# Topical application of Ankaferd Blood Stopper® modifies the healing of colon anastomosis in rats

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## Abstract

Ankaferd Blood Stopper® (ABS) offered as a hemostatic agent is a standardized herbal extract obtained from five different plants. The effects of ABS on colonic anastomosis are unknown. This study was designed to assess potential effects on the anastomosis of left colon in an experimental animal model. Thirty-two male Wistar albino rats were randomized into two groups and subjected to colon anastomosis. The study group subjected to colon anastomosis with topical application of ABS to control of mucosal bleeding at the cut ends of the colon, and the control group subjected to colon anastomosis only. The rats were killed 3 and 7 days postoperatively. Four types of assessment were performed: bursting pressure, bursting wall tension, histopathology, and biochemical analysis. Compared to the control group, ABS used rats displayed a higher bursting pressure ($P<0.05$) and anastomotic hydroxyproline content ($P<0.05$). The use of ABS leads to a significant decrease in malondealdehyde levels ($P<0.05$) and increase in paraoxonase activity ($P<0.05$) at both time points. Histopathological analysis revealed that the use of ABS improves anastomotic healing in terms of reepithelialization, increased neovascularization, diminished ischemic necrosis, and inflammatory infiltration to muscle layer. Topical application of ABS to control of mucosal bleeding at the cut ends of the colon significantly improve the anastomotic wound healing by means of increasing mechanical strength and amount of tissue HPL level.

## Introduction

Colon anastomosis is one of the most widely performed operations in general surgery and the healing of colonic anastomosis has remained a challenging area for surgeons. Leakage from colonic anastomosis remains a major complication of surgery and is associated with a significant increase in mortality and morbidity.

The most widely used cutting instrument is the scalpel, but a scalpel incision is prone to bleeding. Electrosurgical cutting tools are used to incise tissue and these tools also produce simultaneous hemostasis. But there are a number of disadvantages associated with electrosurgery including thermal injury and delayed wound healing.[1-4]

Although bleeding mucosa at the cut ends of the colon is a great sign of adequate perfusion, the anastomotic line must be hemostatic. A hematoma formation at the anastomosis can interfere with healing and lead to leak. Control of mucosal bleeding with sutures has been recommended rather than cautery to prevent a deep burn injury that may lead to delayed leak.[5]

Ankaferd Blood Stopper® (Ankaferd Health Products Ltd., Istanbul, Turkey) offered as a hemostatic agent is a standardized herbal extract obtained from five different plants and it has been approved for the clinical management of external postsurgical and postdental surgical bleedings in Turkey. ABS represents its hemostatic effect by promoting the rapid formation of a protein network covering the classical cascade model of the clotting system without independently acting on coagulation factors and platelets.[6] ABS also has the therapeutic potential to be used for the management of hemorrhages in difficult clinical conditions.[7-9] There is only one study evaluated the effect of ABS, which seems to be an effective hemostatic agent, on wound healing. Isler *et al*.[10] observed that the aplication of ABS decreased the occurence of inflamation and necrosis in early bone healing period. Except this study, the effect of ABS on wound healing has not been investigated thus far.

Considering knowledges about ABS as mentioned above, we hypothesized that ABS may use to control of mucosal bleeding at the cut ends of the colon. Therefore, in this study, we examined potential effects of ABS on the healing of left colon anastomosis in an experimental animal model.

## Materials and Methods

### Animals

This study was established at the Experimental Research Center of Selcuk University with the permission of Ethical Committee and was conducted in accordance with the guidelines in the National Institute of Health's Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 86-23, revised 1985, Bethesda, MD, USA). Thirty-two male Wistar albino rats weighing 250-290 g (range: 270±19 g) were used in this study. A half of the subjects (n=16) were subjected to colon anastomosis only. Control group (group A) was consisted of these untreated 16 rats. The remaining half of the subjects (n=16) were subjected to colon anastomosis with topical application of ABS to control of mucosal bleeding at the cut ends of the colon. The study group (group B) was consisted of 16 rats in which ABS used.

To investigate early and late healing at the anastomosis, half of the subjects in each group was sacrificed on postoperative day 3 (A3 group and B3 group, n=8), and the remaining half was sacrificed on postoperative day 7 (A7 group and B7 group, n=8).

### Surgical procedure

After anesthesia with intramuscular injection of ketamine hydrochloride 50 mg/kg (Ketalar; Parke Davis, Eczacibasi, Istanbul, Turkey) and xylazine 5 mg/kg (Rompun; Bayer AG, Leverkusen, Germany), all of animals were restrained in a supine position, shaved, and 3 cm midline incision was made under sterile conditions. The left colon was transected at the colorectal junction, 2 cm proximal to the peritoneal reflection, in all rats. The scalpel was used as cutting tool. The fecal content of the anastomotic ends were milked out. A standardized end-to-end anastomosis was performed with an interrupted inverting 6/0 polypropylene suture. All anastomoses included 8 equidistant stitches. Only colonic anastomosis was performed in the control group. In ABS group, standart cotton gause soaked 1 ml of ABS was administered to the cut ends of the colon along

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30 second and then anastomosis was performed. The laparotomy was closed in two layers with continuous 4/0 silk sutures. Normal saline solution (25 mL/kg body weight) was injected subcutaneously to prevent postoperative dehydration. After experimentation, all rats were placed in special cages under controlled temperature, moisture with a 12-h light/dark cycle. All animals had free access to a standard laboratory diet and allowed water *ad libitum.*

## Macroscopic healing and measurement of colonic bursting pressure

After animals were sacrificed by means of cardiac puncture, the anterior abdominal wall everted as a U-shaped flap and peritoneal cavity was inspected for adhesions. Wound complications, intra-abdominal abscesses, and evident anastomotic failures were recorded. Intraperitoneal adhesions were graded according to the scale proposed by Knightly *et al.*[11]

At least 4 cm segment with the anastomosis at the center was excised. The anastomotic segment was infused with saline using a digital infusion pump (Abbott LC 5000 infuser, USA) at a rate of 4 mL/min. The other end of the anastomosis was connected via a pressure transducer (Abbott Single Transpact, USA) to monitor (Philips IntelliVue MP20, Netherlands). The pressure in the bowel was monitored. Bursting pressure (BP) was recorded as the highest figure reached before evident saline leakage or sudden loss of pressure. Bursting wall tension (BT) was calculated from Laplace's law using the BP and the anastomotic circumference. The formula is

$BT = BP \times 1.36 \times \text{anastomotic circumference} / 2\pi$.[12]

## Obtaining the samples

Blood samples were centrifuged at 3000 rpm for 10 min and serum aliquots were stored at -80°C for further examinations. After measurement of bursting pressures, a 1 cm of colon segment including the anastomosis was resected and transected longitudinally. Two third of this sample was harvested and kept frozen at 80°C for the measurement of tissue hydroxyproline and malondialdehyde level. The remaning one third was fixed in 10% formalin for histopathological examination.

## Biochemical analysis

### Determination of tissue malondialdehyde

For biochemical analyses, tissue samples were washed with physiological saline. They were then homogenized for 5 minutes (min) in a homogenizer (Ultra-Turrax T25, Germany) with 50 mM cold phosphate buffer pH=7.4 in order to collect 10% homogenate. These homogenates were centrifuged at 6000 g for 10 min to obtain supernatants.

Thiobarbituric acid reactive substances (TBARS) were determined by double heating method of Draper and Hadley.[13]

### Determination of paraoxonase activity

Paraoxonase (PON1) activities were measured by using paraoxon as the substrate. The basal activity of paraoxonase was measured. The rate of paraoxon hydrolysis (diethyl-p-nitrophenylphosphate) was measured by monitoring an increase in absorbance at 412 nm at 37°C on an autoanalyser. The amount of generated p-nitrophenpl was calculated from the molar absorptivity coefficient at a pH of 8.5, which was 18290 $M^{-1}cm^{-1}$.[14] PON1 activity is expressed as U/L of serum.

## Determination of ischaemia modified albumin

The assay method involved adding 50 mL of 0.1% cobalt chloride (Sigma, $CoCl_2.6H_2O$) in $H_2O$ to 200 mL of serum, gently mixing, and waiting 10 min for adequate cobalt-albumin binding. Fifty microliters of dithiothreitol (DTT) (Sigma, 1.5 mg/mL $H_2O$) was added as a colorizing agent and the reaction was quenched 2 min later by adding 1.0 mL of 0.9% NaCl. Using a spectrophotometer at 470 nm, color development with DTT was compared to a serum-cobalt blank without DTT and reported in absorbance units (ABSU).[15]

### Determinetion of hydroxyproline level

Hydroxyproline (HPL) level was carried out in dry tissue of the excised samples through the method described previously by Woessner.[16] Results are expressed as mg/g dry tissue.

## Histopathological examination

Histopathological Examination of the tissues was made by a pathologist included in study and blinded to all information about the groups from the start. The specimens were embedded in paraffin and two slices were obtained from each sample with microtome and stained with Masson trichrome and hematoxylin-eosin. Mucosal ischemia was graded following the scale proposed by Chiu *et al.*[17] Histologic changes of anastomotic wound healing, granulation tissue development, and local inflammatory response were determined according to Houdart *et al.*[18] and Hutschenreiter *et al.*[19] parameters as modified by Garcia *et al.*[20] (Table 1).

## Statistical analysis

The mean bursting pressure, bursting wall tension, tissue HP content, MDA level, IMA

**Table 1. Parameters of histologic changes of anastomotic wound healing, granulation tissue development, and local ınflammatory response.**

| | | | | |
|---|---|---|---|---|
| **A: Mucosal anastomotic reepithelialization** | | | | |
| Grade 0 | Absence of epithelialization on the anastomotic line | | | |
| Grade 1 | Incomplete coating of the anastomotic wound with a single layer of cells | | | |
| Grade 2 | Complete coating of the anastomotic wound with a single layer of cells | | | |
| Grade 3 | Complete reepithelialization with glandular epithelium | | | |
| **B: Inflammatory granuloma and granulation tissue formation** | | | | |
| | **Inflammatory cell presence** | **Neovascularization** | **Fibroblasts** | **Fibrosis formation** |
| Grade 1 | Absence | Absence | Absence | Absence |
| Grade 2 | Slight | Slight | Slight | Slight |
| Grade 3 | Mild | Mild | Mild | Mild |
| Grade 4 | Intense | Intense | Intense | Intense |
| **C: Muscle layer destruction** | | | | |
| | **Ischemic necrosis** | **Muscle layer continuity** | | **Inflammatory infiltration** |
| Grade 1 | Absence | Complete interruption | | Absence |
| Grade 2 | Slight | Muscle synechia | | Slight |
| Grade 3 | Mild | Complete restitution | | Mild |
| Grade 4 | – | – | | Intense |
| **D: Anastomotic wound inflammatory infiltration** | | | | |
| | **Neutrophils** | **Lymphocytes** | **Histiocytes** | **Giant cells** |
| Grade 1 | Absence | Absence | Absence | Absence |
| Grade 2 | Slight | Slight | Slight | Slight |
| Grade 3 | Mild | Mild | Mild | Mild |
| Grade 4 | Intense | Intense | Intense | Intense |

level, PON1 activity were expressed as means (±SD). Statistical comparisons of the data expressed as means (±SD) were analyzed by Mann-Whitney U test. The Chi-squared test was used for the analysis of histopathological parameters and adhesion scores. Statistical significance was set at P<0.05.

## Results

There was no death during surgical procedure in groups.

### Macroscopic healing

Anastomotic leakage and septic complications were not observed. Relaparotomy after the seventh postoperative day showed significantly (P=0.029) higher adhesion formation in Group A7 compared with Group B7 (Table 2). There is no difference between Group A3 and Group B3 in term of adhesion formation (P>0.05).

### Bursting pressure and bursting wall tension

The mean±SD BP was found to be lower in group A3 than group B3 (84.8±11 vs 97.8±6.3 mmHg, P=0.021), and the mean±SD BP was also found to be lower in group A7 than group B7 (182.8±14 vs 201.2±13.2 mmHg, P=0.024). In addition BT was found to be lower in group A than group B in animals killed on postoperative day 3 as well as postoperative day 7 (29.9±3.9 vs 34.4±2.3 dyne 10-3/cm on third day, P=0.027 and 64.6±4.8 vs 71±4.7 dyne 10-3/cm on seventh day, P=0.036). Table 3 demonstrates the group results of BP and BT.

### Biochemical analysis

The HPL level in the anastomotic line was 4.3±0.3 mg/g dry tissue for B7 group and 3.5±0.2 mg/g dry tissue for A7 group. The difference between the A7 and B7 groups was statistically significant (P=0.001). There is no statistically difference between the A3 and B3 groups (2.2±0.5 vs 2.2±0.6 mg/g dry tissue P=0.95).

The animals in Group A3 were found to have significantly higher serum MDA levels compared with the study group animals killed on postoperative day 3 (P<0.05). There is no statistically difference between animals killed on postoperative day 3 in term of IMA levels (P>0.05). The MDA values and IMA levels were found to be higher in A7 group than B7 group (P<0.05). The PON1 activity was significantly higher in Group B than Group A at both time points (P<0.05). Table 4 shows the group results for the biochemical analysis.

### Histopathological examination

#### Chiu scores

The results of mucosal ischemia grading following Chiu scale are listed in Table 5. Differences between groups A3 and B3 were not statistically significant (P>0.05). Group A7 showed higher mucosal ischemia compared with group B7 and this difference was statistically significant (P<0.05).

#### Anastomotic healing examination

Postoperative day 3: The inflammatory infiltration of the muscle layer, muscle layer discontinuity and destruction were higher in the group A than group B (P<0.05). The neutrophil and histiocytes presence in the anastomotic wound was superior in group B than group A (P<0.05).

Postoperative day 7: A significantly higher mucosal anastomotic reepithelialization score

**Table 2. Adhesion formation following Knightly scale.***

| | Group A3 (n) | Group B3 (n) | Group A7 (n) | Group B7° (n) |
|---|---|---|---|---|
| Grade 0 | - | - | - | - |
| Grade 1 | 7 | 6 | - | 4 |
| Grade 2 | 1 | 2 | 5 | 4 |
| Grade 3 | - | - | 3 | - |
| Grade 4 | - | - | - | - |
| Grade 5 | - | - | - | - |

*The Knightly scale: Grade 0: No adhesions; Grade 1: Single, thin easily separable; Grade 2: Less extensive but weak adhesions which withstand traction poorly; Grade 3: Numerous extensive visceral adhesions involving local bowel and omentum; Grade 4 :Numerous extensive visceral adhesions extending to abdominal wall; Grade 5: Adhesion mass with multiple dense adhesions forming localized mass. °Difference between Groups A7 and B7 is significant, P=0.029 $\chi^2$ test.

**Table 3. Bursting pressure and Bursting wall tension results.**

| | Postoperative day 3 | | | Postoperative day 7 | | |
|---|---|---|---|---|---|---|
| | Group A | Group B | P value | Group A | Group B | P value |
| BP (mmHg) | 84.8±11 | 97.8±6.3 | 0.021 | 182.8±14 | 201.2±13.2 | 0.024 |
| BT (dyne 10-3/cm) | 29,9±3.9 | 34.4±2.3 | 0.027 | 64.6±4.8 | 71±4.7 | 0.036 |

**Table 4. Hydroxyproline, MDA, IMA and PON1 results.**

| | Postoperative day 3 | | | Postoperative day 7 | | |
|---|---|---|---|---|---|---|
| | Group A | Group B | P value | Group A | Group B | P value |
| HPL(mg/g dry tissue) | 2.2±0.6 | 2.2±0.5 | 0.95 | 3.5±0.2 | 4.3±0.3 | 0.001 |
| MDA (nmol/g protein) | 6.6±0.6 | 5.7±0.4 | 0.011 | 6.8±1.2 | 5.7±0.7 | 0.020 |
| IMA (ABSU) | 0.220±0.05 | 0.190±0.04 | 0.20 | 0.220±0.04 | 0.170±0.04 | 0.036 |
| PON1 (U/L of serum) | 90.9±12.8 | 109±6.9 | 0.012 | 103.9±16.9 | 133.6±24 | 0.036 |

**Table 5. The results of mucosal ischemia grading following Chiu scale***

| | Postoperative day 3 | | Postoperative day 7 | |
|---|---|---|---|---|
| | GroupA (n) | Group B (n) | Group A (n) | Group B (n) |
| Grade 1 | - | - | 2 | 2 |
| Grade 2 | - | - | - | 6 |
| Grade 3 | 3 | 2 | 6 | - |
| Grade 4 | 2 | 2 | - | - |
| Grade 5 | 3 | 4 | - | - |

*The Chiu scale: Grade 1 = normal mucosal villi; Grade 2 = development of subepithelial Gruenhagen´s space, usually at the apex of the villus, often with capillary congestion; Grade 3 = extension of the subepithelial space with moderate lifting of epithelial layer from the lamina propria; Grade 4 = massive epithelial lifting down the sides of villi, a few tips may be denuded; Grade 5 = denuded villi with lamina propria and dilated capillaries exposed. °Difference between Groups A and B is significant on POD7, P=0.002 $\chi^2$ test.

was detected in group B than group A (P=0.041). The inflammatory cell presence in the granulation tissue was significantly lower in group A than group B (P=0.021). Neovascularization was significantly higher in group B than group A (P=0.002). The muscle layer discontinuity and destruction were higher in the group A than group B (P<0.05) (Figure 1).

## Discussion

The scalpel has traditionally been considered the surgical cutting tool of choice because of its precision, control, preservation of tissue integrity, and superior associated wound healing. However, its primary disadvantage is bleeding; consequently, numerous electrosurgical devices have been developed to provide hemostasis. Although hemostasis is improved, electrosurgical devices suffer from thermal damage to surrounding tissues and impaired wound healing.[1-4]

In the present study, we used ABS to control of mucosal bleeding at the cut ends of the colon and investigated the potential effects of ABS on wound healing of left colon anastomosis in an experimental animal model.

ABS is a folkloric medicinal plant extract product, which has historically been used in Turkish traditional medicine as a haemostatic agent. It is a standardized mixture of the plants *T. vulgaris, G. glabra, V. vinifera, A. Officinarum* and *U. dioica*, each of which has some effect on hematological and vascular parameters, and cellular proliferation.[21-24] To our best knowledge, ABS has not been evaluated in colon anastomosis and the effects of ABS on colon anastomosis are unknown.

Wound healing in the digestive system involves related processes such as haemostasis and inflammation, proliferation–fibroplasia, maturation, and remodeling. Successful colonic wound healing, reducing failure rates, is very important. Delay or problem in any of these stages results with a delay or failure in healing. Although inflammation is an imperative phenomenon for successful wound healing, over-inflammation leads to impaired wound healing due to increased collagenolysis and delayed reepithelization. During this early phase of the healing process anastomotic strength and integrity depend on the suture-holding capacity of the submucosa and anastomosis is theoretically under the greatest risk for leakage. Thereafter, wound strength increases rapidly, between the fifth and seventh days after surgery, collagen synthesis peaks during proliferative phase.[25] For that reason, the third and the seventh postoperative days were used to evaluate the early and late anastomotic wound healing process in our study. On histopathological examination, we detected higher mucosal ischemia, lower inflammatory cell presence and reduced tendency to develop neovascularization in the granulation tissue in group A compared with group B on the seventh day after surgery. The muscle layer discontinuity and destruction were found to be increased in control group at both time points. IMA is produced continuously during ischemic conditions such as myocardial ischemia, muscle ischemia, pulmonary embolism, acute mesenteric ischemia and acute stroke.[26-30] The IMA levels were found to be higher in A7 group than B7 group (0.220±0.04 *vs* 0.170±0.04 ABSU, P<0.05).

Oxidative damage has long been investigated as an adjunct in impaired wound healing. In the present study, MDA and PON1 activities were used as oxidative stress markers. The levels of malondialdehyde in perianastomotic tissues were determined as an indicator of lipid peroxidation.[31] PON1 is an ester hydrolase that has many enzymatic activities. Oxidative stres has been shown to decrease PON1 activity.[32] MDA levels predicting lipid peroxidation was found to be higher, and PON1 activity was determined to be lower in group A at both time points. Many techniques have been developed to assess anastomotic healing. HPL is a good marker in anastomotic healing, because HPL is present only in collagen and elastine in animals. The HPL level in the anastomotic line was 4.3±0.3 mg/g dry tissue for B7 group and 3.5±0.2 mg/g dry tissue for A7 group. The difference between the A7 and B7 groups was statistically significant (P=0.001). Generally, a low HPL level negatively affects wound healing. Assessment of the anastomotic healing mainly depends on mechanical parameters, such as bursting pressure and bursting wall tension, which has been found to be a better reflection of physiological strain demonstrating mechanical healing. Both BP and BT values were significantly lower in group A than group B at both time points.

Adhesions are encountered after many intra-peritoneal surgical procedures. The formation of adhesion might result from mechanical peritoneal damage, tissue ischemia and the presence of foreign materials.[33] The optimal time for quantification of adhesions in rats is 7 or more days after operation.[34] Comert *et al.*[35] had been investigated the effect of ABS on intraabdominal adhesion formation. Although, they did not observe significantly differences in adhesion formation, we detected significantly lower adhesion formation in rats that used ABS after the seventh postoperative day, in this study.

In conclusion, the results of this experimental study revealed that use of ABS to control of mucosal bleeding at the cut ends of the colon significantly improves the wound healing of colonic anastomosis by means of increasing mechanical strength and amount of tissue HPL level. Before starting human studies, further *in vitro* and *in vivo* studies are necessary to evaluate effect of the agent.

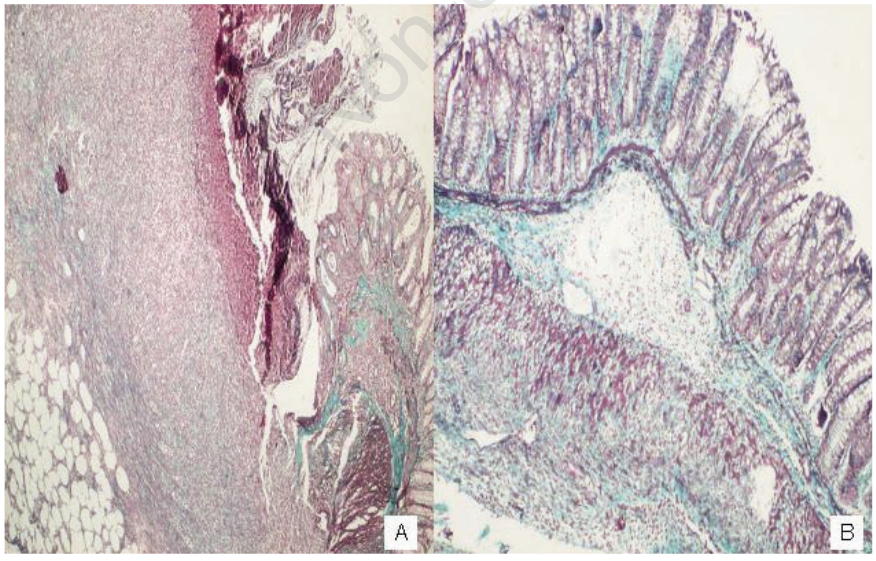


**Figure 1. (A) The muscle layer discontinuity and epithelial destruction in control group, (Mason trichrome X40). (B) Complet epithelization and minimal submucosal edema in ABS group, (Mason trichrome X40).**

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