The Effect of Neem (Azadirachta indica) Leaf Extracts on Interleukin-10 Expression and Histological Score in Dextran Sodium Sulfate-induced Colitis Mice

Riska Habriel Ruslie1*, Darmadi Darmadi2, Gontar Alamsyah Siregar2

1Department of Child Health, Faculty of Medicine, Universitas Prima Indonesia, Medan, North Sumatera, Indonesia; 2Department of Internal Medicine, Faculty of Medicine, Universitas Sumatera Utara, Haji Adam Malik General Hospital, Medan, North Sumatera, Indonesia

Abstract

BACKGROUND: Inflammatory bowel disease (IBD) is a chronic or relapsing inflammation of the intestine which consists of Crohn’s disease and ulcerative colitis. Interleukin (IL)-10 is an anti-inflammatory cytokine which plays important role in the pathogenesis of IBD. Neem (Azadirachta indica) is rich of azadiractoids which possess anti-inflammatory activity.

AIM: The objective of the study was to determine the effect of neem leaf extract on IL-10 expression and histological score in dextran sodium sulfate (DSS)-induced colitis rats.

METHODS: The first phase compared 7 DSS-induced colitis rats (case group) with seven healthy rats (control group). All of them were sacrificed. The second phase compared 28 DSS-induced colitis rats treated with mesalazine (Group I) and 56 rats treated with neem leaf extract (Groups II and III). Seven rats from each group were sacrificed on days 7, 14, 21, and 28. Colon samples were harvested and underwent histopathological examination and immunohistochemical analysis to determine IL-10 expression and histological score.

RESULTS: IL-10 expression and histological score were higher in case group compared to control group. There was similar IL-10 expression between Groups I and II on day 28 while the similarity was observed since day 7 between Groups I and III. Mean histological score was higher in Group II compared to Group I and it was similar between Group I and Group III on days 21 and 28.

CONCLUSION: Neem leaf extract increased the expression of anti-inflammatory cytokine, in particular IL-10, and improved histological score in DSS-induced colitis rats.

Introduction

Inflammatory bowel disease (IBD) is a chronic or relapsing inflammation of the intestine which consists of Crohn’s disease and ulcerative colitis [1, 2]. The etiology of IBD is still uncertain. It is proposed that IBD is the result of excessive immune response toward gastrointestinal microorganisms marked by increased effector T cell activity and/or decreased regulator T cell activity, changed gastrointestinal microorganisms composition, and/or damaged epithelial barrier [3]. Interleukin (IL)-10 is an anti-inflammatory cytokine which plays important role in the pathogenesis of IBD, together with IL-6 and tumor necrosis factor (TNF)-α. IL-10 works in contradictory fashion with IL-6 and TNF-α by decreasing pro-inflammatory cytokines [4]. Human B cell expresses and produces IL-10 after appropriate stimulation such as infection, especially in atopic children [5]. The previous study reported that IL-10 possesses anti-proliferation effect of T helper cells on human intestinal lamina propria [6]. In the other hand, antigen presenting cells produced IL-10 to regulate homeostatic T cell response toward commensal bacteria [7]. The expression of T-cell-derived IL-10 has protective role from colitis in human by controlling inflammation within colonic mucosa [8].

At present, mesalazine is the drug of choice for IBD and is utilized to maintain remission status of patients with ulcerative colitis. Alternative treatments are corticosteroids, immunomodulatory drugs, biological agents, small molecular therapies, and immunosuppressants [2, 9]. Neem (Azadirachta indica) is a plant from Meliaceae family. It is native to India, Myanmar, Bangladesh, Sri Lanka, Malaysia, and Pakistan. It grows in tropical and subtropical regions around the globe. It is rich of azadiractoids, a highly active liminoid terpenoids, which possesses anti-inflammatory and anticancer activity. The extract of neem leaf has protective role from colitis in human by controlling infection within colonic mucosa [8].
trinitrobenzenesulfonic acid-induced colitis rats showed improvement in the disease course. Neem leaf extract was shown to have antibacterial, antioxidant, anti-inflammatory, and immunomodulatory activities [13].

Due to its anti-inflammatory activity, neem is hoped to be an alternative or adjuvant therapy for patients with colitis. This study aimed to determine the effect of neem (A. indica) leaf extract on IL-10 expression and histological score in dextran sodium sulfate (DSS)-induced colitis rats.

**Methods**

**Animals and study design**

This study was conducted in Biological Laboratory and Anatomical Pathology Laboratory of Universitas Sumatera Utara, Medan, Indonesia, between June and September 2019. This study was divided into two phases. The first phase involved 14 male healthy Wistar rats (*Rattus norvegicus*), aged 6–8 weeks old and weighing 30 g, kept at 20–25°C with controlled 12 h light/dark cycle. Laboratory-standardized cages were used to keep the animals with *ad libitum* access to food and water. A half of them (case group) were induced by DSS for five cycles (70 days) to create colitis while the rest (control group) were left without induction. All of them were sacrificed and colon samples were harvested from each mouse. Colon samples were then fixated in phosphate-buffered saline 10% formalin for histopathological examination and immunohistochemical analysis of IL-10.

The second phase was enrolling 84 rats. All of them were divided into three groups. The first group received 7.8 mg of mesalazine daily (Group I), the second received 100 mg/200 g body weight of neem leaf extract twice daily (Group II), and the third received 200 mg/200 g body weight of neem leaf extract twice daily (Group III) [14]. Seven rats from each group were sacrificed on days 7, 14, 21, and 28. Colons samples were harvested from each mouse and utilized for histopathological examination and immunohistochemical analysis as described above. All procedures were conducted according to Helsinki Declaration. This study had been approved by The Institutional Ethics Committee of the Universitas Sumatera Utara, Medan, Indonesia.

**Chemical induction of colitis**

Colitis was induced by administrining 5% of DSS (MP Biomedicals LLC) in five cycles. Each cycle was conducted for 7 days and followed by distilled water administration for 7 days. After five cycles with a total of 70 days, the rats were sacrificed or treated with either mesalazine or neem leaf extract.

**Histopathological examination**

Colon samples were stained using hematoxylin and eosin (H&E) after being paraffin-embedded and cut. H&E-stained colonic sections were coded for blind microscopic assessment of inflammation (i.e., DSS-induced colitis). Histological scoring was based on three parameters. Severity of inflammation was scored as follows: 0 – rare inflammatory cells in the lamina propria; 1 – increased numbers of granulocytes in the lamina propria; 2 – confluence of inflammatory cells extending into the submucosa; and 3 – transmural extension of the inflammatory infiltrate. Crypt damage was scored as follows: 0 – intact crypts; 1 – loss of the basal one-third; 2 – loss of the basal two-thirds; 3 – entire crypt loss; 4 – change of epithelial surface with erosion; and 5 – confluent erosion. Ulceration was scored as follows: 0 – absence of ulcer; 1–1 or 2 foci of ulcerations; 2–3 or 4 foci of ulcerations; and 3 – confluent or extensive ulceration. Values were calculated to give a maximal histological score of 11 [15].

**Immunohistochemical analysis of IL-10**

The paraffin-embedded slides were deparaffinized, rehydrated, and heated on microwave with 0.01 M citrate buffer (pH 6.0) for 30 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min, and then washed with sulfate salt buffer. The specimens were incubated overnight in 4°C, then immune-stained with primary antibody (rabbit polyclonal IgG to bind the rats IL-10) (Wuhan Fine Biotech Co., Ltd., China) in concentration of 1 mg/mL diluted by 1:600. Primary antibody was detected by avidin-biotin peroxidase solution (ScyTek Laboratories, Inc., USA) and signal was visualized using diaminobenzidine (ScyTek Laboratories, Inc., USA). The slides then counterstained with hematoxylin and assessed by two-blinded experienced pathologists from the anatomical pathology Department of Universitas Sumatera Utara. The slides were categorized as 0 for 0–15%, 1 for 15–25%, 2 for 26–50%, and 3 for 51–100% stained cells for IL-10 (Figure 1). Scale 0 and

![Figure 1: Immunohistochemical analysis of interleukin (IL)-10 results. From upper left to lower left slides in clockwise order showed category 0 to 3 of IL-10 expression, sequentially](image-url)
was categorized as negative, while scale 2 and 3 as positive [16].

### Statistical analysis

Fisher’s exact test was used to determine the difference in IL-10 expression between case and control group in the first phase of study. We also conducted Mann–Whitney test to analyze the difference in histological score between both groups. Data from the second phase were analyzed with Fisher’s exact test for IL-10 expression and independent t-test for histological score between the three groups. Statistical analysis was conducted at 95% confidence interval where \( p < 0.05 \) was considered significant.

### Results

#### Phase 1

The positive expression of IL-10 based on immunohistochemical analysis was significantly higher in the case group compared to control group (\( p = 0.005 \)). All rats in the case group expressed IL-10 compared to one mouse in the control group. In addition, median histological score from histopathological examination was significantly higher in case group compared to control group (8 vs. 0, \( p = 0.001 \)) (Table 1).

#### Phase 2

Further analysis was conducted to determine the difference in IL-10 expression and histological score among the three groups with different interventions. For the expression of IL-10, there were significant differences between Group I and Group II on day 7 (\( p = 0.029 \)), 14 (\( p = 0.021 \)), and 21 (\( p = 0.021 \)). The difference was not observed on day 28. There was no difference of IL-10 expression between Group I and Group III. It can be inferred that anti-inflammatory expression is similar in colitis rats after being treated with both mesalazine and 200 mg/200 g body weight of neem leaf extract (Table 2).

We observed significant differences in mean histological score between Group I and Group II on day 7 until day 28. There were significant differences between Group I and Group III on day 7 (\( p = 0.009 \)) and day 14 (\( p = 0.024 \)). No difference was observed on days 21 and 28. In the case group, there was a significant difference in mean histological score between Group I and Group II on day 7 (\( p = 0.029 \)). The difference was not observed on day 28. There was a difference in IL-10 expression between case and control groups (8 vs. 0, \( p = 0.001 \)) (Table 1).

### Discussion

Ulcereative colitis is an idiopathic, chronic, and incurable inflammatory disease affecting colon. Its incidence is high in Western countries. In the past two decades, its incidence is rising in Asia and Middle East with a rate of 0.15–6.5 per 100,000 population. The peak incidence of ulcerative colitis occurs at the age of 20–39 years and 70–79 years. Ulcerative colitis is more common in males compared to females [2]. Colitis raises from several underlying conditions. Abnormal bacterial composition and reduced biodiversity defect in local defense mechanism, and dysregulated immune response plays an important role in the pathogenesis of colitis. Dysregulated immune system here means an imbalance between pro- and anti-inflammatory substances [17], [18], [19]. The damage of intestinal epithelial barrier, as the local defense mechanism, allows intestinal microorganisms to reach the deeper part of intestine and trigger inflammation which is responsible for colitis. IL-10 as an anti-inflammatory cytokine works to maintain gastrointestinal homeostasis by decreasing the production of pro-inflammatory cytokines such as IL-6, IL-12, and TNF-\( \alpha \) [2], [20]. Decreased or absent of IL-10 increased the risk of colitis in the previous study [18]. Imbalance between anti- and pro-inflammatory cytokines leads to more severe inflammation and colitis. Chronic colitis will increase the risk of developing colorectal carcinoma [2], [20]. In the first phase of our study, all rats in the case group expressed IL-10 and had higher median histological score compared to control group. This was in accordance with colitis features as stated in the previous literatures.

#### Table 1: The difference of IL-10 expression and histological score between case and control groups

| Variables | Case group | Control group | \( p \) |
|-----------|------------|---------------|------|
| IL-10 expression, n (%) | | | |
| 0 and 1   | 0 (0.0)    | 6 (85.7)      | 0.005* |
| 2 and 3   | 7 (100.0)  | 1 (14.3)      |      |
| Median historical score, (min-max) | 8 (6 – 9) | 0 (0 – 1) | 0.001* |

*\( p < 0.05 \).

#### Table 2: The difference of IL-10 expression among Groups I, II, and III

| Day | Group I | Group II | \( p \) Group I versus II | Group III | \( p \) Group I versus III |
|-----|---------|---------|--------------------------|----------|--------------------------|
| 7   | 6 (85.7) | 1 (14.3) | 6 (85.7) | 0.005* | 2 (28.6) | 1 (14.3) | 0.103 |
| 14  | 7 (100)  | 0 (0)   | 2 (28.6) | 0.021* | 5 (71.4) | 2 (28.6) | 0.462 |
| 21  | 7 (100)  | 0 (0)   | 2 (28.6) | 0.021* | 6 (85.7) | 1 (14.3) | 1.000 |
| 28  | 7 (100)  | 0 (0)   | 3 (42.9) | 0.070  | 6 (85.7) | 1 (14.3) | 1.000 |

*\( p < 0.05 \).

### Chronic and recurrent nature of ulcerative colitis burdens the health-care resources for treatment. Mesalazine is the standard therapy for inducing and maintaining remission in colitis by modulating the

#### Table 3: The difference of histological score among Groups I, II, and III

| Day | Group I Mean (SD) | Group II Mean (SD) | \( p \) Group I versus II | Group III Mean (SD) | \( p \) Group I versus III |
|-----|------------------|--------------------|--------------------------|---------------------|--------------------------|
| 7   | 3.1 (1.46)       | 7.66 ± 1.35        | <0.001*                  | 5.29 ± 0.11         | 0.009*                  |
| 14  | 2.1 (0.9)        | 5.14 ± 1.34        | <0.001*                  | 3.29 ± 0.77         | 0.024*                  |
| 21  | 1.71 ± 0.49      | 3.71 ± 1.11        | 0.002*                   | 2.43 ± 0.57         | 0.109                   |
| 28  | 1.29 ± 0.28      | 2.86 ± 0.69        | <0.001*                  | 1.71 ± 0.76         | 0.232                   |

*\( p < 0.05 \). SD: Standard deviation.
inflammatory cascade. However, alternative or adjuvant therapy is needed to achieve better outcome [2]. Anti-inflammatory property of neem tree is a promising alternative or adjuvant therapy for colitis [12].

Morris et al. in their study reported that neem extract had significant anticancer effect in oral squamous cell carcinoma. This effect was suggested from the role of neem extract in reducing pro-inflammatory cytokines including IL-6 and TNF-α [10]. Patel et al. also found that neem extract had anti-inflammatory property. Their study regarding neem extract for rats with colon cancer treatment showed that cyclooxygenase-1, IL-6, and TNF-α were significantly decreased after the treatment [12]. Neem leaf extract was also found to increase the activity of natural killer cells. These cells are potent cytotoxic cells particularly against virus-infected cells, intracellular parasites, and tumor cells by increasing production of IL-12 [21]. This study showed an increase of anti-inflammatory cytokines (IL-10) in neem leaf extract colitis rats comparable with mesalazine as gold standard treatment. Further study is needed to assess the inflammatory cytokines profile in neem leaf extract colitis rats. This study showed that IL-10 expression in Group I and II was different in the first 3 weeks. The number of rats which expressed IL-10 in Group II was lower compared to Group I. In the other hand, IL-10 expression was different between Group I and III only in the 4th week. From the second until the 4th week of administration, the expression of IL-10 between both groups was comparable.

Histopathological feature was also improved by administration of neem leaf extract. Gautam et al. examined colon specimen of trinitrobenzenesulfonic acid-induced colitis rats after administration of 50% neem leave extract. The result showed improved histopathological finding comparable to sulfasalazine as control [13]. This is concordance to the result of this study that shows no significant differences between Group I and Group III since the 4th week. No significant differences found between Group I and Group II only in 4th week of administration. This confirms that mesalazine is still the primary choice of treatment in patients with colitis [22].

As the conclusion, neem leaf extract increased the expression of anti-inflammatory cytokine, particularly IL-10, and improved histological score in DSS-induced colitis rats. However, these effects were achieved with a higher dose of neem leaf extract (200 mg/200 g body weight). Studies regarding the combination of mesalazine and neem leaf extract for colitis treatment are mandatory to determine the role of neem leaf extract as adjuvant therapy.

References

1. Matsuoka K, Kobayashi Y, Ueno F, Matsui T, Hirai F, Inoue, et al. Evidence-based clinical practice guidelines for inflammatory bowel disease. J Gastroenterol. 2018;53(3):305-53
2. Sharara AI, Awadhi SA, Alharbi O, Dhabab HA, Mounir M, Salese L, et al. Epidemiology, disease burden, and treatment challenges of ulcerative colitis in Africa and the Middle East. Expert Rev Gastroenterol Hepatol. 2018;12(9):883-97. https://doi.org/10.1080/17474124.2018.1503502
3. Sun M, He C, Cong Y, Liu Z. Regulatory immune cells in regulation of intestinal inflammatory response to microbiota. Mucosal Immunol. 2015;8(5):969-78. https://doi.org/10.1038/mi.2015.49
4. Muzzes G, Molnar B, Tulassay Z, Sipos F. Changes of the cytokine profile in inflammatory bowel diseases. World J Gastroenterol. 2012;18(41):5848-61. https://doi.org/10.3748/wjg.v18.i41.5848
5. Valsecchi C, Tagliacarne SC, Brambilla I, Kkery C, Benazzo M, Montagna L, et al. Detection of IL10-producing B cell (B10) in adenoids of atopic children with adenoidal hypertrophy. Ital J Pediatr. 2018;44(1):30. https://doi.org/10.1186/s13052-018-0471-3
6. Barman S, Kayama H, Okuzaki D, Ogino T, Osawa H, Matsuno H, et al. Identification of a human intestinal myeloid cell subset that regulates gut homeostasis. Int Immunol. 2016;28(11):533-45. https://doi.org/10.1093/intimm/dxw034
7. Liu B. Tonkonogy SL, Sartor RB. Antigen-presenting cell production of IL-10 inhibits T-helper 1 and 17 cell responses and suppresses colitis in mice. Gastroenterology. 2011;141(2):653-62. https://doi.org/10.1053/j.gastro.2011.04.053
8. Barman S, Kayama H, Okuzaki D, Ogino T, Osawa H, Matsuno H, et al. Identification of a human intestinal myeloid cell subset that regulates gut homeostasis. Int Immunol. 2016;28(11):533-45. https://doi.org/10.1093/intimm/dxw034
9. Nielsen OH, Seidelin JB, Munck LK, Rogler G. Use of biological molecules in the treatment of inflammatory bowel disease. J Intern Med. 2011;270(1):15-28. https://doi.org/10.1111/j.1365-2796.2010.02285.x
10. Morris J, Gonzales CB, De La Chapal JJ, Cabang AB, Fountzillas C, Patel M, et al. The highly pure neem leaf extract, SCNE, inhibits tumorigenesis in oral squamous cell carcinoma via disruption of pro-tumor inflammatory cytokines and cell signaling. Front Oncol. 2019;9:980. https://doi.org/10.3389/fonc.2019.00890
11. Hao F, Kumar S, Yadav N, Chandra D. Neem components as potential agents for cancer prevention and treatment. Biochim Biophys Acta. 2014;1846(1):247-57. https://doi.org/10.1016/j.bbamcr.2014.05.012
12. Patel MJ, Tripathy S, Mukhopadhyay KD, Wangjam T, Cabang AB, Morris J, et al. A supercritical CO2 extract of neem leaf (A. indica) and its bioactive limonoid, nimbidol, suppresses colon cancer in preclinical models by modulating pro-inflammatory pathways. Mol Carcinog. 2018;57(9):1156-65. https://doi.org/10.1002/mc.22832
13. Gautam MK, Goel S, Ghatule RR, Singh A, Joshi VK, Goel RK. Azadirachta indica attenuates colonic mucosal damage in experimental colitis induced by trinitrobenzene sulfonic acid. Indian J Pharm Sci. 2013;75(5):602-6. https://doi.org/10.4103/0973-1296.127366
14. Biswas K, Ishita C, Ranajit K, Banerjee UB. Biological activities and medicinal properties of neem (Azadirachta indica A. Juss). Curr Sci. 2002;82(11):1136-45.

15. Laroui H, Ingersoll SA, Liu HC, Baker MT, Ayyadurai S, Charania MA, et al. Dextran sodium sulfate (DSS) induces colitis in mice by forming nano-lipocomplexes with medium-chain-length fatty acids in the colon. PLoS One. 2012;7(3):e32084. https://doi.org/10.1371/journal.pone.0032084
PMid:22427817

16. Lubis M, Siregar GA, Bangun H, Ilyas S. The effect of roselle flower petals extract (Hibiscus sabdariffa Linn.) on reducing inflammation in dextran sodium sulfate-induced colitis. Med Glas (Zenica). 2020;17(2):395-401. https://doi.org/10.17392/1095-20 PMid:3239325

17. Fries W, Salvatore C. Ulcerative colitis: Pathogenesis. Curr Drug Targets. 2011;12(10):1373-82. PMid:21466489

18. Kennedy RJ, Hoper M, Deodhar K, Erwin PJ, Kirk SJ, Gardiner KR. Interleukin 10-deficient colitis: New similarities to human inflammatory bowel disease. Br J Surg. 2000;87(10):1346-51. https://doi.org/10.1046/j.1365-2168.2000.01615.x PMid:11044159

19. Keubler LM, Buettnner M, Hager C, Bleich A. A multihit model: Colitis lessons from the interleukin-10-deficient mouse. Inflamm Bowel Dis. 2015;21(8):1967-75. https://doi.org/10.1097/mib.0000000000000468
PMid:26164667

20. Li B, Alli R, Vogel P, Geiger TL. IL-10 modulates DSS-induced colitis through a macrophage-ROS-NO axis. Mucosal Immunol. 2014;7(4):869-78. https://doi.org/10.1038/mi.2013.103
PMid:24301657

21. Bose A, Baral R. Natural killer cell mediated cytotoxicity of tumor cells initiated by neem leaf preparation is associated with CD40-CD40L-mediated endogenous production of interleukin-12. Hum Immunol. 2007;68(10):823-31. https://doi.org/10.1016/j.humimm.2007.08.002
PMid:17961770

22. Yuan B, Zhou S, Lu Y, Liu J, Jin X, Wan H, et al. Changes in expression and distribution of claudins, increased epithelial apoptosis, and a mannose-binding lectin-associated immune response lead to barrier dysfunction in dextran sodium sulfate-induced rat colitis. Gut Liver. 2015;9(6):743-40. https://doi.org/10.5009/gnl14155
PMid:25717051