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EXPERIMENTAL STUDY

Effect of ankaferd blood stopper on hemostasis and histopathological score in experimental liver injury

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Abstract: *Aim:* To investigate intra-abdominal, local histopathological and hemostatic effects of Ankaferd (ABS) in an experimental liver injury.
Methods: Forty rats were randomly assigned to the primary suture group (PS) (n=15), ABS group (n=15) and control group (n=10). A wedge resection was performed on the left lobe of the liver. In the primary suture group, the liver was sutured with polypropylene material, while in ABS group the liver surface was covered with ABS. Adhesion, histo-pathological scores and hydroxyproline levels were measured postoperatively on day 3 and day 15.
Results: The mean bleeding time was 7.5 sec shorter in the ABS group (6.5 sec-13.5 sec) and 107 sec shorter in PS group. AST, ALT, total bilirubin and ALP values complete blood count (CBC) did not change. Intra-abdominal adhesions were the same in PS and ABS groups on 3rd (2.20±1.30 vs 2.0±1.11) and 15th (1.60±0.54 vs 1.25±0.7) days postoperatively. Histopathological scores were better in the ABS group than in the primary suture group on 3rd (2.5±0.5 vs 5.25±0.2, p=0.006) and 15th (1.65±1.7 vs 3.15±1.0, p=0.025) days postoperatively. Hydroxyproline levels were higher in ABS group on postoperative 15th day (17.12 μ/tissue vs 13.69 μ/tissue; p=0.005).
Conclusion: These data suggest that ABS in experimental liver trauma causes favorable histopathological scores and shorter hemostasis time and higher hydroxyproline levels *(Tab. 2, Fig. 2, Ref. 35)*. Full Text (Free, PDF) *www.bmj.sk.*
Key words: ankaferd, hemostasis, adhesion, histopathological score, liver injury.

Liver bleeding is controlled by non-surgical techniques in 80% of the cases; operative intervention may be required in complex injuries (1). Liver bleedings due to trauma and elective resections are difficult to control and might be life threatening due to location, size, sinusoidal structure, large surface area and excessive sanguination of the liver (2, 3). Manual compression, suture, tamponade with ribbon gauze, resection and packing and topical hemostatic agents are used to control bleeding in liver injuries. However, blood loss and related transfusion problems are still main causes for postoperative morbidity and mortality (4, 5).

Topical hemostatic agents are used to prevent or decrease complicated bleedings. There have been many experimental studies to develop topical hemostatic agents and to use them in different fields of medical practice. These topical hemostatic agents may be in the form of collagen, fibrin and gelatin etc. They usually employ a matrix, activate fibrin formation and form protocoagulant (6).

We attempted to perform an experimental study on effects of using Ankaferd Blood Stopper as a topical hemostatic agent to stop hemorrhage in liver injuries created in the rats on biochemical parameters, local irritation and intra-abdominal adhesions.

Materials and methods

Animal Study

The study was performed at the animal laboratory of School of Medicine in the Suleyman Demirel University after the Ethics Committee approval. 40 rats aging between 16–20 wks and with a body weight of 225–280 g. Animals were fed with standard rodent food and water. The rats were randomly divided into three groups: (1) Primary suture (PS) group consisting of 15 rats; (2) ABS group consisting of 15 rats; and (3) control group (used only for adhesion study) consisting of 10 rats. The experiments were conducted under half-sterile conditions. The rats starved for 24 h during both the pre- and post-operative periods. Anesthesia was induced with 75 mg/kg ketamine HCl and 5 mg/kg

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Tab. 1. Distributions of biochemical and Complete Blood Count measures by groups and days.

		Ankaferd		Primary suture		Control group		
		Mean±SD	Med.	Mean±SD	Med.	Mean±SD	Med.	p[a]
AST	Day 3	252.6±79.5	260.0	199.6±65.8	196.0	158.8±30.8	160.0	0.13
	Day 15	118.2±21.9	120.0	145.0±25.2	147.0	125.8±16.8	128.0	0.08
	p[b]	0.006[d]		0.042[c]		0.076		
ALT	Day 3	152.2±63.0	134.0	112.2±25.6	95.0	119.0±21.1	123.0	0.59
	Day 15	60.6±33.9	43.0	55.3±16.3	57.0	57.4±23.9	59.0	0.98
	p[b]	0.028[c]		0.006[d]		0.009[d]		
Total Bilirubin	Day 3	0.03±0.01	0.03	0.03±0.01	0.04	0.02±0.00	0.02	0.53
	Day 15	0.05±0.03	0.05	0.05±0.03	0.06	0.27±0.2	0.3	0.008**
	p[b]	0.355		0.100		0.008[d]		
Total Protein	Day 3	5.9±0.5	5.8	5.8±0.2	5.8	5.7±0.3	5.6	0.91
	Day 15	6.8±0.4	6.9	6.4±0.7	6.4	6.2±0.5	6.1	0.14
	p[b]	0.018[c]		0.028[c]		0.094		
ALP	Day 3	87.6±8.6	88.0	87.6±36.1	73.0	83.0±18.0	83.0	0.47
	Day 15	166.2±63.2	142.5	142.3±24.8	144.0	119.0±11.2	115.0	0.17
	p[b]	0.006[d]		0.028[c]		0.028[c]		
WBC	Day 3	5.0±2.0	4.5	7.0±3.1	6.5	7.7±3.0	7.3	0.39
	Day 15	6.0±1.8	6.3	7.5±2.3	7.8	8.7±2.4	7.8	0.19
	p[b]	0.599		0.754		0.599		
HB	Day 3	13.6±0.7	13.9	13.4±0.9	13.2	12.8±1.1	12.3	0.41
	Day 15	12.7±1.0	12.9	13.3±0.9	12.9	12.9±1.4	13.2	0.87
	p[b]	0.142		0.833		0.916		
HCT	Day 3	34.9±2.7	35.5	33.6±1.5	33.4	33.4±2.7	33.0	0.40
	Day 15	34.1±2.3	34.5	32.9±0.9	33.0	34.1±2.9	33.0	0.52
	p[b]	0.602		0.530		0.750		

[a]Kruskal Wallis test; [b]Mann Whitney U test; [c]$p<0.05$; [d]$p<0.01$.
SD=Standard Deviation; Med. = Median

xylazine HCl (Rompunr) IM. Then, hair on the abdominal wall was shaved and skin was cleaned by using Betadiner solution. A 3-cm median abdominal incision was made and a 1 cm-long triangular piece was resected from the inferior edge of the left liver lobe (approximately 4 % of the overall liver weight).

In the primary suture group, liver injury was repaired by horizontal matrix sutures (5/0 polypropylene). In the ABS group, ABS was drawn into syringes from 1 MI ampoules and sprayed onto bleeding areas (The sample of ABS (Ankaferd Blood Stopperr; patent number 2007-0-114485; Trend Teknoloji Ilac AS, Istanbul). After the administration of PS and ABS, test groups underwent chronometric measurements to determine bleeding times. Blood samples (1 ml) were taken from the inferior vena cava to determine CBC and blood chemistry values. On post-operative 3rd day, specimens were obtained with U shaped laparotomy incision from rats – 5 in each group – under anesthesia to evaluate effects of ABS on the liver tissue and surrounding tissues.

On the 15th post-operative day, blood samples were obtained from the vena cava inferior and U shaped laparotomy incision was made and liver areas coated with ABS and PS together with the left lobe of the liver were excised in the remaining ten rats in three groups. Then, the rats were sacrificed. Levels of AST, ALT and ALP, the liver enzymes indicating cellular damage, were measured.

Measurements of hydroxyproline levels

Hydroxyproline (HP) assay was made as defined by Jamall et al (7). Briefly, the frozen tissues were dried by filter paper, weighed and divided into tiny pieces, and were hydrolyzed in 6 N hydrochloric acid. The free HP was then oxidized by chloramine T to produce a pyrole-type compound. The addition of Ehrlich's reagent resulted in the formation of chromophore with a wave length of maximum at 558 nm. These producers yielded HP levels equivalent to nanomoles. These absolute measures were proportioned to tissue weights and the results were obtained as μmol/g tissue, the HP content.

Adhesion Study

Development of intra-abdominal adhesions was assessed qualitatively according to Bothin's description (8). The assessment was performed by someone independent from the authors.

Histopathologic examination

Liver tissues obtained from the rats were fixated in 10 % formaldehyde and examined microscopically. After a follow-up period, sections of 5 micron in thickness were obtained from paraffin embedded blocks of the specimens. They were examined under a light microscope. The recovering liver region was sampled. Scoring with light microscopy evaluation was based on the following criteria: 1 = necrosis, 2 = hemorrhage, 3 = cyto-

Tab. 2. Distribution of adhesion scores by groups and days.

	Day 3		Day 15		
	Mean±SD	Median	Mean±SD	Median	p[a]
Ankaferd	2.2±1.3	2	2.0±1.1	2	0.78
Primary suture (PS)	1.6±0.5	2	1.3±0.7	1	0.37
Control	0.0	0	0.2±0.4	0	0.27
p[b]	0.006		0.001		
Ankaferd vs PS	0.50		0.18		
Ankaferd vs Control	0.005		0.001		
PS-Control	0.005		0.006		

[a]Mann Whitney U test; [b]Kruskal Wallis test

plasmic vacuolization, 4 = multinuclear large cells, 5 = fibrovascular structures, 6 = inflammatory exudates. Hepatic regeneration was rated depending on the presence and severity of the parameters.

Scores ranging from 0 to 3 were interpreted as: 0 = absence of any of these parameters, 1 = slight levels, 2 = moderate levels and 3 = high levels. Obtained scores were used to calculate the total histopathological regeneration score as described by Tovar et al (9). High levels indicated that the regeneration had an immature character. Total histopathological regeneration scores were calculated for each group.

Statistical analysis

Data were analyzed with NCSS 2007 & PASS 2008 Statistical Software (Utah, USA) and descriptive statistics, Kruskal--Wallis test and Mann-Whitney U test. $p<0.05$ was considered significant.

Results

None of the rats lost weight during the experiment except in the first 3 days. There was no difference in weight loss between ABS and PS groups ($p>0.05$). There were no differences in eating habits, and vital signs between the groups. None of the rats died because of experimenting.

Bleeding times

The mean bleeding time was 7.5 sec shorter in ABS group (6.15–13.5) and 107 sec shorter in PS group (105 sec–115 sec) ($p=0.001$). ABS was used twice only in one rat to stop bleeding.

Biochemical measurements

AST, ALT, total bilirubin and ALP values were stable. CBC measures were not changed in all three groups on post-operative 3rd and 15th days (Tab. 1).

Measurement of liver tissue hydroxyproline levels

Hydroxyproline levels on post-operative 3rd day were 15.9 µg/tissue ± 0.6 in ABS group and 16.2 µg/tissue ± 3.6 in PS group with no significant difference ($p>0.95$). However, there was a significant difference in hydroxyproline levels on the post-operative 15th day between ABS and PS groups ($p<0.01$). In fact, hydroxyproline levels were 17.0 µg/tissue ± 0.7 in ABS group and 13.2 µg/tissue ± 1.4 in PS groups; ABS group had significantly higher hydroxyproline levels than PS group ($p=0.009$). In addition, while there was no significant difference between hydroxyproline levels on post-operative 3rd and 15th days in PS group ($p>0.05$), hydroxyproline levels on post-operative 15th day were significantly higher than those on post-operative 3rd in ABS group ($p\leq0.05$).

Adhesion scores

There were not any differences in adhesion scores between ABS and PS groups ($p>0.05$); however, both groups had higher adhesion scores than those of the control group ($p<0.01$). Adhesions were loose on post-op day 3, but dense on the day 15. Considering locations of the adhesions, there were more adhesions of the omentum to the target organ in ABS group. However, PS group had more adhesions of the target organ to other organs. There was no significant difference between adhesion scores on post-op day 3 and 15 in ABS and PS groups ($p>0.05$). In addition, there were more adhesive scars and abdominal scar adhesions in ABS group than PS group (Tab. 2).

Histopathological evaluation

Although the difference was not significant ($p>0.05$), ABS group had lower scores for necrosis, fibrosis, inflammation and degeneration than those of in the PS group ($p>0.05$). There was no difference in tissue regeneration between PS and ABS groups. ABS group had more favorable regeneration scores on the post-op day 3 ($p=0.005$) and day 15 ($p=0.015$) than those in the PS group. Necrosis and hemorrhage in the liver decreased on post-op day 3 and local regeneration was completed on post-op day 15. There were acute inflammatory process, inflammation and macrodegeneration in the hepatocytes in the ABS group on post-op day 3. Regeneration was completed in ABS group on post-op day 15. In addition, no hematoma, secondary bleeding signs or local acumination were observed on post-op day 15 (Figs 1 and 2). Specimens obtained from ABS treated local regions did not have any signs suggestive of degeneration and local irritation. As a result, there were no difference in histological features between ABS and PS groups, and a complete regeneration of the liver was achieved. However, there was inflammatory granulation tissue in the deep layers of dermis and focal abscess in PS group on post-op day 3.

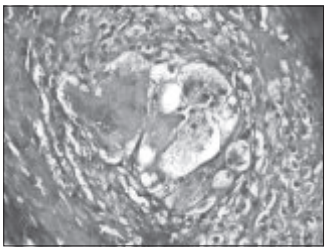

Fig. 1. Liver tissue with multinuclear histiocytic giant cells (H&Ex400).

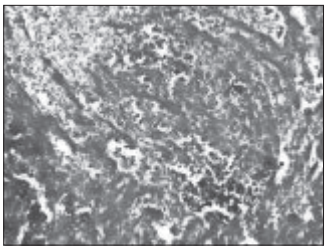

Fig. 2. Liver tissue with fibrinoleukocytic exudation (H&Ex200).

Discussion

A variety of topical hemostatic agents with various characteristics have been developed to stop bleeding especially in large rough body areas where it is difficult to access by mechanical techniques, and cauterization is not applicable (6).

Topical agents were applied in 60 % of hospitals in Japan during liver surgery, without standardized indication. However, very limited number of studies, which were deliberately focused on applications in hepatobiliary tract and liver, were identified (10). Thus, surgeons recommend topical hemostatic agents (11). Several factors, such as vascular features of the affected organ, open or laparoscopic surgical approach, these procedures being daily minor operations, bleeding severity, duration the hemostatic agent's response to bleeding, characteristics of application environment like in wars, emergency and elective surgical approaches are important in clinical practice. Additionally, the cost, applicability, presentation and storage characteristics of topical agents are also important (6, 11, 12). It has been reported that use of these agents has adverse effects (13–16). Therefore, there is an ongoing attempt to search for an ideal topical hemostatic agent (17, 18). Although there have been studies on various topical applications of ABS in clinical practice, there has not been a study on ABS in liver injuries at an experimental setting. Therefore, in the present study, we investigated effects of intra-abdominal use of ABS as a topical hemostatic agent following local excision of the liver. ABS is a composition of plants namely *Thymus vulgaris*, *Glycyrrhiza glabra*, *Vitis vinifera*, *Alpinia officinarum* and *Urtica dioica*. These plants have some specific effects on the endothelium, blood cells, angiogenesis, cellular proliferation, vascular dynamics and intercellular interaction. ABS can exert its effects on both fibrinogen and other proteins possibly through agglutination of these molecules. Blood proteins and blood cells play a role in the ABS-induced network formation (19—23).

There is also an interaction between ABS and fibrinogen as well as other blood proteins. These effects of ABS have been shown to appear without any effects on blood clothing factors levels (coagulation factors II, V, VII, VIII, IX, X, XI and XIII). This finding explains effects of ABS in cases of clothing factor deficiencies (24). There have been experimental studies on ABS use. *In vivo* studies on bleeding time and outcomes in ABS use have shown that ABS shortens bleeding time in cases of amputations treated with warfarin (25). In the present study, ABS significantly decreased bleeding time. Since it is a liquid, it is easy to use in liver injuries in clinical practice. We observed that ABS organized in seconds and occupied the bleeding areas. Consistent with the results of this study, ABS was absorbed in shorter time than other products, such as fibrin glue and cellulose (26).

Bilgili et al on a porcine model found that ABS led to hemostasis in deep skin lacerations and grade II spleen and liver injuries (27). In a major renal trauma model, ABS was found to be effective in stopping bleeding and that there was no inflammation, fibrosis and tissue damage (28). There have been several case reports about ABS use at the emergency situations in humans. ABS has been shown to achieve favorable results in bleedings due to hepatico-jejunostomy in the gastrointestinal canal (29), Dieulafoy's lesion (30), solitary rectal ulcer (31).

It has also been shown that, application of ABS and Surgicel were associated with significant reductions in the blood loss compared to controls, whereas with no significant differences between treatment groups (32). In fact, it took shorter time to stop bleeding and bleeding repeated less frequently in patients treated with ABS. Kurt et al reported that oral and rectal uses of ABS achieved stabilization of bleeding due to massive hematemesis following insertion of a plastic stent used for the treatment of the symptoms of Klatskin tumor (33). Although there have been studies about different uses of ABS, there is no study performed on hemostatic effects of ABS, effects of ABS on histopathological changes, CBC and biochemical parameters in liver injury, and features of adhesions caused by intra-abdominal use of ABS. This present study is the first one on this issue, and we have observed that ABS had no irritant effect or adhesion on macroscopic examination, whereas hydroxyproline levels in the liver and surrounding tissues were higher. We have not observed that intra-abdominal administration of ABS has caused increased fibrosis, granulomatous reaction, adhesion, abscess formation and fistulae due to its local irritant effects. This can be considered as strong evidence that ABS does not cause adhesions, and can be used to treat bleeding due to hepatic and abdominal organ injuries. Measurement of tissue hydroxyproline levels is important to evaluate effects of ABS on local healing. Hydroxyproline is a good indicator of tissue collagen levels. It has been used to determine healing capacity of sutured tissues and in experimental liver transplantation. In the present study, hydroxyproline levels in the liver tissue considerably increased in ABS group, though the increase was not significant. However, this finding may suggest that ABS can increase local healing (34, 35). In addition,

the finding that there was no difference in CBC, total protein, AST, ALT and ALP levels between the two groups supports the evidence that ABS does not have systemic side-effects (Tab. 1). In terms of adhesions, ABS group had lower scores for adhesions, though not significant. There was no significant difference in adhesion scores on post-op day 3 and on post-op day 15 in ABS group ($p>0.05$). Adhesion scores on post-op days 3 and 15 did not significantly differ in PS and control groups, either ($p>0.05$). Studies on effects of ABS on dermis and subdermis revealed no toxicity or local irritation. Consistent with the results of the prior studies, especially phase 1 studies of ABS, we found that ABS had a low side effect profile and did not cause any enzymatic changes. Histopathological examination did not show any local irritant effects of ABS. ABS stopped bleeding earlier than PS, and did not delay recovery. It caused fewer adhesions of the target organ to other organs than PS. In fact, ABS did not worsen the problem of adhesions. It did not create a picture of regeneration different from those previously reported. However, further experimental and clinical studies are needed to elucidate the intra-abdominal effects of ABS. The favorable hemostatic effects of ABS may justify its intra-abdominal use.

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