



The Potential of Vitamin D3 to Repaired Mucosal Injury in Dextran Sulfate Sodium Induced Acute Colitis in Mice

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Abstract

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BACKGROUND: Inflammatory Bowel Disease (IBD) has become an emerging disease worldwide. The treatment of IBD involves two basic principles: Inflammation control and mucosal repair.

AIM: This study evaluates the potential effect of Vitamin D3 in mucosal repair through colon stem cell activation and proliferation.

METHODS: Dextran sulfate sodium (DSS; 5%) was used to induce colitis in mice. Vitamin D₃ at various dosages was then administered as a treatment. The mice were divided into five groups: Control (C-); DSS only (C+); and DSS (5%) plus Vitamin D₃ at 0.2 µg (VD1), 0.4 µg (VD2), or 0.6 µg (VD3) per 25 g body weight as the treatment groups. Immunofluorescence analyses of Lgr5+ expression indicated stem cell activation, and Ki67 expression indicated stem cell proliferation. The disease activity index (DAI), colon length, and histopathological index scores were determined after treatment to assess the inflammation and severity of colitis.

RESULTS: Immunofluorescence analyses showed a gradually increasing expression of Lgr5+ also Ki67 in proportion with high doses group of Vitamin D₃ (p < 0.05). The colon length, DAI scores, and histopathological index scores improved in all groups after Vitamin D₃ treatment (p = 0.05; p = 0.026; and p = 0.029, respectively).

CONCLUSION: Vitamin D₃ has a potential beneficial effect on amplifying intestinal stem cells regulated by Wnt/B-catenin signaling. It is also reduced the inflammatory process proved by the evaluation severity of colitis. It might play an essential role in mucosal repair in IBD.

Introduction

The etiology of inflammatory bowel disease (IBD) is not yet fully understood, but some studies have revealed its potential cause to interact with multiple factors, such as infection, immunological disturbances, genetic alterations, and environment. Patients with IBD have to be hospitalized because of repeated episodes of inflammation, endoscopically mucosal injury, and microscopic ulceration of the colon, resulting in complications even have to undergo surgery [1], [2]. In histopathological assessment cryptitis and crypt abscess usually causing destructive damage of the epithelium, along with infiltration of neutrophils in lamina propria as evidence of inflammation. Recent studies have shown that in patients with IBD, the mucosal injury likely followed by an abnormal immune response. Temporarily, mucosal healing was associated for the improvement in IBD treated patients with decreased need for future corticosteroid treatment [3], [4]. Current proven therapies for achieving mucosal healing include anti-TNF α , salicylic agents, and steroids. Another new therapeutic approach was using mesenchymal stem cells (MSCs) and hematopoietic stem cells to reconstruct the epithelial barrier in IBD. Stem cell

activation to achieve mucosal healing could be performed by transplantation or transfusion but also triggered by several micro-environmental factors, such as inflammation and nutritional cues, including Vitamin D3 [5], [6], [7].

Several strands of evidence support that Vitamin D₃ has a role in maintaining the integrity of the intestinal barrier and immune homeostasis. Vitamin D₃ regulates many cell types by controlling hundreds of genes in a tissue and cell. The previous studies have underlined the potential effects of Vitamin D₃ as an inducer in the differentiation of normal colon epithelial cells and through maintaining the morphology typical of the differentiated epithelial phenotype [4], [8], [9]. Administration of Vitamin D₃ is also thought to activate the Wnt canonical pathway, which could control the proliferation and differentiation of intestinal stem cells (ISC). Wnt pathways rarely investigated in inflammatory colonic tissues. It could be said that the proliferative act by ISC could cover the injury, forming channel-like structure and migrate over. Furthermore, one of the β -catenin gene products, Lgr5+, is a receptor on the cell surface that maintains intestinal epithelial homeostasis and tissue integrity. It could be used as a marker for intestinal epithelium renewal after injury. The signaling activity of stem cells will then continue in the proliferation process

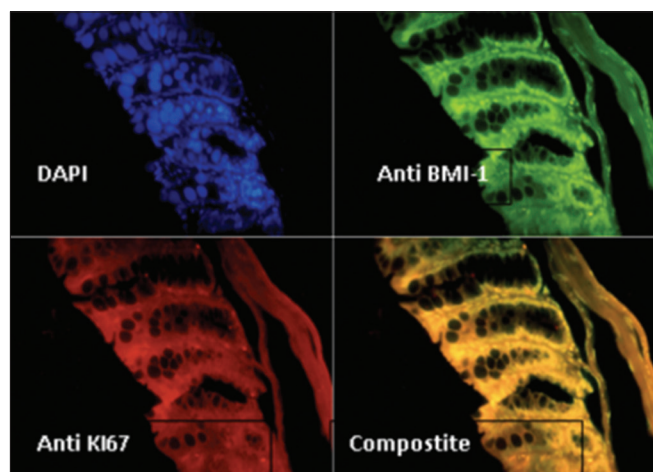


Figure 1: Immunohistochemistry/Immunofluorescence analysis

marked by an increase in Ki-67. Immunofluorescence analysis showed that Ki-67 is widely used as a cell proliferation marker but also highly sensitive to injuries such as IBD [10], [11], [12], [13]. Based on the facts mentioned above, it is necessary to conduct a study regarding the role of Vitamin D₃ in activating, initiating proliferation which will eventually carry out renewal in repairing damaged gastrointestinal mucosal epithelium naturally.

Materials and Methods

Animal studies

Colitis induced by administration of DSS (dextran sodium sulfate; 36,000–50,000 MW; Sigma) drinking water. The DSS used in this study was purchased from MP Biomedical™ (Surabaya, Indonesia). Male C57BL/6 mice of 8 weeks randomly categorized into five groups, either received regular drinking water (control) (C- group); 5% DSS drinking water ad libitum (w/v) for 7 days (colitis) (C+ group); and the other three groups after colitis induction with 5% DSS then were administered Vitamin D₃ in different dosages. The dosages included 0.2 µg (VD1), 0.4 µg (VD2), and 0.6 µg (VD3), respectively, per 25.0 g/day for 7 days. A total of 30 mice were randomly assigned to the control, colitis, and Vitamin D₃ treated group (each group = 5).

Ethics statement

The Ethical Commission of Health Research at the Faculty of Medicine, University of Brawijaya Indonesia, has evaluated and approved the protocol for mice use. Approval ID: no.279/EC/KEPK-S3/10/2019.

Evaluation of disease activity index (DAI)

The combined score of DAI includes weight loss, stool consistency, and occult/gross blood in

the stool. Weight loss was measured on day 2 (in percentage). Stool consistency scored as well-formed scored 0, pasty and semiformal stools scored 2, and liquid stools scored 4. Occult bleeding counted as 0 points for no blood, 2 points for hemoccult positive, and 4 points for gross bleeding. All scores are added up then divided by three according to the number of assessment subjects [14].

Colitis histopathology score

After being euthanized, this study used colon length as a marker of inflammation. The proximal colon was then fixated in 10% formalin and embedded in paraffin; slide 4 µm-thickness sections were stained with hematoxylin and eosin (H and E). Pathological changes were evaluated using the colitis histology index, which consists of four parameters (Table 1) [15].

Table 1: Mouse colitis histology index for the mouse model of colitis

Histological type	Score	Factor	
Goblet cell loss	0	None	1
	1	<10%	
	2	10–50%	
	3	>50%	
Crypt density	0	Normal	2
	1	<10% crypt density	
	2	>10% crypt density	
Crypt hyperplasia	0	None	2
	1	Crypt length increase <2 times	
	2	Crypt length increase 2–3 times	
	3	Crypt length increase >3 times	
Submucosal infiltration	0	None	3
	1	mild cell infiltration	
	2	moderate cell infiltration	
	3	severe cell infiltration	

Immunohistochemistry/Immunofluorescence analysis

Samples of colon epithelial tissue were sliced at 4 µm thickness and immunofluorescence stained to identify (1) colon cells, (2) colon stem cells, (3) activated colon stem cells, and (4) proliferating colon stem cells. 4',6-Diamidino-2-phenylindole fluorescent staining was used to determine the number of nuclei and assess gross colon cell morphology. The expression of Hes-1 is the number of IEC identified by the Hes-1 antibody (sc-166410) visualized with alexa fluor 790, which appears as a red glow and observed by inverted olympus IX71 microscope examination with 40× magnification in five fields of view. Fluorescent Lgr5+ antibody sc-517661 (Santa Cruz Biotechnology, Inc.) was used to identify activated colon stem cells, and fluorescent basic Ki67 antibody sc-23900 (Santa Cruz Biotechnology, Inc.) was used to identify proliferating stem cells. Double immunofluorescence staining was performed using monoclonal rabbit SMAD3-rhodamine and a double-staining blocking kit (anti-Stro-1-FITC), shown in Figure 1. The sample was examined using FluoView FV-1000 (v.1.7; Olympus) by laser scanning confocal microscopy.

Statistical analysis

Data were analyzed with SPSS 13.0 and expressed as mean \pm standard deviation (SD). The comparison of mean variability was conducted by one-way ANOVA analysis. Kruskal–Wallis and Mann–Whitney *post hoc* were applied to compare differences among the groups carried out for non-parametric data. Statistical significance was stated at $p < 0.05$.

Results

Differential dose of Vitamin D₃ in ameliorated inflammation

Mice in the colitis group (C+) had significantly lower colon length as a marker of inflammation than those in the Vitamin D₃-treated group at 0.4 μ g (VD2) and 0.6 μ g (VD3) dose, respectively, (10.14 ± 0.38 ; 9.66 ± 0.16 vs. 8.02 ± 0.54 , $p < 0.01$) (Figure 2c). As in the DAI score, DSS caused hematochezia, weight loss leading to erosions and ulcerations due to the gut inflammation process. We observed that mice induced by DSS had significantly more significant weight loss than that in the control group (C-) from day 3 to day 8, for example, the highest DAI at VD2 and VD3 group (8.00 ± 0.24 ; 7.33 ± 0.26 , $p < 0.01$). However, mice in the Vitamin D₃ group, even in a small dose (VD1), had rapid weight recovery from day 9 to day 14. We found that only the group with middle (VD2) and high dose (VD3) of Vitamin D₃ has DAI scores lowered significantly than those in the colitis group (C+) (1.50 ± 1.19 ; 1.75 ± 0.25 vs. 6.00 ± 1.41 , $p < 0.05$). Other than

that, the group with lower doses of Vitamin D₃ (VD1) did not significantly lower the DAI score (Figure 2a and b).

Histological analysis showed that 5% DSS-induced colitis group (C+) showed extensive ulceration of the epithelial layer, infiltration of inflammatory cells, and crypt damage of colon wall. It is also proved mucosal injury in mice induced with 5% DSS. For example, the inflammation process was proved by the presence of neutrophils infiltrating crypt epithelium compared to the control group (C-) (Figure 3b). This study showed that the histology score in the colitis (C+) group had significantly higher than that in control (C-) group (20.30 ± 0.64 vs. 2.38 ± 0.98 , $p < 0.01$). Nevertheless, mice in the Vitamin D₃ group have compromising to reduce colitis histology index by starting from a low dose, the observation of fewer neutrophils infiltration and cryptitis progresses toward spontaneous healing as the dose of Vitamin D₃ increased. However, mucosal healing has not been seen optimally in the presence of inflammatory cells and ulceration, implying the damage to colon tissues was not completely repaired. The colitis histology index of the VD2 and VD3 groups is significant ($p < 0.01$) lower than the colitis group, indicating the possibility of good therapeutic effect Vitamin D₃ in dose 0.4–0.6 μ g/25.0 g/day.

Differential dose of Vitamin D₃ regenerating intestinal mucosal epithelium

To determine whether administration of Vitamin D₃ could promote proliferation and differentiation of colonic epithelial cells into processed healing injury in colitis, the expression of Ki67 (one of the markers

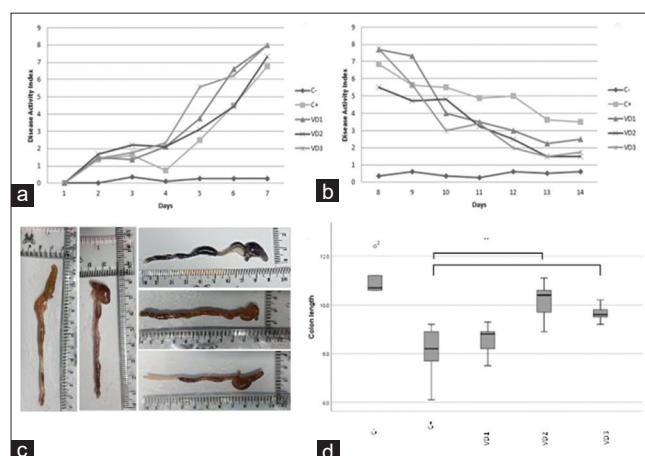


Figure 2: (a) The DAI score increased in mice treated with DSS compared to the untreated control group $*p < 0.05$ compared at day 1–day 7. (b) The DAI scores decreased in the groups given various doses of Vitamin D₃ for 7 days after DSS induction. $n = 5$; $\#p < 0.05$ at day 8–day 14. (c) Colon length measured and a representative of each group. (d) Box plot of colon length showed VD2 and VD3 significant ($p < 0.05$) vs. colitis group.

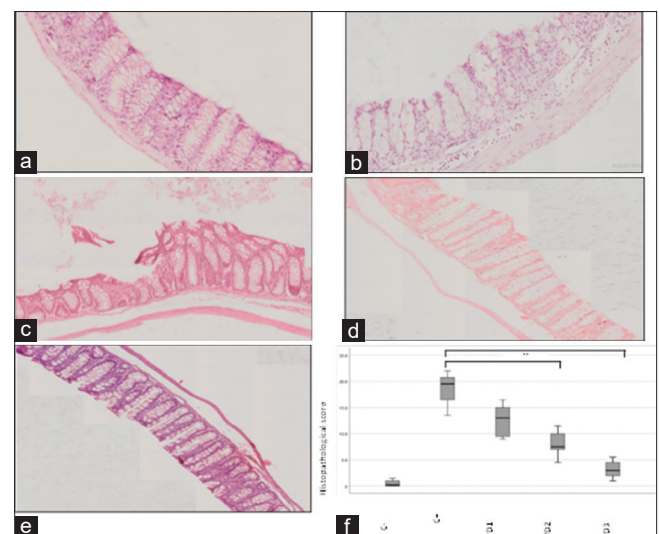


Figure 3: Histopathological images of the colon epithelium from a mouse model of colitis. (Hematoxylin and eosin staining with a magnification of 400 \times , bar=50 μ m). (a) Controls group (C-), (b) Colitis group (C+), (c) DSS colitis treated with low dose Vitamin D₃ (VD1), (d) medium-dose Vitamin D₃ (VD2), (e) high dose Vitamin D₃ (VD3) (f). Comparisons of histopathological scores. Values are expressed as mean \pm SD. $**p < 0.05$.

of cell proliferation) and Lgr5 (one of the markers of differentiation cells crypt base columnar) was examined by immunohistochemistry. Seven days after treated with Vitamin D₃, the expression of Ki67 and Lgr5+ in the VD2 and VD3 group increased significantly compared with colitis group (C+) (Ki67: 277.00 ± 24.32 vs. 162.75 ± 8.68 , $p < 0.05$; Lgr5: $206.25 \pm 7.92 \times 10^3$ vs. $150.75 \pm 11.49 \times 10^3$, $p < 0.05$). Immunofluorescence staining revealed that Lgr5+ expression relatively strong fluorescence intensity and abundant in the basal colonic epithelium. The immunofluorescence analyses showed that leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) were enriched at the bottom of crypts, meanwhile Ki67 shows at the side of crypt colon (Figure 4). We could also note that in control group (C-), these two protein expressions were quite high compared to the colitis group (C+), which defines that colitis disrupts the regulation of body homeostasis to heal injured tissues. These data suggested that Vitamin D₃ may accelerate crypt epithelial cell proliferation and differentiation to promote mucosal healing by the absence of ulceration.

Discussion

ISC are a source of rapid renewal of the intestinal epithelium and beneficial for diseases with the pathophysiology of tissue damage or need to be replaced. ISC showed proliferation and differentiation

activity and were able to restore the intestinal mucosa through Wnt signaling pathway. The Wnt signaling cascade is a key pathway that regulates stem cell homeostasis, inhibition of cell differentiation, and control of Paneth cell development. The Canonical signaling activated by Wnt3a enhances the proliferation of MSCs and previous study found that the expression escalate in IBD colon tissues [16], [17].

Evidence from clinical activities, histopathological and immunofluorescence analysis suggest that Vitamin D may be beneficial against colitis to achieved mucosal healing. In our study, we established that Vitamin D₃ prompted Wnt canonical signaling through Vitamin D receptors (VDR). Leucine rich repeat containing G protein-coupled receptor 5 (Lgr5) is an adult stem cell marker in multiple tissues including intestinal epithelium. Lgr5 could regulate the invasion, migration of colonic cells [18], [19]. Examination of Lgr5+ expression in the DSS-treated mice by immunofluorescence staining showed a proportional increase in expression with the increasing dose of Vitamin D₃, meanwhile also appeared in the control group. In agreement with the previous studies that stated VDR expressed in Lgr5 colon stem cells in human tissue in normal conditions [20]. Temporarily, Ki-67 indicate cell proliferation and differentiation marker and found significantly increased in the previous study compared to non-colitis mice [21]. While the analysis also identified the proliferation index Ki67 was found to slightly decreased in colonic epithelial crypts from damaged mucosa. Mice treated with

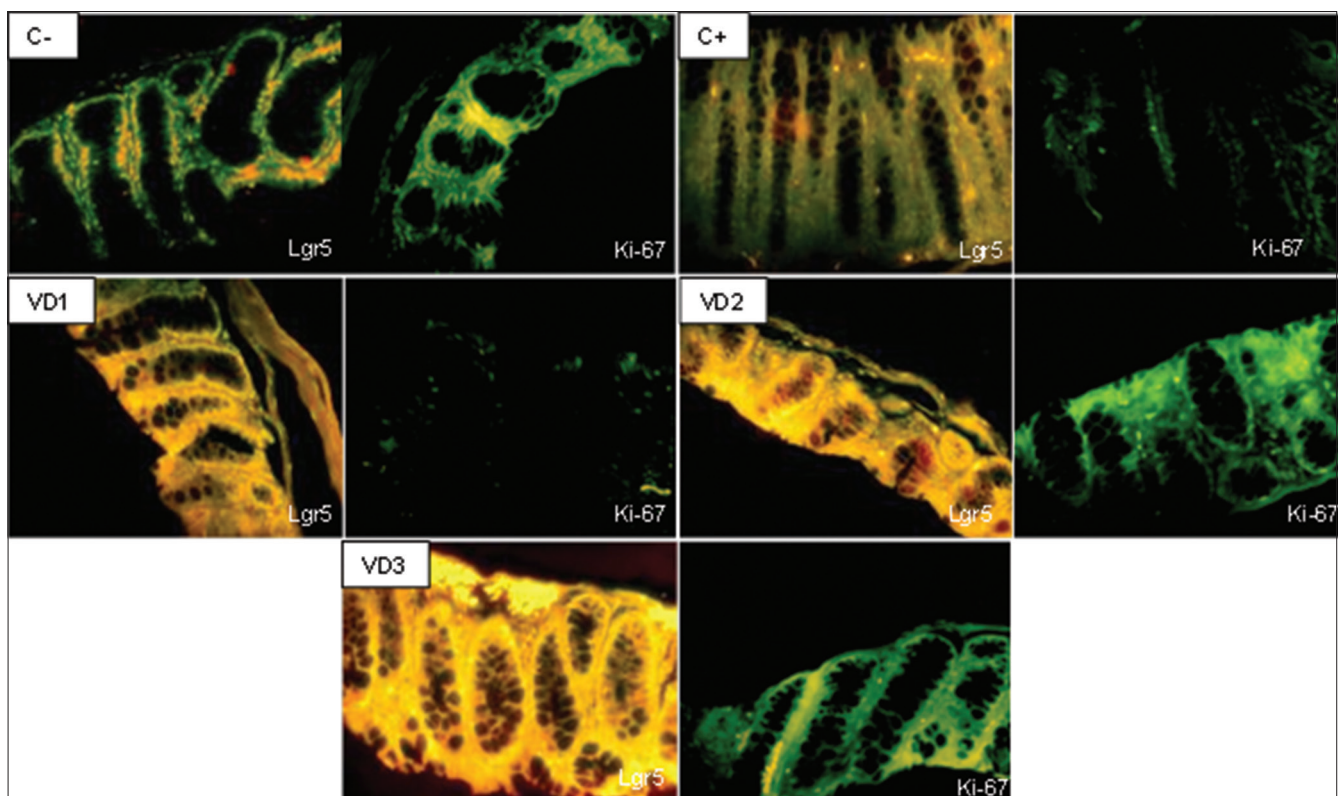


Figure 4: Immunofluorescence staining for stem cells.

Vitamin D₃ had successfully induced mucosal healing by extension of protein Ki-67 in mucosal colon lining. The lower serum Vitamin D₃ levels by colitis condition were in turn implicated in the rapid inactivation of VDR expression in Lgr5+ cells or Ki-67 and a subsequent loss of stem cell function. Therefore, low Vitamin D₃ exposure was considered similar to the stress induced by radiation and chemically induced inflammation, implicating Vitamin D₃ as an extrinsic variable that determines the function of Lgr5+ and Ki-67 in colon stem cells [17], [21], [22], [23]. However, there are limitations to knowing that IHC/IF does not have precise accuracy for counting proteins, and it would be better to use Western Blot or ELISA. Apart from that, research to detect stem cells in the colon, especially for IBD, is still rare, so it requires variable results from another protein expression, especially in the Wnt pathway.

Vitamin D₃ treatment also improved mucosal damage and clinical symptoms in the mouse colitis model. The MCHI score, which identifies the degree of mucosal damage, decreased significantly in response to Vitamin D₃ treatment, while the DAI score increased significantly, confirming that Vitamin D₃ has pleiotropic effects that go beyond its traditional role in calcium homeostasis [24], [25]. Stranded evidenced thought that Vitamin D₃ could improve intestinal brush-border caused by the inflammation process. It was increasing the proliferation of colonic mucosal cells and showed the minimal absence of erosions and ulceration in order to heal [21], [26]. The histopathological appearance at the highest dose of Vitamin D₃ had best view similar to the control group except in goblet cell and submucosal infiltration. Therefore, Vitamin D₃ could repair mucosal injury in mice with colitis but cannot restore the same histological appearance as healthy mucosa.

Conclusions

Wnt signaling pathway activation evolutionarily conserved tissue in colon, which can be transiently activated for wound repair. Vitamin D₃ could have an important role for IBD patient, as it showed significant effects on colon stem cell activation and proliferation in the mouse model of colitis. In addition, direct evidence showing that Vitamin D₃ either influences stemness maintenance or proliferation needs to be demonstrated in further studies.

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