Potential pharmacological mechanism of Colitis treatment by diosmetin

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Abstract

BACKGROUND AND PURPOSE Diosmetin exhibits a series of therapeutic efficacy but little is known of its effects on colitis. EXPERIMENTAL APPROACH In this study, two mouse models of DSS (the concentration of 3% and 5%)-induced colitis and Caco2 and IEC-6 cells were employed. The 16S amplicon sequencing was used to assess Gut microbiota changes by diosmetin. Various physical signs of mice (body weight, colon length and DAI score), proinflammatory cytokines and antioxidant enzymes were tested. KEY RESULTS The results showed that diosmetin can markedly decrease the disease activity index and microscopic colon tissue damage, increase the expression of tight junction protein (Occludin, Claudin-1 and Zo-1) and reduce the secretion of proinflammatory cytokines. And diosmetin also significantly inhibited colon oxidative damage through adjusting the levels of intracellular ROS, mitochondrial ROS, GSH-Px, SOD, MDA and GSH in vitro and in vivo. Furthermore, it was found that diosmetin can modulate the abundance of Bacteroidetes, Actinobacteria, Cyanobacteria and Firmicutes, which were reported to be the crucial bacteria related to inflammatory bowel disease (IBD). CONCLUSIONS AND IMPLICATIONS Our data suggested that diosmetin ameliorated the colitis in mice induced by DSS in the potential mechanism that it alleviates intestinal epithelial barrier damage, inhibits the secretion of proinflammatory cytokines, decreases oxidative stress and modulates gut microbiota. It implies that diosmetin may be a novel candidate to alleviate DSS-induced colitis or a lead compound for future optimization and modification.

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In this study, two mouse models of DSS (the concentration of 3% and 5%)-induced colitis and Caco2 and IEC-6 cells were employed. The 16S amplicon sequencing was used to assess Gut microbiota changes by diosmetin. Various physical signs of mice (body weight, colon length and DAI score), proinflammatory cytokines and antioxidant enzymes were tested.

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The results showed that diosmetin can markedly decrease the disease activity index and microscopic colon tissue damage, increase the expression of tight junction protein (Occludin, Claudin-1 and Zo-1) and reduce the secretion of proinflammatory cytokines. And diosmetin also significantly inhibited colon oxidative damage through adjusting the levels of intracellular ROS, mitochondrial ROS, GSH-Px, SOD, MDA and GSH in vitro and in vivo. Furthermore, it was found that diosmetin can modulate the abundance of*Bacteroidetes, Actinobacteria, Cyanobacteria* and*Firmicutes,* which were reported to be the crucial bacteria related to inflammatory bowel disease (IBD).

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Our data suggested that diosmetin ameliorated the colitis in mice induced by DSS in the potential mechanism that it alleviates intestinal epithelial barrier damage, inhibits the secretion of proinflammatory cytokines, decreases oxidative stress and modulates gut microbiota. It implies that diosmetin may be a novel candidate to alleviate DSS-induced colitis or a lead compound for future optimization and modification.

Keywords: diosmetin, proinflammatory cytokines, oxidative stress, gut microbiota

Introduction

Ulcerative colitis (UC), one of the main forms of inflammatory bowel disease (IBD), has the clinical severe symptoms like diarrhea, abdominal pain and mucus with bloody stools, which indicates the potential attack by cytokines and so on (Tripathi & Feuerstein, 2019; Ungaro, Mehandru, Allen, Peyrin-Biroulet & Colombel, 2017), but little is known about pathogenesis of UC. In recent years, many studies reported that genetic factors, environmental factors, microbial factors and intestinal immune system molecules have promoted the occurrence and development of UC (Ordás, Eckmann, Talamini, Baumgart & Sandborn, 2012). At present, the most used clinical drugs of IBD are aminosalicylic acid and glucocorticoids, however, they both show serious side effects, including the potential damages in liver and kidney (Patel, Barr & Jeejeebhoy, 2009; Zoubek et al., 2019). Therefore, it is urgent clinical needs to develop more effective therapeutic methods or agents and further investigate the underlying mechanisms of UC.

The intestinal epithelial barrier, composed of intestinal mucosa connecting epithelial cells and adjacent cells, is the first natural line of defense (Xu, Becker, Elizalde, Masclee & Jonkers, 2018). In recent years, a large number of evidences have indicated that the pathogenesis of IBD is associated with intestinal epithelial barrier injury (Antoni, Nuding, Wehkamp & Stange, 2014; Jäger, Stange & Wehkamp, 2013). Apical tight junction protein, a closed complex, is mainly formed by the interconnection of transmembrane proteins (Claudin, Occludin) and perimembrane protein (zonula occludens, ZO), which is vital in the control of epithelial barrier function and maintenance of paracellular permeability (Choi, Yeruva & Turner, 2017; France & Turner, 2017). Furthermore, some evidences were reported that inflammation and oxidative stress play critical roles in the pathogenesis of IBD. Reactive oxygen species (ROS) can induce intestinal tissue lipid peroxidation and disruption of intercellular junctions, as well as leukocyte and neutrophil infiltration, and promote the inflammatory process. The accumulation of ROS in the gut can cause death of mice and drosophila melanogaster (Dudzińska, Gryzinska, Ognik, Gil-Kulik & Kocki, 2018; Vaccaro et al., 2020). Therefore, preventing ROS and proinflammatory cytokine accumulation in the gut may be able to alleviate IBD.

In recent years, the relationship between disease and gut microbiotas have recently been intensively studied, which strongly indicate that gut microbiota play critical role in gut disorders, including IBD, obesity, liver disease and colorectal cancer (Hagymási, Bacsárdi, Egresi, Berta, Tulassay & Lengyel, 2018; Marchesi et al., 2016; Peng et al., 2019). Imbalance of gut microbiota can directly disorder tightly connected proteins, which

results in decreasing integrity of intestinal mucosa epithelium and ultimately hurts the mucosal barrier (McNamara, Koutsouris, O'Connell, Nougayréde, Donnenberg & Hecht, 2001; Wang, Li & Ren, 2019). Moreover, gut microbiota can promote the differentiation of immune cells and the production of immune mediators, which regulate intestinal immune function (Li, Leonardi & Iliev, 2019). It is critical to look into the possible effect gut microbiota on IBD and seek the potential treatment.

Diosmetin (3', 5, 7-trihydroxy-4 '-methoxy flavone, $C_{16}H_{12}O_6$), a natural flavonoid compound, is found in citrus species (Roowi & Crozier, 2011). A large number of pharmacological investigations have shown that diosmetin has anti-tumor, anti- acute kidney injury and anti-acute lung injury activities (Chen et al., 2019; Liu, Ci, Wen & Peng, 2018; Yang, Li, Yu, Yi & Huang, 2017). The previous studies also indicated that diosmetin can attenuate oxidative stress and decrease level of proinflammatory cytokine (Mo, He, Zhang, Lei & Luo, 2020; Zaragozá, Villaescusa, Monserrat, Zaragozá & Álvarez-Mon, 2020). In our experiment, we studied and reported the effects and potential molecular mechanisms of diosmetin treating IBD.

Methods

Animal and ethical approval

The C57BL/6 mice (18-22 g) with 4-6 week old were obtained from the Experimental Animal Center at Nankai University (Tianjin, China) (SCXK: 2019 -0001) and maintained under a controlled environment: 23 ± 2°C, 50 ± 5% humidity, and a 12 h/12 h light/dark cycles with free access to food and water, and were fed one week to acclimatize before experiment. All animal care and experimental procedures complied with guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Nankai University (Permit No. SYXK 2019-0001).

DSS-induced IBD

The mice were divided into five groups (n = 10): Control group (0.5% CMC-Na), DSS group (the concentration of 3%), DSS with salazosulfapyridine (SASP, 200 mg/kg/d) group and DSS with diosmetin (25 and 50 mg/kg/d) treatment groups. In the DSS, DSS with SASP and DSS with diosmetin groups, the mice were given 3% DSS (molecular weight of 36–50 kDa, MP Biomedical Solon, OH, USA) in drinking water on days 0–7 and the daily drinking volume of each mouse is calculated as 6mL, and the DSS solution is supplemented to the daily drinking volume the next day., while in the Control group, the mice were given drinking water without DSS. In the DSS with SASP and DSS with diosmetin groups, the mice were given SASP (200mg/kg/d) and diosmetin (25 and 50 mg/kg/d) on the day 8-15, and the mice in the NC and DSS groups were given 0.5% CMC-Na. The second model was also divided into five groups (n = 10): Control group (0.5% CMC-Na), DSS group (the concentration of 3%), DSS with salazosulfapyridine (SASP, 200mg/kg/d) group and DSS with diosmetin (25 and 50 mg/kg/d) treatment groups. In the DSS, DSS with SASP and DSS with diosmetin groups, the mice were given 5% DSS in drinking water on the day 0–7, while in the Control group, the mice were given drinking water without DSS. In the DSS with SASP and DSS with diosmetin groups, the mice were given SASP (50mg/kg/d) and diosmetin (25 and 50 mg/kg/d) on the day 1–7, and the mice in the NC and DSS groups were given 0.5% CMC-Na. During the experiments, body weight and disease activity index (DAI) (13) were measured daily. After mice were sacrificed, the colon tissue and feces samples were collected and colon length was measured.

Histological assessment

The isolated colon tissues were fixed in 10% formalin for one week, embedded in paraffin, and sectioned into 5 μm slices, and then the sections were stained with hematoxylin-eosin (H&E) solution. Finally, images of the stained sections were photographed using a light microscope (Nikon Eclipse TE2000-U, NIKON, Japan) at 200 × magnification.

Immunofluorescence assessment

The effect of diosmetin on expression of ZO-1 was examined by means of immunofluorescence staining in vivo and in vitro. The tissue slices or formalin-fixed cells were incubated with anti-ZO-1 antibodies for overnight

at 4 . Following, washed three times with PBS, fluorescein-conjugated secondary antibody was incubated for 1 h at 37. The cell nuclei were stained with DAPI (5.0 μg/mL). Finally, the samples were photographed by using a confocal microscope (Nikon, Japan), with 400 × magnification.

Cell viability assay

The Caco2 and IEC-6 cells were seeded in 96-well plates at a density of 5 × 10^4cells·mL^{-1} for 24 h and pretreated with various concentrations of diosmetin (0- 400 μM·mL^{-1}) for 6, 12 and 24 h and various concentrations of Lipopolysaccharide (LPS, 0-32 μg·mL-1). Cell viability was measured by the MTT method as described above.

Western blotting assay

The total protein from Caco2 and IEC-6 cells and colon tissues were homogenized using RIPA lysis buffer containing protease and 1 mM phenylmethylsulfonylfluoride (PMSF). The protein concentrations were tested using a BCA Protein Assay Kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. The proteins were separated by SDS-PAGE (7.5–15%), and then transferred to PVDF membranes (Millipore, Massachusetts, USA). Finally, the membranes were detected using enhanced chemiluminescence reagent with a Bio-Spectrum Gel Imaging System, respectively (UVP, California, USA). Experiments were performed at least five time.

Quantitative real-time PCR assay

Total RNA samples were obtained from colon tissues using TRIzol (Invitrogen) reagent following the manufacturer's protocol. cDNA was synthesized by reverse transcription of each RNA sample by First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing). The levels of proinflammatory factor were quantified using Green qPCR SuperMix (TransGen Biotech, Beijing). The GAPDH RNA was used for normalization. The primers used in the present study are indicated in Supporting Information Table S1

Biochemical Analysis

The levels of GSH-peroxidase (GSH-Px), GSH, malonaldehyde (MDA) and superoxide dismutase (SOD) in colon tissues were determined using the specific kits (Beyotime Institute of Biotechnology) according to the manufacturer's instructions.

Measurement of intracellular ROS level

The Caco2 and IEC-6 cells were plated in 24-well culture plates at a density of 5 × 10^4 cells·mL^{-1} and treated with diosmetin (25 and 50 μM) for 2h before challenge with LPS. Then the cells were added in 500 μL of DCFH-DA (10.0 μM) for the detection of ROS, which was photographed by using a confocal microscope (Nikon, Japan), with 400 × magnification. **Mitochondrial ROS level determination**

The Caco2 and IEC-6 cells were plated in 24-well culture plates at a density of 5 × 10^4 cells·mL^{-1} and treated with diosmetin (25 and 50 μM) for 2h before challenge with LPS. Then the cells were added in 1mL MitoSOX Red Mitochondrial Superoxide Indicator (Yesen, Shanghai), incubated at 37 °C in dark for 10 min, washing three times with washing buffer. Finally, the cells were photographed by using a confocal microscope (Nikon, Japan), with 400× magnification.

Microbial community analysis

Mice feces were collected and stored at -80 degC. The mice feces were divided into three groups (n = 5): Control group,DSS group and DSS with diosmetin (50 mg/kg) group, and the 16S rDNA gene sequencing of the feces samples were sent to Center for Genetic & Genomic Analysis, Microread, (Beijing, China) under dry ice conditions. The DNA of total bacteria in mice feces was extracted with QIAamp(r) Fast DNA Stool Mini Kit. Primers corresponding to V3-V4 regions of bacterial 16S rRNA were selected for amplification. Sequencing results and statistical results of sequence data of all samples were based on sequenced reads and operational taxonomic units (OTUs).

Statistical analysis

Data were expressed as mean +- SD and analysed using Graph Pad Prism 7 (Graph Pad Software, Inc., San Diego, CA). Statistical analysis was performed with one-way ANOVA when comparing multiple independent groups. The p value¡0.05 was considered statistically significant.

Material

Diosmetin was obtained from Shanghai yuanye Bio-Technology Co., Ltd. (Shanghai, China). LPS was purchased from Santa Cruz Biotechnology. (Santa Cruz, USA). The BCA Protein Assay Kit, DCFH-DA, GSH-Px, GSH, MDA and SOD kits were obtained from Beyotime Institute of Biotechnology (Shanghai, China). The First-Strand cDNA Synthesis SuperMix kit and Green qPCR SuperMix kit were obtained from TransGen Biotech (Beijing, China). The MitoSOX Red Mitochondrial Superoxide Indicator was purchased from Yesen (Beijing, China). Anti-Occludin, anti-Claudin-1 and anti-ZO-1 were obtained from Proteintech Group, Inc. (Chicago, IL, USA). Anti-GAPDH were obtained from Cell Signaling Technology (Boston, United States).

Result

Diosmetin protects against DSS-induced colitis in vivo

We first evaluated the effects of diosmetin treatment on DSS (the concentration of 3% and 5%)-induced colitis, and the experimental program is shown in Fig. 1A, B. As shown in Fig. 1C, the body weight was decreased apparently and the DAI score increased rapidly with the treatment of DSS. After stopping drinking DSS, the body weight was recovered gradually and the DAI score decreased. As shown in Fig. 1D, compared to DSS only group, the body weight in mice treated with diosmetin was higher and DAI score was lower. As shown in Fig.1E, F, compared with control group, the length of colon significantly shorten in DSS only group, however the length of colon observably become longer in DSS with diosmetin group compared to DSS group. As shown in Figure 1G, H, (200x magnification) the histopathological changes including the inflammatory infiltration, disappearance of goblet cells and superficial epithelial cells and edema between mucosal and muscular layers, in the groups treated with DSS only, were all markedly reversed by SASP and diosmetin.

As shown in Figure 1I, K, the viability of caco2 and IEC-6 cells was increased or decreased without any significant difference compared to that of control group after treatment with diosmetin (25–400 μM) for 6–24 h, which revealed that diosmetin was non-toxic to caco2 and IEC-6 cells under such conditions. As shown in Figure 1G, L, exposure to LPS (0.125–32 μg /mL) for 24 h induced the viability of caco2 and IEC-6 cells slightly increased or decreased without any significant difference compared with the control groups. These results indicated that diosmetin markedly alleviated DSS-induced colitis and was non-toxic to caco2 and IEC-6 cells.

Diosmetin alleviates intestinal epithelial barrier damage in vitro and in vivo

As shown in Fig. 2A, B compared to the control group, the expression levels of tight junction proteins (Occludin and Claudin) in the mice of DSS only group were observably decreased, however, diosmetin and SASP were able to markedly increase the expression of Occludin and Claudin compared to the model group. In addition, the results of immunofluorescence analysis of ZO-1 as shown in Fig. 2C, D, the expression levels of ZO-1 in colon sections were markedly decreased after DSS treatment in vivo compared to the control groups. These levels were significantly up-regulated when the cells were pre-treated with diosmetin and SASP. Next, we analyzed the expression of tight junction proteins, Occludin and Claudin, by Western blot after 24 h exposure to different concentrations (0.1–30μg/ml) of LPS in caco2 and IEC-6 cells. As shown in Fig. 3A, the levels of expression of Occludin and Claudin were obviously decreased in a dose-dependent way for the caco2 and IEC-6 cells in LPS-treated group. Furthermore, the expression level of Occludin and Claudin administrated with LPS (30 μg) was significantly decreased.After pretreatment with diosmetin (25-100 ng/mL) for 24 h, the levels of expression of Occludin and Claudin were obviously increased in caco2

and IEC-6 cells (Fig. 3B). As shown in Fig. 3C, D, the immunofluorescence analysis of ZO-1 exhibited similar results.

Effect ofdiosmetin can reduce pro-inflammatory cytokines in vivo

The effects of diosmetin on the expression of inflammation related genes were studied at mRNA level shown in Fig. 4. diosmetin (50 mg/kg) significantly inhibited the expression of IL-1β, IL-6, cyclooxygenase-2 (COX-2) and IFN-γ (p¡0.05) Compared to 3% DSS only group (Fig4 A-D). Compared to 5% DSS only group, diosmetin (50 mg/kg) significantly inhibited the expression of IL-1β, IL-6, cyclooxygenase-2 (COX-2) and IFN-γ (p¡0.05) (Fig. 4E- H).

Diosmetin suppressed oxidative stress in vitro and in vivo

As shown in Fig. 5A-H, compared with DSS (the concentration of 3% and 5%) only groups, MDA levels in colon tissues were significantly decreased, and the levels of SOD, GSH and GSH-Px were reversely increased by diosmetin. As shown in Fig. 5I, J, the intracellular ROS level of caco2 and IEC-6 cells in LPS group were remarkably increased compared to control group. However, diosmetin significantly decreased ROS level in caco2 and IEC-6 cells compared with LPS group. Next, we evaluated the mitochondrial ROS production in Fig. 5K, L, compare with LPS group, diosmetin markedly decreased mitochondrial ROS level of caco2 and IEC-6 cells.

Effects ofdiosmetin and DSS on gut microbiota

Different Alpha diversity indices, including Shannon-Wiener curve, Simpson index, PD_whole_tree index, Good_coverage index, Chao1 index, observed_species index and Rank Abundance curve of each sample are near flat (Fig. S1), which indicates that the bacterial diversity and sequencing depth and results were sufficient. Alpha diversity index box of difference between groups were shown in Fig. 6A-F. The diversity of DSS group observably decreased (p¡0.05) compared to the control group, however the diversity of DSS with diosmetin group was markedly increased compared to the DSS only group. With 97% sequence similarity, the USearch61 clustering method in QIIME software was used to group the sequence into multiple Operation Taxonomic Unit (OTUs). As shown in Fig. 6G, there were 1629 unique (OTU) found in the control group, 536 in the DSS only group, 397 in the DSS with diosmetin group. Beta diversity analysis, principal component analysis (PCA) was displayed in Fig. 6H, and UniFrac heatmap analysis was displayed in Fig. S2. UniFrac analysis is divided into two measurement methods: weighted unifrac and unweighted unifrac. Unweighted unifrac can detect the existence of variation between samples, while weighted unifrac can further quantitatively detect the variation that occurs on different lineages between samples. PCA and UniFrac heatmap analysis showed that DSS significantly changed the gut microbiota, and on this basis, diosmetin could regulate the gut microbiota. As shown as in Fig. 6 I, J, DSS group decreased the relative abundance of Bacteroidetes and cyanobacteria and increased the relative abundance of Firmicutes compared with the control group in phylum level of bacterial composition, however, DSS with diosmetin group increased the relative abundance of Bacteroidetes and cyanobacteria and decreased the relative abundance of Firmicutes compared with the DSS group.

The changes of the main microbiota at Class, Order, family and Genus level are shown in Fig. S3 and Fig. S4. After DSS treatment, the relative abundancens of*Lachnospiraceae* and *Ruminococcaceae* markedly decreased and the relative abundances of *Bacteroidaceae, Clostridiaceae, Lactobacillaceae* and *Turicibacteraceae* significantly increased compared to the control group. The whole sample microbial community structure was analyzed by Species abundance clustering heatmap at the phylum level shown in Fig. S5.

In order to find maker bacteria with statistical differences among the groups. LEfSe analysis, or LDA Effect Size analysis were used to discover high-dimensional biomarkersand reveal genomic features. The non-parametric factorial Kruskal-Wallis (KW) sum-rank test is used to detect features with significant abundance differences and find Taxa with significant differences from abundance (Puri et al., 2018). As results, 42 dominant OTUs from the 3 groups are displayed. Herein, a total of 3 distinct OTUs were observably reversed by diosmetin intervention (Fig. 7A). The results are also shown in Evolutionary branch graph of LEfSe

analysis (Fig. 7B). AS shown as in Fig. 8, diosmetin treatment and markedly decreased bacteria abundance of*Eggerthella,Flavobacterium* and*Clostridium*and significantly increase bacteria abundance of *Odoribacteraceae, prevotella, Rikenellaceae, Ruminococcus, Coprococcus, Roseburia, Oscillospira, Anaeroplasma and Synergistales* compared with DSS group.

Furthermore, the PICRUSt was used to predicted the discrepancy of functional profiles between different groups (Fig.9). Compared with DSS group, 14 (8 enriched, 6depleted) functional modules were significantly altered ($p < 0.05$) by treatments with diosmetin. Diosmetin mainly caused changes in some metabolism pathways, such as arginine, ornithine and proline interconversion, methanogenesis from acetate and superpathway of tetrahydrofolate biosynthesis. In conclusion, these data illustrated that diosmetin contributed to the functional difference of gut microbiota.

Discussion

DSS-induced colitis results in dysfunction of colon, including severe Intestinal barrier dysfunction, inflammatory infiltration, rectal bleeding, diarrhea, and gut microbiota changes (Dai et al., 2015; Rohwer et al., 2019; Wirtz et al., 2017), which is similar to clinical and histological manifestations of human ulcerative colitis (Li et al., 2020). Several studies have demonstrated the antioxidant agents can be against colitis, through effects on reducing inflammation and rebalancing gut microbiota (Liu et al., 2019; Peng et al., 2019). In this study, we adopted *in vivo* acute and chronic colitis mouse models induced by DSS (the concentration of 3% and 5%).

Diosmetin has already been demonstrated that exert protective effects against colon cancer and LPS induced acute lung injury (Koosha, Mohamed, Sinniah & Alshawsh, 2019; Liu, Ci, Wen & Peng, 2018). In this study, we illustrated that diosmetin can protect against LPS-induced injury to Caco2 and IEC-6 cells *in vitro* and against DSS-induced colitis in mice *in vivo* shown by alleviating histopathological changes and restoring the length of the colon. These data suggested that diosmetin can inhibit DSS-induced colitis.

The disruption of the intestinal epithelial barrier has been found to be relevant to the colitis (Su et al., 2009) which can lead to pathogenic antigen invasion. Thus, new ways of repairing mucosal barrier, improving mucosal healing and reducing mucosal permeability are considered to be potential methods of treating colitis. Among many proteins in mucosal barrier, ZO-1 is a major tightly linked scaffold protein, which associates with the epithelial integrity (Furuse et al., 1994). Occludin is essential for barrier function and tight junction stability (Saitou et al., 2000). Claudin-1 is a transmembrane protein and a component of tight junction strands (Furuse, Fujita, Hiiragi, Fujimoto & Tsukita, 1998). Transmembrane proteins Claudin, Occludin and perimembrane protein ZO form a closed complex. The complex formation and breakage of ZO-1, Claudin-1 and Occludin in intestinal mucosal epithelial cells can affect intestinal epithelial barrier function greatly (Morini, Babini, Barbieri, Baiocco & Ottolenghi, 2017). In our study, the expression levels of Claudin-1 and Occludin can be significantly increased by diosmetin *in vitro* and*in vivo* compared to DSS group. The expression of ZO-1 in immunofluorescence also significantly increased by diosmetin in vitro and in vivo compared to DSS group. These datas showed that diosmetin could inhibit DSS-induced intestinal epithelial barrier dysfunction.

It has been pointed out that the processes of oxidative stress and inflammation be relevant to DSS-induced colitis (Macias-Ceja et al., 2017; Zhao et al., 2019). The MDA is natural products of lipid hydroperoxide of organisms. Oxidative stress causes some fatty acids in cells to be oxidized and broken down into a series of compounds, including MDA, which has been frequently used as an indicator of oxidative stress (Qian et al., 2010). SOD has been pointed out that it can catalyze the disproportionation of superoxide anions to generate hydrogen peroxide (H_2O_2) and oxygen (O^2), which is an important antioxidant enzyme in the body (Cao et al., 2015). GSH and GSH-Px can catalyze hydrogen peroxide and other peroxides to produce water and organic alcohol (Maejima, Kuroda, Matsushima, Ago & Sadoshima, 2011). In our study, we found DSS promoted oxidative stress shown by the low levels of GSH, GSH-Px and SOD and high level of MDA in colon tissue of mice, which were reversed by diosmetin. In addition, diosmetin significantly reduced ROS levels and mitochondrial ROS in Caco2 and IEC-6 cells caused by DSS. Therefore, inhibition of oxidative

stress may be one mechanism by which diosmetin protects against DSS -induced colitis.

The inflammatory cytokines of IL-1β, IL-6, COX-2 and IFN-γ play leading roles in the formation of colitis (Biasi, Astegiano, Maina, Leonarduzzi & Poli, 2011). In addition, these pro-inflammatory cytokines can stimulate the activation of NF-ϰB transcription (Cao et al., 2015). Meanwhile, activation of NF-ϰB pathway further releases proinflammatory cytokines including TNF-α, IL-1β and IL-6 in DSS-induced colitis (Kim, Shin, Chung, Lee, Baek & Lee, 2019). IL-6 can stimulate the secretion of electrolytes from intestinal epithelial cells, result in increased permeability of endothelial cells, prompt the aggregation of neutrophils to the inflammation site and cause IBD (Zhang et al., 2011). Yamamoto (Yamamoto-Furusho, Santiago-Hernández, Pérez-Hernández, Ramírez-Fuentes, Fragoso & Vargas-Alarcón, 2011) have demonstrated that the expression of IL-1β in the lesion site of ulcerative colitis was significantly higher than that in the normal site, and also found that the gene polymorphisms of IL-1β and IL-1RN are relevant to genetic susceptibility and hormone dependence of ulcerative colitis. COX-2 plays a dominant role in the regulation of the inflammatory state by stimulating the biosynthesis of prostaglandins (PG) (Cianciulli, Calvello, Cavallo, Dragone, Carofiglio & Panaro, 2012). IFN-γ is a mediator of the development of DSS-induced colitis (An, Li, Bhang, Song & Youn, 2020). Thus, suppression of these inflammatory cytokine is an important target for the treatment of IBD. Our studies found, diosmetin significantly downregulated the mRNA levels of IL-1β, IL-6, COX-2 and IFN-γ, which suppress the inflammation signalling pathways, it may be another pathway of diosmetin against DSS-induced colitis.

Many studies have shown that gut microbiota is a key factor in the pathogenesis of IBD (Ni, Wu, Albenberg & Tomov, 2017; Zuo, Kamm, Colombel & Ng, 2018). Robert W. Li's study of porcine colitis model demonstrated that mechanistic insights into the attenuation of intestinal inflammation and modulation of the gut microbiome by krill oil (Liu et al., 2020). In the present work, we investigated the differences in gut microbiota of mice among control groups, DSS group and DSS with diosmetin group. Alpha diversity index dilution curve of each sample tend to be flat, indicating that the sequencing result was credible. Alpha diversity index has indicated that microbial diversity in DSS group significantly decreased compared with control group, after diosmetin treatment, the diversity was increased and the community composition was changed. From the level at phylum, DSS group increased the ratio of Firmicutes to Bacteroidetes due to the relative abundance decreased in Bacteroidetes and the relative abundance increased in Firmicutes compared with the control group. The similar phenomena has also been found in other studies (Jeffery et al., 2012; Peng et al., 2019). However, DSS with diosmetin group increased the relative abundance of Bacteroidetes and decreased the abundance of Firmicutes compared with the DSS group.

The LEfSe analysis at OTU level exhibited different dominant bacteria in different groups. Herein, *Eggerthella lenta* is kind of bacterial genera typical for inflammation (Soltys et al., 2020), and it was repressed in the DSS with diosmetin group. Previous studies have found that *Pelomonas*(25%) and *Flavobacterium* (13%) dominated the bacterial composition of patients with a high Crohn's disease activity index, while *Bacteroidetes* had a relatively low abundance (4%). And in this study, diosmetin treatment observably decreased the bacteria abundance of *Flavobacterium* . Our research also found that diosmetin significantly increase bacteria abundance of *Odoribacteraceae, prevotella, Rikenellaceae, Ruminococcus, Coprococcus, Roseburia, Oscillospira, Anaeroplasma and Synergistales* compared with DSS group. These bacteria may become potential biomarkers of colitis in the future. Thus, these data demonstrated that diosmetin can modulate the community of gut microbiota, especially certain specific microbiome. These specific bacteria can be benefit for treating colitis.

In summary, Our studies have demonstrated that diosmetin exerted the therapeutic effects in DSS-induced colitis through several pathways, including reducing the expression levels of pro-inflammatory cytokines (IL-6, IL-1β, IFN-γ and COX2) and oxidative stress, increase expression of tight junction proteins of claudin-1, Occludin and ZO-1 and modulate gut microbiota. These pathways consist of the part of pharmacological mechanism of diosmetin against colitis. Diosmetin can potentially use to treat Colitis or become a lead compound for further optimization.

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Author contributions

H.L.L., Y.Y.W., X.H.L., H.G.Z. and C.Y designed the experiments and wrote the manuscript. H.L.L., Y.Y.W., H.R. and S.S.Z. performed the animal and cell experiments. H.L.L., Y.Y.W. and X.H.L. performed the quantitative real-time PCR and Western blotting assays. Z.W.L and S.S.S. performed the immunofluorescence experiments. R.T.Z. and B.W.M performed the Histopathological section. K.X.L. and J.Y.P. edited the manuscript.

Conflict of interest

The authors declare no conflicts of interest

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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