Original Article

Anti-Inflammatory Efficiency of Ankaferd Blood Stopper in Experimental Distal Colitis Model

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ABSTRACT

Background/Aim: Ankaferd blood stopper (ABS) is a herbal extract that enhances mucosal healing. In this study, we aimed to investigate the efficiency of ABS in the treatment of experimental distal colitis. **Materials and Methods:** Twenty one male albino rats were divided into three groups: Sham control (Group 1), colitis induced by acetic acid and treated with saline (Group 2), colitis induced by acetic acid and treated with ABS (Group 3). At end of the 7th day of induction, all the rats were lightly anesthetized with intramuscular ketamine (8 mg/kg) and thereafter laparotomy and total colectomy were performed. The distal colon segment was assessed macroscopically and microscopically. In addition malondialdehyde (MDA), superoxide dismutase (SOD) and nitric oxide (NO) levels of the colonic tissue and changes in body weight were measured. **Results:** The MDA and NO levels of the colonic tissues and weight loss were significantly higher in Group 2 compared to Group 1 and Group 3. Microscopic and macroscopic damage scores were significantly higher in Group 2 and Group 3 than Group 1 (*P*: 0.001, *P*: 0.004, respectively). Although the microscopic and macroscopic damage scores in Group 3 were slightly lower than Group 2, the difference was not statistically significant. The SOD levels of the colonic tissues were not different between the three groups. **Conclusion:** Weight alterations and high-levels of the colonic tissue MDA and NO suggested that ABS might have anti-inflammatory effects on experimental distal colitis. However, this suggestion was not supported by histopathological findings.

Key Words: Acetic acid, ankaferd blood stopper, experimental colitis, malondialdehyde, nitric oxide, superoxide dismutase

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Ulcerative colitis (UC) is an inflammatory bowel disease (IBD) of uncertain etiology that is characterized by recurring episodes of inflammation primarily involving the mucosal layer and occasionally the submucosa of the colon. As the etiology of UC has not yet been clarified, no actual causal approaches exist. When the disease is confined to the rectum and sigmoid colon it is called distal colitis. Patients with distal UC have a lower response rate to standard therapy as compared with cases with more extended lesions.[1] Moreover, therapy-refractory and chronically active cases still present a therapeutic problem. For this reason, new therapeutic agents are needed for the treatment of distal UC.

Ankaferd blood stopper (ABS) is a herbal extract attained from five different plants *Thymus vulgaris*, *Glycyrrhiza glabra*, *Vitis vinifera*, *Alpinia officinarum* and *Urtica dioica*.[2] It has a therapeutic potential to be used for the management of external hemorrhage and controlling gastrointestinal bleeding associated with various benign and malignant lesions, refractory to conventional anti-hemorrhagic measures.[3,4] ABS, besides its homeostatic activity, has anti-neoplastic actions and *in vitro* anti-infectious affect.[5,6] Several experimental studies investigated the effect of ABS on inflammation and fibrosis in bladder, liver and renal tissues.[7,8]

Oxygen-free radicals and lipid peroxides (oxidative stress) are highly reactive and damaging compounds. Nowadays

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considerable attention has been given to the role of reactive oxygen metabolites in the pathogenesis of IBD. To date there is no clear information about the anti-oxidative effect of ABS. In the published literature, only one study has demonstrated the anti-oxidative effect of ABS treatment. In this study, Hasgul *et al*., has showed that MPO activity, nitric oxide (NO) and tumor necrosis factor-α (TNF-α) levels were significantly decreased by ABS treatment in rats with gastric mucosal injury.[9]

In this study, we aimed to investigate oxidative stress markers and to evaluate the efficiency of ABS in the treatment of experimentally induced distal UC.

MATERIALS AND METHODS

The study was approved by the Institutional Animal Use and Care Committee of Ankara Education and Research Hospital and performed in accordance with the National Institutes of Health Guidelines. Twenty one male Wistar albino rats were used in the study, with a mean age of 4 months and mean weight of 200-280 g. The rats were randomly divided into three groups: Group 1: Sham control group ($n = 7$), Group 2: Colitis treated with saline ($n = 7$), Group 3: Colitis treated with ABS ($n = 7$).

All the animals were fed standard food and water. Twelve hours before the study procedure, feeding was stopped and the rats were only allowed to drink water. On the day of induction, all rats were lightly anesthetized with intramuscular ketamine (8 mg/kg). Rats were in Trendelenburg position during the process and 6F feeding tube was inserted rectally until the tip was 5 cm proximal to the anus. Initially, each rat received a 1-ml saline (0.9%) flush followed by manual palpation of the abdomen to remove any feces. Then 2 ml 4% acetic acid was administered slowly to Group 2 and Group 3. In Group 1 (sham control group) only rectal insertion of feeding tube was performed once a day from day 1 to day 7. In Group 2, rats were treated with daily rectal single dose of saline (2 ml, 0.9% NaCl) via feeding tube for 7 days following the induction of colitis. In Group 3, rats were treated with daily rectal single dose of ABS (2 ml/day) via feeding tube for 7 days following the induction of colitis. Thereafter, all rats were maintained in a head-down position for 60 s to limit expulsion of the solution. On the morning of the 7th day, all rats were weighed and anesthetized with ketamine, xylazene, and euthanized by cervical dislocation. A laparotomy and total colectomy was performed. The lumen of resected specimen was irrigated with 0.9% NaCl. The distal colon segment was then split longitudinally into two pieces and preserved for histological and biochemical analysis.

When tissue samples were obtained, macroscopic damage was scored on a scale of 0-5 modified from a description by Morris *et al*.[10] [Table 1] by the same pathologist who was blinded to the group assignment of the rats. Later the tissue samples were fixed in 10% neutral buffered formalin solution. All samples were embedded in parafin wax and sections were taken and stained with H and E. All sections were evaluated by light microscopy and scored on a scale of 0-10 as described by Wang *et al*.[11] [Table 2] in a blinded fashion by the same pathologist.

Biochemical analyses

Tissues were homogenized in ten volumes of 150 mM ice-cold KCl using a glass teflon homogenizer (Ultra Turrax IKA T-18 Basic Homogenizer) after cutting the tissues into small pieces with scissors (for 2 min at 5000 rpm). The homogenate was then centrifuged at × 5000 g for 15 min. The supernatant was used for analysis. High performance liquid chromatographic analysis was performed with isocratic method using a Shimadzu High-performance liquid chromatography (HPLC) system (Kyoto, Japan) using a

Table 1: Criteria for scoring of gross morphologic damage

Score	Gross morphology
0	No damage
1	Localized hyperemia, but no ulcers or erosions
2	Ulcers or erosions with no significant inflammation
3	Ulcers or erosions with inflammation at one site
4	Two or more sites of ulceration and/or inflammation
5	Two or more sites of ulceration and inflammation or one major site of inflammation and ulceration extending >1 cm along the length of colon

Table 2: Criteria for scoring of microscopic damage

Histological lesion	Score
Ulcer	
None	0
Ulcer <3 mm	1
Ulcer >3 mm	2
Inflammation	
None	0
Mild	1
Severe	2
Granuloma	
None	0
Present	1
Depth of the disease	
None	0
Sub-mucosal layer	1
Muscular layer	2
Serosal layer	3
Fibrosis	
None	0
Mild	1
Severe	2

commercial malondialdehyde (MDA) kit (Immundiagnostik AG, Bensheim, Germany). The first step in determining MDA is a sample preparation with derivatization reagent transforms MDA into a fluorescent product. Afterwards, the pH is optimized and reaction mixture (20 μl) was then chromatographed on a reversed phase C18 column (18.5 mm, 125 mm × 4 mm) at 30°C. The flow rate was 0.8 ml/min. Fluorimetric detection was performed with excitation at 515 nm and emission at 553 nm. The detection limit was 0.15 μmol/l and linearity was up to 100 μmol/l. Protein concentrations of the supernatants were determined by the method of Lowry *et al*.[12] Total superoxide dismutase (SOD) activity was determined according to the method reported by Sun *et al*.[13] The principle of the method is based on the inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine–xanthine oxidase system as a superoxide generator. Activity was assessed in the ethanol phase of the liver homogenate after a 1.0 ml ethanol/chloroform mixture (5/3, v/v) was added to the same volume of the hemolysate and centrifuged. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. Serum NO levels (nitrite + nitrate) were measured, after conversion of nitrate to nitrite by copperized cadmium granules, by a spectrophotometer at 545 nm (Shimadzu, Tokyo, Japan).

Statistical analyses

All results are reported as mean ± standard error of the mean. The statistical analyses were performed using the SPSS® statistical package, version 16.0 for Windows. Due to limited number of rats in each group, non-parametric methods were used for statistical analysis. Kruskal-Wallis variance analysis, which is used to compare the means of three or more groups, was used to determine whether there was a statistical difference between the groups. The Mann-Whitney U test, which is used to compare the means of two groups, was used to determine from which group the significant difference originated. A *P* value of less than 0.05 was considered significant.

RESULTS

At the beginning of the study, there was no significant difference between sham control, acetic acid and colitis treated with ABS groups, according to weight. However, at the end of the study, the weight loss in Group 2 (acetic acid group) was significantly higher than Group 1 (sham control) and Group 3 (colitis treated with ABS) ($P < 0.05$) [Table 3].

Microscopic damage scores and macroscopic damage scores were significantly higher in Group 2 and Group 3 than Group 1 (*P*: 0.001, *P*: 0.004, respectively). There was no significant difference between Group 2 and Group 3, according to microscopic and macroscopic damage scores (*P*: 0.1, *P*: 0.7, respectively). Although, Group 3 (colitis treated with ABS) had lower damages scores (both microscopic and macroscopic) than Group 2, there was no statistical significance [Table 4]. As shown in Figure 1, colitis treated with ABS have lower microscopic scores of colonic inflammation compared to untreated group [Figure 1].

The MDA levels of the colonic tissues were significantly higher in Group 2 ($P = 0.003$). In addition, there was no significant difference in the colonic tissue MDA levels between Group 1 and Group 3 ($P = 0.7$). The SOD levels of the colonic tissues were similar in the three groups ($P = 0.07$). The NO levels of the colonic tissues were significantly higher in Group 2. The results of MDA, SOD, and NO levels of the colonic tissues are summarized in Table 4.

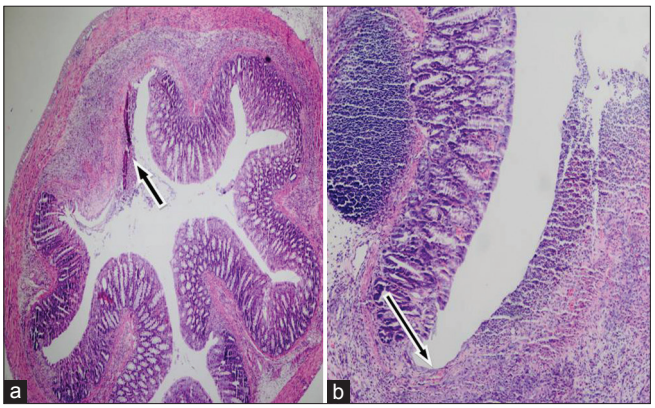

Figure 1: Histological examination of the colonic sections from the rats. (a) Acetic acid group showing the presence of severe inflammation with depletion of goblet cells. (b) Colitis treated with ankaferd blood stopper showing the presence of mild to moderate inflammation

Table 3: Weight changes during the study period

	Initial weight	Weight at 5th day	*P* value
Group 1	241.43±32.17	240.71±38.64	0.8
Group 2	240.86±20.82	225.86±24.89	0.02
Group 3	262.00±35.52	259.33±44.51	0.5

Table 4: Comparison of study groups according to microscopic damage score, macroscopic damage score, colonic tissues malondialdehyde, superoxide dismutase, and nitric oxide levels

	Group 1	Group 2	Group 3
Microscopic damage score	0.0±0.0	2.14±0.69*	1.50±0.54*
Macroscopic damage score	0.0±0.0	1.84±0.69*	1.70±0.63*
MDA	1.28±0.22	1.88±0.12*	1.31±0.22†
SOD	16.09±5.63	12.93±7.86	15.78±6.10
NO	8.06±0.93	11.28±1.14*	8.91±2.88†

*$P<0.05$ versus Group 1, †$P<0.05$ versus Group 2, MDA: Malondialdehyde, SOD: Superoxide dismutase, NO: Nitric oxide

DISCUSSION

In the present study, the colonic tissue MDA and NO levels were significantly lower in the treatment group compared to the experimental colitis group. In addition, we have found no reduction in body weight in rats treated with ABS. In this study, weight alterations and decreased colonic MDA and NO levels suggested that ABS might have anti-inflammatory effects on colitis, however, histological findings did not support this suggestion.

ABS is a standardized mixture of the plants including, *T. vulgaris*, *G. glabra*, *V. vinifera*, *A. officinarum* and *U. dioica*. Each ingredient of this mixture has specific characteristics. *G. glabra* inhibits angiogenesis, decreases vascular endothelial growth factor production and cytokine-induced neovascularization. *G. glabra* also has anti-inflammatory, anti-thrombin, antiplatelet, antioxidant, anti-atherosclerotic, and antitumor activities.[14] *T. vulgaris* has been shown to exhibit varying levels of anti-oxidant activity, which may help to prevent *in vivo* oxidative damage, such as lipid peroxidation, associated with atherosclerosis.[15] Recently Chandrasekeran *et al*., have showed that *G. glabra*, one of the ingredients of ABS, has an anti-inflammatory property through the inhibition of prostaglandin E2 (PGE2), Thromboxane B2 (TXB2) and Leukotriene B4 (LTB4).

ABS has many cellular effects[9,16] and has been shown to affect renal tubular apoptosis based on the level of hemorrhage.[9] The finding of ABS-induced thrombin receptor down-regulation gives an additional clue on the possible mechanism of ABS associated apoptosis modulation at the tissue level.[17] Preliminary findings focusing on *in vitro* anti-neoplastic effects[18] of ABS also prompt to begin for searching the ABS effects at the cellular level in health and in neoplastic diseases. ABS has positive effects on early bone healing together with decreasing inflammation and necrosis and increasing new bone formation.[19]

ABS has also *in vitro* anti-bacterial effects. Ankaferd, besides its hemostatic activity, may also inhibit the growth of bacteria.[20] The mechanism of action regarding the anti-infective effects of ABS is currently unknown. Anti-infective actions of ABS may be related to its homeostatic functions acting on Protease activated receptor-1 (PAR-1), Endothelial protein C receptor (EPCR) and Plasminogen activator inhibitor-1 (PAI-1) affecting distinct steps of coagulation and vascular endothelium. The interrelationships between ABS-induced immune system-driven loss of intestinal goblet cells, anti-infective actions of ABS, and its association with homeostatic molecules remain to be elucidated.

In this study, microscopic and macroscopic damage scores in ABS treated group did not show significant differences when compared to the acetic acid group. Nevertheless, this difference is not statistically significant, colitis treated with ABS have lower microscopic and macroscopic scores of colonic inflammation. In the present study, rats were treated with daily rectal single dose of ABS (2 ml/day) for 5 days. For this reason it can be speculated that short follow-up period and insufficient dose adjustment may lead to inadequate mucosal healing. Further studies with longer treatment periods might be beneficial in examining the effectiveness of ABS in the treatment of distal colitis.

Oxidative stress with tissue damage is the hallmark of cell death. There is increasing evidence that, in certain pathologic states, the increased production and/or ineffective scavenging of such reactive oxygen species may play a crucial role in determining tissue injury. Oxidative stress could be a major contributing factor to the tissue injury and fibrosis. The increased production of reactive oxygen species decreased antioxidant levels and decreased antioxidant enzymes in intestinal mucosa have been shown in IBD.[21,22] Recently, Alzoghaibi *et al*., showed that increased levels of plasma MDA in IBD is an important indication of oxidative stress.[23] In the present study, MDA levels of colonic tissue were significantly decreased in the treatment group. These results provide evidence of an antioxidant action of ABS in the intestine in an experimental distal colitis model. This antioxidant effect may be attributed to herbal mixtures including *G. glabra* and *T. vulgaris*.

NO is a highly reactive free radical, is generated from arginine by an enzymatic pathway originally demonstrated in vascular endothelial cells.[24] It has pro-inflammatory properties by stimulating chemotaxis of neutrophils and monocytes, enhancing the production of cytokines and generating superoxide ions.[25,26] Rachmilewitz *et al*., have shown that the colonic NO level is significantly increased in patients with IBD, more than two-fold higher than in control subjects.[27] In the present study, lower colonic NO levels in treatment group is probably related to the anti-inflammatory activity of ABS.

SOD is an enzyme that repairs cells and reduces the damage done to them by superoxide, the most common free radical in the body. Studies have shown that SOD acts as both an antioxidant and anti-inflammatory in the body, neutralizing the free radicals that can lead to wrinkles and precancerous cell changes. Superoxide and hydrogen peroxide generated by activated leukocytes cause damage to the mucosa in intestinal inflammation.[28] It has been demonstrated that activated leukocytes form reactive oxygen metabolites and oxidative stress is one of the causes for tissue injury during IBDs.[29] In addition, human studies have reported decreased intestinal tissue levels of SOD in patients with IBD.[30] In our study, the SOD levels of colonic tissues were slightly higher in control group and treatment group compared

to acetic acid colitis group. However, this difference was not significant. From a theoretical point according to our findings we hypothesize that topical administration of ABS might lead to increased production of SOD, which results in beneficial effects on intestinal inflammation.

One of the limitations of our study is the relatively short follow-up period, which cannot provide to assess the long-term effect of ABS on colonic inflammation. Another limitation is that we did not examine the inflammatory cytokines such as TNF-α and interleukin-6.

CONCLUSION

In conclusion, ABS might have anti-inflammatory effect in an experimental distal colitis. Further studies with larger groups and longer treatment periods are needed to evaluate the anti-oxidant and anti-inflammatory activity of ABS.

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