

VDR Pathway-Specific Dose-Related Effects of 1,25(OH)₂D₃ on *Salmonella typhimurium*-induced Mouse Colitis

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Abstract

Background: Whether and how $1,25(\text{OH})_2\text{D}_3$ supplementation influences VDRs in experimental mice with colitis remains to be seen. To explore the effect of $1,25(\text{OH})_2\text{D}_3$ on *S. typhimurium* colitis through the VDR pathway and to discover the role of VDR in its action.

Methods: We established a mouse UC model induced by *S. typhimurium*. After streptococcal typhus infection, the mice were fasted for 12 hours. Blood was collected by the eyeball extraction method, and then sacrificed by cervical dislocation, specimens were collected for corresponding indicators.

Results: Mice exposed to *S. typhimurium* infection developed signs of acute colitis. After HE staining were performed on the diseased colons from the mice. high dose VD supplementation, the pathological colonic damage did not improve in the mice, and there was no statistical difference between the groups with VD deficiency ($P>0.05$). VDR expression in the UC group treated with Salmonella was higher than that in the control group, a statistically significant difference ($P<0.01$). Compared with the VDD+UC group, VDR expression rose in both the LVDS+UC group and the HVDS+UC group, with VDR protein expression being highest after high dose VD supplementation ($P<0.01$). Compared with the control and the UC groups, the VDR mRNA expression level in the VDD+UC group was significantly higher, and the colon VDR mRNA expression level decreased after active VD supplementation (Fig.7C).

Conclusions: Our data suggest the need for defining the accurate $1,25(\text{OH})_2\text{D}_3$ dose limits that induce an anti-inflammatory effect as current data indicate that higher doses would produce an inflammatory response.

Introduction

Inflammatory bowel disease (IBD) is a chronic and spontaneously relapsing disease featured by extensive inflammation of the gastrointestinal tract. IBDs mainly comprise ulcerative colitis (UC) and Crohn's disease (CD), and IBD has become one of the most difficult benign diseases of the digestive system to diagnose and treat. Considering that IBD emerged in Western countries throughout the middle of the 20th century, its emergence in developing nations over the past 25 years, including in China, India, and North Africa, suggests that this epidemiological evolution is related to lifestyle Westernization and industrialization [1–3]. The existing epidemiological data from China shows that both the incidence and prevalence of IBD are rising. According to the statistics from the Chinese Center for Disease Control and Prevention in 2014, the total number of Chinese IBD cases between 2005 and 2014 was about 350,000, but worryingly, by 2025 the number of IBD patients in China is expected to reach 1.5 million [4]. Although the precise etiology of IBD is unknown, recent scientific literature suggests that there is an exaggerated immune response to commensal gut bacterial flora along with the influence of genetic and environmental factors, leading to imbalanced mucosal homeostasis. Alteration of the gut microbiota, or dysbiosis, is closely linked to initiation or progression of IBD [5]. This dysregulation of mucosal immunity in the gut of IBD patients causes an overproduction of inflammatory cytokines such as IL-17 and trafficking of

effector leukocytes into the intestine, resulting in uncontrolled inflammation and damage to the intestinal epithelial barrier. However, signaling pathways, such as the IL-23/IL-17 pathway, also play a role in the pathogenesis of IBD as shown from both model animal and human studies [6, 7]. The traditional therapy for IBD mainly involves antibiotics, hormone steroids and immunosuppressive drugs such as azathioprine, all of which can have side effects and potential long-term toxicity [8]. At present, no complete cure for IBS is possible, but combined pharmacological and nutritional therapy may assist with disease remission [9].

For over a century, vitamin D (VD) has been considered a key modulator of calcium, phosphate and bone development homeostasis. However, the discovery of the VD receptor (VDR) in most tissues and cells, even in intestinal mucosa throughout the small intestine from the duodenum to ileum and large intestine (including the cecum and colon), has provided new sight into the function of VD apart from its traditional roles [10]. VDR belongs to the superfamily of nuclear hormone receptors, through which VD confers its most biological effects, and as a transcriptional factor its existence in immune cells and immune organs indicates there is immune regulation of VD. Recent studies suggest that VD may have immunosuppressive effects, both in animal models of colitis and in human trials. In particular, VD suppresses dendritic and T-cell functions by inhibiting pro-inflammatory cytokine production. VD insufficiency is associated with the active phenotype of IBD. It has also been suggested that VD supplementation may ameliorate several immune-related diseases including multiple sclerosis, experimental autoimmune encephalomyelitis and IBD [11–13]. Recently, the role of VD and VDR in most chronic diseases such as cardiovascular disease, diabetes, cancers, autoimmune diseases, and infectious diseases among others, has been the subject of much attention as well as its role in regulating gene transcription [14–16]. Relevant studies have reported that VDR expression in the intestinal epithelium is particularly important for preventing IBD [17]. Genome-wide association studies (GWASs) have revealed that VDR may play an important role in over 47 common diseases including colorectal cancer and immune-related diseases [18]. However, there is evidence that VDR agonists may exert therapeutic efficacy in experimental colitis by inhibiting the activities of peripheral blood mononuclear cells and pro-inflammatory cytokine-producing TNF- γ and IL-12/23p40 lymphocyte enriched lamina propria mononuclear cells [19].

Overall, VD deficiency (< 20 ng/mL) and insufficiency (20–30 ng/mL) affects more than one billion people worldwide [20]. There is a significantly increased rate of IBD-related hospitalizations in the northern USA states compared with the southern ones, although this is not fully explained by differences in ultra violet light exposure [21]. It is possible that VD inhibits T cell proliferation and suppresses IFN- γ and IL-17 production by T cells. Conversely, VD is known to increase IL-4 production from T cells [22]. In CD and UC patients, 25(OH)D₃ serum levels are lower than in healthy controls [23]. It is also reported that VD insufficiency may be linked to a number of immune-related diseases [24–26]. One study has shown that VD deficiency in UC patients accelerates the disease's activity, a finding in accordance with VD insufficiency possibly predisposing mice to dextran sulfate sodium (DSS)-induced colitis [27]. However, some studies have shown that serum VD is much lower in IBD patients than in healthy people, which may

be related to too little sun exposure and/or malabsorption of VD and fat because VD is fat-soluble and mainly acquired by skin exposure to sunshine. VD deficiency has been hypothesized to cause IBD because IBD patients are known to have an increased incidence of VD deficiency, and this is independently associated with a lower health-related quality of life in CD subjects [28].

A number of studies have suggested that $1,25(\text{OH})_2\text{D}_3$ has important effects on the immune system. Indeed, VD deficiency has been linked to IBD in both humans and animal disease models. VDR expression may be influenced by $1,25(\text{OH})_2\text{D}_3$, whose genomic actions are regulated through the VDR. In addition, as VDRs are present on at least 38 different bodily tissues, including intestinal and colonic tissues [29], many non-skeletal tissues also express VD activating enzymes, thereby permitting local production of $1,25(\text{OH})_2\text{D}_3$. Whether and how $1,25(\text{OH})_2\text{D}_3$ supplementation influences VDRs in experimental mice with colitis remains to be seen.

The DSS modeling method we often use has certain defects for the study of bacterial infections and intestinal flora, so in the present study we employed the bacteria-induced colitis model. *Salmonella typhimurium*, a food source for Gram-negative bacilli, can cause human gastroenteritis [30]. The mechanism of inducing intestinal inflammation may involve the expression of T-cell-dependent cytokines such as IFN-gamma, IL-22 and IL-17, and IL-23 plays a very important role in initiating this immune response [31]. Therefore, we established a mouse UC model induced by *S. typhimurium* to explore the effect of $1,25(\text{OH})_2\text{D}_3$ on *S. typhimurium* colitis through the VDR pathway and to discover the role of VDR in its action.

Materials And Methods

Animals

Specific-pathogen-free female C57BL/6 mice (3-week-old) were purchased from Hunan Slack Penmark Laboratory Animal Technology [License No. SCXK (Xiang) 2011-0003]. Mice were housed under laboratory conditions with a 12h-light/12h-dark cycle and had free access to water all the time. Temperature and relative humidity were controlled at 21 ± 1 °C and $55\pm 5\%$, respectively. All animal handling was performed with the approval of the Ethics Committee of Shanxi Medical University. After 1 week of adaptive feeding, the mice were randomly divided into 5 groups according to their body weights: normal group (control), model group (UC), VD deficiency group (VDD+UC), low-dose VD supplement group (LVDS+UC) and high-dose VD supplement group (HVDS+UC), with eight mice in each group.

Experimental Feeding and Medium

Both basic feed (rich in VD) and experimental feed (lacking VD) were purchased from the Research Diet Company (USA), numbers D10001 and D08090903N, respectively. The feed formulas are shown in Table 1. LB liquid medium contained peptone 10g/L, yeast 5g/L, NaCl 10g/L, and was preserved at 4 °C after autoclave sterilization. AGAR (final concentration, 2%) was added to the LB liquid medium for solidification, followed by sterilization by high-pressure steam and storage at 4 °C. MacConkey medium,

which contained tryptone 20g/L, bile salt 1.5 g/L, sodium chloride 5 g/L, lactose 10g/L, neutral red 0.03g/L, AGAR 14g/L, crystalline violet 0.001 g/L, at pH 7.1±0.2, was autoclaved and stored at 4 °C.

VD Deficiency Model Preparation

The control and UC groups both received normal feed and normal illumination, whereas the VDD+UC group, the LVDS+UC group and HVDS+UC group received feeds lacking VD. Experiments were conducted under the yellow light of filtered ultraviolet feeding to avoid VD production from the skin. After 7 weeks, the mice were randomly sampled for the two different feeding methods, and serum from tail vein blood samples from eight mice were separated from the whole blood, and the resulting serum was used to determine the serum 25(OH)D₃ levels.

Mouse UC Model Preparation and Active VD Intervention

1. *typhimurium* ATCC14028 was activated and inoculated into fresh LB broth medium at 37 °C for 18h overnight. The UC animal model was prepared with reference to the literature [32]. After fasting for 4 h, except for the normal control group, each mouse was given a fasting gavage of 0.1 ml sterile water containing 7.5 mg of streptomycin solution, and the normal control group was given an equal amount of sterile water, after which the mice could drink and eat freely. After streptomycin treatment for 20 h, the mice were fasted for 4 h. Each mouse in the experimental group was given 1×10^6 colony forming units of *S. typhimurium* (in 100µl of LB suspension liquid) on an empty stomach, while the control group received the same amount of sterile LB solution. After continued fasting for 2 h, the mice received food and water freely. After 5 d of *Salmonella* infection, the LVDS+UC group and the HVDS+UC group were supplemented with 1 µg/kg and 10 µg/kg 1,25(OH)₂D₃ (0.1 ml), respectively, and the other three groups were administered the same amount of phosphate buffered saline (PBS) containing 0.12% ethanol solvent by gavage for continuous supplementation for 3 d.

Sample Collection and Index Determination

Body weights and clinical and behavioral signs in the mice were closely monitored during and after streptococcal typhus infection (e.g., mental condition, hair, diarrhea, and hematochezia). After fasting for 12 h, each mouse was anesthetized with sodium pentobarbital (60mg/kg) intraperitoneally, and blood was collected by the eyeball extraction method. The blood was left at room temperature for 20 min, and centrifuged after coagulation (2000× g, 20 min). Serum was separated and stored in -20°C for later use. The mice were killed by cervical dislocation, and their livers and spleens were aseptically separated and weighed, and stored separately at 4°C for later use. Each colon was separated and cut along the longitudinal axis of the mesenteric margin. The intestinal contents were removed with pre-cooled saline, and the residual liquid on the surface was sucked dry with filter paper. We immediately noted whether there were signs of colon adhesion, congestion, ulceration, or other abnormalities, a gross morphology damage score was applied, and the length and weight of each colon was measured. Tissue from each diseased colon was collected, and a conspicuous location on the lesion was selected (i.e., colon lesions in the normal control group were located in the same location as those in the model group) and then fixed

in 10% formaldehyde solution. Conventional paraffin-embedded sectioning was conducted and the resulting sections were stained with hematoxylin-eosin (HE), made into pathological sections, and another part of the colon tissue was stored at -80°C for later use.

Index Evaluation and Methods

Macroscopic colonic damage was assessed using the scoring system of Wallace and Keenan [33], which takes into account the area of inflammation and the presence or absence of ulcers. The criteria used to evaluate the macroscopic damage were based on a semi-quantitative scoring system. The features were graded as follows: (0), no ulcer, no inflammation; (1), no ulcer, local hyperemia; (2), ulceration without hyperemia; (3), ulceration and inflammation at one site only; (4), two or more sites of ulceration and inflammation; and (5), ulceration extending over > 2 cm.

The diseased colonic tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections ($5\ \mu\text{m}$) were deparaffinized with xylene and stained with HE according to standard procedures. Referring to Bergstrom's method [34], the colonic histopathological scoring criteria were as follows: the tissue sections were assessed for submucosal edema (0=no change; 1=mild; 2=moderate; 3=profound), epithelial hyperplasia (scored based on the percentage above the height of the control where 0=no change; 1=1–50%; 2 =51–100%; 3 =>100%), epithelial integrity (0=no change; 1=<10 epithelial cells shedding per lesion; 2=11–20 epithelial cells shedding per lesion; 3=epithelial ulceration; 4=epithelial ulceration with severe crypt destruction) and neutrophil and mononuclear cell infiltration (0=none; 1=mild; 2=moderate; 3=severe).

***Salmonella* Burden in the Liver and Spleen**

For enumeration of *S. typhimurium*, the mouse tissues were prepared as previously described [34]. Briefly, spleens and livers were removed aseptically from each mouse, weighed separately, and homogenized in PBS at 4°C using a Potter homogenizer. Each homogenate was diluted 1000 to 10000 times in an LB medium gradient, and $100\ \mu\text{l}$ of each diluted homogenate was applied to a MacConkey AGAR plate containing streptomycin ($50\ \mu\text{g}/\text{ml}$). After 37°C incubation overnight, the number of *S. Typhimurium* were counted and the *Salmonella* content in the original solution was determined according to the dilution factor.

Determination of Serum VD, Cytokines and Calcium Concentrations, and VDR Content

The levels of murine $25(\text{OH})\text{D}_3$, $1,25(\text{OH})_2\text{D}_3$, IL-23, and IL-17 in the serum were quantified using commercially available enzyme-linked immunosorbent assay kits (ELISA; JRDUN Biotechnology, China) according to the manufacturer's instructions. The serum calcium concentration was evaluated by the calcium-cresol phthalein colorimetric assay according to the manufacturer's instructions (Nanjing Jiancheng, China). The VDR protein content in the colon tissues from the mice was determined by immunohistochemistry and finally analyzed with Image-Pro Plus (6.0 program), and VDR-specific mRNA expression in the colon tissues was determined by real-time quantitative PCR (RT-PCR).

Statistical Analysis

Data are expressed as the mean \pm standard deviation (mean \pm SD) using SPSS 22.0 software. Data were analyzed by one-way ANOVA. $P \leq 0.05$ was considered statistically significant. When ANOVA suggested a significant difference ($P < 0.05$), post hoc comparisons of the individual mean values were conducted by least significant difference testing. The exact Mann-Whitney U test was used for bacterial numeration.

Results

The mouse model of VD deficiency was successfully established

Mice fed a VD deficient diet under yellow light were VD deficient at 7 weeks post set up. The serum level of 25(OH)D₃ (ng/ml) was 7.09 ± 0.60 , a value lower than that of mice of the same age that were given normal light and a normal diet rich in VD (18.22 ± 2.06) ($P < 0.001$). Mice raised on a VD deficient diet showed decreased circulating levels of calcium compared with mice on the VD sufficient control diet (2.25 ± 0.19 vs. 2.93 ± 0.22 mmol/L, $P < 0.001$). Mice in the control group displayed normal activity and food intake, no significant body weight change, smooth hair, active spirit, granular stool, and no diarrhea or bloody stool. Mice treated with *Salmonella* had varying degrees of weight loss, diarrhea, hematochezia, and decreased mental and physical activity.

Effect of VD on colitis severity, colon length, weight and histology

Mice infected with *S. typhimurium* developed acute colitis that even resulted in acute mortality. One VDD+UC group mouse infected with *Salmonella* died within 5 d of infection and another died the following day. Two mice in the UC group died on days 6 and 7, comparatively later than that of the VDD+UC group. Mice in the HLVDs+UC group were relatively resistant to *Salmonella*, and only one died on day 6 (Fig.1). No mice in the control and LVDS+UC groups died during the experiment.

To investigate whether VD deficiency and 1,25(OH)₂D₃ supplementation would affect bacteria-induced colitis, we monitored the mouse body weights every day. As expected, the body weight significantly decreased in the bacteria-infected UC mouse group, regardless of 1,25(OH)₂D₃ supplementation (Fig.2A). However, compared with the HVDS+UC group, the LVDS+UC group displayed a lower body weight loss (-3.11 ± 1.01 vs. -1.86 ± 0.87 , $P = 0.016$). There was no difference in weight change detected between the UC and VDD+UC groups (-2.7 ± 1.44 vs. -2.85 ± 0.95 , $P = 0.741$).

The weight and colon length were measured as inflammation markers, revealing that although there was no difference between the weight and colon length, the weight to colon length ratio showed significant differences among the groups (Fig.2B). The ratio in the UC group was much higher than that in the other three groups except for the VDD+UC group ($P < 0.001$). Remarkably, supplementation with 1,25(OH)₂D₃ decreased the colon wet weight index. Unlike the body weight, the weight and length ratio of the colon in the LVDS+UC and HVDS+UC groups was similar, showing no visual difference ($P > 0.05$).

However, the spleens and livers from the bacterial infection groups were much bigger and redder than those from the control group. Furthermore, the spleen weight for the VDD+UC group was heavier than that for the UC group feed on a control diet, while the liver weight was not. Supplementation with $1,25(\text{OH})_2\text{D}_3$ at a dose of $1\mu\text{g}/\text{kg}$ reduced the spleen and liver weights (Fig.2C-D).

Mice exposed to *S. typhimurium* infection developed signs of acute colitis, with diarrhea being observed first followed by fecal blood and weight loss. To assess whether VD affected the bacteria-induced colon damage, we observed the colon morphology. Macroscopic analysis of the colons revealed a striking degree of hyperemia, necrosis, and inflammation compared with the control group, and the macroscopic score for the colon showed that the injury score of mice treated with streptomycin and *Salmonella* increased significantly, and was higher than that of the normal group (Fig.4A). Administering the active form of VD ($1,25(\text{OH})_2\text{D}_3$) significantly improved the macroscopic scores after 3 d of continuous administration, with the colons showing a massive reduction in colitis associated hyperemia and inflammation when the $1\mu\text{g}/\text{kg}$ dose was used, whereas no significant change was observed in the $10\mu\text{g}/\text{kg}$ dose group. The severity of bacteria-induced colonic inflammation and ulceration was further evaluated by histological examination. Normal paraffin sections and HE staining were performed on the diseased colons from the mice to observe the histopathological features of each group (Fig.3). Microscopically, the epithelial cells in the colonic tissues from the control group were neatly arranged, the lamina propria glands were tightly regular, and the mucosa and submucosa were clear with abundant goblet cells. No inflammatory cell infiltration or ulceration was observed. By contrast, the UC and VDD+UC groups colon mucosal tissues showed epithelial cell loss, disordered gland arrangements, and the mucosa and submucosa both displayed a large degree of inflammatory cell infiltration, visible as erosion and as ulcers, and in glands, with a generalized structural disorder, impaired epithelial integrity, and goblet cell loss, with the VDD+UC mouse group displaying more colonic mucosa impairment compared with the UC group ($P<0.01$, Fig.4B). After low dose VD intervention, the mice showed decreased epithelial cell loss, orderly arrangement of lamina propria glands, significantly reduced inflammatory cell infiltration, decreased erosion and ulceration, and improved condition, a statistically different finding from that of the VDD+UC group (Fig.4). However, after high dose VD supplementation, the pathological colonic damage did not improve in the mice, and there was no statistical difference between the groups with VD deficiency ($P>0.05$, Fig.4).

Serum VD content and calcium

As expected, high dose $1,25(\text{OH})_2\text{D}_3$ supplementation produced higher calcium, $25(\text{OH})\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$ levels compared with the other four mouse groups (Fig.5A-C). Undoubtedly, the calcium and $1,25(\text{OH})_2\text{D}_3$ levels in the VDD+UC group were dramatically lower. There were, however, no observed differences in serum calcium and $25(\text{OH})\text{D}_3$ between the control and the LVDS+UC group.

Bacterial burdens

To investigate whether $1,25(\text{OH})_2\text{D}_3$ could improve pathogen burden during *S. typhimurium* infection, we aseptically isolated the colon, cecum, spleen and liver from each mouse. We observed significantly increased loads of *S. typhimurium* in the intestines, and even in the spleens and livers from the VDD+UC and HVDS+UC groups (Fig.6). These results show that low dose $1,25(\text{OH})_2\text{D}_3$ administration can improve the damage induced by *S. typhimurium*. Interestingly, the high dose $1,25(\text{OH})_2\text{D}_3$ -treated mice carried more culturable *S. typhimurium* in their intestinal tissues, spleens and livers.

Expression of IL-17 and IL-23 in mouse serum

We measured the serum-specific pro-inflammatory cytokines IL-17 and IL-23 protein levels by ELISA. Serum IL-17 and IL-23 were significantly elevated in the colitis animals (Fig.7A-B). As we expected, mice fed a VD deficient diet and infected with bacteria had elevated IL-17 and IL-23 levels ($P<0.01$) compared with the UC group. However, these pro-inflammatory markers were drastically suppressed by $1,25(\text{OH})_2\text{D}_3$ treatment in the LVDS+UC and HVDS+UC groups. It is worth mentioning that the level of serum-specific IL-23 expression in the LVDS+UC group was similar to that in the control group. However, IL-23 expression in the HVDS+UC group was even lower than the normal level seen in the control group.

Colon-specific VDR protein and mRNA expression levels

The VDR protein content in the colon tissues from the mice was immunohistochemically detected (Figure 8). A small amount of VDR protein stained positive in the colon tissues of the normal control group mice, which was yellow-brown and mainly distributed in the cytoplasm of the colonic epithelial cells. While VDR expression was positive in the colonic mucosa epithelium of the UC group, VDR positive expression was weak and the staining was shallow in the VDD+UC group. In contrast, VDR protein showed deep staining and its expression was more apparent in the LVDS+UC group, while VDR staining in the colon epithelial cells of the mice supplemented with high dose VD was dark brown.

Analysis of the average optical density value for positive expression of VDR protein showed that VDR expression in the UC group treated with *Salmonella* was higher than that in the control group, a statistically significant difference ($P<0.01$). Compared with the UC group, VDR expression was lower in the VDD+UC group ($P<0.01$), and compared with the VDD+UC group, VDR expression rose in both the LVDS+UC group and the HVDS+UC group, with VDR protein expression being highest after high dose VD supplementation ($P<0.01$) (Table 2). Compared with the control and the UC groups, the VDR mRNA expression level in the VDD+UC group was significantly higher, and the colon VDR mRNA expression level decreased after active VD supplementation. The result differs from that for the protein expression level (Fig.7C).

Discussion

An increasing body of evidence has linked diet to IBD [35]. There is growing evidence that VD is a potent immune modulator. In the present study, we sought to determine whether VD deficiency and active VD supplementation by oral administration would affect inflammatory responses in a bacteria-induced

mouse colitis model. Expectedly, we found that VD deficiency exacerbates colitis. In addition, low dose $1,25(\text{OH})_2\text{D}_3$ treatment attenuated the macroscopic and histological damage scores and decreased serum IL-17 and IL-23 pro-inflammatory cytokine levels. However, its administration at high dose (0.2 $\mu\text{g}/20\text{g}/\text{d}$) did not significantly affect the macroscopic and histological damage scores or the intestinal bacterial burden. Despite its immunosuppressive effect on IL-17 and IL-23, high dose $1,25(\text{OH})_2\text{D}_3$ treatment did not ameliorate the clinical disease manifestations, the reason for which is unclear. Additionally, in the VD-deficient bacteria infected mice the colitis appeared to be severer with extensive bacterial colonization and high level IL-17 and IL-23 pro-inflammatory cytokine expression.

Given the inconsistency of our findings with the general belief that VD is anti-inflammatory and protects against immune-related diseases, the interpretation and implications of these results need to be addressed carefully. It is well documented that $1,25(\text{OH})_2\text{D}_3$ plays a protective role in mucosal barrier homeostasis and regulates the gut microbiome in DSS-induced mouse colitis [36-38]. The C56BL/6 mice in our study received each $1,25(\text{OH})_2\text{D}_3$ daily (0.2 $\mu\text{g}/25\text{g}/\text{d}$) by intragastric administration for 14 d. The $1,25(\text{OH})_2\text{D}_3$ was dissolved in normal saline. We used PBS with 0.12% ethanol as vehicle to promote $1,25(\text{OH})_2\text{D}_3$ absorbance in the intestine, which may lead to a high level of $1,25(\text{OH})_2\text{D}_3$ in the serum. Notably, in the HVDS+UC group, the colonic IL-17 level was much lower than in the LVDS+UC group. This seems to be paradoxical to that reported previously where $1,25(\text{OH})_2\text{D}_3$ (0.5 $\mu\text{g}/\text{kg}$) was found to suppress Th17 T-cell responses *in vivo* and impair mucosal host defenses against *Citrobacter rodentium*, while protecting mice against acute DSS-induced colitis [39].

VD deficiency is associated with a number of immune-mediated disorders, including type 2 diabetes, multiple sclerosis, and IBD [40-42]. VD metabolite levels are regulated by negative feedback from various enzymes and receptors, suggesting that it acts more like a hormone than just a vitamin. VDR, a nuclear factor receptor superfamily member, is key to the role played by active VD, mainly mediating the biological effects of VD on target cells [43, 44]. VDR is widely found in various bodily tissues and cells and is highly expressed in the intestinal tract [45]. $1,25(\text{OH})_2\text{D}_3$ binds to intracellular VDR, enters the nucleus, forms a complex with the retinoid X receptor, and then binds to the VD response element on the promoter to regulate the expression of various target genes, promote cell proliferation and differentiation, thereby regulating the immune response [46]. VD regulates the intestinal microbial composition and deficiency of $1,25(\text{OH})_2\text{D}_3$ or VDR leads to biological imbalance, making the intestinal tract more vulnerable to damage [15]. One GWAS found that the VDR gene is significantly correlated with overall microbial variation and individual classification [47], and a conditioned knockout of VDR resulted in intestinal ecological disorders, including increased bacterial loads, increased levels of *Escherichia coli* and Bacteroidetes, and decreased butyrate-producing bacteria, leading to severe colitis [48]. In fecal samples from VDR knockout mice, lactobacillus was depleted, while clostridium and Bacteroidetes were enriched [49]. Other studies have also confirmed that the interaction between the VD axis and the intestinal microbiome is associated with a higher susceptibility to colitis in VDR knock-out mice, as compared with normal mice from the same colony, and was also associated with a bacterial imbalance characterized by more Proteus and fewer Firmicutes, similar to the results observed with IBD patients [38].

In the present study, the diet formulated by The Research Diet Company of the USA was adopted to feed the mice with VD deficiency, and the mice were housed under non-ultraviolet yellow light to avoid the production of VD through the skin, so as to establish a mouse model of VD deficiency. Because of their VD deficiency, the expression levels of serum IL-17 and IL-23 in the VDD+UC group after treatment with *S. typhimurium* were significantly higher than those in the UC group that were fed a normal feed with *S. typhimurium*, indicating that VD deficiency caused increased expression of IL-17 and IL-23 pro-inflammatory cytokines. The spleen weight and pathological score in the VDD+UC group was higher than that in the UC group, indicating that VD deficiency aggravated the bacteria-induced inflammation of the colon. After infecting the mice with bacteria, the serum 25(OH)D₃ content in the UC group did not differ from that of the control group, indicating that the serum VD levels in the UC group were not affected by the bacterial infection. In mice deficient in VD, low doses of 1,25(OH)₂D₃ were given to improve the colitis of the LVDS+UC group, while the clinical manifestations of UC were not improved in the HVDS+UC group. Moreover, we also found that during bacterial infection, the expression of VDR mRNA and protein in the colon tissues of the UC group was higher than that of the control group, indicating that bacteria may promote a dysfunctional VDR state and stimulate an increase in VDR expression in the colon tissues. VDR protein expression in the VDD+UC group may be lower than that in the UC group because VD is lacking, possibly confirming that VD deficiency leads to decreased VDR expression. The expression of VDR protein in the colon tissues increased significantly after different doses of 1,25(OH)₂D₃ were administered to the mice. We hypothesized that VD acts as a VDR receptor agonist in a dose-dependent manner. However, the mRNA level expression of VDR differs from that of the protein level. Our interpretation hinges on the fact that gene expression is divided into two levels: transcription and translation, namely, the mRNA level and protein level. The time and location of transcription and translation of eukaryotic gene expression are spatio-temporal and after transcription post-transcription processing, transcript degradation, translation, post-translation processing and modification occur at several levels. Therefore, the transcription level and the translation level will not be exactly the same. Active VD acts through the VDR receptor and inhibits the expression of IL-17 and IL-23 inflammatory cytokines. However, high doses of VD may over-inhibit IL-17 and IL-23 by increasing the expression level of VDR protein. This expression, in turn, causes the body to fail to make a timely and effective inflammatory response to intestinal pathogens, thereby aggravating inflammation.

We observed in our study that low dose VD supplementation ameliorates pro-inflammatory effects, but did not completely reverse the features of intestinal inflammation. In this research, we used *S. typhimurium* as a bowel-specific pathogen to induce colitis in mice rather than chemicals like DSS or 2,4,6-trinitrobenzene sulfonic acid as numerous studies have indicated that intestinal commensal bacteria are responsible for the development of colitis [50, 51]. *Salmonella* is a leading cause of gastrointestinal disease worldwide [52]. Moreover, recent studies have further shown that *Salmonella* infections can cause a disease profile and the type of strong inflammatory responses that resemble those of human colitis [53, 54]. As far as we know, this is the first time that liver and spleen inflammation in a mouse colitis model has been reported, and further investigations will be needed to unravel the potential mechanism.

Conclusion

In summary, in this study we have reported the effect of VD deficiency and supplementation with different doses of $1,25(\text{OH})_2\text{D}_3$ on the inflammatory response in a mouse colitis model induced by *S. typhimurium*. We found that VD deficiency can lead to severe colitis and a low dose of $1,25(\text{OH})_2\text{D}_3$ may ameliorate the bacteria-induced colitis we observed, while a high dose does not. While the mechanisms and clinical significance of the effect of $1,25(\text{OH})_2\text{D}_3$ require further investigation, our data suggest the need for defining the accurate $1,25(\text{OH})_2\text{D}_3$ dose limits that induce an anti-inflammatory effect as current data indicate that higher doses would produce an inflammatory response. Future research might establish VDR signaling as a new target for treating bacterial infections and IBD.

Abbreviations

IBD: Inflammatory bowel disease

UC: ulcerative colitis

CD: Crohn's disease

VD: Vitamin D

VDR: VD receptor

GWASs: Genome-wide association studies

DSS: dextran sulfate sodium

PBS: phosphate buffered saline

HE: hematoxylin-eosin

RT-PCR: real-time quantitative PCR

Declarations

Availability of data and materials

The datasets herein used and analyzed are available from the corresponding author on reasonable request.

Authors' contributions

Fubin Qiu and Lijuan Zhang designed the research and developed the overall research plan. Ying Zhang, Chunyan Li and Xiaoyan Zhang conducted the research; Fubin Qiu and Lijuan Zhang analyzed the data

and wrote the paper. All authors read and approved the final manuscript. All authors contributed to the critical revision of the manuscript and final approval of the version to be published. All authors agree to be accountable for all aspects of the work by ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Ethics declarations

Ethics approval and consent to participate

The protocol was approved by the Ethical Committee of Shanxi Medical University, China.

Consent for publication

Not applicable.

Competing interests

All authors have no potential conflicts of interest to disclose. Fubin Qiu has received scientific grants from the National Natural Science Foundation of China and Science Foundation of Shanxi Province of China.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest.

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Tables

Table 1: the formula of the control diet (D10010) and vitamin D deficient diet (D08090903N)

Product #	D10001		D08090903N	
	gm%	kcal%	gm%	kcal%
Protein	20	21	20	21
Carbohydrate	66	68	65	67
Fat	5	12	5	12
Total		100		99
kcal/gm	3.90		3.90	
Ingredient	gm	kcal	gm	kcal
Casein	200	800	200	800
DL-Methionine	3	12	3	12
Corn Starch	150	600	150	600
Sucrose	500	2000	500	2000
Cellulose, BW200	50	0	50	0
Corn Oil	50	450	50	450
Mineral Mix S10001	35	0	35	0
Vitamin Mix V10001	10	40	0	0
Vitamin Mix V13201 (No Vit D)	0	0	10	40
Choline Bitartrate	2	0	2	0
Total	1000	3902	1000	3902

Table 2 average optical density of VDR positive protein expression in colon tissues of each group

group	n	VDR
Control	8	1.34±0.047
UC	8	2.38±0.059 ^b
VDD+UC	8	1.33±0.048 ^d
LVDS+UC	8	2.44±0.049 ^{bef}
HVDS+UC	8	2.78±0.056 ^{bdf}

Note: a:P<0.05, b:P<0.01, compared with the control group; c:P<0.05, d:P<0.01, compared with the UC group; e:P<0.05, f:P<0.01, compared with VDD+UC group

Figures

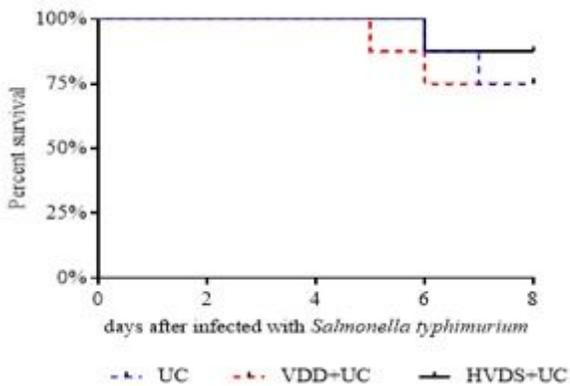


Figure 1

Vitamin D deficiency induces mortality of mice infected with *S. typhimurium* (n=8). Survival percentage of mice in the control and LVDS+UC group was not shown, with 100% survival. The control and UC groups were fed the control diet, while the remaining group fed the vitamin D deficient diet. The LVDS+UC group and HVDS+UC group were supplemented with 0.02µg/20g/d and 0.2µg/20g/d 1,25(OH)2D3, respectively. Two mice in UC group and VDD+UC group were dead, leading to 75% survival. Moreover, there was only one died in HVDS+UC group while none was dead in the control and LVDS+UC group.

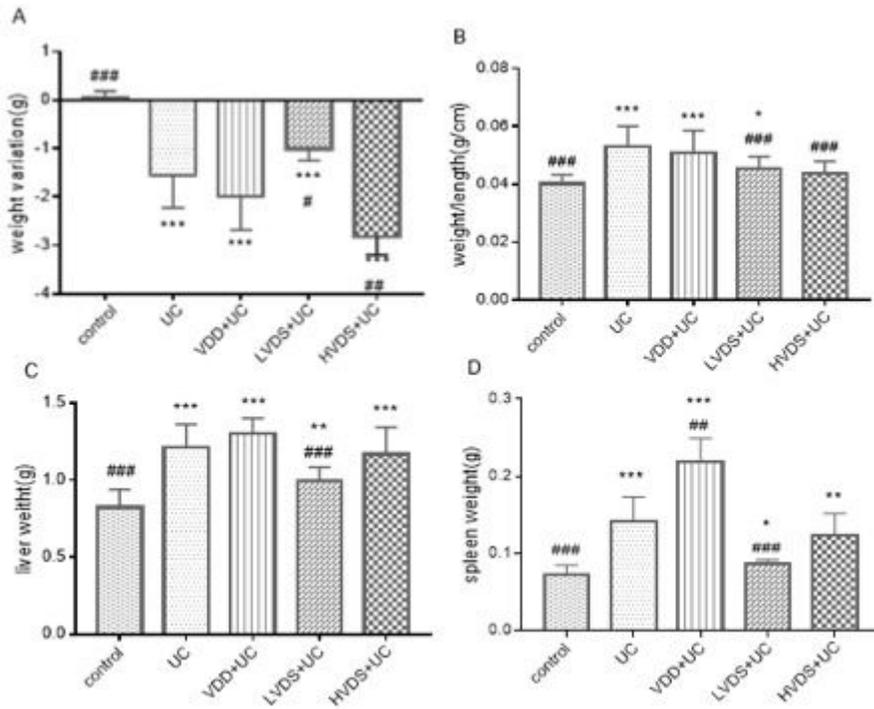


Figure 2

The impact of different vitamin D levels on body weight and some organs in mice. A: Ultimate body weight change in mice with or without *Salmonella* infection; B: the wet weight index of colon, ration of weight and length. C: weight of spleen and liver among groups. Values are means \pm SD. Control group: n=8; UC group: n=6; VDD+UC group: n=6; LVDS+UC group: n=8; HVDS+UC group: n=7. ***P<0.001, **P<0.01, * P<0.05 vs. control group; and ###P<0.001, ## P<0.01, # P<0.01 vs. UC group.

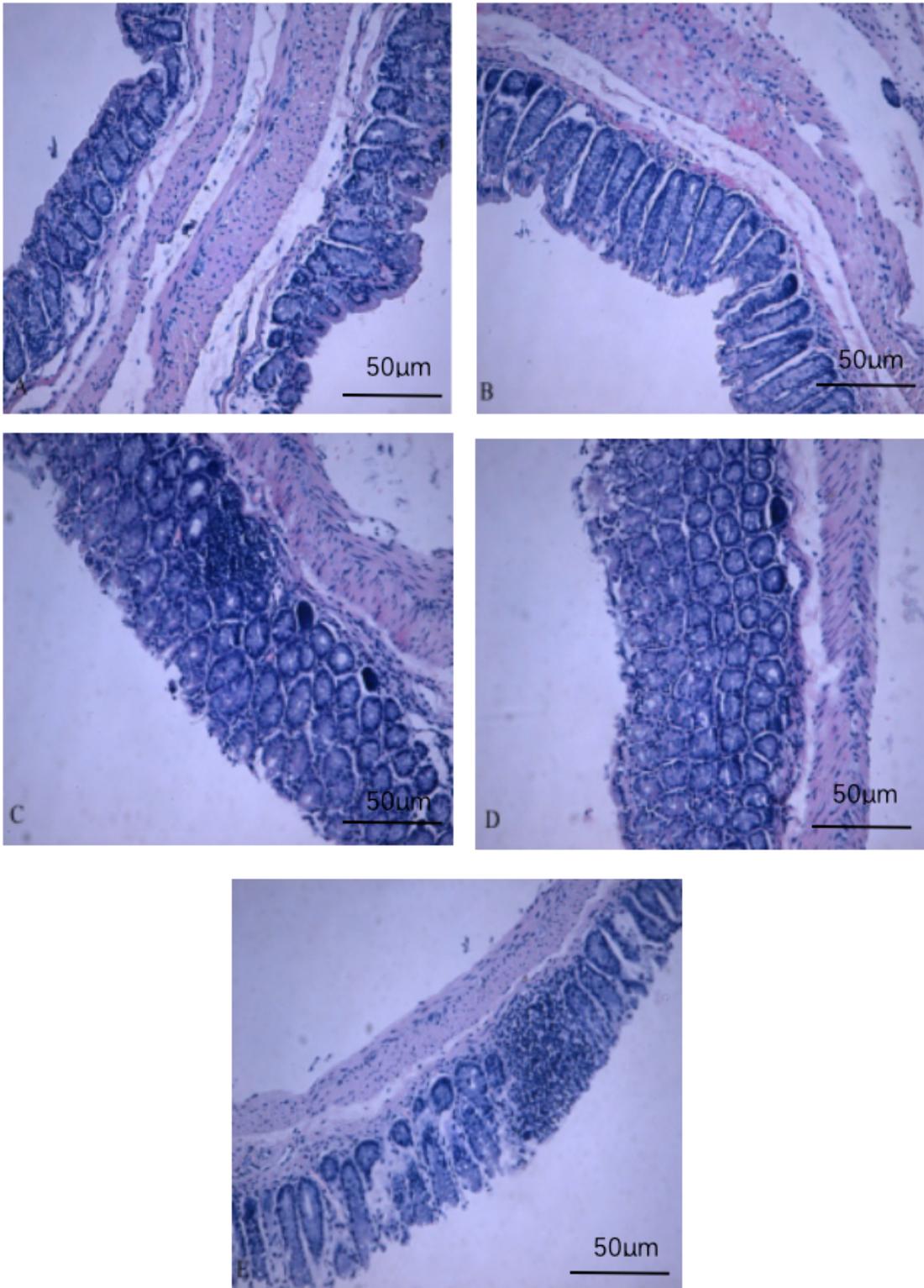


Figure 3

HE staining of colon tissue (X400). A: control group; B: UC group; C: VDD+UC group; D: LVDS+UC group; E: HVDS+UC group.

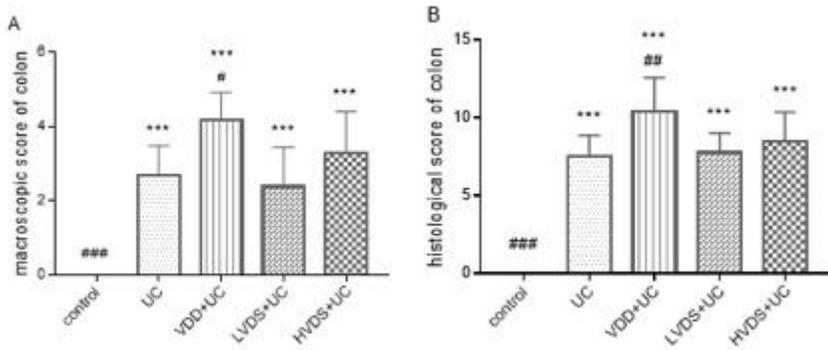


Figure 4

Macroscopic and histologic scores of colons. Macroscopic and microscopic analysis of colons reveals colitis in streptomycin-pretreated mice infected with *S. typhimurium*. Values are means \pm SD. Control group: n=8; UC group: n=6; VDD+UC group: n=6; LVDS+UC group: n=8; HVDS+UC group: n=7. ***P<0.001, **P<0.01, * P<0.05 vs. control group; and ###P<0.001, ## P<0.01, # P<0.01 vs. UC group.

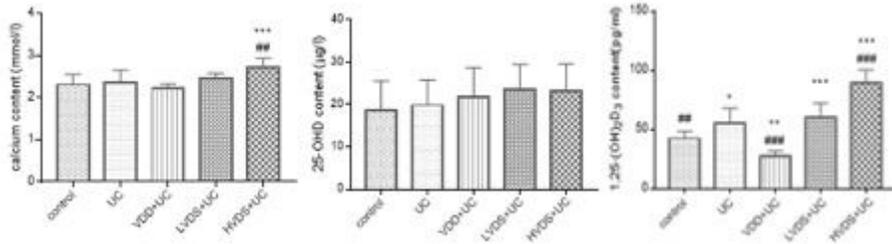


Figure 5

Levels of calcium, 25(OH)D₃ and 1,25(OH)₂D₃ in mice serum. Vitamin D deficiency caused lower level of calcium, decreased content of 1,25(OH)₂D₃. In addition, LVDS and HVDS reversed this situation. Values are means \pm SD. Control group: n=8; UC group: n=6; VDD+UC group: n=6; LVDS+UC group: n=8; HVDS+UC group: n=7. ***P<0.001, **P<0.01, * P<0.05 vs. control group; and ###P<0.001, ## P<0.01, # P<0.01 vs. UC group.

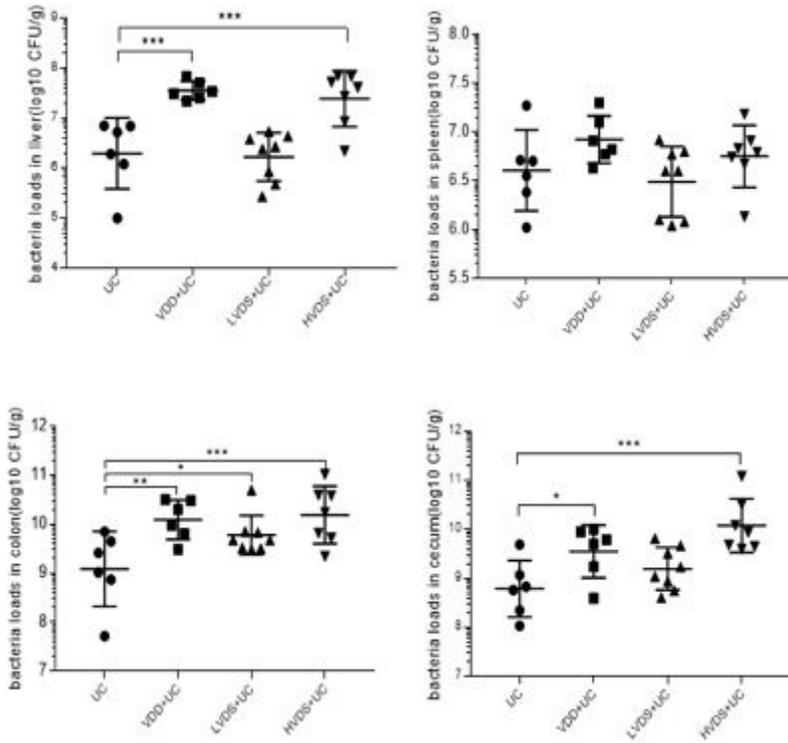


Figure 6

Effect of vitamin D on the colonization of the intestine and internal organs by *S. typhimurium*. HVDS may increase bacteria loads in intestine, liver and spleen compared with LVDS+UC group. Mice fed with a vitamin D free diet gained much more bacteria number, consequently exaggerated colitis. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

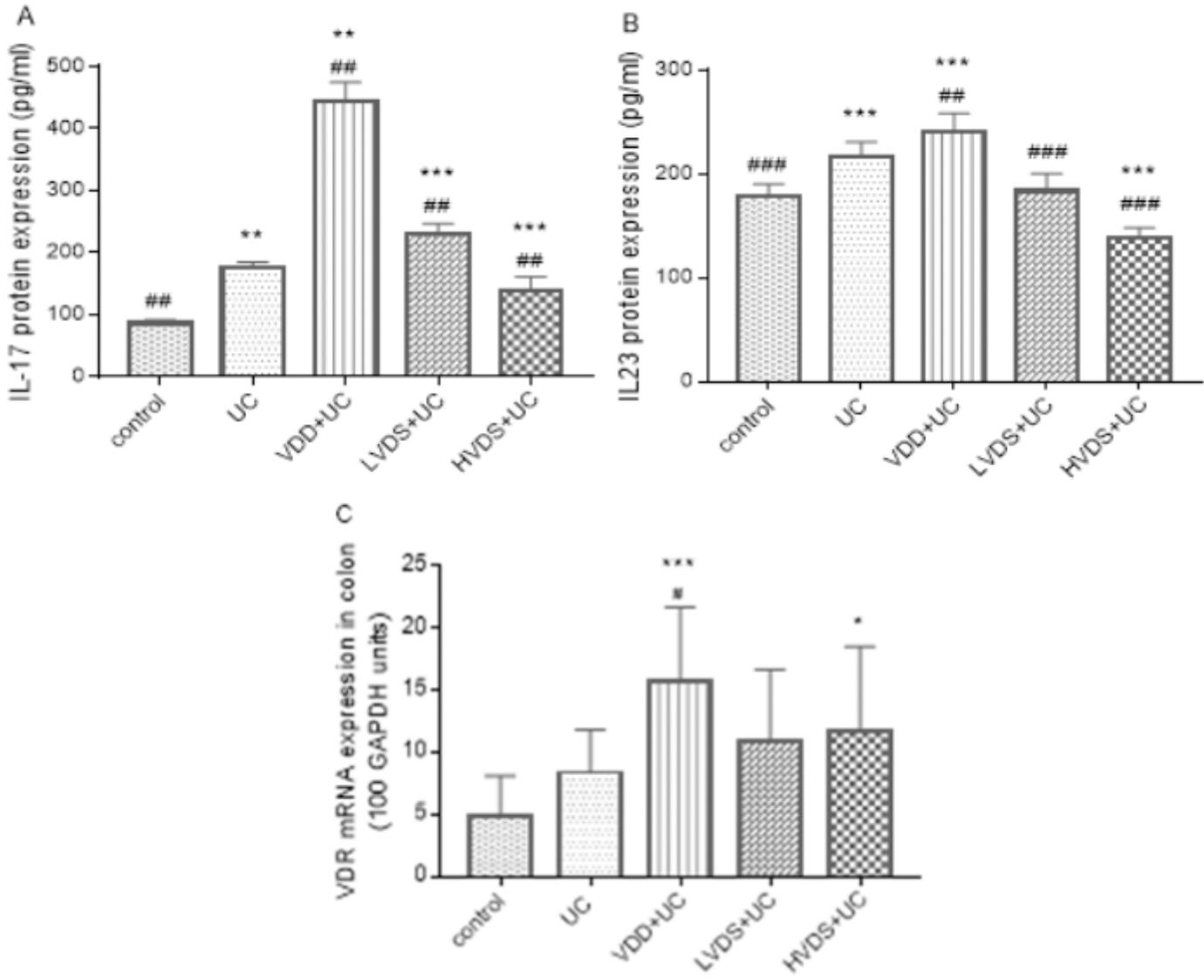


Figure 7

Expression levels of VDR in colonic tissue, and IL-17, IL-23 mRNA in serum. Control group: n=8; UC group: n=6; VDD+UC group: n=6; LVDS+UC group: n=8; HVDS+UC group: n=7. ***P<0.001, **P<0.01, * P<0.05 vs. control group; and ###P<0.001, ## P<0.01, # P<0.01 vs. UC group.

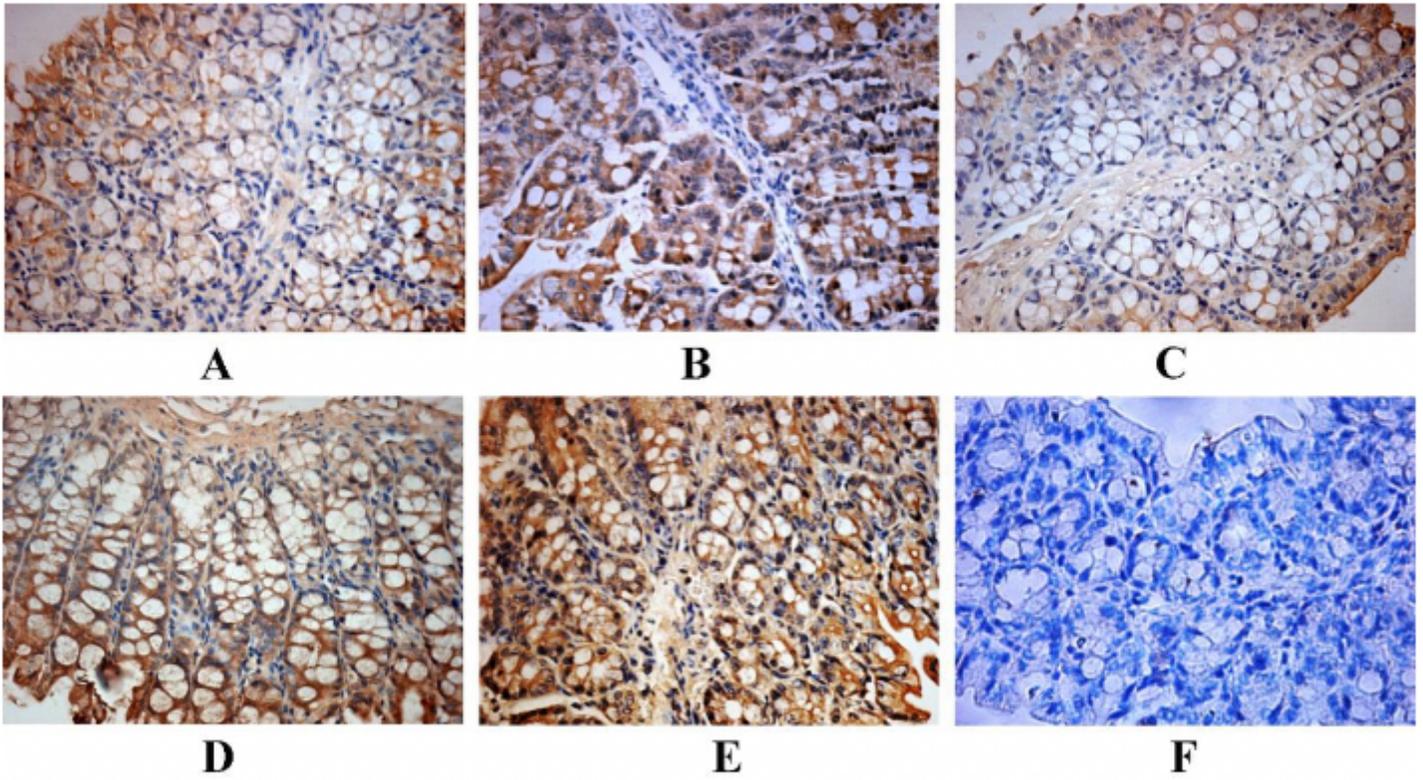


Figure 8

System mice colon tissue VDR protein immunohistochemical detection results (X400). A: control group; B: UC; C: VDD+UC; D: LVDS+UC; E: HVDS+UC; F: blank control