



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

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Abstract

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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 possesses 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 its seed, leave, flower, and fruit has long been used as cancer treatment for centuries [10], [11], [12]. A study by Gautam *et al.* using neem leaf extract in

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, was 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. Colon sample was 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 administering 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. Endogen 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

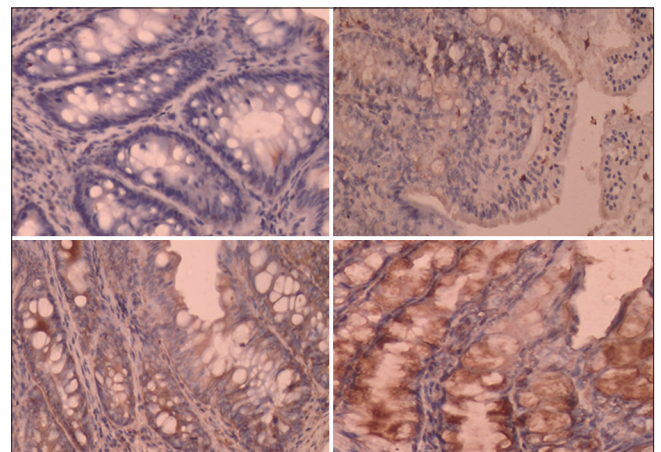


Figure 1: Immunohistochemical analysis of interleukin (IL)-10 results. From upper left to lower left slides in clockwise order showed category 0 to 3 of IL-10 expression, sequentially

1 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

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 histological score, (min-max)	8 (6–9)	0 (0–1)	0.001*

* $p < 0.05$.

20 between those groups. This means that histological score in colitis rats is similar after receiving mesalazine and 200 mg/200 g body weight for 3 weeks (Table 3).

Discussion

Ulcerative 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- α [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 2: The difference of IL-10 expression among Groups I, II, and III

Day	Group I n (%)		Group II n (%)		p Group I versus II	Group III n (%)		p Group I versus III
	2 and 3	0 and 1	2 and 3	0 and 1		2 and 3	0 and 1	
7	6 (85.7)	1 (14.3)	1 (14.3)	6 (85.7)	0.029*	2 (28.6)	5 (71.4)	0.103
14	7 (100)	0 (0)	2 (28.6)	5 (71.4)	0.021*	5 (71.4)	2 (28.6)	0.462
21	7 (100)	0 (0)	2 (28.6)	5 (71.4)	0.021*	6 (85.7)	1 (14.3)	1.000
28	7 (100)	0 (0)	3 (42.9)	4 (57.1)	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)	7.86 \pm 1.35	<0.001*	5.29 \pm 0.11	0.009*
14	2.1 (0.9)	5.14 \pm 1.34	<0.001*	3.29 \pm 0.77	0.024*
21	1.71 \pm 0.49	3.71 \pm 1.11	0.002*	2.43 \pm 0.57	0.109
28	1.29 \pm 0.28	2.86 \pm 0.69	<0.001*	1.71 \pm 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 1st 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 leaf extract. The result showed improvement 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 1st 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.

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