Diet-Induced Hyperhomocysteinaemia Increases Intestinal Inflammation in an Animal Model of Colitis

Siying Zhu, a,b† Jin Li, a,b† Yuntao Bing, b† Wenfeng Yan, b Youqing Zhu, a,b Bing Xia, a,b Min Chen a,b

†These authors contributed equally to this work.

Abstract

Background: Hyperhomocysteinaemia [HHcy] is a common phenomenon observed in patients with inflammatory bowel disease [IBD]. Homocysteine is a pro-inflammatory molecule and has been identified as a risk factor for cardiovascular and cerebral diseases. Whether HHcy contributes to the chronic inflammation of the colon in IBD has rarely been explored. The aim of this study was to investigate the effect of HHcy on dextran sulphate sodium [DSS]-induced colitis.

Methods: Wistar rats were randomly divided into eight groups: [1] Control; [2] HHcy; [3] p38 inhibitor; [4] DSS; [5] HHcy + DSS; [6] HHcy + DSS+p38 inhibitor; [7] HHcy + DSS [21 days]; and [8] HHcy + DSS + folate [21 days]. Colitis was induced by 5% DSS. HHcy was induced by the normal rodent diet containing 1.7% methionine. The mRNA expression of interleukin 17 [IL-17] was detected by qRT-PCR. The protein expressions of IL-17, retinoid-related orphan nuclear receptor-γt [RORγt], p38 MAPK, phosphorylated-p38 MAPK, cytosolic phospholipaseA2 [cPLA2], phosphorylated-cPLA2, and cyclooxygenase 2 [COX2] were detected by immunoblot analysis.

Results: The rats of the HHcy + DSS group had significantly higher myeloperoxidase [MPO] activity, DAI score, and histological score. HHcy significantly increased the plasma concentration, the colonic mRNA, and the protein levels of IL-17. HHcy also activated p38 MAPK and cPLA2, and increased the protein levels of COX2 and RORγt as well as the plasma level of prostaglandin E2 [PGE2]. Folate supplementation down-regulated homocysteine-induced IL-17 and RORγt expressions.

Conclusions: HHcy aggravated DSS-induced colitis by stimulating IL-17 expression via the p38/cPLA2/COX2/PGE2 signalling pathway. The folate supplementation may represent a novel approach to treating the chronic intestinal inflammation of IBD exacerbated by HHcy.

Keywords: Homocysteine; interleukin 17; inflammatory bowel disease

1. Introduction

Inflammatory bowel disease [IBD], which includes Crohn’s disease [CD] and ulcerative colitis [UC], is a chronic inflammatory disorder of the gastrointestinal tract. Patients with inflammatory bowel disease [IBD] carry an increased risk of developing thromboembolic complications. Although the exact aetiology of IBD remains unknown, numerous recent studies suggested that T helper type 17 [Th17] cells play a critical role in the pathogenesis of IBD.1,2,3 Th17
cells originate from naïve CD4+ T cells and mainly secrete interleukin 17 [IL-17] cytokines when stimulated by transforming growth factor-β and interleukin 6 [IL-6].

Homocysteine is a non-protein sulphur-containing amino acid. It is biosynthesised from methionine and degraded by remethylation or the trans-sulphuration pathway. The level of homocysteine is influenced by its nutritional determinants, including folate, vitamin B12 [Vit B12], and vitamin B6 [Vit B6], as well as the genetic polymorphisms of enzymes involved in its metabolic pathway. Elevated levels of homocysteine are a common phenomenon observed in the plasma, as well as in the colonic mucosa, of patients with IBD. It is well known that homocysteine is a pro-inflammatory molecule and plays a critical role in the pathogenesis of several chronic inflammatory diseases, including atherosclerosis, cerebrovascular and heart diseases and rheumatoid arthritis.

Hyperhomocysteinaemia [HHcy] is also recognised as a risk factor for arterial and venous thrombosis. It is possible that homocysteine is implicated in the pathogenesis of IBD. One study in vitro showed that treating human intestinal microvascular endothelial cells [HIMECs] with homocysteine resulted in vascular cell adhesion molecule 1 [VCAM-1] up-regulation, monocyte chemotactic protein-1 [MCP-1] and p38 mitogen-activated protein kinase [MAPK] phosphorylation. Whether homocysteine contributes to the inflammation of colon of IBD in vivo has rarely been explored.

Therefore, we aimed to study whether homocysteine could aggravate the colonic inflammation of experimental colitis in rats induced by dextran sulphate sodium [DSS] and its potential mechanisms.

2. Materials and Methods

2.1. Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Wuhan University. The protocols were approved by the Committee on the Ethics of Animal Experiments of Wuhan University. All surgery was performed under sodium pentobarbital anaesthesia, and all efforts were made to minimise suffering.

2.2. Animals

Specific pathogen-free 2- to 3-month-old Wistar male rats [200–250 g] were supplied by the Experimental Animal Center of Zhongnan Hospital of Wuhan University. The animals were given free access to food and water, and were kept under a constant room temperature of 22°C and a 12-h light/dark cycle. They were allowed to acclimatise to these conditions for at least 5 days before the experiment. Animals were humanely sacrificed if they reached a predetermined experimental end point [loss of ≥ 20% body weight and significant deterioration of body condition].

2.3. Induction of colitis and hyperhomocysteinaemia

Rats were randomly divided into eight groups: [1] Control group; [2] HHcy group; [3] p38 inhibitor group; [4] DSS group; [5] HHcy + DSS group; [6] HHcy + DSS + p38 inhibitor group; [7] HHcy + DSS [21 days] group; and [8] HHcy + DSS + folate [21 days] group. The total number in each group was 10. The numbers of dead and of remaining rats in each group are shown in Table 1. The minimum number of remaining rats was 6 in groups [5], [7], and [8]. Then 6 rats were randomly selected from other groups and used for analysis.

HHcy was induced by giving rats the normal rodent diet containing 1.7% methionine for the first 2 weeks. Colitis was induced by the addition of 5% [w/v] DSS [molecular weight 36–50 kD; Sigma, USA] to the drinking water during the second week. The p38 inhibitor [SB203580, R&D Systems, USA] was dissolved in 200 μl of physiological saline and administered intraperitoneally [5 μmol/kg body weight] at 60 h following DSS induction, and twice daily during the second week. The rats of group [5] were treated with a volume of 200 μl physiological saline intraperitoneally as a control group. The rats of group [8] were treated with folate [0.071 mg/kg, Maidihai Pharmaceuticals, Beijing, China] by gavage during the third week. The rats of group [7] were fed with physiological saline by gavage as a control group [Figure 1].

The rats were sacrificed by cervical dislocation under anaesthesia. Blood samples were obtained from the carotid artery. Colon samples were removed, opened longitudinally and then flushed with phosphate buffer. A portion of colonic tissue was fixed in 4% paraformaldehyde and embedded in paraffin, and then stained with HE for histological analysis. Another portion of colonic tissue was snap-frozen in liquid nitrogen and stored at -80°C for further analysis.

2.4. Disease activity index and histological score

Disease activity index [DAI] was used to evaluate the severity of intestinal inflammation. It was calculated based on body weight, stool consistency, and occult or gross bleeding of the rats as shown in Table 2.

The 5-μm thick sections were stained with HE. All slices were reviewed by two pathologists in a blinded fashion. Intestinal inflammation was quantitated based on a histological score, as described by Berg et al. [0 = no change from normal tissue; 1 = low levels of mononuclear cells infiltration in mucosa with little epithelial damage; 2 = moderate levels of mononuclear cell and neutrophil infiltration, detachment of crypt glands, mucin depletion from goblet cells, and occasional detachment of the epithelium; 3 = high levels of mononuclear cells and neutrophils infiltrating the submucosa, crypt abscesses present, and epithelial disruption with ulceration; and 4 = severe infiltration of neutrophils in mucosa, loss of crypts, and epithelium lost or completely detached].

2.5. Myeloperoxidase activity assay

Myeloperoxidase [MPO] activity was measured within 1 week of harvest of the colonic tissues according to the MPO assay kit manufacturer’s instructions [Jiancheng BioEngineering, Nanjing, China]. The change in absorbance at 460 nm was measured using a Multiskan™ FC Microplate Photometer [Thermo Scientific, USA]. One unit of MPO activity is defined as degrading 1 μmol of hydrogen peroxide at 25°C and MPO activity of tissue is expressed as units per gram protein [U/gprot].

2.6. ELISA assays

Plasma samples were used to determine the concentration of homocysteine [Hcy], IL-17, prostaglandin E2 [PGE2], Vit B12, and folate

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Table 1. The number of remaining and dead rats in each group.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day14 Number remaining [dead]</th>
<th>Day21 Number remaining [dead]</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1] Control</td>
<td>10 [0]</td>
<td>/</td>
</tr>
<tr>
<td>[2] HHcy</td>
<td>10 [0]</td>
<td>/</td>
</tr>
<tr>
<td>[3] p38 inhibitor</td>
<td>10 [0]</td>
<td>/</td>
</tr>
</tbody>
</table>

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by an enzyme-linked immunosorbent assay [ELISA] kit [Elabscience Biotech, China]. Procedures followed the manufacturer’s instructions.

2.7. RNA extraction and quantitative real-time reverse-transcription polymerase chain reaction

Total RNA was extracted from the colonic tissue using the Trizol reagent [Invitrogen, USA]; 5 µg of total RNA was reverse transcribed using a cDNA synthesis kit [Fermentas, USA]. Quantitative real-time reverse-transcription polymerase chain reaction [qRT-PCR] was performed in an ABI 7900 RT-PCR system [Applied Biosystems, USA], and β-actin mRNA was used as an internal control. The following primers were used for the qRT-PCR analysis:

**IL-17:**

- sense 5′- ACAGTGAAGGCGAGCGGTACT-3′;
- antisense 5′- GCTCAGAGTCCAGGGAAG-3′.

**β-actin:**

- sense 5′-CAGATGGAGGGCAGGCTCATC-3′;
- antisense 5′-TGAACCTCTATGCCAACACAGT-3′.

The 20-µl reaction mixture consisted of 10 ng of cDNA, 10 µl SYBR-Green/ Flourescein qPCR Master Mix [2×, Fermentas, USA], and 100 µM of the specific primers [Jingsirui Molbiol, Wuhan, China]. PCR cycling conditions were: initial denaturation for 10 min at 95°C; 40 cycles of denaturation at 95°C for 30s, and annealing/extension at 60°C for 30s. The cycle threshold [Ct] indicated the fractional cycle number at which the PCR product was first detected above a fixed threshold. All samples were analysed in triplicate. The relative mRNA levels were determined using the 2^−ΔΔCT method.

2.8. Immunoblot analysis

Colonic tissues were homogenized in the lysis buffer [50 mM Tris, pH 7.4, 1% sodium dodecylsulphate, and 10 mM EDTA] containing a protease inhibitor cocktail [Sigma, USA], and centrifuged at 15,000 x g for 15 min. The protein concentration in the supernatant was assayed using the Bradford method. Protein [50 µg] was loaded and separated on 10% SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked with 5% non-fat milk in tris-buffered saline for 2h and then incubated with antibodies overnight at 4°C. To detect IL-17, retinoid-related orphan nuclear receptor-γt [RORγt], p38 MAPK [p38], phosphorylated-p38 MAPK [p-p38], cytosolic phospholipaseA2 [cPLA2], phosphorylated-cPLA2 [p-cPLA2], and cyclooxygenase 2 [COX2] proteins, the membranes were probed with polyclonal rabbit-rat antibody IL-17 [1:400, Santa,

Figure 1. The schema of animal models of colitis and hyperhomocysteinaemia. Hyperhomocysteinaemia was induced by giving rat rodent food containing 1.7% methionine. Colitis was induced by the addition of 5% [w/v] DSS to the drinking water. The p38 inhibitor was given intraperitoneally at 60 h following DSS administration. Folate was administered by gavage. Rats were sacrificed for analysis at Day 14 and Day 21.

Table 2. Disease activity index [DAI] scoring system.

<table>
<thead>
<tr>
<th>Score</th>
<th>Weight loss [%]</th>
<th>Stool consistency</th>
<th>Occult/gross bleeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>1</td>
<td>1–5</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>2</td>
<td>5–10</td>
<td>Loose stools</td>
<td>Haemoccult positive</td>
</tr>
<tr>
<td>3</td>
<td>10–20</td>
<td>Loose stools</td>
<td>Haemoccult positive</td>
</tr>
<tr>
<td>4</td>
<td>&gt;20</td>
<td>Diarrhoea</td>
<td>Gross bleeding</td>
</tr>
</tbody>
</table>

Scores were tallied for each category and then divided by three to obtain the DAI.
9. Fluorescent immunocytochemistry

The 5-μm paraffin-embedded sections were deparaffinised and rehydrated using standard protocols. Antigen retrieval was performed by incubating slides for 15 min at 95°C in the sodium citrate buffer [10 mM, pH 6.0]. Sections were blocked with goat serum for 30 min at room temperature. For IL-17 protein detection, the slides were incubated with polyclonal rabbit anti-rat antibody against IL-17 [1:100, Abcam, USA] or isotype control antibody [1:100, Abcam, USA] overnight at 4°C. Sections were washed with PBS and incubated for 1 h at room temperature with Cy3-conjugated goat anti-rabbit IgG [1:100, Boster Biotech, China]. Sections were then washed with PBS and blocked with goat serum for 30 min at room temperature. Then, sections were washed and incubated with 4',6-diamidino-2-phenylindole [DAPI] to visualise nuclei, for 5 min. Stained tissue sections were imaged on a fluorescence microscope [NIKON Eclipse 80i, Japan].

10. Flow cytometry analysis

Lamina propria lymphocyte [LPL] suspensions were obtained as previously described.25 Colons were flushed in PBS and opened longitudinally. Intestinal pieces were incubated in HBSS without Ca\(^{2+}\)/Mg\(^{2+}\) containing 10 mM HEPES, 2 mM EDTA, 1 mM DTT, and 5% FCS for 2×15 min at 37°C with shaking at 250 rpm. Then, intestinal pieces were digested in HBSS containing Ca\(^{2+}\)/Mg\(^{2+}\), 5% FCS, 1.5 mg/ml Collagenase VIII [Sigma, USA], and 0.1 mg/ml DNase I [Thermo Scientific, USA] for 45 min at 37°C with shaking at 250 rpm. LPLs were purified using 40% and 80% Percoll gradients [Sigma, USA] for 45 min at 95°C. After centrifuging at 1000 × g for 20 min at 20°C without brakes. The LPLs were washed and resuspended in culture medium [RPMI 1640 containing 5% FCS] for flow cytometry analysis.

Cells were stimulated with 5ng/ml PMA [eBioscience, USA], 250ng/IL ionomycin [eBioscience, USA], and 2 μM monensin [eBioscience, USA] for the last 4h of culture. Cells were harvested and stained with FITC-labelled CD4 antibody [eBioscience, USA]. Then, cells were fixed with intracellular fixation buffer [eBioscience, USA], permeabilised with permeabilisation buffer [eBioscience, USA], and stained with PE-labelled IL-17 antibody [eBioscience, USA] and RORγt antibody [Abcam, USA]. Cells were analysed using a FACS Calibur flow cytometer [BD Biosciences, USA] with CellQuest Pro\textsuperscript{TM} software on a Macintosh 650 computer. The threshold of positivity was defined beyond the non-specific binding observed in the presence of a relevant isotype control antibody.

11. Statistical analysis

Data were presented as the mean ± standard deviation [SD]. The statistical significant differences among groups were analysed using one-way analysis of variance [ANOVA] for overall comparisons or Student’s unpaired t-test for individual comparisons. A value of p <0.05 was considered significant. Statistical analysis was performed using SPSS 17.0 software [SPSS for Windows version 17.0, USA].

3. Results

3.1. Confirmation of DSS-induced colitis and methionine diet-induced hyperhomocysteaemia

Treatment with DSS induced a colitis characterised by body weight loss, elevated DAI and histological score, and increased MPO activity. There was no difference in daily water consumption among different groups. The body weight of rats began to decrease after treating with DSS for 2 days [Figure 2A]. DAI is a well confirmed scoring system to quantify intestinal inflammation, and has been shown to be correlated with tissue damage scores and histological lesions.3,14 DAI started to increase on the second day and reached maximum at the sixth day after initiation of DSS treatment [Figure 2B]. Colonic MPO activity was measured as an indicator of neutrophil infiltration and subsequent inflammation. As shown in Figure 2C, the MPO activity of the DSS group was significantly increased as compared with the control group [1.36 ± 0.04 vs 0.33 ± 0.02 U/gprot, p < 0.001]. DSS treatment significantly increased the histological score [Figure 2D]. Colon showed severe destruction of the epithelial layer, mononuclear cell and neutrophil infiltration, detachment of cryp glands, and mucin depletion from goblet cells after DSS administration [Figure 2E].

Levels of homocysteine were measured in the blood samples of the rats [Table 3]. As expected, the methionine diet significantly increased the plasma concentration of homocysteine in the HHcy group [HHcy: 13.57 ± 0.66 vs Control: 3.33 ± 0.13 μmol/l, p < 0.001] and the HHcy + DSS group [HHcy + DSS: 13.47 ± 0.36 vs DSS: 3.74 ± 0.14 μmol/l, p < 0.001]. As shown in Table 3, the methionine diet also decreased the plasma concentration of folate and VitB12 in the HHcy group [folate, HHcy: 33.67 ± 1.40 vs Control: 50.65 ± 0.81 nmol/l, p < 0.001; Vit B12, HHcy: 321.78 ± 11.89 vs Control: 447.00 ± 59.54 pmol/l, p < 0.001] and the HHcy + DSS group [folate, HHcy + DSS: 33.04 ± 1.13 vs DSS: 49.69 ± 1.48 pmol/l, p < 0.001; Vit B12, HHcy + DSS: 336.69 ± 10.84 vs DSS: 439.12 ± 31.52 pmol/l, p < 0.001]. Of note, DSS treatment had no influence on the plasma concentration of homocysteine, folate, or Vit B12.

3.2. Hyperhomocysteaemia aggravated DSS-induced colitis

HHcy aggravated the severity of colitis induced by DSS. We found that the rats had more significant body weight loss, higher DAI and histological score, and higher MPO activity in the HHcy+DSS group than in the DSS group. Compared with the DSS group, the body weights were significantly decreased by 2.1% [95.00 ± 1.10% vs 97.00 ± 1.55%, p = 0.027], 2.8% [91.33 ± 1.37% vs 94.00 ± 2.37%, p = 0.038] and 4.8% [86.33 ± 1.75% vs 90.66 ± 2.73%, p = 0.008] in the HHcy + DSS group on Days 4, 5, and 6 of after DSS administration [Figure 2A]. The DAI scores in the HHcy + DSS group were significantly increased on the fourth day [HHcy + DSS: 5.67 ± 0.52 vs DSS: 4.33 ± 0.52, p = 0.001], the fifth day [HHcy + DSS: 9.67 ± 0.52 vs DSS: 5.33 ± 1.37, p < 0.001] and the sixth day [HHcy + DSS: 10.67 ± 0.52 vs DSS: 8.00 ± 0.89, p < 0.001] after DSS treatment [Figure 2B]. The MPO activity in the HHcy + DSS group was also higher than that in the DSS group [1.56 ± 0.05 vs 1.36 ± 0.04 U/gprot, p < 0.001] [Figure 2C]. As shown in Figure 2D, the histological score...
was significantly increased [3.50 ± 0.55 vs 2.33 ± 1.03, \( p = 0.004 \)] in the HHcy + DSS group compared with the DSS group. The rats in the HHcy + DSS group had more severe infiltration of neutrophils in mucosa, loss of crypts, and epithelium loss compared with those in the DSS group [Figure 2E].

Treating rats with a methionine diet also induced a mild inflammation, as shown by significant body weight loss and MPO activity up-regulation in the HHcy group compared with the control group [Figure 2A and C]. Although the histological score of the HHcy group was higher than that of the control group, the difference was not significant [Figure 2D].

3.3. Homocysteine activated the p38/cPLA2/COX2/PGE2 signalling pathway

Numerous previous studies showed that homocysteine might promote inflammation by activating p38 MAPK signalling pathway.\(^{16}\) The p38 MAPK is an upstream regulator of cPLA2 and can activate cPLA2 by phosphorylating it on Ser\(^{505} \).\(^{17}\) Upon the activation of cPLA2, arachidonic acid will be released from the membrane phospholipids. The arachidonic acid can be further converted into prostaglandins by COX2. Therefore, we postulated that the p38/cPLA2/COX2/PGE2 signalling pathway might be implicated in the intestinal inflammation aggravated by homocysteine.

### Table 3. Concentration of homocysteine, vitamin B12 and folate in the plasma of rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Hcy [( \mumol/l )]</th>
<th>Vit B12 [pmol/l]</th>
<th>Folate [nmol/l]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.35 ± 0.13</td>
<td>447.00 ± 59.54</td>
<td>50.65 ± 0.81</td>
</tr>
<tr>
<td>HHcy</td>
<td>13.57 ± 0.66*</td>
<td>321.78 ± 11.89*</td>
<td>33.67 ± 1.40*</td>
</tr>
<tr>
<td>DSS</td>
<td>3.74 ± 0.14</td>
<td>439.12 ± 31.52</td>
<td>49.69 ± 1.48</td>
</tr>
<tr>
<td>HHcy + DSS</td>
<td>13.47 ± 0.36#</td>
<td>336.69 ± 10.84*</td>
<td>33.04 ± 1.13*</td>
</tr>
<tr>
<td>HHcy + DSS + p38 inhibitor</td>
<td>13.39 ± 0.61</td>
<td>320.32 ± 15.19</td>
<td>31.84 ± 0.55</td>
</tr>
<tr>
<td>HHcy + DSS [21 days]</td>
<td>12.08 ± 0.38</td>
<td>363.50 ± 15.28</td>
<td>32.84 ± 1.49</td>
</tr>
<tr>
<td>HHcy + DSS + folate [21 days]</td>
<td>8.43 ± 0.56*</td>
<td>389.19 ± 21.91*</td>
<td>59.54 ± 1.27*</td>
</tr>
</tbody>
</table>

Results were expressed as mean ± SD.

*\( \text{Hcy vs control, } p < 0.05 \).
#\( \text{HHcy + DSS vs DSS, } p < 0.05 \).
†\( \text{HHcy + DSS + p38 vs HHcy + DSS, } p < 0.05 \).
Ø\( \text{HHcy + DSS + folate [21 days] vs HHcy + DSS [21 days], } p < 0.05 \).
As shown in Figure 3A and B, the phosphorylated level of p38 MAPK protein was significantly increased by 1.5-fold [3.26 ± 0.30 vs 2.23 ± 0.46, p = 0.001] in the HHcy + DSS group compared with the DSS group. The phosphorylated level of cPLA2 protein was significantly increased by 1.4-fold [4.73 ± 0.63 vs 3.31 ± 0.56, p = 0.002] in the HHcy + DSS group, as compared with the DSS group [Figure 3A and C]. As shown in Figure 3A and D, the protein level of COX2 in the HHcy+DSS group was higher [5.28 ± 1.46 vs 1.40 ± 0.35, p < 0.001] than that in the DSS group. The plasma concentration of PGE2 was significantly increased in the HHcy + DSS group compared with the DSS group [132.25 ± 2.28 vs 101.79 ± 1.36 pg/mL, p < 0.001 Figure 3E].

To confirm that the increased expressions of cPLA2, COX2, and PGE2 in the HHcy group were induced by the activation of p38 MAPK, we treated rats with the p38 inhibitor [SB203580]. We found that p38 inhibitor treatment significantly decreased the phosphorylated level of cPLA2 protein [2.24 ±0.36 vs 4.73 ± 0.63, p < 0.001] [Figure 3A and C], the protein expression of COX2 [3.64 ± 0.86 vs 5.28 ± 1.46, p = 0.039] [Figure 3A and D], and the plasma level of PGE2 [94.85 ± 0.79 vs 132.25 ± 2.28 pg/mL, p < 0.001] [Figure 3E]. The phosphorylated level of cPLA2 protein and the protein expression of COX2 in the p38 inhibitor group was not different from that in the control group [Figure 3A, C, and D]. These results demonstrated that p38 MAPK was the upstream regulator of cPLA2 and COX2, and HHcy aggravated the intestinal inflammation through the p38/cPLA2/COX2/PGE2 signalling pathway.

3.4. Homocysteine increased the expressions of IL-17 and RORγt
Th17 cells and their cytokine IL-17 played a critical role on the pathogenesis of IBD.19,35 We found that homocysteine increased the expression of PGE2. Several previous studies demonstrated that PGE2 could regulate the differentiation and activation of Th17 cells, which mainly secreted IL-17.20,21 Therefore, we investigated whether homocysteine could stimulate the expression of IL-17 at the mRNA and protein levels.

The plasma IL-17 concentration in the HHcy + DSS group was significantly increased with the DSS group [184.75 ± 4.40 vs 122.77 ± 2.72 pg/mL, p < 0.001] [Figure 4A]. We found that the colonic mRNA and protein levels of IL-17 were also significantly increased by 1.3-fold [10.20 ± 0.57 vs 7.72 ± 0.29, p < 0.001] and 1.2-fold [14.61 ± 2.26 vs 11.72 ± 1.93, p = 0.038] in the HHcy + DSS group, compared with the DSS group [Figure 4B, C, and D]. Similar to the findings of immunoblot analysis, immunohistochemistry showed an increased colonic expression of IL-17 in rats of the HHcy + DSS group, compared with the DSS group [Figure 4F]. Those findings were also confirmed by flow cytometry analysis, which showed that the expression of IL-17 within CD4+ T cells was significantly increased in the HHcy + DSS group compared with the DSS group [6.42 ± 0.62% vs 4.47 ± 0.52%, p = 0.014 Figure 4G and I].

Moreover, the plasma concentration of IL-17 in the HHcy + DSS + p38 inhibitor group was decreased [114.54 ± 3.35 vs 184.75 ± 4.40 pg/mL, p < 0.001] in comparison with that in the HHcy + DSS group [Figure 4A]. The colonic mRNA and protein expressions of IL-17 in the HHcy + DSS + p38 inhibitor group were significantly decreased by 53.8% [4.71 ± 0.65 vs 10.20 ± 0.57, p < 0.001] and 34.3% [9.61 ± 0.80 vs 14.61 ± 2.26, p < 0.001], compared with the HHcy + DSS group [Figure 4B, C, and D]. The protein expression of IL-17 in the p38 inhibitor group showed no difference with that in the control group [Figure 4C and D]. As shown in Figure 4E, immunohistochemistry revealed that colonic expression of IL-17 was decreased in the HHcy + DSS + p38 inhibitor group compared with the HHcy + DSS group. Administration of p38 inhibitor significantly decreased the expression of IL-17 by 24.6% [4.84 ± 0.35 vs 6.42 ± 0.62, p = 0.019] within CD4+ T cells, compared with the HHcy + DSS group [Figure 4G and I].

Focusing on the transcriptional regulation of IL-17 response, RORγt is a key regulator in the differentiation of Th17 cells.22 The protein level of RORγt in the colon was significantly increased by 1.1-fold [14.61 ± 0.99 vs 12.79 ± 1.10, p = 0.013] in the HHcy + DSS group compared with the DSS group [Figure 4C and E]. Similar to the results of immunoblot analysis, flow cytometry analysis also showed that the expression of RORγt within CD4+ T cells in the

Figure 3. Homocysteine activated the p38/cPLA2/COX2/PGE2 signalling pathway. [A] Immunoblotting analysis of the p38 MAPK, phosphorylated-p38 MAPK [p-p38], cPLA2, phosphorylated-cPLA2 [p-cPLA2], and COX2 protein in the colon of rats; [B, C, and D] quantitative analysis of the protein levels of p-p38, p-cPLA2/cPLA2, and COX2; [E] concentration of PGE2 in plasma. *Hcy vs control, p < 0.05; †HHcy + DSS vs DSS, p < 0.05; ‡HHcy + DSS + p38 vs HHcy + DSS, p < 0.05.
Figure 4. Homocysteine increased the expressions of IL-17 and RORγt. [A and B] The plasma and mRNA levels of IL-17; [C] immunoblotting analysis of the IL-17 and RORγt protein in the colon of rats; [D and E] quantitative analysis of the expression of IL-17 and RORγt protein; [F] immunocytochemistry of IL-17 expression in colonic mucosa of rats (fixed cells were labelled with the IL-17 antibody [red], and DNA counterstained with DAPI [blue], scale bar 10 μm); [G and H] expression of IL-17 and RORγt within CD4+ T cells analysed by flow cytometry; [I and J] quantitative analysis of IL-17 and RORγt expression within CD4+ T cells. *Hcy vs control, p < 0.05; †Hcy+DSS vs DSS, p < 0.05; ††Hcy+DSS+p38 vs Hcy+DSS, p < 0.05.
HHcy + DSS group was more significantly increased than that in the DSS group [9.98 ± 0.35 vs 6.53 ± 0.64 %, \( p = 0.001 \), Figure 4H and J]. The p38 inhibitor also decreased the expression of ROXY in the colon as shown by immunoblot analysis [11.38 ± 1.27 vs. 14.61 ± 0.99, \( p = 0.001 \)] [Figure 4C and E] and within CD4+ T cells as shown by flow cytometry analysis [7.51 ± 0.47 vs. 9.98 ± 0.35, \( p = 0.002 \)] [Figure 5B and D]. However, the protein level of ROXY in the p38 inhibitor group showed no change, compared with the control group [Figure 4C and E].

Taken together, these results showed that HHcy significantly increased the plasma concentration, as well as mRNA and protein, levels of IL-17 in the colon through the activation of p38 MAPK.

3.5. Folate ameliorated homocysteine-aggravated colitis in rats

Many studies found that the elevated plasma concentration of homocysteine observed in patients with IBD was inversely correlated to circulating level of folate.22,24 One study showed that treating the patients with IBD with 400 µg of folate could significantly decrease the plasma level of homocysteine.23 Another study reported that folate supplement was effective in inhibiting the homocysteine-triggered VCAM-1 and MCP-1 production in vitro.26 In our study, we found that the plasma levels of folate and Vit B12 were significantly decreased in the HHcy and HHcy + DSS group as compared with the control and DSS groups. We hypothesised that folate supplementation could exert a beneficial effect by inhibiting homocysteine-mediated inflammatory events.

On Day 14, six of the remaining 12 rats were treated with folate by gavage. We found that folate supplementation significantly decreased the MPO activity, the DAI score, and the histological score. Administration of folate significantly decreased the DAI scores on Day 15 [9.00 ± 2.19 vs 11.00 ± 0.00, \( p = 0.049 \)], Day 16 [6.00 ± 1.10 vs 7.00 ± 0.98, \( p = 0.049 \)], and Day 17 [5.00 ± 0.00 vs 6.17 ± 0.98, \( p = 0.016 \)] [Figure 5B]. The MPO activity [0.86 ± 0.05 vs 1.21 ± 0.05 U/gprot, \( p < 0.001 \)] and histological score [2.17 ± 0.75 vs 3.67 ± 0.52, \( p = 0.002 \)] were also decreased in the HHcy + DSS + folate [21 days] group compared with the HHcy-DSS [21 days] group [Figure 5C and D]. As shown in Figure 5E, the epithelium loss, loss of crypts, and high levels of neutrophil infiltration were improved after folate treatment. However, no difference was found between the HHcy + DSS + folate [21 days] group and the HHcy + DSS [21 days] group in terms of body weight [Figure 5A].

3.6. Folate inhibited the p38/cPLA2/COX2/PGE2 signalling pathway activated by homocysteine

The phosphorylated level of p38 MAPK protein was significantly decreased by 30.5% [0.69 ± 0.13 vs 1.00 ± 0.20, \( p = 0.011 \)] in the HHcy + DSS + folate [21 days] group compared with the HHcy + DSS [21 days] group [Figure 6A and B]. Folate supplementation also significantly decreased the phosphorylated level of cPLA2 protein [0.57 ± 0.05 vs 1.00 ± 0.14, \( p < 0.001 \)], and the protein expression of COX2 [0.69 ± 0.09 vs 1.00 ± 0.18, \( p = 0.004 \)] [Figure 6A, C, and D]. Meanwhile, the plasma concentration of PGE2 was significantly lower [93.82 ± 3.32 vs 109.60 ± 4.24 pg/ml, \( p < 0.001 \)] in the HHcy + DSS + folate [21 days] group than that in the HHcy + DSS [21 days] group [Figure 6E]. These results suggested that folate might ameliorate the inflammation in colon aggravated by homocysteine via inhibiting the p38/cPLA2/COX2/PGE2 signalling pathway.

Folate down-regulated homocysteine-induced IL-17 and RORγε expressions. We found that folate treatment decreased the plasma concentration of IL-17 [107.10 ± 4.69 vs 135.56 ± 4.70 pg/ml, \( p < 0.001 \)] [Figure 7A] and the protein expression of IL-17 in the colon [0.62 ± 0.09 vs 1.00 ± 0.13, \( p < 0.001 \)] [Figure 7B and C]. Fluorescent immunocytochemistry analysis showed that the visualisation of IL-17 in the colon revealed reduced expression in the HHcy + DSS + folate [21 days] group [Figure 7E]. Folate administration also significantly decreased the expression of IL-17 within CD4+ T cells [3.67 ± 0.43 % vs 4.77 ± 0.47 %, \( p = 0.042 \)] [Figure 7F and H].

![Figure 5](image-url) Folate supplementation ameliorated homocysteine-aggravated colitis. [A] Body weight; [B] DAI score; [C] MPO activity of colonic tissues; [D] histological scores; [E] representative cross-sections of the transverse colon [HE staining, magnification x400, scale bar 100 µm]. HHcy + DSS + folate [21 days] vs HHcy + DSS [21 days], \( p < 0.05 \).
As shown in Figure 7B and D, the colonic protein expression of RORγt in the HHcy + DSS + folate [21 days] group was significantly decreased by 38.2% [0.62 ± 0.15 vs 1.00 ± 0.08, p < 0.001] compared with the HHcy + DSS [21 days] group. The RORγt expression within CD4+ T cells was inhibited by folate [7.08 ± 0.19% vs 8.28 ± 0.66%, p = 0.040] in Figure 7G and I.

4. Discussion

IBD is characterised by chronic relapsing inflammation of the intestine, and is also associated with increased risk of thrombosis.27,28 Hyperhomocysteinaemia is a common phenomenon observed in patients with IBD.29 Homocysteine is a pro-inflammatory molecule associated with chronic inflammation and risk of cardiovascular and cerebrovascular diseases.30-32 Homocysteine is also identified as a confirmed risk factor for thrombosis.33-35 Based on the current literature, it has been postulated that homocysteine might play an important role in the pathogenesis of IBD.36 However, whether homocysteine contributes to the intestinal inflammation in IBD and its underlying mechanisms still remains unclear. A previous in vitro study reported that homocysteine promoted the inflammatory reaction of HMECs in vitro.22 Our study showed that homocysteine aggravated the inflammation of the colon induced by DSS in rats. Although one study reported that hyperhomocysteinaemia, induced by a vitamin B-deficient diet, reduced DSS-induced disease activity in mice, both our previous study and another two studies showed that hyperhomocysteine-aggravated colitis, which was similar to the results of our current study.35,32,33,34 DSS-induced acute colitis is a Th1-Th17 mediated acute inflammation, characterized by Th17 [IL-17], monokine [IL-6, tumor necrosis factor-α] and chemotactic [keratinocyte-derived chemokine] profile.33 We found that HHcy exacerbated DSS-induced colitis by promoting the production of IL-17 via the p38/cPLA2/COX2/PGE2 signalling pathway. Folate supplementation ameliorated homocysteine-aggravated colitis in rats by decreasing the concentration of HHcy, and inhibiting the p38/cPLA2/COX2/PGE2 signalling pathway.

A recent study showed that p38 MAPK signalling pathway played an important role in the intestinal inflammation of IBD, and the activity of p38 MAPK increased in the colon of patients with IBD.34 Consistently, we also found that the phosphorylated level of p38 MAPK was significantly increased in the rats of the DSS groups. In addition, our study showed that homocysteine significantly increased the phosphorylated level of p38 MAPK protein in colitis induced by DSS. The p38 gene locates in the major IBD susceptibility region 3 on chromosome 6.37,38,39 It plays a key role in intestinal inflammation, cytokine production, and T cell activation in IBD.40 Homocysteine increased p38 MAPK phosphorylation possibly by the inactivation of regulatory protein phosphatase and/or direct oxidation of thiol residues of the enzyme.41,42 It is well known that p38 is the upstream regulator of cPLA2 and can activate this enzyme by phosphorylating it on Ser118,19. Previous studies showed that the activity of cPLA2 was markedly increased in the colon of rats treated with DSS, and in the colonic mucosa of patients with active IBD.33,44 Our study demonstrated the same results. More importantly, our study showed that homocysteine significantly increased the phosphorylated level of cPLA2 protein during DSS-induced colitis. In addition, we found that p38 inhibitor significantly suppressed the phosphorylated level of cPLA2, and attenuated homocysteine-aggravated colitis, suggesting that cPLA2 was regulated by p38 MAPK, and homocysteine might aggravate the intestinal inflammation by activating the p38/cPLA2 signalling pathway. In line with our results, one study in vitro also showed that homocysteine stimulated the sustained phosphorylation of platelet p38 MAPK, which led subsequently to the phosphorylation of cPLA2 in platelet hyperactivity.16 The phosphorylation of cPLA2 will lead to the production of arachidonic acid, which is the substrate of cyclooxygenases.45

COX2 represents the inducible form of cyclooxygenase. It can convert arachidonic acid to prostaglandins, such as PEG2, when stimulated by proinflammatory cytokines.46 It has been found that COX2 was increased in DSS-induced mice, and its mRNA level increases with increasing endoscopic disease activity in patients with IBD.46,47 Nimesulide, a selective COX2 inhibitor, significantly
decreased the production of PGE2, and prevented development of inflammatory changes in animal models of IBD. 48 Similarly, we also found that the expression of COX2 and PGE2 were increased during DSS-induced disease in rats. These results further demonstrated that homocysteine aggravated the intestinal inflammation through activating the p38/cPLA2/COX2/PGE2 signalling pathway. Cytokines are key immunoregulatory modulators of IBD pathology, which play important roles in the innate and adaptive immune responses in mucosal inflammation. 49 Highly differentiated Th17 cells and IL-17 are abundant in the intestinal mucosa of patients with IBD. 50, 51 PGE2 directly promotes differentiation and proinflammatory functions of human and murine Th17 cells. 21 Our study showed that homocysteine significantly increased the plasma concentration,
as well as the colonic mRNA and protein expressions of IL-17. Th17 cells are the new lineage of effector CD4+ T cells, in addition to the classical Th1 and Th2 cell lineages. Production of IL-17 is one of the major features of Th17 cells. RORγt is necessary and sufficient for IL-17 expression and Th17 cell differentiation in both mouse and human CD4+ T cells. RORγt attempts to target Th17 cells through small molecule inverse agonists and IL-23/IL-23R signaling. Previous study showed that RORγt was increased in the T cells of lamina propria in patients with CD. In line with the previous study, we found that DSS treatment significantly increased the protein level of RORγt, and homocysteine further augmented its level in DSS-induced colitis. Meanwhile, we observed that p38 inhibitor could significantly suppress the plasma level and the protein and mRNA levels of IL-17, as well as the protein level of RORγt. Noubade et al. also demonstrated that activation of the p38 MAPK signalling pathway in CD4+ T cells plays an important role in regulating IL-17 production at the translational level. Taken together, these results might suggest that homocysteine increased the production of IL-17 through activating the p38/cPLA2/COX2/PGE2 signalling pathway.

Homocysteine metabolism includes trans-sulphuration to cystathionine and remethylation to methionine, which requires folate and vitamin B12 as the co-factors of enzymes. Folate is the most important determining factor of homocysteine concentration in the plasma. The homocysteine observed in patients with IBD and animal models of IBD was often associated with lower folate level. The folate deficiency among patients with IBD could result from inadequate intake, higher requirements due to bowel inflammation, or malabsorption induced by sulphasalazine treatment. Of note, folate ameliorated homocysteine-aggravated colitis in a stepwise fashion. The DAI score was only slightly decreased on the first and second day of folate administration, then the change became more significant after treatment with folate for another 2 days. Our results showed that folate supplementation decreased the plasma concentration of homocysteine and ameliorated homocysteine-aggravated colitis by inhibiting the production of IL-17 via the p38/cPLA2/COX2/PGE2 signalling pathway. In addition, folate deficiency would predispose patients with IBD to colon cancer. Therefore, we proposed that all patients with IBD, especially those with high levels of homocysteine and low levels of folate, should receive daily folate supplementation to protect against homocysteine-associated inflammation and folate deficiency-related cancer risk.

In conclusion, our study showed that homocysteine aggravated DSS-induced colitis by stimulating the expression of IL-17 via the p38/cPLA2/COX2/PGE2 signalling pathway. Folate could inhibit the p38/cPLA2/COX2/PGE2 signalling pathway, and attenuate homocysteine-aggravated colitis in rats. Folate supplementation might represent a novel target for future therapy in patients with IBD, especially for those with elevated levels of homocysteine.

**Conflict of Interest**

There is no financial conflict of interest to declare for any of the authors.

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**Author Contributions**

Conceived and designed the experiments: SZ, JL, YB, YZ, and MC; performed the experiments: SZ, JL, YB, and WY; analysed the data: SZ, JL, YB, and WY; contributed reagents/materials/analysis tools: SZ, JL, YB, and WY; wrote the paper: SZ, JL, YB, WY, YZ, BX, and MC.

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