Administration of Vitamin D3 Improves Hemoglobin Level by Regulating TNF-α and IL-6 in DSS-induced Colitis Mice

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BACKGROUND: Anemia is frequently found in ulcerative colitis (UC) patients and assumed to be related to inflammatory process. Vitamin D3 (VD) is known to have anti-inflammatory and immunomodulatory effects. It also has the potential to be an alternative treatment of the inflammatory process that occurs at UC, however its mechanism has not been clearly established. This study aimed to assess the effect of VD on histopathology and hemoglobin levels in UC through its regulation in tumor necrosis factor (TNF)-α and interleukin (IL)-6.

METHODS: Total samples of 24 mice were divided equally into Sham group, UC group, UC+VD group (given 3% dextran sodium sulfate (DSS) followed by VD), and VD+UC group (given VD followed by 3% DSS). Mouse Colitis Histology Index (MCHI) was used to measure histopathological changes. Immunohistochemical staining was used to observe expression of TNF-α and IL-6 in colon. Evaluation of anemia was determined by hemoglobin levels.

RESULTS: Based on MCHI scores, significant epithelial damage was found in colon sample of UC group (8.25±3.05) compared to Sham (0.33±0.26), UC+VD (2.33±1.07), and VD+UC group (2.83±0.75) (p<0.05). Significant lower numbers of TNF-α were found in Sham (27.33±3.42), UC+VD (36.33±1.86), and VD+UC group (36.68±1.86) compared with UC group (44.66±4.87) (p<0.05). Significant less IL-6 expression was found in Sham (18.05±2.96), UC+VD (24.78±0.79), and VD+UC group (25.09±2.79) compared to UC group (38.85±3.51) (p<0.05). Differences in hemoglobin levels were significantly lower in UC group (11.85±0.97) compared to Sham (14.25±0.47), UC+VD (13.68±0.68), VD+UC group (13.52±1.07) (p<0.05).

CONCLUSION: VD significantly reduced pro-inflammatory cytokines, increased mucosal repair, and improved hemoglobin levels.

KEYWORDS: colitis, ulcerative, interleukin-6, tumor necrosis factor-alpha

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Introduction

Ulcerative colitis (UC) is characterized by diffuse inflammation of the colon and rectum mucosa.(1) Based on colonoscopy data from Saiful Anwar General Hospital in 2010-2014, 176 patients were diagnosed with UC or 8.2% of the total colonoscopy patients.(2) A combination of genetic, environmental, and microbial factors causes colitis by disrupting immune system regulation in the form of changes in the balance of T helper cells to Th1 and Th17 compared to Th2.(3) If not treated properly, colitis can cause various complications such as anemia, as one of the most common extraintestinal complications.(4) The discharge of blood from the digestive tract continuously resulting in iron deficiency is what usually causes anemia in colitis.(5) Increased activity of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and...
interleukin (IL)-6 also can cause anemia in colitis. TNF-α increases ferritin production which reduces the amount of iron for erythropoiesis, and IL-6 initiates the formation of hepcidin as a negative regulator of iron absorption and iron release. These mechanisms disrupt the erythropoietin (EPO) signaling process and reduce the regulation of EPO receptors. Thereby EPO resistance is induced in erythroid progenitor cells.(5,6) Several studies have found low levels of vitamin D in colitis patients. Vitamin D deficiency plays a critical role in the severity of colitis by causing an increase in pro-inflammatory cytokines, decreased expression of tight junction, and bacterial clearance of intestinal epithelium.(7,8)

Vitamin D refers to a group of fat-soluble secosteroids, and the primary compounds are vitamin D3 (cholecalciferol) and vitamin D2 (ergocalciferol). The biggest abundant source of vitamin D in our body is cholecalciferol synthesis in the skin through direct sun exposure. Ultraviolet-B (UVB) from the sun is absorbed by the skin that activates the synthesis of vitamin D.(9) The active form of vitamin D, vitamin D3 or calcitriol is known to take part in regulating various inflammatory cytokines so that an equilibrium can be achieved between pro-inflammatory cytokines (TNF-α, IL-6) and anti-inflammatory cytokines.(10-13) Then, with the controlled expression of various inflammatory cytokines, hepcidin formation can be inhibited to prevent the occurrence of anemia.(14)

### Methods

The mice used in this study was obtained from Pusvetma, Surabaya, Indonesia. Treatment of animal models, immunohistochemistry (IHC) staining for TNF-α and IL-6, hematoxylin-eosin (H&E) staining for measurement of epithelial damage, and measurement of hemoglobin level were all conducted at Faculty of Medicine, Universitas Brawijaya. Ethical clearance was obtained from the Ethics Committee of Faculty of Medicine, Brawijaya University, with number: 434/EC/KEPK/12/2017.

**Treatment of Mice**

This study used twenty-four 8 weeks old male Balb/c mice weighing 17-25 grams. The mice were randomly divided into 4 groups after 1 week of acclimatization. Distilled water was given to the sham group for 14 days. Colitis group was given 3% of dextran sodium sulfate (DSS) (Cat No. ab146569, Abcam, Cambridge, UK) for 7 days and followed by distilled water for 7 days. The first treatment group (UC+VD) was given 3% of DSS for 7 days and followed by vitamin D3 (Oscal, Calcitriol, Dankos Farma, Jakarta, Indonesia) at a dose of 0.2 ug/25 g/day via intrastrac for 7 days. The second treatment group (VD+UC) was given vitamin D3 at a dose of 0.2 ug/25 g/day via intrastrac for 7 days and followed by 3% of DSS for 7 days. After DSS induction and vitamin D3 administration in the treatment group were completed, all mice were dissected to collect blood samples and colonic tissue. The colitis condition was confirmed by the epithelial damage in histopathological colon after dissection. The distal colon was taken to make paraffin block and sliced using a microtome. Last, H&E and IHC staining were observed using the blind method.

**Histopathological Analysis**

A 5-mm distal colonic portion was formed in paraffin and H&E stain. The Mouse Colitis Histology Index (MCHI) score was used to measure colonic epithelial damage. MCHI = (1 x Goblet cells disappear) + (2 x crypt density) + (2 x crypt hyperplasia) + (3 x submucosal infiltration).(15)

**Hemoglobin Analysis**

Blood samples were taken and were diluted in the WBC chamber then measured by Spectrophotometry, through optical pathways in the white blood cell (WBC) room. Then the reading was carried out at $\lambda$ 532 nm with a spectrophotometer using the Hematology Analyzer ABX Micro 60 (Horiba ABX SAS, Irvine, California, USA), which can be used to analyze hemoglobin levels in mice. Hemoglobin levels were expressed in grams per deciliter (g/dL).

**IHC Staining of TNF Alpha and IL-6 Expression**

Colon parts were fixed in neutral buffer formalin and embedded in paraffin. Then, they were deparaffinized, as well as sequentially rehydrated using xylol, absolute ethanol, 90% ethanol, 80% ethanol, 70% ethanol, sterile distilled aqua, 3% H$_2$O$_2$, and blocking buffer. The parts were examined with Rabbit polyclonal TNF-α (Cat No. bs-2081R, Bioss, Woburn, USA) and IL-6 (Cat No. bs-0782R, Bioss, Woburn, USA), then incubated overnight at 4°C. After incubation, they were thoroughly washed with phosphate buffer saline (PBS) for 5 minutes and repeated three times. Subsequently, the colon parts were incubated with secondary antibody at room temperature for 60 minutes, then with horseradish peroxidase at room temperature for 40 minutes. Reaction products were visualized by diaminobenzidine (Nichirei Bioscience Inc, Tokyo, Japan) and hematoxylin. Then, cells expressing TNF-α and IL-6 in the form of
cells with a flat, brownish core were observed under the microscope in 20 fields of view with 400x magnification. Last, the mean scores were collected.

**Statistical Analysis**

Data were expressed as mean±standard error of the mean (SEM). Comparisons between the 4 groups (Sham, UC, UC+VD, and VD+UC) were analyzed using analysis of variance (ANOVA) test. A comparison between two different groups was carried out using the Post Hoc LSD test. Kruskal-Wallis and Post Hoc Mann-Whitney tests were used for abnormal and non-homogeneous data. The \( p \)-values<0.05 were considered statistically significant. All statistical analyses were conducted using IBM SPSS software ver. 23.0 (IBM Co., Armonk, NY, USA).

### Results

**Vitamin D3 and Reduction of Epithelial Damage**

The mice model of colitis was induced by 3% of DSS through drinking water. Epithelial damage increased in the UC group compared to other groups (Figure 1). In Figure 1, two observers found significant epithelial damage in colon samples of UC group, marked by the loss of goblet cells, decreased cryptic density, hyperplasia, and leukocytes infiltration into sub-mucosa in the UC colon. Comparisons of MCHI scores of the samples showed significant increases in UC (8.25±3.05) compared to sham (0.33±0.26), UC compared to UC+VD (2.33±1.07), and UC compared to VD+UC (2.83±0.75) \( (p<0.05) \). However, there was no significant difference found between Sham, UC+VD and VD+UC groups \( (p>0.05) \).

**Hemoglobin Level**

This research evaluated colitis-induced anemia to determine the potential of vitamin D3 in effectively increasing hemoglobin levels. The mean hemoglobin levels of mice after the treatment were measured (g/dL) (Table 1). The levels of hemoglobin in UC group (11.85±0.97) decreased compared to other groups. ANOVA test results showed significant differences between each treatment in the parameter measured at \( \alpha=0.05 \). Table 2 showed that UC+VD group (13.68±0.68) and VD+UC group (13.52±1.07) had a significant increase in hemoglobin effect at a 95% confidence level compared to UC group. There was no significant difference found between Sham, UC+VD and VD+UC groups \( (p>0.05) \).

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**Figure 1. Light microscopy observation of the colon tissue samples of mice following haematoxylin and eosin staining.** Black arrow: goblet cell; Blue arrow: decreased crypte density; Red arrow: hyperplasia crypte; Yellow arrow: leukocytes infiltration. Black bar: 100µm.
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| Groups            | Post Hoc LSD | p-value# |
|-------------------|--------------|----------|
|                     | Sham | UC | UC+VD | VD+UC |               |
| Sham (14.25±0.47)  | -    | 0.000* | 0.252 | 0.143  | 0.000         |
| UC (11.85±0.97)   | 0.000* | -   | 0.001* | 0.002* |               |
| UC+VD (13.68±0.68) | 0.252 | 0.001* | -     | 0.732  |               |
| VD+UC (13.52±1.07) | 0.143 | 0.002* | 0.732 | -      |               |

*Significancy was tested with ANOVA (p<0.05).

**Table 1. Multiple comparisons of hemoglobin levels.**

### Vitamin D3 and TNF-α Expression

TNF-α (cell/field of view) expression of colonic samples after IHC staining (Figure 2) in Sham (27.33±3.42), UC+VD (36.33±1.86), and VD+UC (38.68±3.68) groups showed a significant smaller number of TNF-α cells compared to UC (44.66±4.87) (p<0.05). Meanwhile, there were no significant differences between the UC+VD and VD+UC groups (p> 0.05) (Table 2).

### Vitamin D3 and IL-6 Expression

As shown in Figure 3, the number of cells expressing IL-6 (cells/field of view) decreased significantly in Sham (18.01±2.96), UC+VD (24.78±0.79), and VD+UC (25.09±2.79) groups compared to UC group (38.85±3.51) (p<0.05). Meanwhile, there was no significant difference between the UC+VD group and VD+UC group (p>0.05) (Table 3).

**Figure 2. Results of immunohistochemistry staining to observe numbers of cells that express TNF-α.** Yellow arrow: TNF-α+ cells (cells with a flat, brownish core). Black bar: 20µm.

**Figure 3. Results of immunohistochemistry staining to observe numbers of cells that express IL-6.** Yellow arrow: IL-6+ cells (cells with a flat, brownish core). Black bar: 20µm.

| Groups            | Mann Whitney | p-value# |
|-------------------|--------------|----------|
|                     | Sham | UC | UC+VD | VD+UC |               |
| Sham (27.33±3.42)  | -    | 0.002* | 0.002* | 0.002* | 0.000         |
| UC (44.66±4.87)   | 0.002* | -   | 0.002* | 0.041* |               |
| UC+VD (36.33±1.86) | 0.002* | 0.002* | -     | 0.589  |               |
| VD+UC (38.68±3.68) | 0.002* | 0.002* | 0.589 | -      |               |

*Significancy was tested with Kruskal Wallis (p<0.05).
Discussion

At present, various treatments do not meet the full remission of the ulcerative condition and cause complications like anemia. Anemia is frequently found in patients at the time of diagnosis and correlates with the severity of the disease. Anemia in colitis can occur as a consequence of impaired absorption of iron, continuous blood loss in the acute phase, or even both. As shown in this study, we found significant differences in hemoglobin levels among colitis mice compared to the Sham group. Hemoglobin levels increased with vitamin D3 administration, which suggested that vitamin D3 played a role in preventing or improving anemia as a complication of colitis.

Vitamin D3 is known as an immunomodulation agent in homeostasis and inflammation disease. Vitamin D3 reduced the expression of TNF-α and IL-6 colon in this study. As previously known, vitamin D3 can decrease the activation of NF-κB in a vitamin D receptor (VDR)-dependent manner. Vitamin D also blocks the TNF-α-induced NF-κB activation. The deletion of VDR also ameliorates the regulation of inflammation by reducing IκBα production. IκBα inhibits the NF-κB activity by preventing its translocation into the nucleus. Then, the levels of IL-6 may affect hemoglobin levels by increasing hepcidin production. Increased hepcidin production is inversely correlated with circulating iron levels. It binds ferroportin, the iron exporter, to prevent the release of iron from enterocyte into circulation. This can be one of the underlying mechanisms of anemia in colitis because IL-6 production increases significantly and may lead to iron deficiency.

Previous studies have shown that vitamin D supplementation in clinical trials of UC patients significantly improved patients' quality of life, based on Inflammatory Bowel Disease Questionnaire-9 (IBDQ-9) and Simple Clinical Colitis Activity Index Questionnaire (SSCAIQ) questionnaires. In this study, vitamin D3 administration improved the mucosal improvement, which was evaluated by MCHI score. This scoring system is a newly developed

Table 3. Multiple comparisons of IL-6 expression.

| Groups                  | Sham | UC  | UC+VD | VD+UC | p-value* |
|-------------------------|------|-----|-------|-------|----------|
| Sham (18.05±2.96)       | -    | 0.002* | 0.002* | 0.009* | 0.000    |
| UC (38.85±3.51)         | 0.002* | -   | 0.002* | 0.002* |          |
| UC+VD (24.78 ±0.79)     | 0.002* | 0.002* | - | 0.937  |          |
| VD+UC (25.09±2.79)      | 0.009* | 0.002* | 0.937 | -      |          |

*p-value was tested with Kruskal Wallis (p<0.05).
tool to evaluate mucosal damage and is claimed to be as reliable as endoscopic evaluation in pre-clinical studies. The finding of increased mucosal improvement in this study indicated the role of vitamin D3 in preventing or treating anemia in colitis, as it reduced the possibility of continuous blood loss. Blood loss itself was included in the calculation of Mayo score to determine the severity of the disease with other endoscopic and clinical scales used. 

This study would reveal that there are other benefits of vitamin D3 administration to anemia, as one of the most frequent complications of ulcerative colitis. However, further research is required to understand the mechanism of vitamin D3 in the regulation of hepcidin production to increase hemoglobin levels. Further evaluation is needed to determine vitamin D3 levels in circulation, its toxicity, and other aspects to determine the most beneficial pathway as a therapeutic or preventive target in the development and severity of ulcerative colitis.

### Conclusion

Before and after the colitis induction, vitamin D3 administration significantly reduced the expression of pro-inflammatory cytokines, especially TNF-α and IL-6. This treatment also improved the mucosal repair process, as showed in the histology studies and observed with the MCHI score. Moreover, vitamin D3 administration in this study improved anemia condition in UC, which was proved by significantly higher hemoglobin levels in the treatment groups. However, all these benefits of vitamin D3 still require further research to determine its therapeutic window and toxicity.

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