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Leucovorin effect on 2,4,6-trinitrobenzenesulfonic acid (TNBS) colitis in rats

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This study examined whether leucovorin, used in colon cancer treatment, promotes healing of colonic tissue in a model of inflammatory bowel disease. Ten-week-old male Wistar rats were divided into five groups: Group 1 (control) received no medication; Group 2 received leucovorin 10 mg/kg. Colitis was induced in Groups 3–5 with 25mg 2,4,6-trinitrobenzene sulfonic acid (TNBS) in 37% ethanol; Groups4–5 received intraperitoneal leucovorin 3 or 10 mg/kg for 3 days. Macroscopic scoring and histopathological evaluation were performed. The colon weight-to-length ratio increased from 92.30 ± 3.29 (control) to 169.33 ± 12.69 in the TNBS group (P<0.001). This ratio improved to 140.14 ± 9.16 with 10 mg/kg leucovorin (P<0.01), but not with 3 mg/kg (166.94 ± 11.98, P<0.001). Macroscopic score fell from 2.88 ± 0.35 (TNBS) to 1.88 ± 0.13 with 10 mg/kg LV (P<0.05). Histopathologic evaluation showed a dose-dependent reduction in total score: 15.5 (TNBS), 13.0 (LV3, P>0.05) and 11.38 (LV10, P<0.05); significant decreases were seen in cellular infiltration and muscle thickening (P<0.05). Leucovorin promotes dose-dependent healing in a colitis model and may serve as an adjuvant therapy. This study supports further investigation of higher doses and longer regimens to determine maximal efficacy.

Keywords: Folic acid, Drug repurposing, Gut inflammation, Rational drug therapy, Vitamin B9

Introduction

Inflammatory bowel disease (IBD) is a group of chronic conditions causing long-term inflammation in the intestines. The two main forms of IBD are Crohn's disease (CD) and ulcerative colitis (UC)[1]. IBD can develop after surgery or medication use. It is also a common complication of many rheumatic diseases or cancer treatments. Dysfunction of gastrointestinal smooth muscle contributes to symptoms; diarrhea, abdominal pain, and often blood loss seen in the clinic[1,2].

The 2,4,6-trinitrobenzene sulfonic acid (TNBS) colitis model has been established as an animal model for understanding the mechanisms of pathogenesis and developing new treatment principles for inflammatory bowel diseases in humans[1,3]. It has been stated that in this model ileal functions were also deteriorated due to inflammatory mediators[2].

Folic acid (vitamin B9) is one of the vitamins that cannot be synthesized by mammalian cells and must be obtained from external sources[4]. It is a key component in the treatment of megaloblastic anemia, plays a vital role in preventing fetal neural tube defects during pregnancy, and is also utilized to protect neurons in alcohol coma. Its active metabolite, leucovorin (LV; folinic acid), finds clinical application in mitigating the adverse effects of methotrexate, phenytoin, aminopyrine, pyrimethamine, and trimethoprim[4-6]. Leucovorin is a component of the standard first-line treatments, FOLFOX and FOLFIRI regimens, to protect healthy cells during colon cancer chemotherapy[7]. It has been stated that folic acid deficiency is linked to colon, breast and prostate cancer[4]. Interestingly, a single dose of 250 mg/kg folic acid is used to induce animal model of acute kidney failure[8].

This research could help to determine benefits associated with the use of folinic acid in the TNBS colitis model in rats.

Materials and Methods

Animals

This research was approved by the Aydin Adnan Menderes University Animal Experiments Committee (HADYEK 64583101/2024/49). Ten-week-old male Wistar rats (200–220 g) were obtained from the animal laboratory of Aydin Adnan Menderes University.

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The induction of colitis was performed under the ketamine (50 mg/kg) and xylazine (5 mg/kg) anesthesia by 2,4,6-trinitrobenzenesulfonic acid (TNBS, Sigma-Aldrich, Interlab, Turkey) as described in previous studies. TNBS was dissolved in 37% ethanol to a concentration of 25 mg/kg and administered intrarectally 8 cm proximal to the anus using a catheter[3,9]. Animals were maintained in a head-down position for several minutes to prevent any leakage of solution.

The doses of 3 mg/kg and 10 mg/kg LV (Folinato de calcio Teva®, Pharmachemie BVSwensweg, Netherlands) were selected based on a logarithmic scale to ensure a clear characterization of the dose-response relationship, as 3-fold increments are more effective in capturing significant shifts in pharmacological activity than linear increments. The LV dose range was determined in light of previous research demonstrating the efficacy of similar ranges (6–8 mg/kg) in rodent models of methotrexate-induced neurotoxicity and chemotherapy-related studies[10-12]. By employing these doses, we aimed to align our experimental design with validated literature while investigating potential dose-dependent responses.

Animals were randomly assigned to 5 groups, with the following treatments:

Control group

Serum physiologic was applied intrarectally, and subsequently administered intraperitoneally for 3 days.

LV10 group

The highest dose of leucovorin (10 mg/kg) was given intraperitoneally for 3 days to demonstrate the safety of treatment agent on colonic mucosa.

TNBS group

TNBS was applied via the intrarectal route only on the first day of the study, serum physiologic was given intraperitoneally for 3 days.

TNBS+LV3 group

TNBS was applied only the first day of the study; leucovorin 3 mg/kg was administered intraperitoneally for 3 days.

TNBS+LV10 group

TNBS was applied only the first day of the study, leucovorin 10 mg/kg was administered intraperitoneally for 3 days.

The animals were sacrificed on the 3rd day after colitis induction, under ketamine (50mg/kg) and xylazine (5mg/kg) anesthesia.

Clinical follow-up

Rats were weighed on the first and the last day of the experiment to calculate the body weight change ratio. At the end of study, whole colonic segment was taken out under the ketamine (50 mg/kg) and xylazine (5 mg/kg) anesthesia, weight to length ratio of the colon was calculated[1,13].

Colon segments were opened along the mesenteric border and gently rinsed with serum physiologic. Macroscopic scoring of colonic mucosa: 0= Not any macroscopic changes; 1= Mucosal erythema only; 2= Mild mucosal oedema, slight bleeding or small erosions; 3= Moderate oedema, bleeding ulcers or erosions; 4= Severe ulceration, erosions, oedema, and tissue necrosis[1,3,9].

Histopathologic evaluation

The distal colon of the rats was fixed in 10% neutral buffered formalin and processed for routine histological analysis. Paraffin-embedded tissue samples were sectioned at 4μm thickness using a rotary microtome. These sections were then stained with hematoxylin and eosin (H & E) and examined under a light microscope (Olympus BX51) at x10 magnification. Loss of mucosal architecture, cellular infiltration, muscle thickening and goblet cell depletion were evaluated. We adapted microscopic scoring methods for our three-day experimental study; ulceration and necrosis were used instead of crypt abscesses (Table 1A)[9].

Statistical Analysis

Body weight was evaluated by percent changes among the groups. The distribution of data was evaluated using the Shapiro-Wilk test. Parametric data were analysed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. For non-parametric data, Mann–Whitney U test was used for evaluating differences between individual groups. Data are expressed as mean ± standard error of the mean (SEM). Values of $P<0.05$ was accepted as significant.

Results

Clinical follow-up

All the clinical findings have been shown in Table 2. Body weight changes of the control and healthy LV10 groups were not remarkable (0.06±0.02 % and 0.80±0.24 %, respectively). All rats in the three TNBS groups lost weight. The percent change in the

body weight of the rats in the TNBS group was -4.20±1.66 %, in the TNBS+LV3 group was -6.08±1.39 % (P<0.05), and in the TNBS+LV10 group was -7.38±1.76 % (P<0.05).

Colon weight increased from 1.79 ± 0.08 grams in the control group to 2.66 ± 0.19 grams in the TNBS group (P<0.01). The colonic weight of the LV10 group was 1.59 ± 0.07 grams. Treatment with 3 mg/kg LV was not effective (2.70 ± 0.16 grams; P<0.001 vs. control) on weight; however, the dose of 10 mg/kg LV tended to decrease the weight to 2.38 ± 0.14 grams (P<0.001 vs. control)

Colon length significantly decreased with the development of the colitis model (from 19.44 ± 0.47 cm to 15.75 ± 0.16 cm, P<0.001). Treatment with 3 mg/kg LV did not change this shortening (16.33 ± 0.28 cm; P<0.001); however, 10 mg/kg LV administration was partially effective (17.13 ± 0.44 cm, P<0.01 vs. control; P<0.05 vs. TNBS group).

The colon weight-to-length ratio was significantly increased in the TNBS group (169.33 ± 12.69) compared to the control group (92.30 ± 3.28, P<0.001). Treatment with 10 mg/kg LV on healthy tissue was ineffective on this parameter (82.43 ± 4.11). This ratio was decreased by treatment with 10 mg/kg LV (140.14 ± 9.16, P<0.01), but not with 3 mg/kg of LV (166.94 ± 11.98, P<0.001).

The treatment of colitis with LV dose-dependently decreased the macroscopic scoring compared to the TNBS group (2.88 ± 0.35, P<0.001 vs. control). The LV3 group's score was 2.44 ± 0.37 (P<0.001 vs. control), and the LV10 group's score was 1.88 ± 0.12 (P<0.001 vs. control, P<0.05 vs. TNBS group).

Histopathologic evaluation

Due to the intensive acute phase of the inflammation on day 3, crypt abscesses could not be able to evaluate. Instead, ulceration and necrosis were scored along with the other parameters, as shown in Table 1A.

Administration of TNBS significantly damaged all parameters in the colonic tissue compared to the control group (P<0.001). Treatment with 3 mg/kg LV was not effective in preventing tissue damage. Treatment with 10 mg/kg LV for 3 days significantly decreased the cell infiltration score from 3 to 2.25 (P<0.01). The muscle thickness score was lowered from 2.33 to 1.38 (P<0.05). The decrements in the scores for mucosal loss, goblet cell depletion, ulceration, and necrosis were not statistically significant with the treatment, although total healing scores were better than in the 3 mg/kg treatment group. Total score of TNBS group dropped down from 15.5 to 13 in LV3 group (P>0.05); further decreased to 11.38 in LV10 group and became statistically significant (P<0.05) (Table 1B; Fig 1).

Discussion

In the present study, we considered the possible beneficial effect of leucovorin treatment on the IBD model and we followed the clinical signs and histopathological findings.

Reduced bioavailability of folate has been hypothesized to be correlated with colon cancer risk in ulcerative colitis patients. A clinical trial showed that giving a daily folinic acid supplement (15 mg) might help regulate rectal cell growth in people with

Table 1 (A). — A. Modified Criteria for Histological Scoring of Damage: Ulceration and Necrosis Used Instead of Crypt Abscesses (Adapted from Appleyard and Wallace)

Colitis score	Loss of mucosal architecture	Cellular infiltration	Muscle thickening	Goblet cell depletion	Ulceration	Necrosis
0	Absent	Absent	Absent	Absent	Absent	Absent
1	Under 5%	Mild	Mild	Mild	Mild	Mild
2	5-10%	Moderate	Moderate	Moderate	Moderate	Moderate
3	Above 10%	Extensive	Extensive	Extensive	Extensive	Extensive

Table 1(B). — Microscopic scoring of histopathological lesions in all groups. LV10: 10 mg/kg Leucovorin, TNBS: 2,4,6-trinitrobenzenesulfonic acid; TNBS+LV3: 3 mg/kg Leucovorin treatment; TNBS+LV10: 10 mg/kg Leucovorin treatment

Group	Loss of mucosal architecture	Cellular infiltration	Muscle thickening	Goblet cell depletion	Ulceration	Necrosis	Total score
Control	0.4	0.6	0.4	0.2	0	0	1.6
LV10	0.44	0.78	0	0.78	0	0	2
TNBS	2.83***	3***	2.33***	2.5***	2.33***	2.5***	15.5***
TNBS+LV3	2.11	2.56	2.11	2.22	2.11	1.89	13
TNBS+LV10	2.13	2.25##	1.38#	1.88	2	1.75	11.38#

Values are mean histopathological scores (n=5–9). (*** P<0.001 vs. Control group; #P<0.05, ## P<0.01 vs. TNBS group)

Table 2 — Clinical parameters change of all groups. LV10: 10mg/kg Leucovorin, TNBS: 2,4,6-trinitrobenzenesulfonic acid; TNBS+LV3: 3mg/kg Leucovorin treatment; TNBS+LV10: 10mg/kg Leucovorin treatment.

	Body Weight Change %	Colon Weight (g)	Colon Length (cm)	Weight /Length of colon (mg/cm)	Macroscopic score
Control	0.06±0.02	1.79±0.08	19.44±0.47	92.30±3.29	0±0
LV10	0.80±0.24	1.59±0.07	19.33±0.16	82.43±4.11	0±0
TNBS	-4.20±1.66	2.66±0.19**	15.75±0.16***	169.33±12.69***	2.88±0.35***
TNBS+LV3	-6.08±1.39*	2.70±0.16***	16.33±0.28***	166.94±11.98***	2.44±0.38***
TNBS+LV10	-7.38±1.76*	2.38±0.14***	17.13±0.44**#	140.14±9.16**	1.88±0.13***#

Values are mean ± SEM from 5-9 rats. (*P<0.05, **P<0.01, *** P<0.001 vs. Control group) (# P<0.05 vs TNBS group)

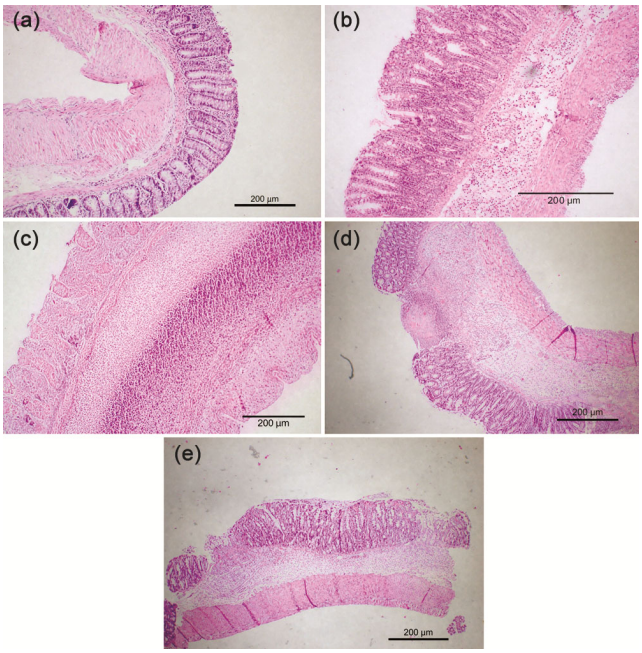

Fig. 1 — Magnifications x10; (a) Normal colonic tissue of the subject in the control group; (b) Slight inflammation observed in a sample from the Leucovorin 10 mg/kg group; (c) Transmural necrotic areas in the colonic wall of the subject in the TNBS group; (d) A focal ulcerated area in the subject from the TNBS+LV3 group; (e) Regenerative findings, mild inflammatory changes, mild mucosal architectural loss, and slight goblet cell loss in the colonic tissue of the subject in the TNBS+LV10 group.

long-term ulcerative colitis[14]. Inspired by this study, we aimed to evaluate whether folinic acid could serve as a convenient and affordable option for adjuvant treatment in clinical settings for IBD. To mimic a real clinical scenario, we administered LV treatment at the beginning of the symptoms, alongside TNBS administration. Folinic acid is sometimes used in the context of many clinical situations, for example to prevent drug side effects such as methotrexate. It is included in the colon cancer treatment regimens (FOLFOX, FOLFIRI), but its role is not clear in wound healing as a direct treatment for the disease itself.

LV alone did not result in weight loss. Its combination with TNBS did not prevent weight loss; paradoxically it slightly increased and made the weight loss significant compared to TNBS alone. LV might have a similar effect to dexpanthenol which is used for intestinal atony[15]; which increases ileal peristaltic movements and emptying of the gut. Also, it might have a potential additive metabolic burden or altered inflammatory-metabolic interactions as vitamin when both agents are administered together which needs further evaluation.

TNBS is widely used for IBD studies. Macroscopic evaluation was performed based on the presence of oedema, erythema, bleeding, erosion, and ulceration. Administration of TNBS resulted in a 49% increase in colon weight and a 19% shortening of its length. The harmful effects of TNBS were further revealed by the histopathological parameters. For microscopic evaluation, we assessed a loss of mucosal architecture, cellular infiltration, muscle thickening, and goblet cell depletion. Its administration also caused severe ulceration and necrosis. The development of this model was consistent with previous literature[1,3,9].

While the colon weight of the TNBS-administered group significantly increased by 49%, the LV3 group's elevation was 51%, LV10 group's elevation was only 33%. Similarly, the 19% shortening of colonic tissue in the TNBS group was partially prevented, with a 16% shortening in LV3 group and a significant prevention resulting in only a 12% in the LV10 group. In our study, a significant 25% reduction in cellular infiltration, in other words active inflammation, was observed in the colons of rats treated with 10 mg/kg LV on the third day after TNBS administration. In contrast, no statistically significant difference in inflammation was detected in the group treated with 3 mg/kg LV, where healing was only 15%, compared to the TNBS-only group. This may be attributed to the dose-dependent effect of leucovorin. Johnson *et al.*[2] showed increased levels of cyclo-

oxygenase-2 (COX-2) and prostaglandin E2 (PGE2) in both the inflamed and non-inflamed sites of the TNBS colitis model. Macroscopic damage score of the colon and tissue nitric oxide (NO) level have been found higher on the third day compared to the seventh day after TNBS application[3]. This finding shows the involvement of surrounding tissues in the inflammatory process. On the other hand, LV treatment has been found highly effective in preventing kidney and liver damage in a life-threatening LPS-septic shock model. This effect is achieved through antioxidant mechanisms and by decreasing inflammatory mediators such as NO, Tumor Necrosis Factor (TNF)-alpha, and Interleukin (IL)-1b levels[6]. A 16-week diet of 15 mg/kg/day of folic acid recovered endothelial dihydrofolate reductase in STZ-diabetic rats. This enzyme is crucial for preserving endothelial function and inhibiting atherosclerosis[16]. Based on findings from studies on sepsis and diabetes, folic acid alone may be capable of combating severe inflammation through a variety of underlying mechanisms. An earlier study suggested that high-dose folate promotes the scavenging of peroxynitrite-derived radicals and hypothesized that it is useful in clinical inflammatory disorders[17].

Studies have shown that during gut inflammation, immune cells release molecules called cytokines, including IL-1β, TNF-α, and IL-12. These cytokines are believed to impair the contractility of gut smooth muscle by suppressing L-type Ca2+ channels, intracellular signaling molecules, and C-kinase potentiated protein phosphatase-1 Inhibitor (CPI-17), ultimately leading to decreased myosin light chain (MLC) phosphorylation[2]. It has been shown that expression of thin-filament associated proteins such as α-tropomyosin, smoothelin, h2-calponin, and h-caldesmon is increased in the TNBS colitis model on the third day. Chronic inflammation of the colon causes both muscle hyperplasia and hypertrophy. These changes ultimately lead to an alteration in the tissue's cellular architecture[18]. Therefore, in the present study, we also evaluated muscle thickness. The statistically significant difference in thickness between the groups suggests a 41% reduction in chronic irritation in the 10 mg/kg LV-treated group. The decrease in muscle thickness was only 9% in the low-dose LV group.

Although ulcer and necrosis scores in the LV10 group decreased by 14% and 30%, respectively, these changes did not reach statistical significance. The slight recovery in the scores for mucosal loss, goblet cell depletion, ulceration, and necrosis was not significant, but it does overall support the macroscopic healing score in our clinical findings. The evaluated parameters for both macroscopic and microscopic healing significantly decreased the total scores. Macroscopic healing reached a score of 35%, while microscopic healing score was 27% in the 10 mg/kg LV treatment group**.** Moreover, Moens *et al*.[19] reported that oral pre-administration of 10 mg/day folate to rats preserved myocyte viability following a cardiac ischemia-reperfusion protocol by reducing oxidative stress. A recent paper reported that folic acid supplementation restored methionine cycle metabolism, and promoted peripheral nerve injury repair by regulating dynamin 3-AKT pathway[20]. Similar to these studies, we have shown that 10 mg/kg LV also protected the colonic cells. However, we suggest that extending the treatment duration or using higher doses of LV may yield statistically significant outcomes in future studies. Importantly, the overall score of macroscopic and histopathological evaluation was lower in the LV10 group compared to the TNBS group, supporting our hypothesis regarding the potential therapeutic benefits of higher-dose LV treatment.

In addition, it has been reported that patients with IBD often have difficulty absorbing nutrients including vitamin B9. Their main medication, sulfasalazine treatment, can also contribute to a deficiency of this vitamin[21]. Genetic engineering involves the production of folate-producing probiotic strains that serve as therapeutics for intestinal pathologies, and complement anti-inflammatory and antineoplastic treatments[22]. Therefore, LV treatment not only resubstitutes folic acid in the body, but it also has a beneficial anti-inflammatory effect that should be considered for the healing of ulceration and necrosis.

While our study provides strong clinical and histopathological evidence of the anti-inflammatory effects of LV, the absence of complementary biochemical markers (e.g., cytokine profiles or oxidative stress parameters) represents a limitation. Future studies incorporating these quantitative measures will further elucidate the molecular mechanisms underlying the observed effects.

Conclusion

The results of the present study suggest that affordable and readily available LV administration may offer additional dose-dependent therapeutic value in mitigating colitis conditions.

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Conflict of interest

The authors declare that they have no competing interests.

Approved by the following research ethics committee

Aydin Adnan Menderes University for Animal Experiments (HADYEK 64583101/2024/49).

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