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Original Article

Effects of hydroxychloroquine on the mucosal barrier and gut microbiota during healing of mice colitis

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Abstract: Objectives: The present study aimed to evaluate the impact of hydroxychloroquine (HCQ) on the mucosal barrier and gut microbiota during the healing of mice colitis. Methods: The body weight, colon length, colon Hematoxylin-Eosin (H&E) staining, occult blood in feces and serum inflammatory factor levels were measured to evaluate the function of HCQ on inflammatory process in colitis mice. The Alcian blue staining, immunohistochemistry, immunofluorescence and serum FITC-Dextran assay were performed to assess the intestinal mucosal permeability. And the composition and expression differences of intestinal microorganisms in feces were analyzed with 16S rDNA sequencing for exploration of HCQ impact on gut microbiota in colitis. Results: The results showed that the administration of HCQ did not significantly alter the body weight, colon length, or fecal occult blood of the mice. However, HCQ treatment did lead to recovery of the structure and morphology of the intestinal mucosa, increased expression of tight junction proteins (E-cadherin and Occludin), decreased permeability of the intestinal mucosal barrier, increased serum IL-10, and decreased level of tumor necrosis factor-alpha (TNF-α). Additionally, HCQ was found to increase the abundance of Euryarchaeota, Lactobacillus_murinus and Clostridium_fusiformis, while decreasing the abundance of Oscillibacter, uncultured_Odoribacter, Bacterioidetes and Muribaculum. Conclusions: These findings support that HCQ plays a role in the treatment of mice colitis possibly by altering the gut microbiota.

Keywords: HCQ, intestinal injury, gut microbiota, mucosal barrier

Introduction

Inflammatory bowel disease (IBD) is a recurrent and long-lasting inflammation disorder of the gastrointestinal tract, caused by an imbalance of the immune system in the intestinal mucosa (IM) [1]. It is estimated that more than one million Americans and two and a half million European individuals have IBD. IBD has become a global health problem, with its frequency increasing especially in industrial nations in the Middle East, Asia, and South America [2]. Two subclasses of IBD have been established, namely Crohn disease (CD) and ulcerative colitis (UC). Existing theory of IBD causation suggests interplays among risk factors such as immune system, microbes and environment in hosts who are genetically vulnerable [1]. IBD induces alterations in intestinal tissues and dysfunction of the enteric barrier of epithelium. Despite an incomplete understanding of IBD etiology, the relationship between disease development and the microbiota of the intestines is well established [3]. Thus, identifying effective drugs to repair intestinal injury and regulate gut microbiota is an essential strategy toward IBD treatment.

Lysosomotropic drugs, such as hydroxychloroquine (HCQ), are alkaline in nature and accumulate in lysosomes, where they disrupt significant biological processes by increasing the pH level [4]. HCQ is currently considered an important drug for treating rheumatic disorders in clinics, especially rheumatoid arthritis and systemic lupus erythematosus [4]. Previous studies have shown that HCQ has health benefits, including anti-inflammatory and immunomodulatory effects [5]. Specifically, HCQ can reduce the levels of proinflammatory cytokines produced by peripheral mononuclear blood cells by inhibiting toll-like receptor (TLR) signaling in endosomes. These cytokines include interleukin-1 (IL-1), IL-2, IL-6, interferon-gamma (IFN-γ),

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and tumor necrosis factor-alpha (TNF-α) [6]. As a result, HCQ can decrease aberrant immune response activation mediated by TLR signaling, thereby reducing inflammatory symptoms in individuals with rheumatic diseases [7]. Recent studies have also described the effects of HCQ on metabolism, platelets, and neoplasms [8].

Many studies have shown the efficacy of HCQ in treating rheumatic autoimmune disorders (RADs), malaria, cancers, and more [9]. However, there is limited research on the potential correlation between HCQ and IBD. Therefore, our study aimed to investigate whether HCQ can be effective in treating IBD by repairing intestinal injury and regulating gut microbiota.

Materials and methods

Animal experiment protocol

The animal protocol of this experiment prior to commencement was approved by Jiangsu University's ethics committee (13436). The Center of Animal Laboratory at Jiangsu University (Jiangsu, China) supplied male C57BL/6J mice weighed about 25 g per mouse (10 weeks old), wherein they were acclimatized to laboratory environment without pathogens at 22±3°C temperature and 40-60% humidity. We allocated the mice into 3 groups at random with each group comprising of 7 mice, wherein they were studied for a period of 3 weeks. When the mice suffered from rapid loss of 15-20% of body weight, complete loss of appetite for 24 hours or poor appetite (less than 50% of normal) for 3 days and being unable to feed and drink by himself, they were euthanized. While mice in CON group were given demineralized water, their counterparts in DSS and HCQ group received 2% DSS in their drinking water for 7 days. After that, the drinking water of mice in DSS group was replaced by demineralized water, while those in HCQ group were supplemented with HCQ (5 mg/mL) in their drinking water.

All the mice were given standard food and euthanized within 3 weeks. Mice were anesthetized with pentobarbital and then euthanized by carbon dioxide asphyxiation. About 500 μl of blood was collected by orbital puncture and serum was separated by centrifugation at 3,000 rpm, for 15 minutes at 4°C. The tissues of colon were collected and stored in a freezer (-80°C). Prior to freezing in -80°C for 16S rDNA sequencing, the intestinal feces were removed and stored in liquid nitrogen tank.

Enzyme-linked immunosorbent assay (ELISA) for cytokines

The concentrations of cytokines, including IL-4 (QZ-10258), IL-6 (QZ-10260), IL-10 (QZ-10235), and TNF-α (QZ-10225), were measured in the serum using commercial ELISA kits (all purchased from Jiubang Biotech, Fujian, China) according to the manufacturer's protocols.

Analysis of morphology and immunohistochemical (IHC)

Phosphate buffered solution (PBS) was used to wash para-formaldehyde (4%)-fixed tissues before dehydration with ethanol (70%, 95% and 100%) and anhydrous alcohol. Later, we sectioned (5 μm thick) the colon tissues after they have been made transparent by xylene, coupled with paraffin embedment. Later, staining of the sections was accomplished with Alcian Blue (Leagene, DG0041) and hematoxylin-eosin (H&E) (SolarBio, DSS120).

We carried out IHC with the under listed antibodies anti-Claudin-1 (1:200, 28674-1-AP, Proteintech, USA), anti-Occludin (1:200, EPR-20992, Abcam, UK), anti-E-Cadherin (1:200, 24E10, CST, MA, USA) and Rabbit two-step detection kit (PV-9001, ZSGB-BIO, Beijing, China). All-night incubation of paraffinized sections with primary antibody was carried out at 4°C after which we incubated for 20 minutes with accompanying secondary antibody at 37°C. Staining of the nuclei with hematoxylin and subsequently with DAB was carried out in accordance with manufacturer's protocol. Optical microscope was employed to examine the sectioned tissues.

Fluorometric assay of FITC-dextran 4

Mice were fasted for 12 hours before the experiment. Mice in each group were given FITC-dextran 4 500 mg/kg by gavage. After 2 hours, the mice were anesthetized with pentobarbital for imaging *in vivo*. After four hours, mice were anesthetized with pentobarbital and euthanized by carbon dioxide asphyxiation. FITC-dextran 4 in serum was determined at the excitation wavelength of 493 nm and the emission wavelength of 518.5 nm.

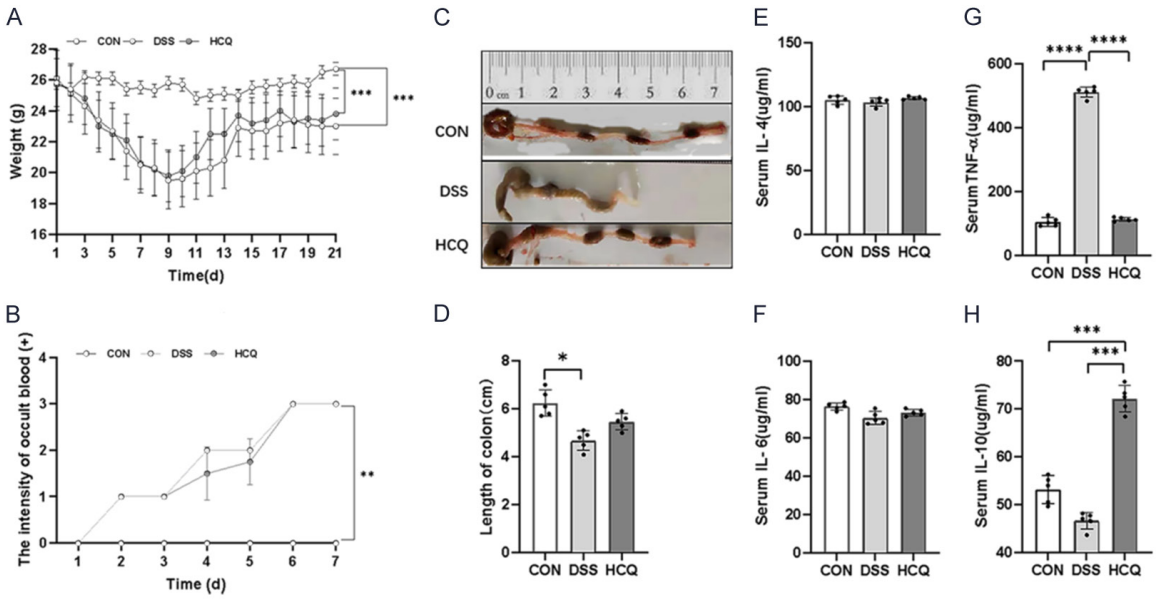

Figure 1. Effects of hydroxychloroquine (HCQ) on body weight, length of colon, serum inflammatory cytokines concentrations and occult blood in the feces of colitis mice. A: Body weight in the entire groups during administration period. B: Occult blood in the feces of mice during modeling. C, D: The HCQ effect on the length of colon. E-H: Serum concentrations of IL4, IL-6, TNF-α and IL-10 in colitis mice after treatment of HCQ. Comparisons: $^{*}0.01<P<0.05$; $^{**}0.001<P<0.01$ and $^{***}P<0.001$.

Analysis of gut microbiota

Based on protocol of the manufacturer, we performed sequencing of samples on the platform Illumina NovaSeq, which was derived from LC-Bio. In analyzing species diversity complexness, we applied alpha diversity via five indicants, namely Observed species, Goods coverage, Chao1, Simpson and Shannon. The indicants employed in this work were computed via QIIME2. In particular, calculation of beta diversity was accomplished with QIIME2, while plotting of data was performed with R-package. We calibrated the sequence with Blast, wherein SILVA database was used to annotate each illustrative sequence. Implementation of other diagrams was done with R-package (V3.5.2).

Statistical analysis

The data were presented as means and standard error of mean (SEM). Group differences were analyzed using unpaired Student's t-test, while Kruskal-Wallis test was used for the evaluation of more than two datasets. Spearman's ρ (rho) was employed for the analysis of correlations. GraphPad Prism 8 software was used for data analysis, and statistical significance was accepted at $P<0.05$.

Results

The effect of HCQ on body weight of colitis mice

We performed daily measurements of the body weight of mice and observed a significant decrease in weight in the DSS group compared to the CON group ($P<0.001$, **Figure 1A**). Furthermore, we found that the weight of mice in the HCQ group was significantly lower than that in the CON group ($P<0.001$, **Figure 1A**), but there was no significant difference between the HCQ group and the DSS group.

The effect of HCQ on the length of colon and the intensity of occult blood

HCQ group and DSS group had a significant increase in fecal occult blood compared to the CON group during the period of colitis induced with DSS ($P<0.01$, **Figure 1B**). The mice in the DSS group also had a significantly shorter colon length compared to the CON group ($P<0.05$, **Figure 1C**). However, there were no significant

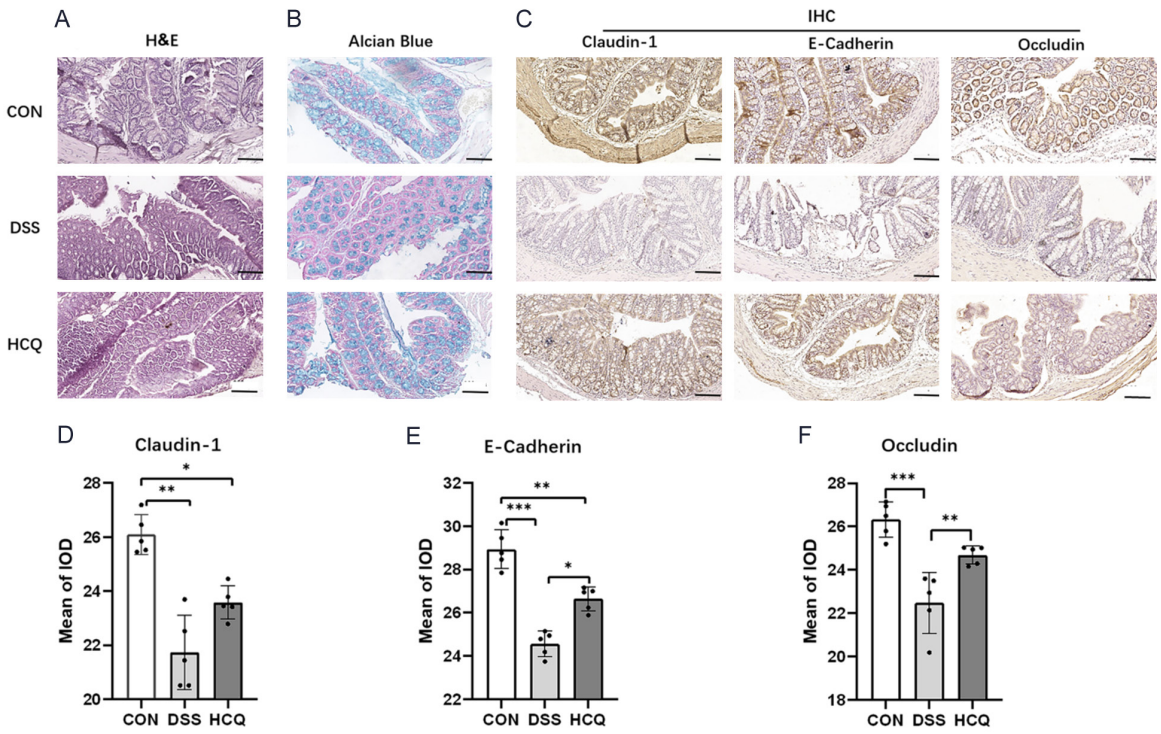

Figure 2. The effect of HCQ on morphology of intestinal mucosa. A: Hematoxylin-Eosin (H&E) staining of colon in mice of CON, DSS and HCQ groups, bar = 200 μm, magnification = 10×. B: Alcian Blue staining of intestinal mucosal in mice of CON, DSS and HCQ groups, bar = 200 μm, magnification = 10×. C: Proteins of tight-junction expression (Claudin-1, E-Cadherin and Occludin) in colon with IHC, bar = 200 μm, magnification = 10×. D-F: Immunohistochemical (IHC) staining intensity of Claudin-1, E-cadherin and Occludin in colonic tissue of mice. Comparisons: *0.01<*P*<0.05; **0.001<*P*<0.01 and ***P<0.001.

differences in colon length between the HCQ group and the CON group (**Figure 1D**).

The effect of HCQ on serum inflammatory cytokines

The results show that there was no significant difference in serum IL-4 and IL-6 concentrations among the three groups (**Figure 1E**, **1F**). However, there was a marked increase in serum TNF-α concentrations in mice of the DSS group compared to those in the CON and HCQ groups (P<0.001, **Figure 1G**). Notably, a higher serum IL-10 concentration was observed in the HCQ group compared to those in the CON and DSS groups (P<0.001, **Figure 1H**).

The effect of HCQ on the morphology of intestinal mucosa

Figure 2A shows the intact colon structure of mice in the CON group, with clear mucosa and orderly arranged glands and no obvious inflammatory cell infiltration. In contrast, the colon structure of mice in the DSS group was severely damaged, with an unclear intestinal wall structure, disorderly arranged glands, and plentiful neutrophils and lymphoplasma cell infiltrations in the mucosa and submucosa. Crypt structure was disrupted, and goblet cells were lost. Mice in the HCQ group showed significant improvement compared to the DSS group. Inflammatory cells in the intestinal mucosa were reduced, the intestinal wall structure became clear, glands were arranged orderly, and the crypt shape recovered, with an increase in goblet cells. Additionally, the thickness of mucus in the DSS group was substantially decreased compared to those in the CON group, as shown by Alcian Blue staining of sectioned tissues. Notably, HCQ-treated mice demonstrated an increase in mucus production compared to those in the DSS group (**Figure 2B**).

Localization and expression of Claudin-1, E-Cadherin, and Occludin were assessed in the epithelium of the enteric mucosa to investigate the healing of HCQ in colitis mice (**Figure 2C**).

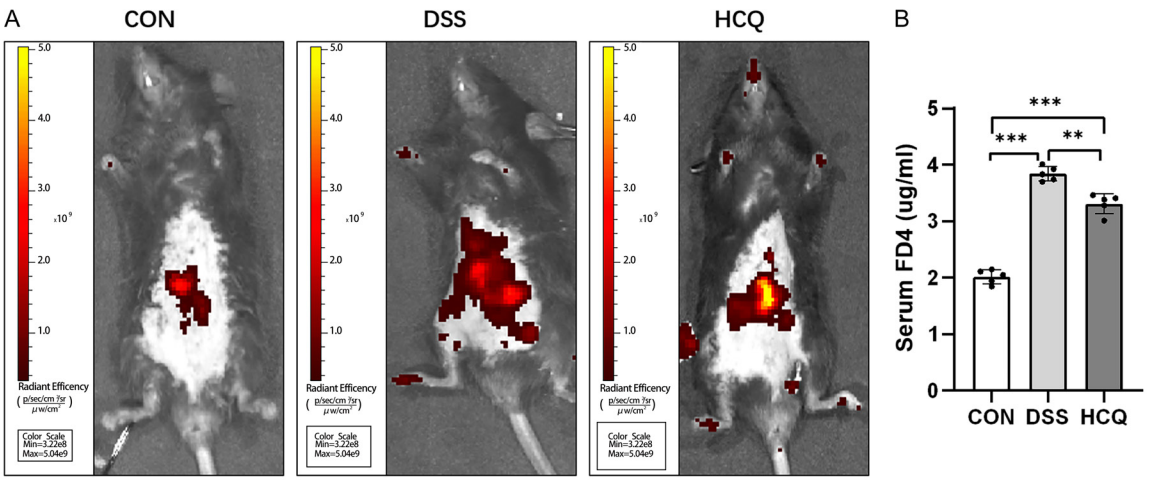

Figure 3. The effect of HCQ on barrier of intestinal mucosa in colitis mice. A: *In vivo* imaging was performed to observe the leakage of FITC-dextran 4. B: Serum fluorescence intensity of FITC-dextran 4. Comparisons: *0.01<*P*<0.05; **0.001<*P*<0.01 and ***P<0.001.

Notably, the proteins were mainly expressed in the cytosol of epithelial cells. Compared to the CON group, there was a significant decrease in the expression of these proteins in the DSS group (Claudin-1: P<0.01, E-Cadherin: P<0.001, Occludin: P<0.001, **Figure 2D-F**). However, HCQ treatment resulted in a significant increase in the expression of E-Cadherin and Occludin (E-Cadherin: P<0.05, Occludin: P<0.01, **Figure 2D-F**).

The effect of HCQ on intestinal mucosal permeability in colitis mice

After administering FITC-dextran 4 to mice via gavage, the leakage of the compound was observed using *in vivo* imaging two hours later. The results showed that colonic mucosal permeability was significantly higher in the DSS group compared to the CON group. However, the HCQ group demonstrated significantly less leakage area and lower permeability compared to the DSS group (**Figure 3A**). Furthermore, the serum FD4 content was significantly increased in both the DSS group and the HCQ group compared to the CON group (P<0.001, **Figure 3B**). However, serum FD4 was significantly lower in the HCQ group compared to the DSS group (P<0.01, **Figure 3B**).

The effect of HCQ on gut microbiota diversity

We measured several alpha diversity indexes to analyze alterations in gut microbiota diversity between the DSS and HCQ groups. As shown in the charts, the Chao1 and Observed_otus indexes represented the number of species contained in a community (**Figure 4A**, **4B**), while the Simpson and Shannon indexes represented diversity and uncertainty (**Figure 4C**, **4D**). After treatment with HCQ, we observed a significant decrease in Chao1 and the Observed_otus index, while insignificant differences were observed in the Simpson and Shannon indexes (P<0.01, **Figure 4A-D**). Both principal component analysis (PCA) and principal coordinates analysis (PCoA) are commonly used methods to analyze beta diversity, which represent differences in species between different communities. In our study, we observed that HCQ treatment led to an increase in the differences in intestinal species between the groups, as demonstrated by the PCA and PCoA plots (**Figure 4E**, **4F**).

The effect of HCQ on gut microbiota species abundance

The results of the comparison of gut microbiota species abundance in DSS group and HCQ group showed that at the family level, there was a significant increase in the abundance of *Muribaculceae, Tannerellaceae, Bacterioidetes, Oscillospiraceae*, and *Odoribacteraceae* in the DSS group compared to the HCQ group (P<0.05 for all). Additionally, there was a lower abundance of *Euryarchaeota* (P<0.05) in the DSS group compared to the HCQ group (**Figure 5A**, **5B**). At the genus level, the abundance of *Muribaculum, Parabacteroides, Bacterioidetes,*

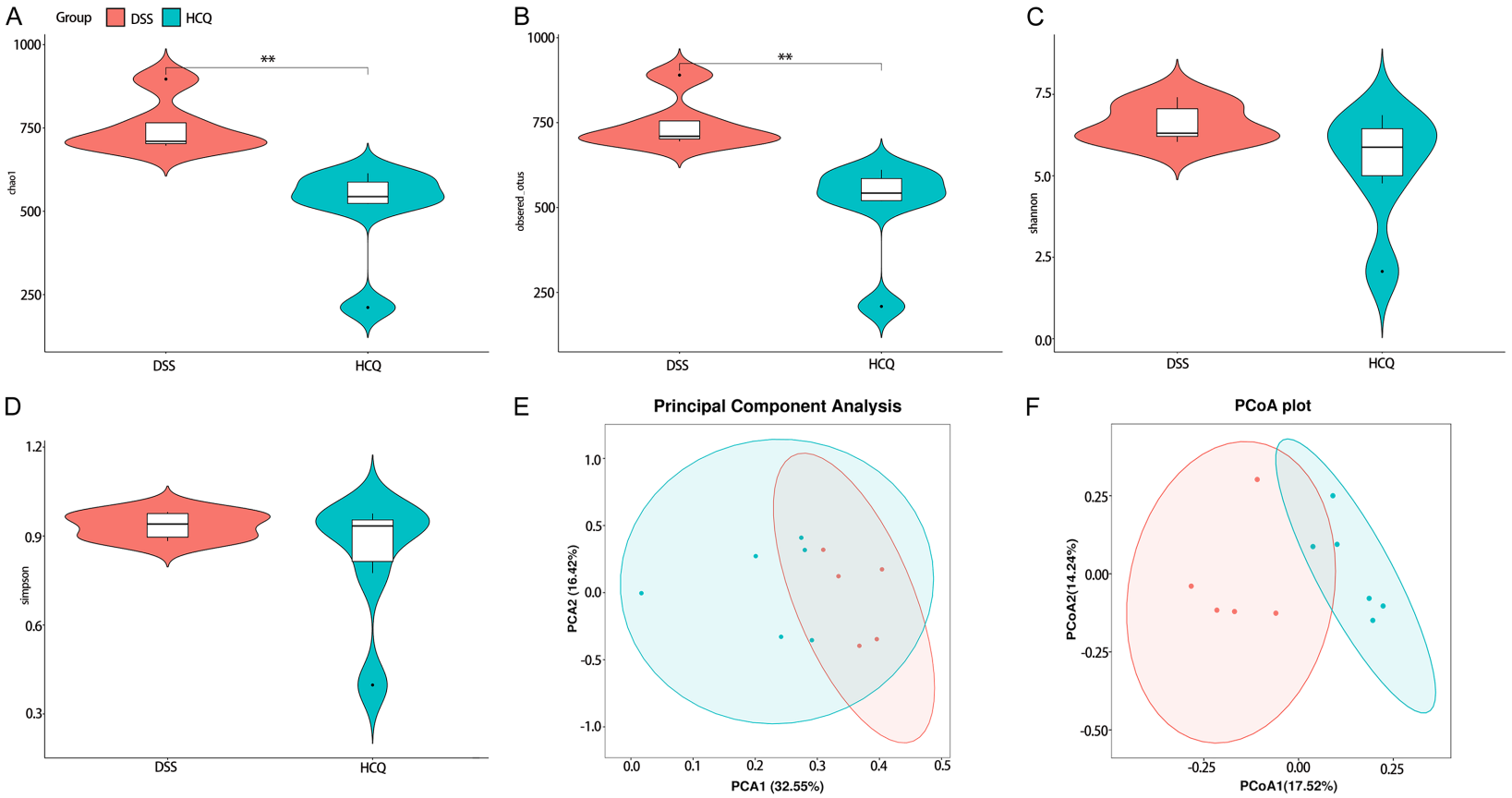

Figure 4. HCQ effects on the gut microbiota diversity. A: Index of Chao1. B: Index of Observed_otus. C: Index of Shannon. D: Index of Simpson. E: Analysis of principal component. F: Analysis of principal coordinates. Comparison: $^{**}0.001<P<0.01$.

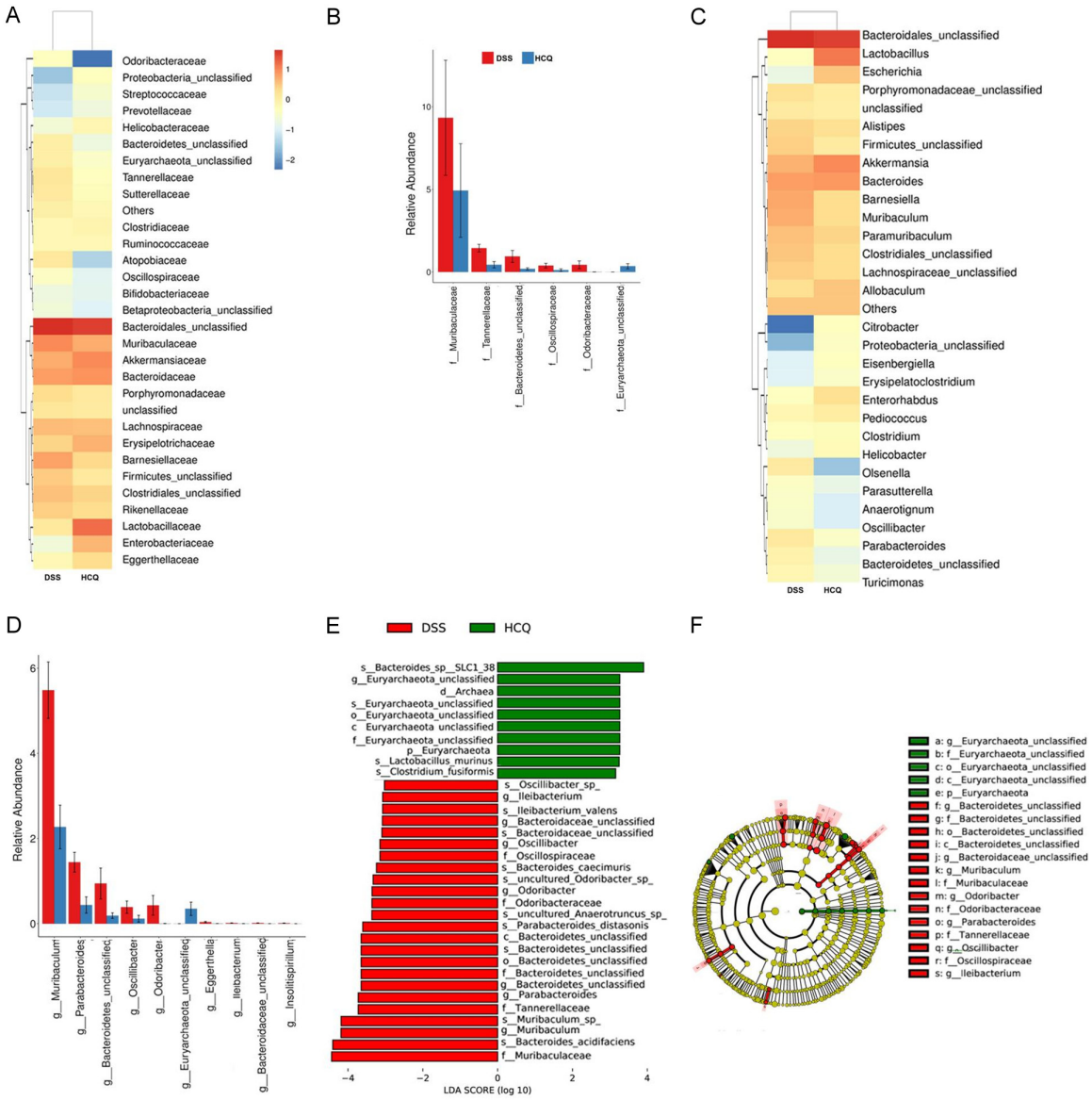

Figure 5. HCQ effects on gut microbiota species abundance. A: Heatmap at family level. B: Differential analysis of mice gut microbiota at family level. C: Heatmap at genus level. D: Differential analysis of mice gut microbiota at genus level. E: LDA of discriminative. F: Cladogram at phylogenic levels.

Oscillibacter, and *Odoribacter* were markedly increased ($P<0.05$ for all) in the DSS group compared to the HCQ group. Conversely, the abundance of *Euryarchaeota* was lower in the DSS group compared to the HCQ group ($P<0.05$ for all) (**Figure 5C**, **5D**).

The LDA effect size (LEfse) mainly aims to compare two or more groups, wherein it is used to identify species that significantly differ in terms of abundance within distinct groups (biomarker) (**Figure 5E**, **5F**). In this study, LEfse analysis was used to compare the differences in species abundance between DSS and HCQ groups at the species level. The results showed that HCQ treatment was associated with an increase in abundance of *Euryarchaeota*, *Lactobacillus_murinus* and *Clostridium_fusiformis*, while the abundance of *Oscillibacter*, *uncultured_Odoribacter*, *Bacterioidetes* and *Muribaculum* were lower in HCQ group compared to DSS group. These species could potentially serve as biomarkers for distinguishing between the two groups.

Discussion

HCQ is a well-known anti-inflammatory drug that has been shown to inhibit inflammatory reactions through various mechanisms [6]. Studies have demonstrated that HCQ can inhibit the generation of Reactive oxygen species (ROS) and proinflammatory mediators in macrophages induced by activated Toll-like receptor 4 (TLR4) [10]. HCQ may also suppress the activation of the NOD-like receptor thermal protein domain associated protein 3 (NLRP3) inflammasome *in vivo* and *in vitro*, which is an innate immune system cytoplasmic component that influences the initial response to inflammation [11]. Additionally, HCQ can interfere with the assembling of NADPH-oxidase (NOX) in the endosomes, which is involved in many prothrombotic and inflammatory pathways [12]. In this regard, we detected serum concentrations of inflammatory cytokines in colitis mice. Our results showed that HCQ treatment increased serum IL-10 concentration and decreased serum TNF-α level, indicating that HCQ treatment could reduce inflammatory reaction. The results are consistent with earlier works [13, 14]. When pathogenic microorganisms invade the body, they activate themselves by recognizing receptors on the surface of antigens, and by enhancing phagocytosis, secrete various proinflammatory cytokines and anti-inflammatory factors to clear pathogens, so as to clear pathogens and control the disease process inflammatory cytokines, such as TNF-α, IL-6 and IL-10, the main factors involved in the regulation of immune response in IBD [15, 16]. However, we found that HCQ had no significant effect on the length of colon, body weight, or intensity of fecal occult blood in the colitis mice. Gastrointestinal bleeding and loss of body weight are common clinical symptoms of IBD [17], thereby relieving IBD patients from these symptoms is an important aspect of the disease treatment [18].

Inflammation and damage to the gastrointestinal epithelium can lead to dysfunction of the mucosal barrier, resulting in barrier defects. FITC-dextran 4 molecules can cross the damaged intestinal epithelial cells and be measured in the blood after oral administration, with serum FITC-dextran 4 concentration serving as an index to measure the degree and severity of intestinal mucosal barrier dysfunction in paracellular permeability [19-21]. This study showed that HCQ treatment can reduce damage to the intestinal mucosal barrier induced by DSS via reducing colonic mucosal permeability.

Goblet cells play an important role in generating intestinal mucus, which is essential for protecting the intestinal epithelium [22]. Tight-junction proteins expressed in endothelia and epithelia form barriers to the para-cellular channel, thereby influencing the permeability of the tight-junction [23]. Loss of tight-junction proteins can lead to intestinal epithelial dysfunction [24]. Our study demonstrated that supplementing HCQ to colitis mice increased the proportion of goblet cells, expression of E-Cadherin, and production of intestinal mucus. Additionally, the shape of colonic crypt and intestinal wall were restored, suggesting that HCQ can heal the damage of structure and morphology of the colon in colitis mice.

The gut microbiota has been found to be associated with the progression of chronic diseases such as certain types of tumors, metabolic syndrome, and obesity [25-27]. While the exact pathological process of IBD is still not fully understood, scientists generally agree that it involves an abnormal immune response induced by changes in the intestinal microbiota [28]. Specifically, it has been consistently observed that bacterial diversity in the intestinal microbiota is reduced in IBD patients, with an increase in *Proteobacteria* and a decrease in *Firmicutes* [29].

The results showed that HCQ significantly reduced the diversity of intestinal microbiota and the abundance of *Bacteroides, Muribaculum* and *Odoribacter* but increased the abundance of *Lactobacillus_murinus, Clostridium_fusiformis*, and *Euryarchaeota*. Of particular note, Bacteroides species are important pathogens in clinical settings. While they have a beneficial relationship with the host when present in the intestines, they can cause serious pathology if found outside the gut [30, 31]. In a mouse model of glucan sodium-induced colitis, the relative abundance of *Muribaculaceae* was negatively correlated with proinflammatory cytokines and positively correlated with the expression level of tight junction proteins. Therefore, bacterial species belonging to the

Muribaculum genus appears to be important for maintaining the normal condition of the mouse gut [32]. *Odoribacter*, on the other hand, not only plays an excellent role in reducing intestinal inflammation but also promotes intestinal maturation [33]. Moreover, studies have shown that *Lactobacillus_murinus* can effectively reduce inflammatory reactions [34]. This species has also been reported to successfully colonize the rodent gut and possess protective properties against necrotizing enterocolitis [26, 35]. However, there are few studies about *Euryarchaeota* and *Clostridium_fusiformis* in gut diseases.

Taken together, our results suggest that supplementing HCQ to colitis mice can reduce inflammatory reactions, heal the damage of the structure and morphology in the colon. Furthermore, we observed that HCQ could promote the abundance of some intestinal probiotics and reduce some pathogens.

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Disclosure of conflict of interest

None.

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