

## Original Research

# Effects of the Folk Medicinal Plant Extract Ankaferd BloodStopper on the Healing of Colon Anastomosis: An Experimental Study in a Rat Model

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### Abstract

**Objectives:** Ankaferd BloodStopper (ABS) is a topical hemostatic agent that modulates the inflammatory response and accelerates wound healing. The aim of this study was to determine the effects of ABS on the colon anastomosis wound healing in a rat model.

**Methods:** Thirty-two Wistar albino rats were divided into four groups as follows: Group A (n=8), left colonic anastomosis plus ABS treatment (sacrificed on the 3<sup>rd</sup> day); Group B (n=8) (control), left colonic anastomosis (sacrificed on the 3<sup>rd</sup> day); Group C (n=8), left colonic anastomosis plus ABS treatment (sacrificed on the 7<sup>th</sup> day); and Group D (n=8) (control), left colonic anastomosis (sacrificed on the 7<sup>th</sup> day). All rats were sacrificed at the end of the experiment to assess the anastomosis integrity and the presence of perianastomosis abscesses, peritonitis, and adhesions. Additionally, the bursting pressure and hydroxyproline (OH-pyrolone) levels were determined, and a histopathologic evaluation of the perianastomosis tissue was conducted.

**Results:** The mean bursting pressure on Day 7 was significantly higher than that on Day 3 in the ABS group (p=0.017). Overall, the bursting pressure was higher in animals treated with ABS than in the control animals, although the difference was not statistically significant. The OH-pyrolone levels of both ABS groups were significantly higher than in the control groups. The mean OH-pyrolone level on Day 7 was higher than that on Day 3 in the ABS-treated animals (p=0.038).

**Conclusion:** ABS increases collagen formation and neovascularization, and it has a positive impact during the colon anastomosis healing in an experimental model of wound healing.

**Keywords:** Ankaferd BloodStopper; colon anastomosis; wound healing.

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Colorectal cancer is the fourth most common cause of cancer deaths worldwide,<sup>[1]</sup> and the anastomosis leakage is one of the most important complications of colorectal surgery. Despite the recent advances in surgical techniques and technologic devices, the anastomosis leakage occurs in up to 37% of patients with colorectal cancer.<sup>[2]</sup> The

main risk factors of leakage include patient-related factors, such as severe anemia, diabetes mellitus, immunosuppression, history of radiation therapy, sepsis, malnutrition, hypoalbuminemia, and hypovolemia, as well as issues with the surgical techniques including excessive suture tension, inadequate perfusion of the anastomosis edges, distal lu-

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minimal obstruction, and abdominal contamination with faecal content.<sup>[3]</sup> A tension-free technique and intact blood perfusion guarantee the stability of anastomosis.

Ankaferd BloodStopper (ABS) is a topical hemostatic agent for the management of hemorrhage where conventional methods to stop hemorrhage are ineffective.<sup>[4]</sup> ABS contains extracts from five different plants (*Thymus vulgaris*, 5 mg/100 ml; *Glycyrrhiza glabra*, 9 mg/100 ml; *Vitisvinifera*, 8 mg/100 ml; *Alpiniaofficinarum*, 7 mg/100 ml; and *Urtica dioica*, 6 mg/100 ml). ABS modulates the inflammatory response via its effects on endothelium, neovascularization, and cytokines.<sup>[4-6]</sup> ABS also accelerates wound healing by increasing the cellular and vascular proliferation,<sup>[7]</sup> and healing of colonic anastomoses depends on these proliferative processes. In this study on an experimental rat model, we aimed to evaluate the efficacy of ABS on wound healing in colon anastomosis.

## Methods

Thirty-two female Wistar albino rats with the mean weight of 235 g (200–275 g) obtained from the Laboratory of Experimental Animals at Biological Experiments with Living Animals laboratory of Istanbul University, Istanbul, Turkey were used in this study. The rats were provided with a regular laboratory rat diet and tap water with a 12-hour day/night light cycle at room temperature of 24 °C–26 °C. Four groups containing 8 rats each were formed. The rats were housed 2/per cage and were allowed to move freely.

Randomization of the rats into groups was achieved using computer-generated random numbers. None of the animals died during the study. The study design and all procedures were approved by the Ethics Committee of the Experimental Medicine Research Institute at Istanbul University.

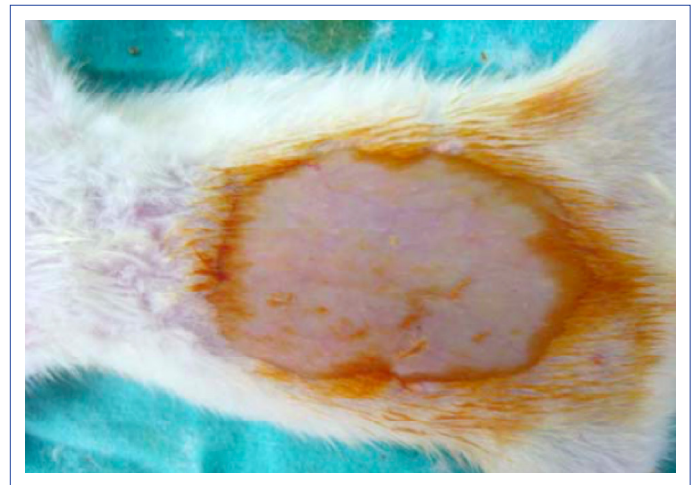
For the study, there were four groups formed as follows: Group A (n=8), left colonic anastomosis plus ABS treatment (sacrificed on the 3<sup>rd</sup> day); Group B (n=8) (control), left colonic anastomosis (sacrificed on the 3<sup>rd</sup> day); Group C (n=8), left colonic anastomosis plus ABS treatment (sacrificed on the 7<sup>th</sup> day); and Group D (n=8) (control), left colonic anastomosis (sacrificed on the 7<sup>th</sup> day).

All rats were sacrificed to assess the anastomosis integrity, and the presence of perianastomosis abscesses, peritonitis, and adhesions. In addition, the bursting pressure and hydroxyproline (OH-proline) levels were determined, and the histopathologic evaluation of the perianastomosis tissue was performed.

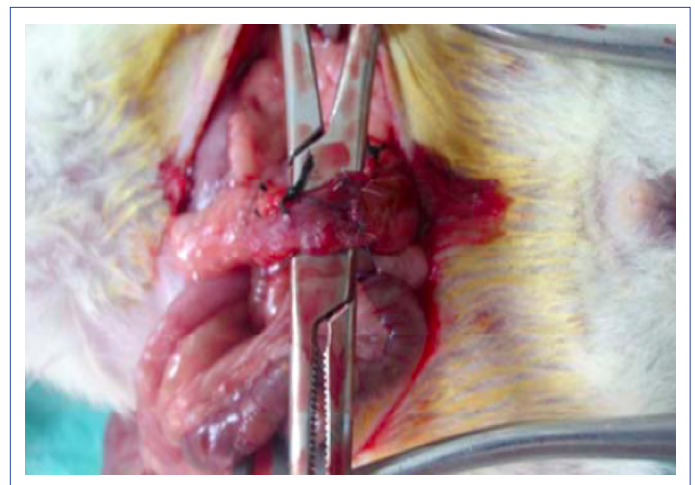
## Surgical Procedure

All rats were fasted for 8 hours before surgery, which was performed under sterile conditions. All colon anastomoses

were performed by the same uninformed/blind surgeon, using six sutures. General anesthesia was administered with 50 mg/kg ketaminehydrochloride intraperitoneally and 5 mg/kg xylazinehydrochloride intramuscularly. Next, the anterior abdominal wall was shaved with an electric razor and wiped with povidone iodine to obtain antiseptic conditions (Fig. 1). The peritoneal cavity was exposed with a 4 cm midline abdominal incision. The left colon was mobilized, and a full-layer cut was created in a 90° angle to the longitudinal axis of the colon. After the exploration of the abdominal cavity, animals with gastrointestinal anomalies and intra-abdominal tumors were excluded from the study. Next, end-to-end colocolic anastomosis was performed using a nonabsorbable mono filament 6/0 polypropylene inverting sutures (Fig. 2), and 0.1 cc of ABS was wiped on the anastomosis line. The abdominal wall was closed with 3/0 silk sutures (Fig. 3). At the end of the procedure, 15 ml of sterile saline was injected subcutaneously for postoper-



**Figure 1.** Shaved and cleansed abdominal wall.



**Figure 2.** Completed anastomosis.



**Figure 3.** Closed skin of the rat.

ative hydration. Animals were fasted for postoperative 24 hours, after which they were started on standard diet and drinking water.

### Evaluation of Adhesions

The uninformed/blind surgeon made the postmortem examination that was conducted to grade adhesions on a scale from 0 to 3 according to the method introduced by van Deer Ham et al.<sup>[7]</sup> (Table 1).

### Measurement of Colonic Anastomosis Bursting Pressure

The uninformed/blind surgeon made the bursting pressure measurements, which were obtained within 3 minutes of sacrifice. First, the anastomosis integrity was determined by exposing the anastomosis line and removal of the adhesions in the surrounding tissues. Next, two cuts, one 2 cm proximal and the other 2 cm distal to the anastomosis site, were made to obtain a 4-cm-long colonic segment. Faecal content of the colon was removed with physiologic saline solution. The distal end of the excised segment was ligated using 4/0 silk sutures after an infusion set connected to a sphygmomanometer was inserted into the proximal end of the colon segment. The resected colon segment was then placed in a beaker filled with physiological saline solution

and inflated with air. The intraluminal pressure was increased in 10 mmHg increments and maintained for 10 seconds. The appearance of air bubbles was used to record the bursting pressure in mmHg. Notably, bursting occurred at the anastomosis line in all samples. The bursting pressure of rats with the anastomosis leakage was determined as 0 mmHg; these animals were excluded from the study. Next, the anastomosis segment was cut across the longitudinal axis and divided into two half rings; one half was placed in 10% formalin solution for histopathological evaluation, and the other half was stored at  $-80^{\circ}\text{C}$  for the OH-proline measurement.

### Histopathological Evaluation

The formalin-fixed colonic segments were sectioned, stained with hematoxylin and eosin, and analyzed with light microscopy at the 200 $\times$  magnification by the same pathologist who was blinded to the treatment groups. The infiltration of inflammatory cells (leukocyte count), activity of fibroblast cells, neoangiogenesis, and collagen content were measured using the modified Ehrlich and Hunt scale by Philips et al.<sup>[8]</sup>

### Tissue OH-Proline Assay

The tissue concentrations of OH-proline, which represent perianastomosis collagen levels, were measured using the spectrophotometric method by Bergman and Loxley.<sup>[9]</sup> The values were expressed as the microgram amount of OH-proline per milligram of tissue ( $\mu\text{g}/\text{mg}$ ).

### Statistical Analysis

Descriptive statistics including the frequency distribution and the mean and standard deviation were used. The Kruskal–Wallis variance analysis was used to determine intergroup differences; paired groups were compared using the Mann–Whitney U test for analyses yielding significant results. P-values less than 0.05 were considered statistically significant. The Statistical Package for Social Sciences for Windows 11.0 was used for all statistical analyses.

### Results

A statistically difference with regard to the anastomosis dehiscence and intra-abdominal abscess was not observed between the groups (Table 2).

Comparison of the tested variables between the ABS (Groups A–C) and control (Groups B–D) groups are shown in Table 3, 4. Acute inflammation, fibroblast activation, and neovascularization scores did not significantly differ between the groups. However, collagenization scores were significantly higher on both Days 3 and 7 after surgery in the ABS group.

**Table 1.** Adhesion grading scale

0:	No adhesions
1:	Minimal adhesions, mainly between the omentum and the anastomosis site
2:	Moderate adhesions, i.e., between the anastomosis site and the omentum or between the anastomosis site and a loop of the small bowel or the abdominal wall
3:	Severe and extensive adhesions, i.e., between the anastomotic site and several loops of the small bowel and the abdominal wall, including the abscess formation

**Table 2.** Evaluation of anastomosis dehiscence, intra-abdominal abscess, intestinal obstruction, adhesion score

	Control group n (%)	ABS group n (%)	p
Anastomoses dehiscence			
3 <sup>rd</sup> day			
No	7 (100)	7 (87.5)	≤1.000
Yes	0 (0)	1 (12.5)	
7 <sup>th</sup> day			
No	8 (100)	8 (100)	≤1.000
Yes	0 (0)	0 (0)	
<sup>d</sup> p	0.192	0.462	
Intra-abdominal abscess			
3 <sup>rd</sup> day			
No	6 (85.8)	6 (87.5)	≤1.000
Yes	1 (14.2)	1 (12.5)	
7 <sup>th</sup> day			
No	8 (100)	7 (87.5)	≤1.000
Yes	0 (0)	1 (12.5)	
<sup>d</sup> p	0.462	1.000	

<sup>a</sup>Kruskal Wallis Test; <sup>b</sup>Mann Whitney U Test; \*p<0.05; <sup>c</sup>Fisher-Freeman-Halton Exact Test; <sup>d</sup>Fisher's Exact Test.

**Table 3.** The comparison between ABS and control group via histopathological analysis in day3

Variable	ABS (Day 3) (mean±SD)	Control (Day 3) (mean±SD)	p
Acute inflammation*	3.5±1.06	3.37±0.51	<sup>a</sup> 0.256
Fibroblast activation*	2.5±0.75	2.25±0.46	<sup>a</sup> 0.289
Collagen*	2.87±0.35	2.37±0.51	<sup>a</sup> 0.046
Neovascularization*	2.75±0.46	2.62±0.51	<sup>a</sup> 0.602

\*Ehrlich and Hunt scale score. <sup>a</sup>Kruskal-Wallis Test; SD: Standard Deviation.

**Table 4.** The comparison between ABS and control group via histopathological analysis in day 7

Variable	ABS (Day 7) (mean±SD)	Control (Day 7) (mean±SD)	p
Acute inflammation*	3.5±0.53	3.75±0.46	<sup>a</sup> 0.317
Fibroblast activation*	3.12±0.64	2.75±0.46	<sup>a</sup> 0.199
Collagen*	3.37±1.06	2.75±0.46	<sup>a</sup> 0.040
Neovascularization*	2.87±0.35	2.62±0.51	<sup>a</sup> 0.264

\*Ehrlich and Hunt scale score; <sup>a</sup>Kruskal-Wallis Test; SD: Standard Deviation.

The mean values of the bursting pressure, adhesion score, and OH-proline level are presented in Tables 5, 6, and 7, respectively. The bursting pressure of one rat from the control group on Day 3 was determined as 0 mmHg, and it was excluded from the study. In addition, briefly, the mean

**Table 5.** The comparison between ABS and control group via bursting pressure, adhesion score, OH-pyroline level in day 3

Variable	ABS (Day 3) (mean±SD)	Control (Day 3) (mean±SD)	p
Bursting pressure (mmHg)	91.25±39.07	61.25±9.9	<sup>b</sup> 0.66
Adhesion score <sup>†</sup>	1.5±0.53	1.62±0.51	<sup>b</sup> 1.00
OH-pyroline (µg/mg)	564.62±59.93	369.5±51.53	<sup>b</sup> 0.001

<sup>†</sup>Adhesions were graded according to system developed by van Der Ham et al. <sup>b</sup>Mann Whitney U Test; SD: Standard Deviation.

**Table 6.** The comparison between ABS and control group via bursting pressure, adhesion score, OH-pyroline level in day 7

Variable	ABS (Day 7) (mean±SD)	Control (Day 7) (mean±SD)	p
Bursting pressure(mmHg)	193±28.75	165±53.45	<sup>b</sup> 0.34
Adhesion score <sup>†</sup>	1.87±0.35	2.12±0.35	<sup>b</sup> 0.31
OH-pyroline (µg/mg)	614.12±81.68	457.25±85.35	<sup>b</sup> 0.007

<sup>†</sup> Adhesions were graded according to system developed by van Der Ham et al. <sup>b</sup>Mann Whitney U Test; SD: Standard Deviation.

**Table 7.** The comparison between ABS day 3 and ABS day 7 group via bursting pressure, adhesion score, OH-pyroline level

Variable	ABS (Day 3) (mean±SD)	ABS (Day 7) (mean±SD)	p
Bursting pressure (mmHg)	91.25±39.07	193±28.75	<sup>b</sup> 0.017
Adhesion score <sup>†</sup>	1.5±0.53	1.87±0.35	<sup>b</sup> 0.63
OH-pyroline (µg/mg)	564.62±59.93	614.12±81.68	<sup>b</sup> 0.038

<sup>†</sup> Adhesions were graded according to system developed by van Der Ham et al. <sup>b</sup>Mann Whitney U Test; SD: Standard Deviation.

bursting pressure on Day 3 was significantly higher than that on Day 7 in the ABS group (p=0.017) (Table 7). Overall, the bursting pressure was higher in animals treated with ABS than the control animals, although the difference was not statistically significant.

The OH-proline levels of both ABS groups were significantly higher than control groups. The mean OH-proline level on Day 7 was higher than that on Day 3 in the ABS-treated animals (p=0.038).

## Discussion

Anastomotic leakage remains a challenge associated with morbidity and mortality in patients undergoing colorectal surgery. Leakage at the site of anastomosis was also reported to contribute to an increase in the length of hospital stays and medical costs.<sup>[10-12]</sup> Treatment of anastomosis leakage is difficult and may require re-laparotomy, with fre-

quent necessity of stoma formation. Therefore, the prevention of anastomosis leakage remains the most critical issue. However, nonmodifiable risk factors such as an advanced age, comorbid conditions, and emergency surgery hinder these efforts,<sup>[3]</sup> highlighting the need for new treatment strategies to prevent anastomosis leakage. To this end, numerous experimental and clinical studies were conducted to achieve better wound healing after colon anastomosis.<sup>[3, 12-14]</sup> Numerous studies have demonstrated the hemostatic effect of ABS, which shortens the bleeding time and the amount of blood loss.<sup>[4-6, 17]</sup> The main mechanism underlying this effect of ABS is an encapsulated protein network formation and increased erythrocyte aggregation.<sup>[15]</sup> Additionally, ABS increases vascular proliferation by reducing tissue necrosis.<sup>[16]</sup> Several studies have also shown the anti-inflammatory effects of ABS.<sup>[4-7, 16, 17]</sup> Interleukins are common to both the inflammatory/anti-inflammatory and the coagulation cascades that are targeted by ABS.<sup>[3]</sup> The most important factor in anastomotic healing is collagen, which forms the tensile strength of submucosal connective tissue.<sup>[18]</sup> Increased inflammation may cause excessive collagenolysis, and the anti-inflammatory function of ABS might inhibit collagenolysis during tissue healing.<sup>[15]</sup> OH-proline levels can be used to assess collagen formation during wound healing. OH-proline is a component of collagen, which was shown to be positively correlated with the collagen amount and strength of anastomosis.<sup>[19]</sup> In the current study, the OH-proline levels were significantly higher in the ABS-treated animals; furthermore, the OH-proline levels were higher on Day 7 compared to those on Day 3 among the animals treated with ABS. These results suggest that ABS might increase the strength of anastomosis by inducing collagen formation. ABS might also decrease collagenolysis via an anti-inflammatory action, which requires further studies.

In clinical practice, the physical strength of anastomosis is not an ideal parameter to assess the healing of colonic anastomosis;<sup>[20]</sup> nonetheless, bursting pressure was used as an indirect method to assess the anastomotic integrity. We found that bursting pressure of the animals treated with ABS is higher than control groups, whereas there was no significant difference in the bursting pressures between the ABS-treated and the control groups.

Fibroblasts and neovascularization promote synthesis of collagen and mucopolysaccharides to increase anastomotic strength.<sup>[21]</sup> The first endothelial cells are seen 48 hours after the beginning of inflammatory process, and this inflammatory process ends on the 6th or 7th day.<sup>[22]</sup> The fibrinopurulent exudate fills anastomotic space in peritoneal sepsis. This exudate reduces the collagen synthesis and formation of new blood vessels and, as a result, anastomo-

sis heals by secondary intention.<sup>[23]</sup> In the presented study, fibroblast activity and neoangiogenesis were not statistically different between the control and ABS groups. We believe that the anti-inflammatory and antioxidant activity of ABS promotes the endothelial cell activity.

There was no difference in neovascularization and fibroblast activity and indicators of fibroblast activity between the ABS and the control groups in the current study. However, histopathological evaluation might not be ideal to assess overlooked ultrastructural changes, and an evaluation by electron microscopy may provide more information on fibrosis and neovascularization. In addition, the short duration of observation in the current study might not have allowed for ultrastructural changes to become evident. Future studies with a longer treatment time that utilize more advanced techniques such as electron microscopy should allow for a better assessment of ultrastructural changes.

The current study findings did not reveal a clear clinical application for ABS in increasing the strength and safety of anastomosis; however, based on our finding of ABS-mediated increase in collagenization. In accordance with the study by Cancan et al.<sup>[19]</sup> which investigated the same issue regarding ABS, we also revealed that ABS has a positive impact in the healing of colonic anastomosis performed under optimal surgical conditions, but further studies are required to elucidate the role of ABS in anastomotic healing under septic conditions.

## Conclusion

ABS increases the collagen formation and neovascularization, and it has a positive impact on the colon anastomosis healing in an experimental rat model of wound healing. Thus, ABS promotes colonic healing and provides a safer and stronger anastomosis than suturing alone.

Although further studies are needed to define the mechanism of the ABS impact in the anastomotic healing of colon, our results are promising for clinical use of ABS to improve the safety of colonic anastomoses.

## Disclosures

**Ethics Committee Approval:** This study was approved by the Biological Experiments with Living Animals Ethic Committee of Istanbul University, Istanbul, Turkey.

**Peer-review:** Externally peer-reviewed.

**Conflict of Interest:** None declared.

**Authorship Contributions:** Concept – U.E.; Design – U.E., B.C.; Supervision – M.U.; Materials – U.E.; Data collection &/or processing – U.E.; Analysis and/or interpretation – U.E., M.F.F.; Writing – U.E., M.F.F.; Critical review – B.C., M.U.

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