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Original Article

Vitamin D3 induces stem cell activation via Lgr5-Bmi1 expression and improving mouse colitis histology index

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Abstract

Conventional therapy for inflammatory bowel disease using long-term anti-inflammatory drugs does not seem to provide optimal results. Adjuvant therapy using vitamin D3 is believed to have an essential role in repairing the colonic mucosa through the activation of colonic stem cells. The aim of this study was to demonstrate the effect of vitamin D3 in mucosal repair through stem cell activation, marked by leucin-rich repeat-containing G protein-coupled receptor 5 (Lgr5) and B lymphoma Mo-MLV insertion region 1 (Bmi1) expression and decrease the mouse colitis histology index (MCHI) score. In this study, 50 *Mus musculus* strain BALB/c were divided into five groups: negative control group, colitis group, and colitis groups with vitamin D3 administration of 0.2 mcg, 0.4 mcg, and 0.6 mcg per 25 g body weight for seven days. Dextran sulfate sodium (DSS) 5% was used to induce colitis. Lgr5-Bmi1 expression was measured using immunodoublestain fluorescent labeling method. Our data suggested that administration of vitamin D3 significantly increased expression of Lgr5-Bmi1 in the colonic mucosa. The colitis group treated with the highest dose of vitamin D3 (0.6 mcg/25 gram) showed the lowest MCHI score (3.60±0.64) while the lowest dose of vitamin D3 had the highest MCHI score (12.60±1.47). In conclusion, by stimulating stem cells, vitamin D3 administration stimulates mucosal regeneration, as demonstrated by upregulated expression of Lgr5-Bmi-1.

Keywords: Colitis, mucosa, histology, vitamin D3, stem cell

Introduction

Inflammatory bowel disease (IBD) is a chronic autoimmune inflammatory disease of the gastrointestinal tract, and classified into two clinical forms: ulcerative colitis and Crohn's disease. Clinical signs of this condition include persistent diarrhea, lower gastrointestinal bleeding, weight loss, and abdominal pain, as well as symptoms beyond the gastrointestinal tract: fever, joint pain, or vision problems. Chronic inflammation in IBD also increases the risk of developing colorectal cancer [1-4]. Multiple factors play a role in the pathogenesis of IBD, including genetic susceptibility triggered by environmental factors, gut microbial patterns, and immunological factors [3,4]. Dietary factors, allergic tendencies, psychosocial problems, and metabolic inadequacies all also have a part in the etiology of IBD [3,4].

According to most studies, IBD is caused by damage to the intestinal epithelial lining and an accumulation of inflammatory cells in the submucosa [5-7]. Multiple ulcers will form when the epithelial layers erode, allowing antigens to penetrate the intestinal lumen and enter the

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submucosal layer. These antigens will cause pro-inflammatory cytokines to be released in the submucosal layer, resulting in local inflammatory response. Due to extensive mucosal damage, the regeneration process of epithelial cells at the afflicted locus fails, resulting in the loss of a substantial number of stem cells [5-7].

Typical treatment of IBD using anti-inflammatory drugs, such as aminosalicylate, steroids, and long-term immunosuppressive drugs, does not always give the best results. In recent years, vitamin D3 has been frequently used as adjuvant therapy in adult patients. Vitamin D3 has been found to play a role in the immune system via the Wnt/β-catenin pathway by suppressing the expression of pro-inflammatory cytokines while increasing that of anti-inflammatory cytokines [8-11]. In addition, vitamin D3 can potentially limit cell proliferation and retain cells in an undifferentiated state via the Wnt/β-catenin pathway, hence preventing colon cancer [9,12-14]. Evidently, stimulation of the Wnt/β-catenin pathway in intestinal cells can boost transcriptional activity as well as stimulate cell proliferation and maturation [15].

There are two types of stem cells in the gastrointestinal mucosa: those with leuchine-rich repeat-containing G protein-coupled receptor 5 (Lgr5) expression and those with B lymphoma Mo-MLV insertion region 1 (Bmi-1) expression on their surface. Lgr5 stem cells have a fast cleavage rate and are involved in the normal regeneration process. Meanwhile, Bmi-1 stem cells only activate when the cell is injured [7]. Activation of the Wnt/β-catenin pathway will increase the expression of Lgr5 and Bmi-1, which will then continue in the proliferation process characterized by an increase in Ki67 expression, an increase in Hes1 levels as a marker of enterocyte progenitors, and an increase in cytokeratin 20 as a marker for enterocytes maturation [15]. The purpose of this study was to prove that increased expression of Bmi-1 and Lgr5 in response to vitamin D3 therapy can reduce the mouse colitis histology index (MCHI) in an IBD animal model.

Methods

Study setting

An experiment study using post-test only design was conducted using fifty male *Mus musculus* strain BALB/c. Colitis induced mice were given vitamin D3 at three different doses for six days after which the blood was collected to determine the vitamin D3 levels and colons were collected to measure Bmi-1 and Lgr5 expression and MCHI score.

Development of colitis animal model

Dextran sulfate sodium (DSS) 5% was to induce colitis. Dextran sulfate sodium solution was made using 5 g of DSS powder (molecular weight 40 kDa) dissolved in 1000 cc of distilled water, then was added to the mice drinking water for seven days (was given on day 1 to day 7).

Animal model and study groups

A total of 50 mice were divided into five groups of ten mice each: negative control (non-colitis model, without DSS 5% and vitamin D3); positive control (colitis model, given DSS 5% alone without vitamin D3); and three experimental groups: E1, DSS 5% and vitamin D3 0.2 mcg/25 g body weight (BW); E2, DSS 5% and vitamin D3 0.4 mcg/25 g BW; and E3, DSS 5% and vitamin D3 0.6 mcg/25 g BW. Dextran sulfate sodium 5% to induce colitis was given on day one to day seven. Vitamin D3 was administered orally with a gastric tube from day 8 to 14 following DSS 5% treatment. On the 15th day, euthanasia was carried out by administering ketamine 80 mg body weight intravenously and distal third of the colon were collected from each animal.

Vitamin D3 level measurement

Serum vitamin D3 levels (25(OH)D) from intracardiac blood samples were measured using enzyme immunoassay (DiaSorin Inc, Stillwater, MN, USA). Intracardiac blood was collected on day 15 before the animals were euthanized.

Mouse colitis histology index (MCHI) scoring system

MCHI scores were determined by histological examination of colon and are based on four components, as shown in **Table 1**. Percentage of goblet cells involvement, crypt

hyperplasia, and submucosal infiltration were examined and calculated manually by histopathologist and well-trained medical doctor under a microscope.

Table 1. Mouse colitis histology index (MCHI) scoring system

Feature graded	Grade	Factor
Percentage of goblet cells involvement	0 None 1<10% 2 10–50% 3>50%	1
Crypt damage	Normal <10% ≥10%	2
Crypt hyperplasia	None Slight increase in crypt length 2–3x increase in crypt length >3x increase in crypt length	2
Submucosal infiltration	None Single-cell infiltration Moderate cell infiltrations significant amount of cell infiltrations	3

Histological examination and measurement of stem cells expression

Colonic mucosal specimens were prepared using immunohistochemical techniques. Briefly, the tissues were washed with phosphate-buffered saline (PBS) to remove contaminants before being fixed in 10% formalin. The specimens were then dehydrated with graded alcohol, cleaned with xylol twice, and infiltrated with soft paraffin at 48°C; each step lasted 60 min. The specimens were then blocked with hard paraffin and let to stand for a day. The next day, it was affixed to the holder and sliced 4 µm thick with a rotary microtome before being mounted on a 5% gelatin object glass.

Bmi-1 and Lgr5 expressions were measured using immunodoublestain fluorescent labeling technique under a microscope. Slides were washed three times using PBS pH 7.4 for 5 min before and after incubation. Slides were incubated in 0.2% triton-X 100 for five times, incubated for unspecific protein blocking using 2% bovine serum albumin (BSA) for 30 min at room temperature, incubated using mouse monoclonal anti Bmi1-FITC conjugated (SC-390443 FITC, Santa Cruz Biotechnology, Dallas, TX, USA) and mouse monoclonal anti Lgr5-phycoerythrin conjugated (SC-390630 FITC, Santa Cruz Biotechnology, Dallas, TX, USA) for one night at 4°C, followed by incubation using 4′,6-diamidino-2-phenylindole (DAPI) (D9542, Sigma-Aldrich, MI, USA) for five min. The slides were dried and observed.

Statistical analysis

The expressions of Bmi-1 and Lgr5 were calculated using Image-J software, and the data obtained were analyzed using the Kruskal-Wallis and post hoc Mann-Whitney tests. Differences in serum vitamin D3 levels and MCHI scores between groups were analyzed using one-way ANOVA at the 95% confidence level (p<0.05) with α=0.05 and post hoc test.

Results

Serum vitamin D3 levels

Although there was a significant difference between groups (p=0.001), the serum vitamin D3 levels had a disproportionate with increasing doses of vitamin D3 given (**Table 2**).

Table 2. Levels of serum vitamin D3 among study groups

Group	Experimental detail	Serum vitamin D3 (ng/mL) (mean±SD)
C–	Negative control	72.609±11.9
C+	Dextran sulfate sodium (DSS) 5% only	53.96±7.95
E1	DSS 5% + vitamin D3 0.2 mcg/25 g	119.7±23.9
E2	DSS 5% + vitamin D3 0.4 mcg/25 g	41.45±1.22
E3	DSS 5% + vitamin D3 0.6 mcg/25 g	48.13±2.9

Lgr5-Bmi1 expression

Using immunofluorescence staining, colon cells are visualized in blue, Bmi-1 expressing cells are visualized in green, and Lgr5 expressing cells are visualized in yellow. Normal stem cells express Bmi1 on their surface meanwhile while activated cells express Lgr5 protein. According to the immunofluorescence pictures, positive control group (C+) had fuzzier green and yellow luminescence than the negative control group (C-). The representative of Lgr5-Bmi1 expression in each study group are presented in **Figure 1**. There was a significant difference in Lgr5-Bmi1 expression in E3 group with positive control group (p=0.021) and E2 group (p=0.029) (**Table 3**).

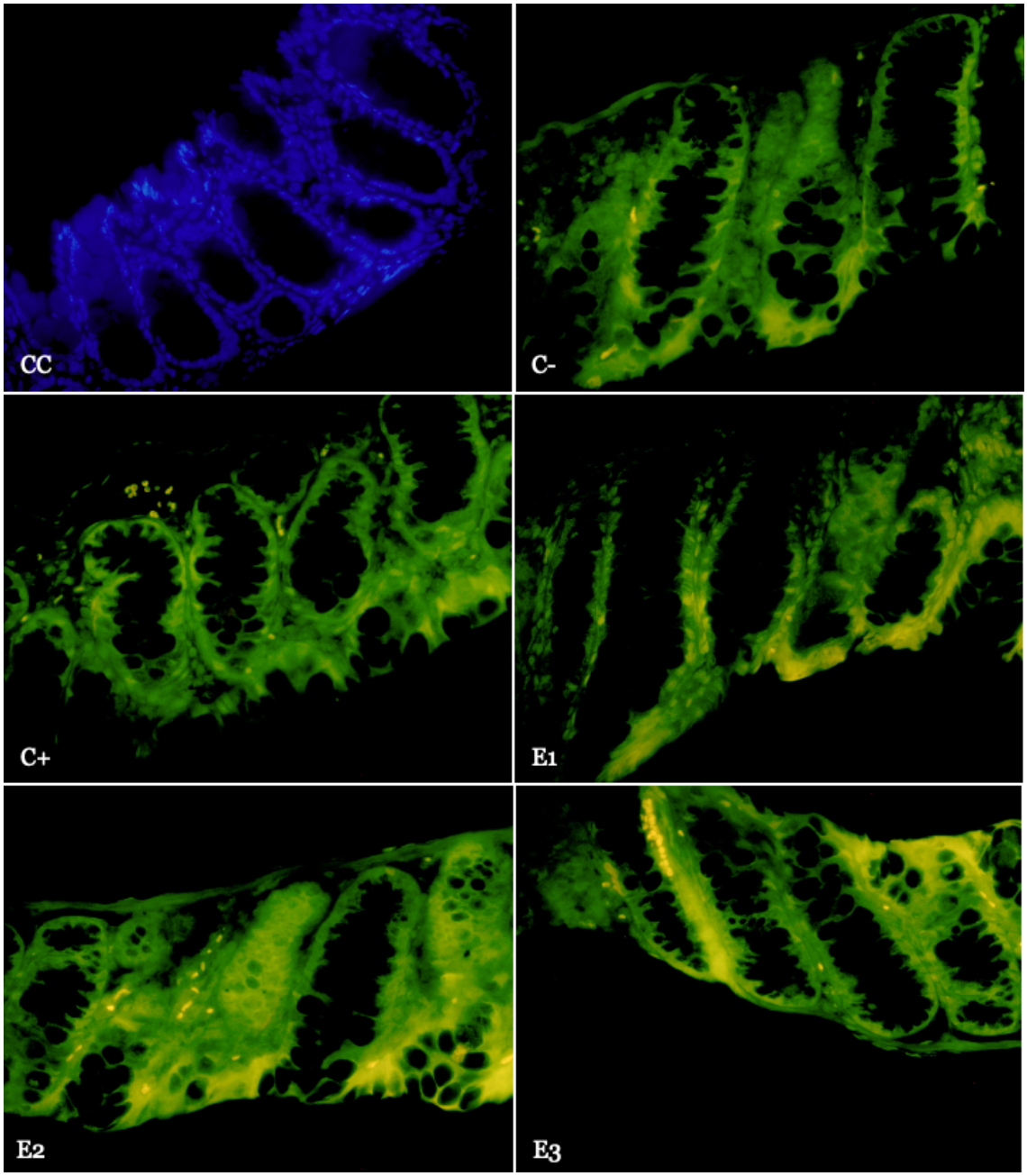

Figure 1. Representative of Lgr5-Bmi1 expression in each group by immunofluorescence staining. CC: healthy colon cells group; (C-): control negative group, mice that are not given any treatment; (C+): positive control group, mice that are given DSS 5% only; E1: group of mice given DSS 5% and 0.2 mcg/25 g vitamin D3; E2: group of mice given DSS 5% and 0.4 mcg/25 g vitamin D3; and E3: group of mice given DSS 5% and 0.6 mcg/25 g vitamin D3.

Table 3. Lgr5-Bmi1 expression analysis among groups

Group	Experimental details	Number of cells expressing Lgr5-Bmi1 (mean±SD)
C–	Negative control	148.25±50.38
C+	Dextran sulfate sodium (DSS) 5% only	150.75±11.49*
E1	DSS 5% + vitamin D3 0.2 mcg/25 g	111.00±10.78
E2	DSS 5% + vitamin D3 0.4 mcg/25 g	164.25±11.18*
E3	DSS 5% + vitamin D3 0.6 mcg/25 g	206.25±7.92

*Statistically significantly at p=0.05 compared to E3 group

Histology

Compared to those of the negative control group, the DSS-induced group's histopathological showed significant submucosal infiltration, crypt hyperplasia, and crypt damage. These characteristics all improved as vitamin D3 dosage increased. The E1 group (0.2 mcg of vitamin D3) showed moderate submucosal infiltration, mild crypt hyperplasia, and a slight decrease in crypt damage (**Figure 2**). Although there was no hyperplasia in the E2 group (vitamin D3 0.4 mcg), there was still minimal submucosal infiltration and crypt damage. In contrast, in the E3 group (vitamin D3 0.6 mcg) there was no crypt hyperplasia, crypt damage, or submucosal inflammatory cell infiltration in the submucosa (**Figure 2**).

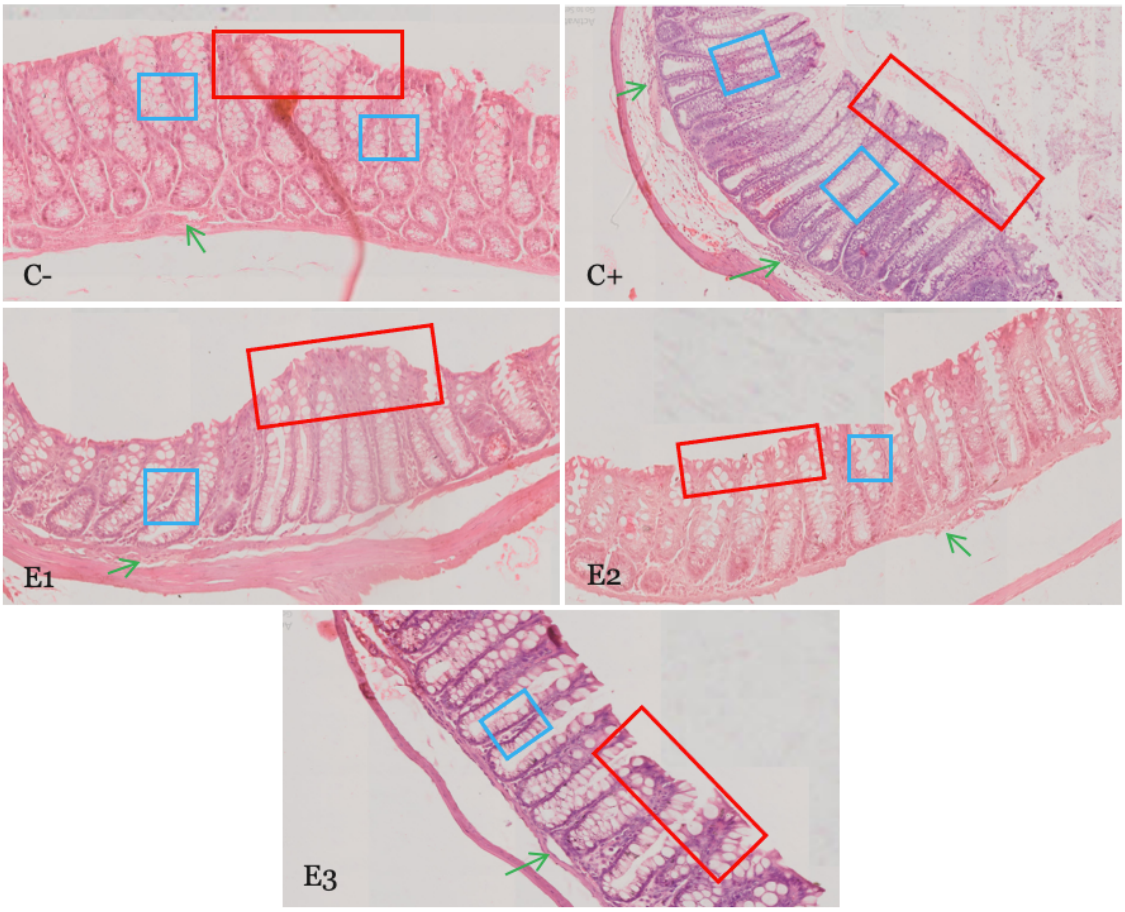

Figure 2. Representative of colon histopathological picture of each study group. Staining using hematoxylin and eosin with 400x magnification. (C-): crypt density is still good (blue square), there is no hyperplasia (red square), and no submucosal infiltration (green arrow); (C+): it shows crypt density damage (blue square), hyperplasia of the crypts (red square), and a large amount of submucosal infiltration (green arrows); E1: crypt density slightly increased (blue square), there is mild hyperplasia of crypts (red square), and moderate submucosal infiltration (green arrow); E2: there is still mild damage to crypt density (blue square), mild submucosal infiltration (green arrow), but no hyperplasia (red square); E3: there is no crypt density damage (blue square), no crypt hyperplasia (red square), and no inflammatory cell infiltration in the submucosa (green arrow).

Mouse colitis histology index (MCHI) score

The negative control group had the lowest MCHI score (0.60±0.29), the positive control group had highest score (20.3±0.64) (**Table 4** and **Figure 3**). The mean MCHI in E1 group (vitamin D3 0.2 mcg) was 12.60±1.47; the MCHI decreased with increasing dose of vitamin D3 (to 9.20±0.86 for vitamin D3 0.4 mcg group or E2) and the lowest mean was in the E3 group (vitamin D3 0.6 mcg/25 g) with 3.60±0.64 (**Table 4**).

Table 4. Mouse colitis histology index (MCHI) scores among groups

Group	Experimental details	MCHI score Mean±SD
C–	Negative control	0.60±0.29
C+	Dextran sulfate sodium (DSS) 5% only	20.3±0.64
E1	DSS 5% + vitamin D3 0.2 mcg/25 g	12.60±1.47
E2	DSS 5% + vitamin D3 0.4 mcg/25 g	9.20±0.86
E3	DSS 5% + vitamin D3 0.6 mcg/25 g	3.60±0.64

The mean MCHI score was then analyzed using one-way ANOVA followed with pos-hoc analysis (**Figure 3**). There were significant differences in MCHI scores between the negative control group and the positive control (p<0.001), E1 (p<0.001), and E2 group (p<0.001). Meanwhile, the positive control group also had significant differences with E1 (p<0.001), E2 (p<0.001), and E3 group (p<0.001). There was no significant difference between the negative control and E3 groups (p=0.150) (**Figure 3**).

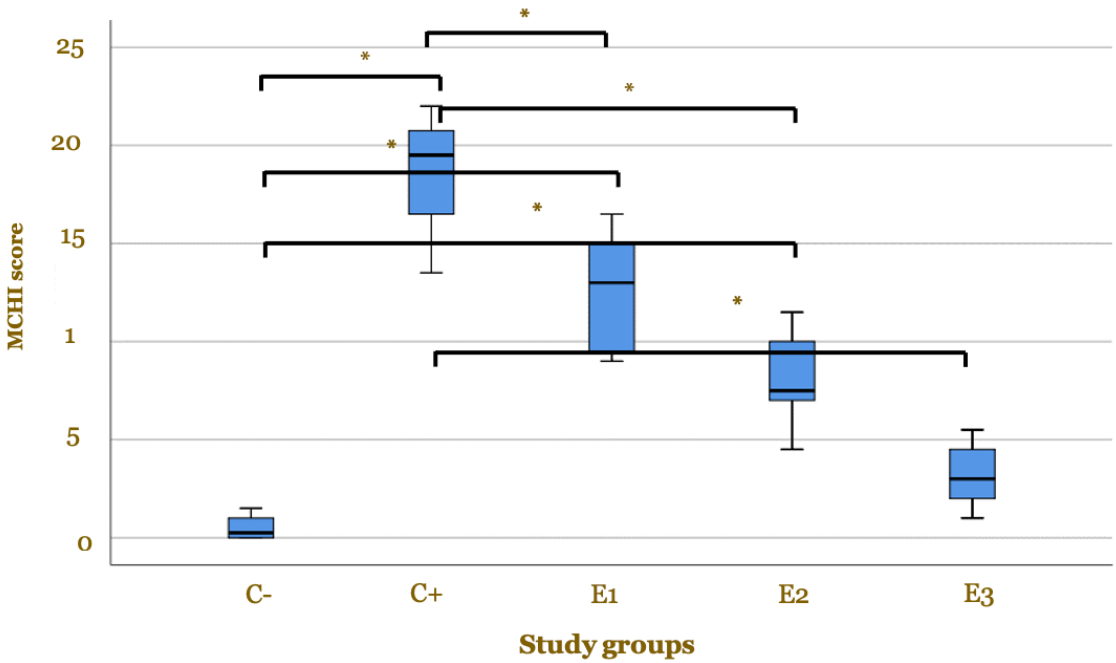

Figure 3. Post-hoc analyses showing the comparison of mouse colitis histology index (MCHI) scores between study groups. Asterisk indicates statistically significant at p=0.05.

Discussion

This study demonstrated that vitamin D3 significantly reduced the MCHI by increasing the expression of Lgr5-Bmi1, which activates colonic stem cells. Vitamin D3 increased the expression of Lgr5-Bmi1, and the expression increased most significantly in the E3 group. It was also discovered that the higher the dose of vitamin D3 given, the greater the mean MCHI score decreased. There was a significant difference in MCHI scores between the positive control group and all other groups (p<0.001), which indicated that vitamin D3 could repair the damage of the colonic mucosa. There was no statistically significant difference between the negative control group and the vitamin D3 0.6 mcg/25 g group (p=0.150). Thus, it can be concluded that 0.6

mcg/25 g vitamin D3 can improve the condition of the colonic mucosa until relatively close to normal.

This is consistent with previous studies that have explained the role of Lgr5+ in the repair of the gastrointestinal mucosa, one of which was a study by Peregrina *et al*. in which mice given a diet of 0.11 IU/g vitamin D3 had lower Lgr5+ expression (17%) than mice given a dose of 2.3 IU/g vitamin D3 (23%) [16]. Lgr5 and Bmi-1 have been widely recognized as markers of gastrointestinal stem cells that comprise enterocyte cell layers in villi. Lgr5 is known to have routine cleavage activity and functions in intestinal mucosal homeostasis for normal regeneration processes, while Bmi1 stem cells are only activated during cell injury [7]. The ability of Lgr5 and Bmi-1 to produce cell populations in colonic crypts depends on dietary intake of vitamin D3 and vitamin D3 receptors (VDRs) [16].

Previous studies have shown that active metabolite of vitamin D3 (1,25(OH)2D3) can stimulate hair follicle development by inducing β-catenin via VDRs [15,17]. As a result, numerous transcription-related genes are thought to be regulated by Wnt/β-catenin. The stimulation of the Wnt/β-catenin signaling pathway is responsible for the enhanced expression of Bmi-1 and Lgr5. In embryological development, carcinogenesis, and epithelial-to-mesenchymal transition, these signaling pathways in the gut mucosa govern epithelial cells to withstand injury and facilitate repair after damage [15,18]. Wnt signaling increases the quantity of β-catenin in its active form via the canonical pathway, followed by an increase in intracellular β-catenin, which finally binds to T-cell factor/lymphoid enhancer factor (TCF/LEF) located on the Wnt responsive element in the promoter region of DNA [19]. This binding triggers the Wnt target gene to express Bmi-1 and Lgr5, which then moves to the cell surface and increases the amount of Lgr5 and Bmi-1 receptors [19]. Increased Lgr5 and Bmi-1 expression will induce the production of Ki67, the secretory progenitor intestinal stem cells, which initiates cell formation and proliferation [20]. In the colon, mesenchymal stem cells stimulate progenitor cells to promote epithelial regeneration but are also lodged in injured tissue and can differentiate into colonic interstitial cells. The expression of cytokeratin 20, a filamentous protein found in the mucosa of the intestine, urothelium, and Merkel cells, facilitates this differentiation process. Cytokeratin 20 is expressed during the maturation of enterocytes and goblet cells in the intestinal villi [15,19].

In this study, serum vitamin D3 levels were inconsistent with increasing doses of vitamin D3 supplementation due to several limitations. Many factors may contribute to this. First, vitamin D3 supplementation only lasted for seven days. A previous study stated that vitamin D3 supplementation significantly increased serum vitamin D3 levels with a minimum of six weeks of administration [21]. Another factor is the possibility that in acute inflammatory conditions, serum vitamin D3 levels might decrease more rapidly with higher doses of vitamin D3 supplementation, because 25(OH)D3 in serum will be immediately converted to its active form, 1,25(OH)D3, which will then interact with VDR in tissues and modulate stem cell expression in injured tissues [15,22]. Finally, it's possible that increasing 25(OH)D has a threshold effect, accelerating the catabolism of 25(OH)D to 24,25(OH)D, the catabolic form of 25(OH)D. According to earlier study, an initial ratio of 24,25(OH)D:25(OH)D was a predictor of response to vitamin D3 therapy [21]. Future studies must verify this hypothesis since this study did not measure 24,25:25(OH)D [21].

Treatment of multipotent stem cells with vitamin D3 is a promising approach to enhance mucosal healing in IBD patients as it utilizes the unique ability of the Lgr5 and Bmi-1 expression to promote tissue regeneration. However, one limitation of our study is that many potential intermediary and confounding factors, such as the genes that might be implicated as targets or the genes that encode and activate advanced pathways, remain unexplored. This is an essential topic that should be explored in the future.

Conclusion

Vitamin D3 administration promotes colonic mucosa regeneration by activating stem cells, as evidenced by increased expression of Lgr5 and Bmi-1. Therefore, vitamin D3 supplementation could be considered as the primary or adjuvant treatment for colitis.

Ethics approval

This study was approved by Health Research Ethics Committee, Faculty of Medicine, Universitas Brawijaya, with the number of ethical approval 270/EC/KEPK-S3/10/2019.

Competing interests

Authors have no conflict of interest to be declared.

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Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

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