon tissues were performed to detect the expression of CD73, F4/80, occludin, claudin-1 and ZO-1. Briefly, anti-occludin antibody (1:750 dilution; Servicebio, GB111401), anti-claudin-1 antibody (1:1000 dilution; Servicebio, GB15032), anti-ZO-1 antibody (1:1000 dilution; Servicebio, GB115686), anti-F4/80 antibody (1:1000 dilution; Servicebio, GB113373) and anti-CD73 antibody (1:200 dilution; abcam, ab175396) were used as the primary antibodies. The primary antibodies were incubated at 4°C for 12h. HRP-conjugated anti-rabbit secondary antibody (1:200 dilution; Servicebio, GB23303) was used as second antibody and incubated at room temperature for 1h. Panoramic and Case Viewer 2.4 software (3DHISTECH, Hungary) were used for image acquisition and analysis.

## Serum Interleukin-6 (IL-6) Detection

The blood of mice was collected. After centrifugation (5000rpm, 4°C, 10min), the serum was collected. The serum IL-6 levels were detected using ELISA assay (VAL604G, Novus, USA) according to the instruction. First, 100µL serum (diluted 3 times with calibrator dilution) was added into well and incubated for 2h at room temperature; Second, 100µL detection antibody was added and incubated for 2h at room temperature; Third, 100µL streptavidin-HRP was added and incubated for 30min at room temperature; Fourth, 100µL 3,3',5,5'-Tetramethylbenzidine substrate was added and incubate for 20min at room temperature; Finally, 50µL stop solution was added and OD<sub>450</sub> was measured immediately.

## Measurement the Activity of Antioxidant Enzyme

For MDA (S1031S, Beyotime, China) and GSH (S0059S, Beyotime, China) detection, the intestinal tissues were lysed by RIPA. The lysate was collected and used to MDA/GSH detection. For SOD detection, the intestinal tissues were lysed by SOD sample preparation solution from Total Superoxide Dismutase Assay Kit (S0101S, Beyotime, China). The supernate was separated for test. The levels of MDA, GSH and SOD were detected according to the operating instructions of kit.

## Quantitative Real-Time PCR Analysis (qRT-PCR)

Total RNA from cells and colon histiocytic suspension were extracted by using Trizol reagent (TAKARA, Japan, Cat: 9109). The cDNA was generated by using Prime Script™ RT Master Mix (ACCUBATE BIOLOGY, China, AG11728). qRT-PCR reaction system was prepared using BlasTaq™ 2X qPCR Master Mix (abm, Canada, Cat: G891). The reaction was performed on Qiagen Amplifer (Germany, Rotor-gene QMDX 5). All operations was according to the manufacturer's instructions. The mRNA expression levels in different groups were calculated using GAPDH as an internal standard. The primers sequences are listed in Table 1.

**Table 1** Primer Sequence

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
Mouse CD73	CTGAGCGCTCTACTACCACA	AACAGCACGTTGGGTCTTC
Claudin-1	GGCTTCTCTGGGATGGATCG	CCCCAGCAGGATGCCAATTA
Occludin	TCTTTCCTTAGGCGACAGCG	AGAGTACGCTGGCTGAGAGA
ZO-1	CGTTCGGGGAAAGTTACGTG	GTGGGACAAAAGTCCGGGAA
IL-6	ACAAAGCCAGAGTCCTTCAGAG	TGTGACTCCAGCTTATCTCTTGG

## Cell Culture

The mouse intestinal epithelial cell line-MODE-K was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Cat: 12800-017) plus 10% fetal bovine serum (FBS, ExCell Bio, Cat: FSS500) at 37°C with 5% CO<sub>2</sub> in a humidified incubator. The cells were treated with APCP (100µM) and inosine (3mM) for 48h and then incubated with LPS for 6h to induce inflammation.

## Adenosine and Inosine Detection

High performance liquid chromatography mass spectrometry (LC-MS; Shimadzu, Japan) was employed to measure the levels of serum adenosine and inosine. Electrospray ionization was employed for detection in the positive ion mode. The multiple reaction monitoring mode (MRM) was utilized as the scanning mode. Transitions were monitored: for adenosine, from the m/z 268 precursor ion to the m/z 136 product ion (268 > 136); for inosine, 268.9>137.1; and for lamivudine (the internal standard) (Cat: 156082, TOPSCIENCE, Shanghai, China), 230 > 112. The analytical data were processed using the labsolution 5.81 (Shimadzu, Japan).

## Statistical Analysis

All quantitative results were presented as mean ± standard deviations (SD). All data analyses were performed by using GraphPad prism 8.0. *T*-test was used to analyze the differences between two independent samples. One-way analysis of variance (ANOVA) test was used for the comparison of multiple groups. *P*<0.05 was considered to be statistically significant.

## Results

### CD73 Blockade Aggravated Disease Progression in DSS-Induced Colitis Mice Models

The experimental flowchart for mice experiment was presented in [Figure 1A](#). Firstly, we detected the expression of CD73 in colon tissues from DSS-induced colitis mice by qRT-PCR and immunohistochemistry. We observed that the CD73 expression was significant decreased in colitis mice compared with control mice ([Figure 1B](#) and [C](#)).

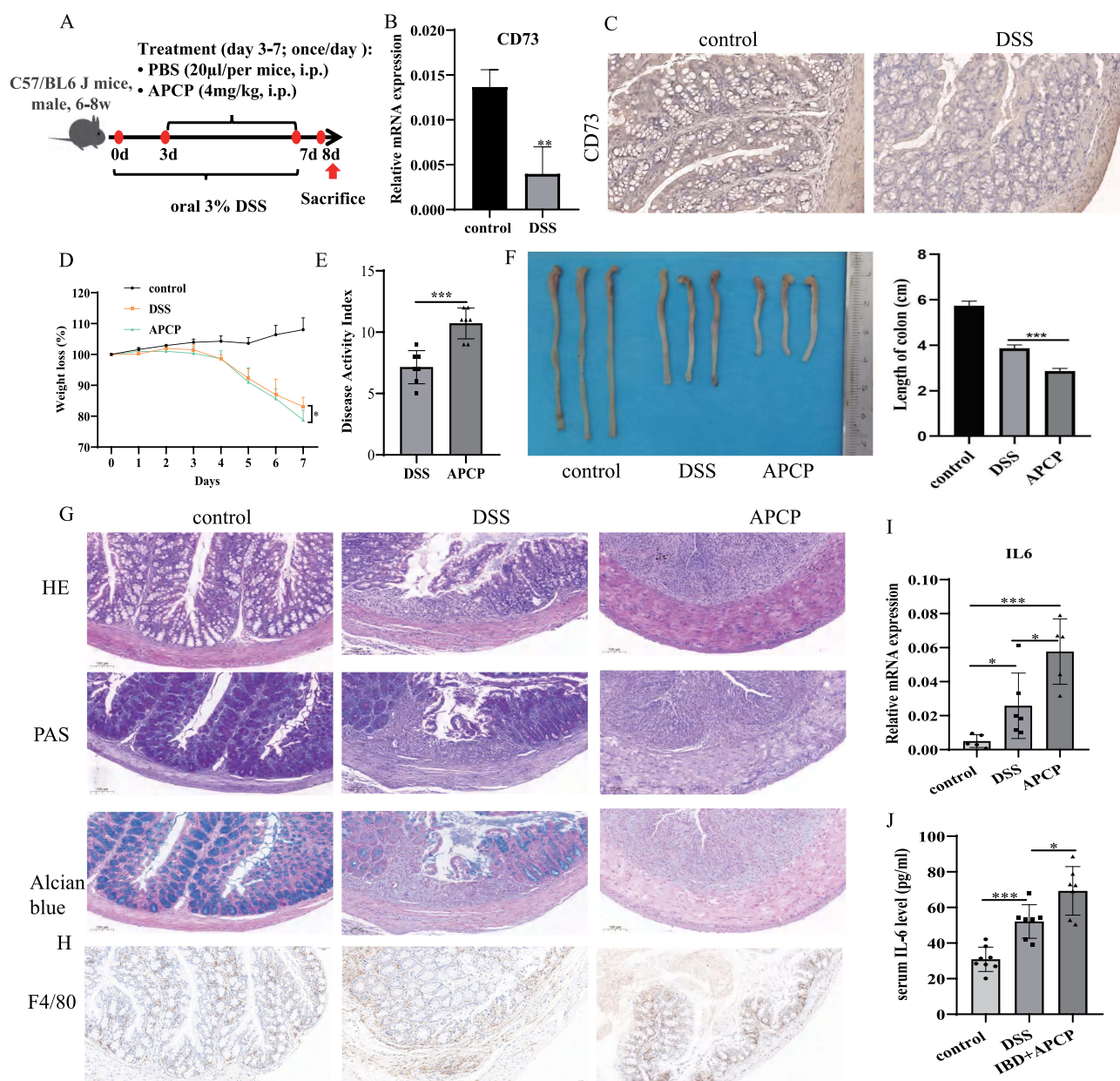
To further investigate the role of CD73 in colitis, the effects of CD73 inhibition with APCP were examined in DSS-induced colitis mice. After 1 week intervention, mice in DSS group showed significant weight loss, which were further exacerbated by APCP treatment ([Figure 1D](#)). The DAI scores were higher in APCP treatment group compared to the DSS group ([Figure 1E](#)). APCP treatment also led to more severe colon shortening ([Figure 1F](#)). Hematoxylin-eosin (H&E), PAS and Alcian blue staining were used to evaluate the inflammation infiltration and colon damage.

We observed that DSS induced the necrosis and exfoliation of mucosal epithelium, goblets cells depletion, tissue disorganization, and immune cells infiltration. APCP treatment could exacerbate the severity of tissue damage and inflammation cell infiltration ([Figure 1G](#)). IHC staining for F4/80 showed markedly increased macrophage infiltration following APCP treatment ([Figure 1H](#)). Notably, IL-6 levels were significant higher in APCP treated DSS-induced colitis mice compared to control and DSS induced mice (Control: 30.88 ± 6.841, DSS group: 52.13 ± 9.438, APCP group: 69.33 ± 13.61pg/mL, [Figure 1I](#) and [J](#)).

### APCP Inhibited the Colonic Antioxidant Abilities and Aggravated the Loss of Tight Junction Proteins in Colitis Mice

To assess the effect of APCP intervention on colonic oxidative stress in DSS treated mice, we measured colonic MDA levels along with SOD and GSH activities. Compared with the control group, the DSS-induced colitis mice showed elevated MDA levels, which were further increased following APCP treatment (Control: 4.199 ± 0.532, DSS group: 5.513 ± 0.825, APCP group: 9.553 ± 1.574 µM/mg, [Figure 2A](#)). Moreover, the activities of SOD (Control: 0.423 ± 0.019, DSS group: 0.320 ± 0.012, APCP group: 0.245 ± 0.033 U/mg) and GSH (Control: 15.110 ± 1.657, DSS group: 11.570 ± 1.842, APCP group: 9.365 ± 0.6951 mU/mg, [Figure 2B](#) and [C](#)) in DSS-induced colitis mice were reduced compared with control groups, which was the lowest in APCP treated mice ([Figure 2B](#) and [C](#)).

To assess the integrity of intestinal epithelial structure and barrier function, we investigated the expression of tight junction proteins in colon tissues. We observed that DSS treatment could reduce the expression levels of claudin-1,

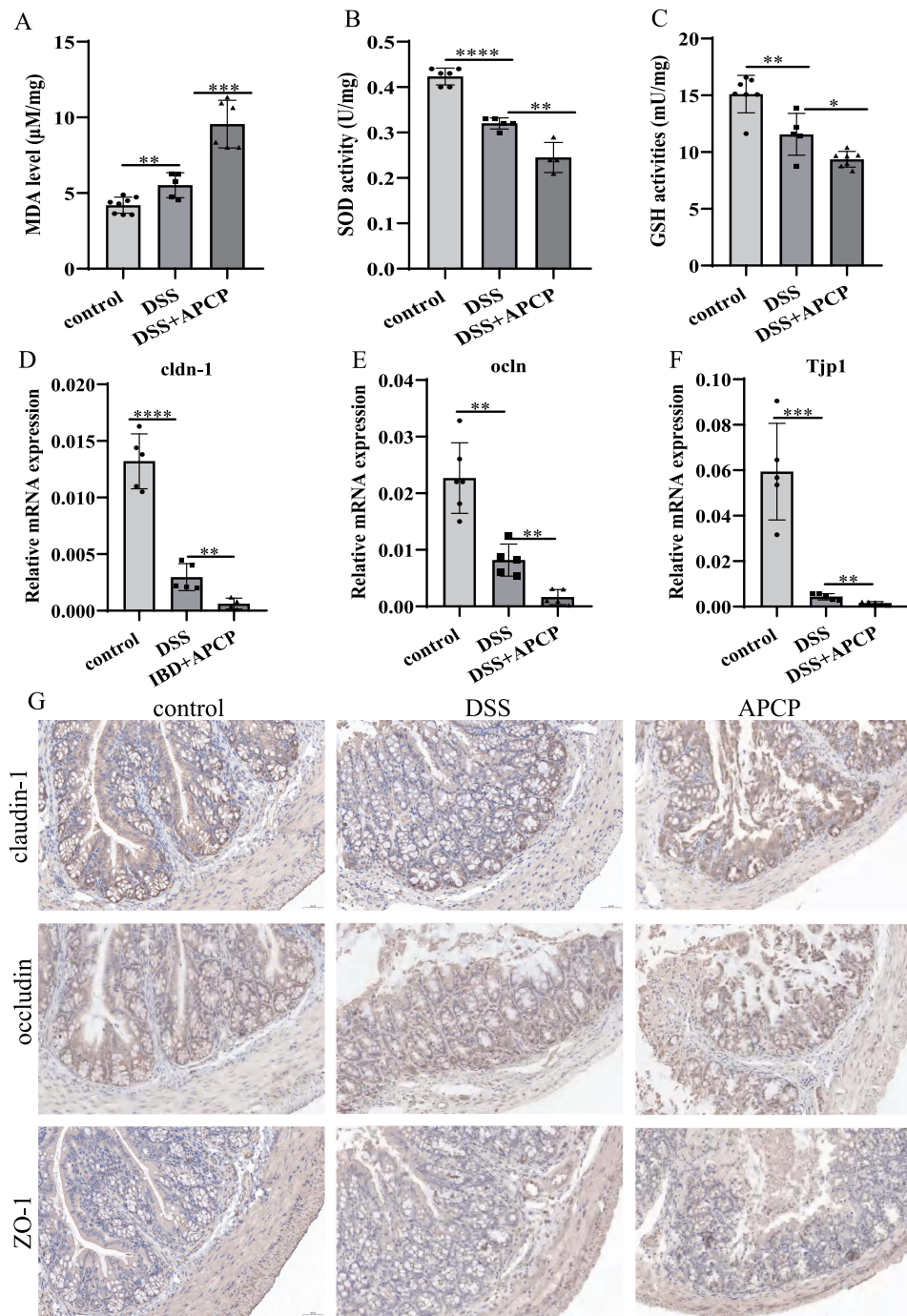


**Figure 1** CD73 blockade aggravated disease progression in DSS-induced colitis mice. **(A)** low chart of animal experiments. **(B and C)** The expression levels of CD73 in colon tissues from control and DSS treated mice were analyzed by qRT-PCR and immunohistochemical (IHC) staining analysis. Scale bar, 50µm; magnification, 20x. **(D–F)** Body weight change, DAI and colon length in mice models. **(G)** The inflammation infiltration and colon damage of control, DSS and APCP treated group were detected by HE, PAS and Alcian blue staining. Scale bar, 100µm; magnification, 10x. **(H)** The infiltration of macrophage was measured by F4/80 staining. Scale bar, 50µm; magnification, 20x. **(I and J)** The expression levels of IL-6 in colon tissues from control, DSS and APCP treated mice were analyzed by qRT-PCR. \* $P < 0.05$ , \*\*\* $P < 0.0005$ .

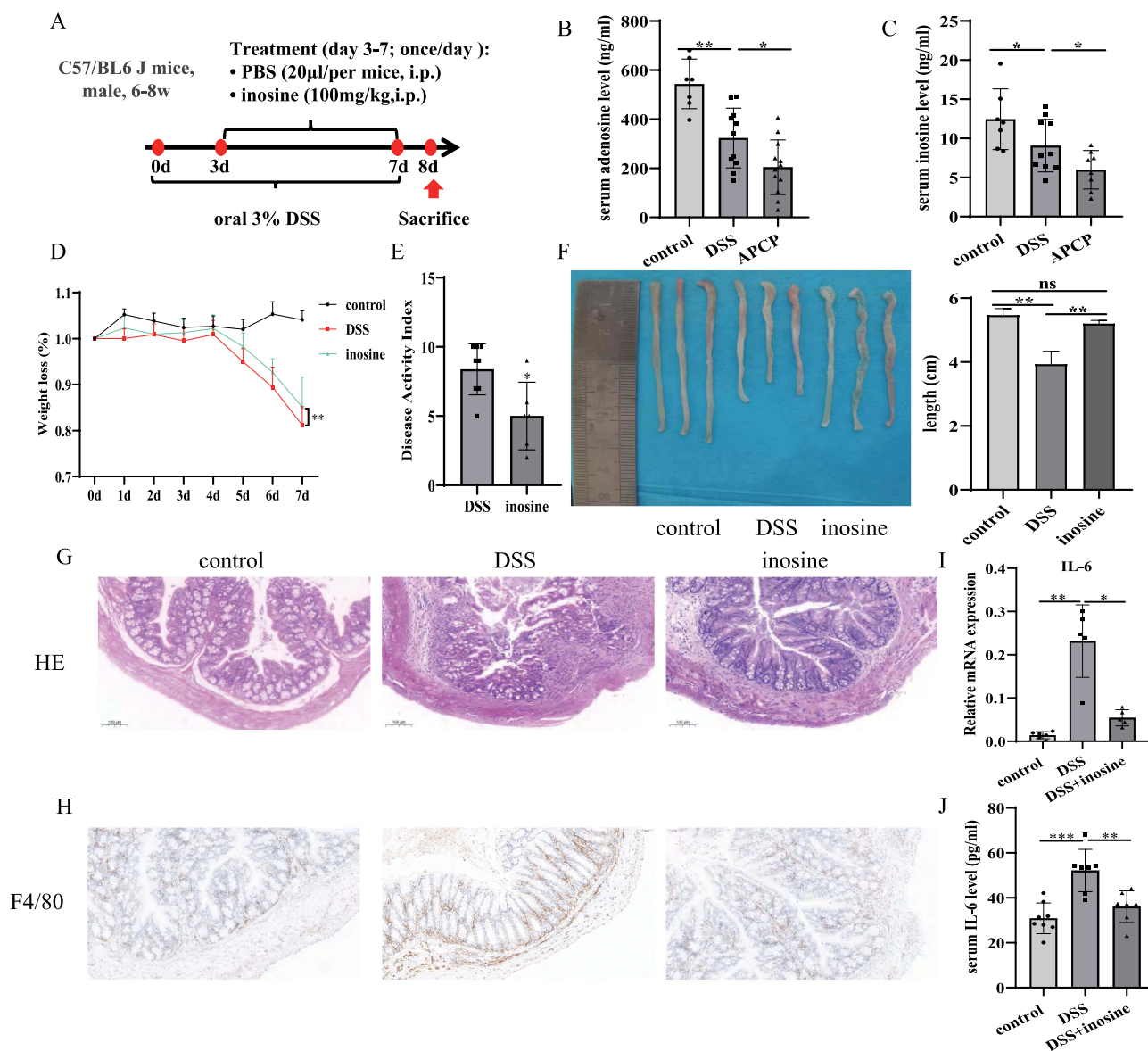
occludin and ZO-1, which were more decreased in APCP treated mice (Figure 2D–F). Moreover, IHC analysis further confirmed the decreased protein expression of claudin-1, occludin and ZO-1 in APCP treated mice (Figure 2G). These finding suggested that the CD73 inhibition exacerbates disruption of intestinal epithelial structure integrity and barrier function.

## Inosine Alleviated Disease Activity in DSS Induced Mice

The experimental flowchart for mice experiment was shown in Figure 3A. CD73 catalyzes the production of adenosine, which could be further degraded into inosine. Accumulating evidences indicates that both adenosine and inosine play significant roles in immunoregulation.<sup>15,16</sup>



LC-MS revealed decreased serum levels of adenosine (Control:  $543.9 \pm 101$ , DSS group:  $323.3 \pm 122$ , APCP group:  $204.4 \pm 111.4$  pg/mL) and inosine (Control:  $12.440 \pm 3.879$ , DSS group:  $9.074 \pm 3.353$ , APCP group:  $5.987 \pm 2.465$  pg/mL) in DSS-induced colitis mice, which were more reduced in APCP treated DSS-induced colitis mice (Figure 3B and C). In this study, we further investigated the effect of inosine on colitis mice. Compared with DSS induced mice, we observed that inosine i.p. significantly alleviated the extent of weight loss, reduced the DAI scores, and ameliorated colon shortening (Figure 3D–F). H&E staining showed that inosine i.p. could ameliorate the severity of colon damage and immune cell infiltration (Figure 3G).

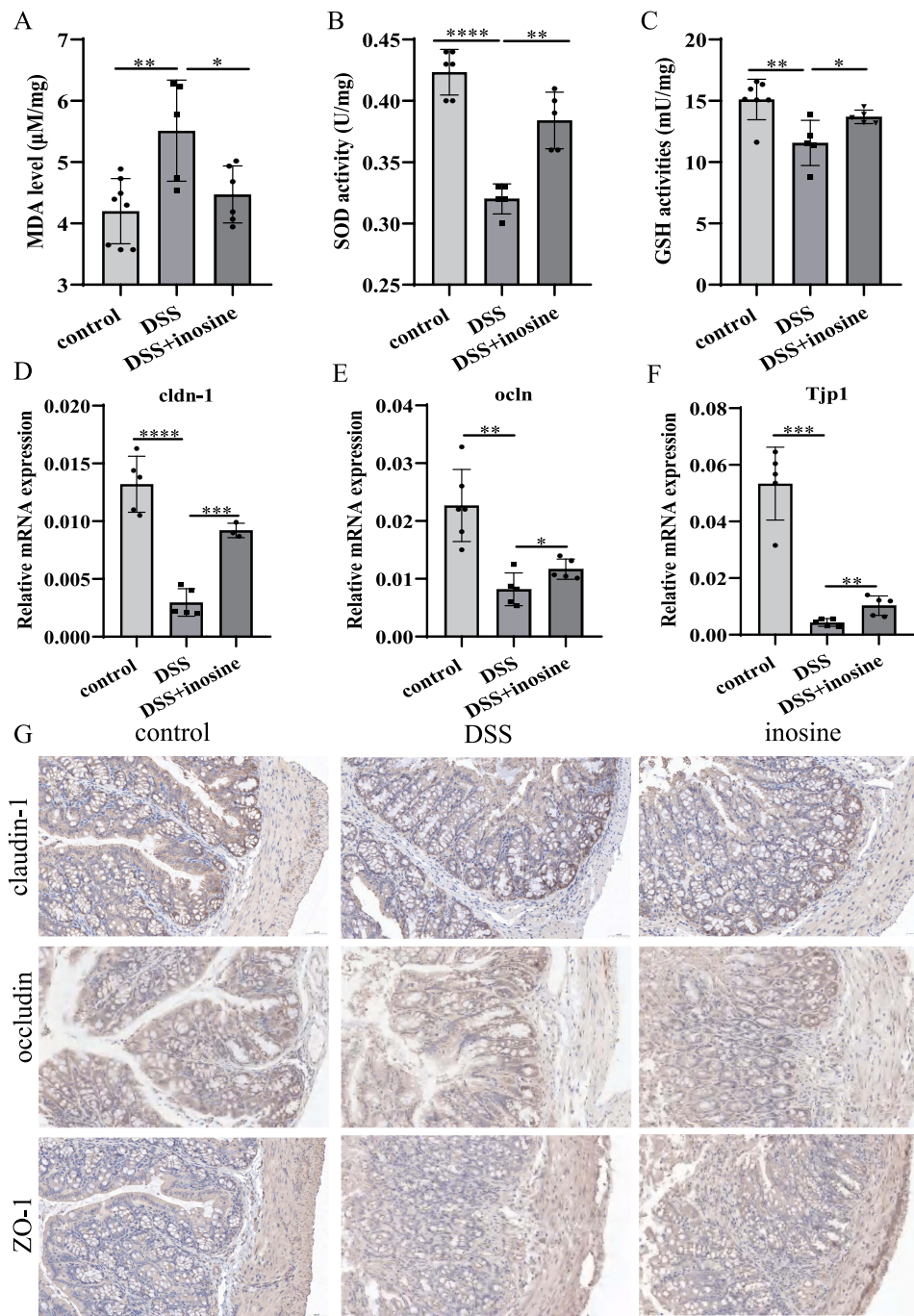


**Figure 3** Inosine alleviated disease activity in DSS induced mice. **(A)** Flow chart of animal experiments. **(B and C)** Serum adenosine and inosine levels in control, DSS and ACP treated mice. **(D–F)** Body weight change, DAI and colon length in mice models. **(G)** The inflammation infiltration of control, DSS and inosine treated group were detected by HE staining. Scale bar, 100 $\mu$ m; magnification, 10x. **(H)** The infiltration of macrophage was measured by F4/80 staining. Scale bar, 50 $\mu$ m; magnification, 20x. **(I and J)** The expression levels of IL-6 in colon tissues from control, DSS and inosine treated mice were analyzed by qRT-PCR. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.0005$ .

Moreover, we observed a significant decrease in macrophages infiltration in inosine treated DSS-induced colitis mice (Figure 3H). Additionally, IL-6 levels in serum and colon tissues were significantly increased by DSS treatment, while were reduced by inosine treatment (Control:  $30.880 \pm 6.841$ , DSS group:  $52.13 \pm 9.438$ , Inosine group:  $36.070 \pm 7.039$  pg/mL, Figure 3I and J).

## Inosine Promoted the Colonic Antioxidant Abilities and Prevented the Loss of Tight Junction Proteins in Colitis

The effects of inosine i.p. on oxidative stress and tight junction proteins in colon tissues were investigated. Compared with the control group, the DSS-induced colitis mice exhibited elevated MDA levels, which were significantly reduced by inosine treatment (Control:  $4.199 \pm 0.532$ , DSS group:  $5.513 \pm 0.825$ , Inosine group:  $4.473 \pm 0.464$   $\mu$ M/mg, Figure 4A). Additionally, SOD and GSH activities were significantly decreased in the DSS group, whereas inosine i.p.

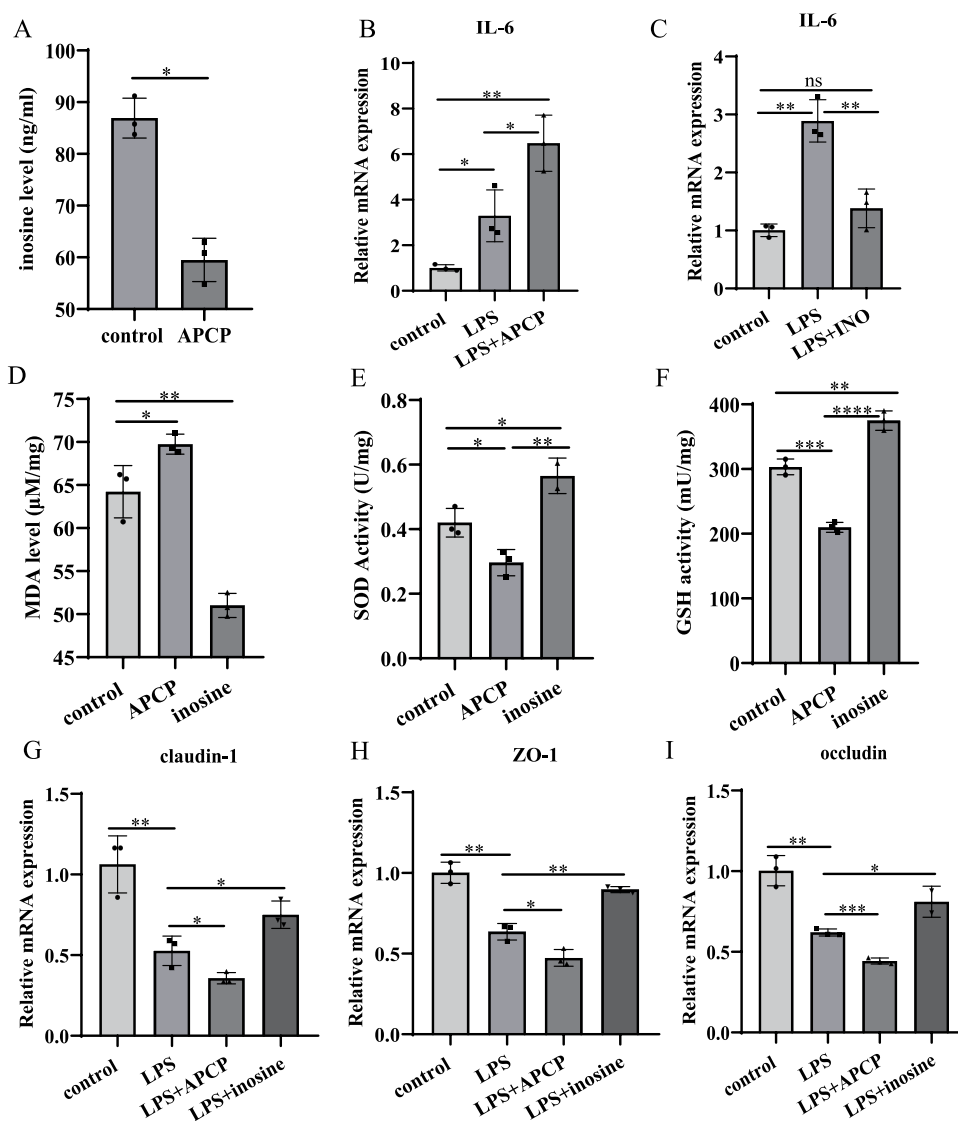


**Figure 4** Inosine promoted the Colonic Antioxidant Abilities and prevented the loss of tight junction proteins in colitis. (A–C) The effect of inosine intervention on the colonic oxidative stress in colon tissues. (D–G) The expression levels of tight junction proteins in colon tissues from control, DSS and inosine treated mice were analyzed by qRT-PCR and immunohistochemical (IHC) staining analysis. Scale bar, 50µm; magnification, 20x. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.0005, \*\*\*\**P* < 0.0001.

significantly elevated the activities of SOD (Control:  $0.423 \pm 0.019$ , DSS group:  $0.320 \pm 0.012$ , Inosine group:  $0.383 \pm 0.021$  U/mg) and GSH (Control:  $15.110 \pm 1.657$ , DSS group:  $11.570 \pm 1.842$ , Inosine group:  $13.690 \pm 0.552$  mU/mg, **Figure 4B** and **C**) in colon tissues. Furthermore, inosine treatment significantly elevated the expression of claudin-1, occludin and ZO-1 (**Figure 4D–G**).

## The Opposite Effects of APCP and Inosine on Intestinal Epithelial Cell

In MODE-K cells, we observed that APCP treatment reduced inosine production (Control:  $86.920 \pm 3.839$ , APCP group:  $59.5 \pm 2.465$  ng/mL, **Figure 5A**). LPS stimulation significantly increased IL-6 expression, which were further enhanced by APCP treatment. In contrast, inosine suppressed IL-6 expression (**Figure 5B and C**). Antioxidant capacity assessment revealed that APCP treatment markedly increased MDA levels, whereas inosine treatment attenuated this effect (Control:  $64.220 \pm 3.033$ , DSS group:  $69.740 \pm 1.162$ , Inosine group:  $51.010 \pm 1.389$   $\mu$ M/mg, **Figure 5D**). Moreover, after APCP treatment, SOD and GSH activities were markedly reduced. However, inosine treatment significantly enhanced the activities of SOD (Control:  $0.420 \pm 0.044$ , DSS group:  $0.296 \pm 0.041$ , Inosine group:  $0.565 \pm 0.055$  U/mg) and GSH (Control:  $303.2 \pm 12.2$ , DSS group:  $209.8 \pm 7.659$ , Inosine group:  $374.9 \pm 14.980$  U/mg, **Figure 5E and F**). The expression levels of claudin-1, occludin, and ZO-1 were significantly reduced by APCP treatment, whereas inosine treatment could restore their expression. (**Figure 5G–I**). Taken together, the impacts of APCP and inosine on MODE-K were completely opposite, consistent with the results from mice experiments.



**Figure 5** The opposite effects of APCP and inosine on intestinal epithelial cell. **(A)**: Serum inosine levels in APCP treated MODE-K cells. **(B and C)** The expression levels of IL-6 in control, LPS, APCP and inosine treated MODE-K cells were analyzed by qRT-PCR. **(D–F)**: The effect of APCP and inosine intervention on the oxidative stress in MODE-K cells. **(G–I)**: The expression levels of tight junction proteins in control, LPS APCP and inosine treated MODE-K cells were analyzed by qRT-PCR. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.0005$ , \*\*\*\* $P < 0.0001$ .

## Discussion

Inflammatory bowel disease (IBD) is characterized by intestinal inflammation and is associated with structural disruption of the intestinal epithelium.<sup>17</sup> Immune disorder and inflammation are implicated in the whole process of IBD. CD73 plays a critical role in immune regulation. In this study, we first demonstrated that blocking CD73 could exacerbate the disease activity in DSS-induced colitis mice, as indicated by increased DAI scores, reduced colon length, more severe intestinal pathological, and elevated IL-6 levels.

Similarly, Zhu's study indicated that Moxibustion could relieve gut inflammation in DSS-induced mice by enhancing the expression of CD39 and CD73.<sup>18</sup> Notably, Shao and colleague's study demonstrated that knocking out CD73 diminishes the therapeutic efficacy of mesenchymal-like endometrial regenerative cells in treating colitis.<sup>19</sup> However, in contrast to our study, Wang et al's study showed that CD73 blockade markedly alleviated DSS-induced colitis in mice, as characterized by reduced weight loss, diarrhea and bloody stool.<sup>8</sup> Moreover, Liu et al's study showed that CD73 blockade suppressed the severity of colitis-associated tumorigenesis with attenuated weight loss, longer colons, lower tumor number and smaller tumor size compared to the model group.<sup>20</sup>

Secondly, we found that CD73 blockade significantly reduced adenosine and inosine levels *in vivo*. Thus, we assumed that adenosine and inosine might be involved in IBD progress. Inosine injection into DSS-mice was used to evaluate the effect of inosine on IBD. As expected, inosine significantly alleviated DSS-induced colitis, as characterized by lower DAI score, longer bowel length, milder intestinal pathology and lower inflammatory cytokines levels. Supportingly, Zhu et al's study demonstrated inosine treatment could improve gut ecosystem and intestinal barrier function.<sup>21</sup> Guo et al's study showed gut microbiota-derived inosine supplementation attenuates colitis through NF- $\kappa$ B and Nrf2 signaling.<sup>22</sup> Notably, although different studies had disagreements about the role of CD73 in colitis, which could be due to the functional diversity of CD73 itself; however, the alleviated effects of inosine on IBD were consistent among different studies. To date, several therapeutic approaches, including anti-TNF- $\alpha$ , corticosteroids, amino salicylates, and antibiotics, have been employed to alleviate IBD. However, long-term use of these medications may give rise to a series of side effects, such as high blood pressure, headaches, diabetes, liver damage, and nausea.<sup>22,23</sup> Inosine is commonly employed as a hepatoprotective and anti-inflammatory agent. Our study indicated that inosine could be a potential therapeutic option for colitis, which needed to be verified by clinical trial in IBD patients. Notably, considering that the lack of a sustained delivery system for adenosine, which led to a high lethality rate in our preliminary experiment, we did not examine the effect of adenosine in DSS-induced mice.

Thirdly, we explored the impact of CD73 blockade and inosine on oxidative stress, both *in vivo* and *in vitro*. Oxidative stress is a crucial pathophysiological factor during IBD development. An overabundance of oxidative stress can undermine the integrity of intestinal barrier.<sup>24</sup> MDA can influence cell mitosis by damaging the DNA structure,<sup>25</sup> which has been regarded as an indicator of oxidative stress and involved in IBD progress.<sup>26</sup> Sahoo's research revealed that the colonic MDA level was increased in IBD.<sup>10</sup> In this study, we found that CD73 blockade led to an increase in MDA. The antioxidant enzymes have the ability to reduce oxidative stress, thereby protecting intestinal cells from oxidative damage.<sup>22</sup> Superoxide dismutase (SOD) could catalyze the conversion of superoxide radicals into oxygen and hydrogen peroxide, which could reduce oxidative damage.<sup>27</sup> GSH can protect cells from free radicals and pro-oxidant damage.<sup>10</sup> Zhang et al's research found the SOD activity was significantly decreased in DSS-induced colitis mice.<sup>28</sup> Grabias's study discovered that enhancing GSH activity can decrease oxidative stress in the colonic mucosa.<sup>29</sup> We observed CD73 blockade significantly reduced SOD and GSH activities. Antioxidants have been proposed as a potential alternative therapy of anti-inflammatory/immunomodulatory drugs for patients with uncomplicated gastrointestinal disorders.<sup>30</sup> In this study, we discovered that inosine could alleviate oxidative stress, through down-regulating MDA and up-regulating SOD and GSH activities.

Fourthly, intact intestinal and mucosal barriers are critical in maintaining colon homeostasis.<sup>31</sup> Tight junction proteins are important for intestinal tract barrier.<sup>12,32</sup> Decreased tight junction proteins lead to a reduction in the firmness of the intestinal barrier and contribute to colitis development.<sup>11</sup> In this study, we explore the impact of CD73 blockade and inosine on claudin-1, occludin, and ZO-1. We observed that APCP treatment decreased claudin-1, occludin, and ZO-1, in both colon tissues and intestinal epithelial cells. Claudin-1 is important for maintaining the integrity of epithelial tight junctions and mainly regulates intestinal permeability.<sup>33</sup> Decreased Claudin-1 lead to the hyperpermeability of intestinal epithelial cells.<sup>34</sup> Severe intestinal leakage was observed in mice with intestinal epithelial-specific knockout of the occludin,<sup>35,36</sup> while the overexpression of

Ocln promotes the epithelial barrier function in vitro.<sup>37</sup> Moreover, ZO-1 regulates epithelial proliferation and mucosal repair. ZO-1 deficiency disturbed mitotic spindle orientation induced abortive proliferation in intestinal epithelial cells.<sup>12</sup> In this study, we observed that inosine can boost the expression of tight junction proteins. Thus, these effects of inosine regulate oxidative stress and tight junction proteins could explain the alleviative effects of inosine on IBD disease. Notably, there were other biomarkers for assessing the structural integrity of the intestinal epithelium, including desmosomes, adherens junctions and cytoskeleton.<sup>38</sup> Except for these, Hempstock's et al also evaluated the paracellular permeability of ions by using Ussing chamber technique.<sup>39</sup> The effect of inosine on structural integrity can be tested by using the above method in further study.

In summary, this study explored the function and mechanism of CD73 in IBD, thereby offering a novel theoretical foundation and potential therapeutic strategy for IBD. Nevertheless, this study has certain limitations. Firstly, the intervention of inosine's effects on IBD disease was performed only in mice model. The potential therapeutic action of inosine on IBD patients needed to be verified by clinical trial in further study. Secondly, gut microbiota plays important roles during IBD progress. Gut bacteria constitute a vital niche within the gastrointestinal tract and play an essential role in maintaining intestinal microecological balance. Compared with healthy individuals, IBD patients exhibit reduced bacterial diversity, diminished levels of anti-inflammatory bacteria, and an expansion of pro-inflammatory bacterial species.<sup>40</sup> However, the influences of CD73 blockade and inosine were not investigated in this study, which needed to be explored in further study.

## Conclusion

In conclusion, our studies revealed that CD73 played a significant role in the progress of DSS-induced colitis via regulating the level of inosine. Inosine could be a potential drug for IBD therapy, which need further verification by clinical trial. Moreover, the alleviative efficacy of inosine on DSS-induced colitis associated with decreased oxidative stress levels, elevated antioxidants levels and tight junction.

## Data Sharing Statement

All data are available through the corresponding author.

## Ethics Statement

This animal study protocol was reviewed and approved by Fourth Military Medical University's Institutional Animal Care and Use Committee (No.20250036). All the experiments were performed and analysed in accordance with ARRIVE guidelines and guidelines for the ethical review of laboratory animal welfare (GB/T35892-2018) issued by China.

## Author Contributions

All authors made a significant contribution to the work reported. Lan Yang: Conception and written the article; Xi Wang: Acquisition of data and written the article; Fang Lin: Execution and draft the article; Chong Liu: Analysis, interpretation and draft the article; Yan Liu: acquisition of data and draft the article; Qing-chun Deng: Analysis, interpretation, written the article and substantially revised the article; Zhao-wei Gao: Study design and substantially revised the article. All of the authors agreed on the Journal of inflammation research to which the article has been submitted. All the authors reviewed and agreed on all versions of the article before submission, during revision, the final version accepted for publication, and any significant changes introduced at the proofing stage. All of the authors agree to take responsibility and be accountable for the contents of the article.

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## Disclosure

The authors declare that they have no conflict of interest.

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