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RESEARCH ARTICLE

Efficacy of Ankaferd Blood Stopper in Bleeding Control in Experimental Partial Splenectomy Model

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Summary

Topical hemostatic agents are used as auxiliary agents for bleeding post trauma and during operation. Ankaferd Blood Stopper (ABS) is an hemostatic agent that is produced in Turkey. We planned to demonstrate the efficacy of this agent in experimental splenectomy model and to compare it with Fibrin Glue (Tisseel®) used in clinics. Forty Wistar Albino female rats were divided in 5 groups. Group 1, Sham; Group 2, Control; Group 3, Suture; Group 4, Fibrin Glue (Tisseel®) and Group 5, Ankaferd Blood Stopper. Partial splenectomy bleeding model was applied to rats after initial hematocrit values were measured. Bleeding in each group was controlled by a different method. Bleeding times of the rats were determined. All the rats were sacrificed on the 5th day after the last hematocrit values were measured. Existence of intraabdominal hematoma and adhesiveness were evaluated and splectonomy was applied for pathological examination. Groups were compared with respect to bleeding time and blood loss and Group 5 and Group 2 were determined to be more effective than Group 3 but Group 4 was determined to be equivalent. Fewer side effects at significant levels were detected in terms of fibrosis and coagulation necrosis during pathological assessment. In conclusion, in spleen bleedings when compared with fibrin glue, Ankaferd Blood Stopper is equally effective and safe and its price is lower.

Keywords: *Ankaferd Blood Stopper, Bleeding control, Fibrin Glue (Tisseel®), Partial splenectomy*

Deneysel Parsiyel Splenektomi Modelinde Kanama Kontrolünde Ankaferd Blood Stopper'ın Etkinliği

Özet

Topikal hemostatik ajanlar travma sonrası ve operasyon esnasındaki kanamaların durdurulmasına yardım amacıyla kullanılmaktadır. Ankaferd Blood Stopper (ABS) Türkiye'de üretilmiş bir hemostatik ajandır. Çalışmamızda deneysel splenektomi modelinde bu ajanın etkinliğinin ortaya konulması ve klinikte kullanılan Fibrin Glue (Tisseel®) ile karşılaştırılması planlandı. Bu çalışmada 40 adet Wistar Albino dişi sıçan kullanıldı. Randomizasyon yöntemi ile 5 eşit grup oluşturuldu. Grup 1, Sham; Grup 2, Kontrol; Grup 3, Dikiş; Grup 4, Fibrin Glue (Tisseel®) ve Grup 5, Ankaferd Bloob Stopper. Çalışmamızda sıçanlara başlangıç hematokrit değeri bakıldıktan sonra parsiyel splenektomi kanama modeli uygulandı. Her grupta farklı yöntem ile kanama kontrolü sağlandı. Gruplardaki tüm sıçanların kanama sürelerine bakıldı. Beşinci gün tüm sıçanlar son hematokrit değeri bakıldıktan sonra sakrifiye edildi. Batın içi hematom varlığı, yapışıklık değerlendirilerek patolojik inceleme için splenektomi uygulandı. Gruplar kanama süresi ve kan kaybı açısından karşılaştırıldı. Grup 5, Grup 2 ve Grup 3'e göre daha etkin, Grup 4 ile ise eşdeğer bulundu. Patolojik değerlendirmede fibrozis ve koagülasyon nekrozu açısından anlamlı düzeyde daha az yan etki saptandı. Sonuçta Ankaferd Blood Stopper'ın fibrin glue ile eşdeğer etkinlikte, güvenli ve daha düşük maliyetli olduğu sonucuna varıldı.

Anahtar sözcükler: *Ankaferd Blood Stoper, Kanama kontrolü, Fibrin Glue (Tisseel®), Parsiyel splenektomi*

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INTRODUCTION

The reason of many splenectomies nowadays is spleen bleeding formed as a result of elective spleen surgeries applied due to medical reasons and trauma [1]. Spleen blunt is the second most frequently injured organ in abdominal trauma and missed spleen injury is the most frequently preventable cause of death in patients with trauma [2]. In these operations, total splenectomy and post operation are significantly important surgical and medical problems. Therefore, studies related with partial preservation of spleen and control of splenic bleeding after trauma continues [3,4]. Especially topical hemostatic agents are used for this purpose frequently, Ankaferd Blood Stopper is a medicinal plant extract. The number of studies regarding this material is limited yet [5-7]. The object of this study is present the hemostatic efficacy of ABS in partial splenectomy model by comparing with the other hemostasis methods.

Mortality of nonoperative treatment of spleen injuries was approximately 100% at the beginning of 1900's. Thus splenectomy in splenic injuries was a treatment option recognized by everybody. This approach came into question when King and Schumacher informed that 5 children developed lethal infection after splenectomy [8].

Today many patients with solid organ injury may be treated without laparatomy by means of advanced visualization techniques [9]. When non operative treatments in solid organ injuries yielded better results than expected, emphasis was put on this treatment model. As postperative infection risk of splenectomy is high in splenic injuries, approaches preserving spleen come into prominence [10].

Many topical hemostatic agents are available at present (bovine collagen, bovine thrombin, autologous plasma, Fibrine Glue etc.) [3]. One must decide which one to use in the light of type, cost of procedure performed, severity of bleeding and personal experience.

Ankaferd Blood Stopper displays haemostatic effect through vital physiological erythrocyte aggregation in "Protein network" environment independent from classical coagulation cascade. This process substantiates in milli-seconds *in vitro* environment and in seconds *in vivo* environment [11,12]. Crucial proteomic components of the ABS-induced erythroid-protein network have been revealed by Demiralp et al.[13].

General haemostatic and biochemical tests have shown that the mentioned network was formed via the interaction between Ankaferd Blood Stopper and proteins in blood; but primarily via the products interaction with fibrinogen. The tests also showed that red spheres provide vital aggregation. At this stage, the process of holding hemorrahgage at a certain level to allow tissue repair is primarily related with protein erythrocyte interaction. Blood cells too take their part in the network. Independent of the physiological haemostatic process tissue factor related to blood coagulation, Ankaferd Blood Stopper, developes without effecting this system. Therefore Ankaferd Blood Stopper is not only effective on patients with normal haemostatic values, but also on those who are primary and secondary homestasis defective.

ABS is a standartized mixture of plants *Glycrrhiza glabra, Vitis vinifera, Urtica dioica, Thymus vulgaris, Alpinia officinarum* [14]. 100 ml product contains; *Urtica dioica* 6 mg, *Vitis vinifera* 8 mg, *Glycrrhiza glabra* 9 mg, *Alpinia officinarum* 7 mg, *Thymus vulgaris* 5 mg [15].

Ankaferd Blood Stopper provides hemostasis rapidly irrespective of clotting factor. It is determined that Ankaferd Blood Stopper is effective and safe in tooth bleeding of patients using hemophilic, diabetic, anti coagulant, antiagregant [11,12].

Ankaferd has no special storage conditions as Fibrin Glue. It is not prepared within a cold chain so it does not require any special process or equipment. It is also advantegous due to its low cost. It may be used for emergency cases because no preliminary preparation is needed.

The aim of the study was to present the advantages of Ankaferd Blood Stopper already used in ambulances and emergency services for external bleeding in partial splenectomy model with respect to efficacy, side effects and cost.

MATERIAL and METHODS

This study was conducted in Istanbul University DETAE laboratory, on rats taken from Istanbul University Experimental Medical Research Institution (DETAE), with the approval given by Istanbul University Animal Experiments Local Ethics Committee (Approval Number: 73/2009).

A total of 40 Wistar-Albino female rats were used in the study. 10 mg/kg intraperitoneal, one dose Ketamine HCl (Ketalar® vial, 50 mg/ml, Eczacibasi Medicine and Commerce A.C. Istanbul) and 5 mg/kg subcutaneous Xylazine HCl (Rompun® vial, 23.32 mg/ml, Bayer Turkish Chemistry Industry Ltd. C., Istanbul) anesthesia were administered to rats in an operating room. All operations on rats were done under anesthesia. During the operations all rats were treated humanistically according to Guide for the Care and Use of Laboratory Animals. 5 groups were formed with 8 rats in each with a randomization method [(Group 1, Sham; Group 2, Control; Group 3, Suture; Group 4, Fibrin Glue (Tisseel®, Eczacıbaşı) and Group 5, Ankaferd Blood Stopper® (Ankaferd Drug Inc., Istanbul-Turkey)].

The rats under anesthesia were weighed with precision

balance and weights were recorded. Initially blood was taken for hematoctit measurement near the tale vein of every rat with the help of 26 G angiocut (BD Neoflon®, Helsingborg, Sweden) into capillary tubes. Hairs of abdominal front wall of all rats were removed. 3 cm midline incision was done after surgical zone disinfection was perfomed with povidone iodine solution (Isosol®, Merkez Lab. İlaç San). Spleen was explored and 1cm partial splenectomy enclosing two end polar arteries in spleen lower pole, was applied to rats from all groups. Bleeding times of rats in Group 2 were measured without applying bleeding control. 4/0 rapid vicrylle omental patch was applied to rats in Group 3 for bleeding control and their bleeding times were measured. Preprepared Fibrin Glue solution was sprayed to rats in Group 4 via special double ject syringe to control bleeding and bleeding time and solution used until bleeding stopped were measured. Ankaferd Blood Stopper® fluid form was sprayed via syringe of insulin to rats in Group 5 to to control bleeding and bleeding time and solution used until bleeding stopped were measured. Laporotomy incisions of all rats were closed with continious 3/0 skin and intracutaneous skin closure was performed with 3/0 rapid vicryl. Then subcutaneous injection of 10 cc normal saline was administered to rats in all groups. No exitus was seen during study due to surgery and anaesthesia.

Laparotomy was administered to rats on the 5th day by abdomen front wall surgical area disinfection after blood was drawn under anesthesia from tail vein for control hematocrit measurement. Adhesiveness was evaluated quantitatively according to Bothin Scale [16]. Hematoma and collection were recorded in abdomen. Rats were sacrified by cutting Vena cava inferior. Spleens of all rats were taken out for histopathological examination. The scale developed was; 0: no finding; 1: mild severity; 2: moderate severity; 3: severe.

Pathological Examination: Tissue samples of spleen were fixed with 10% formalin. H&E stained preparations were evaluated with light microscope. It was examined with respect to degeneration of red pulp, extramedullary hemopoiesis, congestion, hemosiderin accumulation, necrosis, suppurative inflammation, fatty degeneration in pancreas, hypogranulation, coagulation necrosis and fibrosis. All pathological criteria were evaluated as; 0: none, 1: mild; 2 moderate; 3: severe. Total scores were printed.

Statistical Analysis: SPSS (Statistical Package for Social Sciences) for Windows 15.0 program was used for statistical analysis. Descriptive statistics were used for data evaluation and Kruskal Wallis test was used when comparing parameters among groups. Group differences were determined using Mann Whitney U test. Wilcoxon test was used for the recurrent measurements. Findings were considered significant at P<0.05 level.

RESULTS

There was no statistically significant difference in average weights of rats measured at the beginning and on the 5th day. The average weights of rats at the beginning and end are shown in *Table 1*.

There was no statistically significant difference in initial average hematocrit values of rats in all groups (P>0.05)**.** There was no statistically significant difference found between hematocrit values measured at the begining and on the 5th day of Group I (Sham), Group IV (Fibrin Glue) and Group V (Ankaferd) (P>0.05). We detected statistically significant difference between hematocrit values measured at the begining and on the 5th day of Group II (Control) and Group III (Suture) (P>0.05). Average hematocrit values of all groups are presented in *Table 2*.

The mean bleeding times of rats in the groups were measured as; Group 2: 68.875 sec, Group 3: 60.125 sec, Group 4: 11.5 sec, Group 5: 10.875 sec. (P=0.000). Bleeding times of Group 4 and Group 5 were determined to be statistically lower than Group 2 and Group 3 (P=0.000). No statistically significant difference was observed between Group 2 and Group 3 (P=0.234) and between Group 4 and Group 5 (P=0.879).

When evaluated in terms of cost, approximately 0.2 cc hemostatic agent was used per one rat in Group 4 and Group 5. While cost of ankaferd is 0.186 USD/rat in Group 5, cost of fibrin glue was calculated as 12.59 USD in Group 4.

Table 1. *Average weights of groups*
Tablo 1. *Grupların ortalama ağırlıkları*

Group	Outset * (Medium+/-Sd)/gram	5th Day ** (Medium+/-Sd)/gram
1	269.50+/-14.9	267.00+/-15.6
2	252.30+/-14.1	248.20+/-11.5
3	262.50+/-18.3	261.80+/-23.8
4	270.10+/-30.1	258.60+/-27.8
5	267.80+/-29.4	255.80+/-28.4

Sd: *Standard deviation, * P=0.500, ** P=0.551*

Table 2. *Average hematocrit values of groups*
Tablo 2. *Grupların ortalama hematokrit değerleri*

Group	Outset * (Medium+/-Sd)/gram	5th Day ** (Medium+/-Sd)/gram	P
1	46.3+/-1.6	45.7+/-1.9	>0.05
2	44.7+/-1.2	38.8+/-1.6	**<0.05**
3	45.2+/-2.0	40.7+/-1.9	**<0.05**
4	46.3+/-1.8	42.5+/-2.1	>0.05
5	45.3+/-1.4	41.6+/-1.4	>0.05

Sd: *Standard deviation, * P=0.500, ** P=0.551*

Existence of adhesion in Group 1 was found to be significantly lower than the other groups (p=0.01). When total adhesion scores were compared adhesion in Group 3 was determined to be significantly higher than the orher groups (P=0.00). Assessment of adhesion forms of groups are presented in *Table 3*.

Histopathological Findings: Expansion in red pulp in all groups was significantly higher than sham group (P=0.001) but congestion and hemosiderin was significantly lower in Sham group (P=0.001). Similarly necrosis, suppurative inflammation, fatty degeneration in pancreas, and hypo-granulation was significantly lower in sham group than the other groups (P<0.05). We could not determine any statistically significant difference between groups with respect to extramedullery hemopoiesis (P=0.110). In accordance with these findings inflammation was determined to be significantly higher in Group 3, Group 4 and Group 5 when compared with Group 1 and Group 2 (P<0.05). No significant difference in terms of inflammation was determined between Group 4 and Group 5 (P>0.05). We could not determine statistically significant difference between Group 4 and Group 5 with respect to histopathology (P>0.05). Histopathological findings of groups are presented in *Table 4*.

When severity of coagulation necrosis examined, focal and widespread coagulation necrosis was observed in all groups except Group 1. Altough incidence of focal necrosis in Group 5 was higher no statistically significant difference was observed (P>0.05). Incidence of widespread coagulation necrosis in Group 2 and Group 4 was significantly higher than Group 5 (P<0.05). Evaluation of severity of coagulation necrosis of groups is presented in *Table 5*.

When severity of fibrosis of groups were evaluated, altough focal fibrosis frequency in Group 2 and Group 5 was more than the others, no statistically significant difference was determined (P>0.05). Altough incidence of widespread fibrosis in Group 4 was the highest, it was only determined to be statistically higher than Group 2 and Group 5 (P<0.05). Evaluation results of fibrosis severity of groups is presented in *Table 6*.

Table 3. *Assessment of adhesion forms of groups (%)*
Tablo 3. *Grupların yapışıklık biçimlerinin değerlendirilmesi*

Adhesion Zone	Group 1	Group 2	Group 3	Group 4	Group 5	P
Adhesion existence	50	100	100	100	100	0.01
Between omentum and target organ	0	100	62.5	87.5	62.5	0.00
Omentum abdominal scar	50	25	12.5	25	12.5	0.40
Between omentum and other sites	0	0	62.5	25	25	0.01
From adnexa to target organ	0	0	0	50	12.5	0.08
Adnexa abdominal scar	0	0	0	0	12.5	0.30
From adnexa to other places	0	0	0	0	0	
Adhesive tape between any two organs	0	0	0	0	0	
Target organ abdominal scar	0	0	0	0	0	
Target organ abdominal wall	0	0	0	0	0	
Target organ intestine	0	0	0	12.5	25	0.42
Target organ liver	0	12.5	75	0	37.5	0.02
Adhesion in any other organ	0	50	62.5	25	75	0.01
Total Adhesion Score (Avr±Std)	0.8+/-0.7	2.7+/-0.7	3.8+/-0.6	3.1+/-0.9	3.2+/-0.8	0.00

Table 4. *Histopathological evaluation of groups (mean score)*
Tablo 4. *Grupların histopatolojik değerlendirilmesi (ortalama skor)*

Histopathological Evaluation	Group 1	Group 2	Group 3	Group 4	Group 5	P
Expansion in red pulp	1.4	2.0	2.0	2.4	2.4	0.001
Congestion	1.3	2.5	2.5	2.5	2.8	0.001
Hemosiderin	1.6	2.3	2.9	2.8	2.6	0.001
Extramedullery hemopoiesis	1.1	1.4	1.4	1.1	1.0	0.110
Necrosis	0	0.3	0.9	2.0	1.9	0.003
Suppurative inflammation	0	0	0.6	0.6	0.5	0.022
Fatty degeneration in pancreas	0	0.8	0.3	0	0.6	0.048
Lipogranulation	0	0.9	2.0	2.3	2.0	0.000

Table 5. *Severity of coagulation necrosis of groups (%)*
Tablo 5. *Grupların koagülasyon nekrozu şiddeti (%)*

Group	Coagulation Necrosis (%)		
	(-)	Focal	Generalize
1	100	0	0
2	0	62.5	37.5
3	0	75	25
4	0	62.5	37.5
5	0	100	0
P=0.01			

Table 6. *Evaluation of severity of fibrosis in groups (%)*
Tablo 6. *Grupların fibrozis şiddetinin değerlendirilmesi (%)*

Group	Coagulation Necrosis (%)		
	(-)	Focal	Generalize
1	100	0	0
2	0	100	0
3	0	75	25
4	0	62.5	37.5
5	0	100	0
Total	20	67.5	12.5
P=0.01			

DISCUSSION

Splenectomy was applied as an easy and safe method for spleen injury for many years. However developments within the last 30 years have limited the position of splenectomy in spleen traumas. General state of the patient and severity of injury, influence the selection of treatment approach.

One of the alternatives for total splenectomy is surgery preserving spleen. Some researchers state that success rate of spleen preserving approaches in blunt and penetrant originated spleen traumas does not decrease below 50% [17].

Canturk et al.[18] confirmed hematocrit was one of the factors for determining prognosis in patients with intraabdominal bleeding. Therefore, in our study the change in hematocrit value was used as a parameter for efficiency rating.

Many methods were tried for preventing spleen p*arenchymal bleeding*. There is also hemostatic material application among these methods. Fibrin Glue is one of the topical agents used frequently. Kuzu et al.[11] used autologous Fibrin Glue in partial splenectomy model and determined that it was effetive. Karakaya et al.[12] found out Ankaferd decreased bleeding amount and time in experimantal liver laceration. Similar results with two studies were obtained in our study.

Crucial proteomic components of the ABS-induced erythroid-protein network have been revealed by Demiralp et al.[13]. Essential erythroid proteins (spectrin alpha, actin-depolymerization factor, NADH dehydrogenase [ubiquinone] 1 alpha subcomplex, mitochondrial NADP [+] dependent malic enzyme) and the required adenosine triphosphate (ATP) bioenergy are included in the protein library of ABS. While it stops bleeding, ABS forms a strong network very quickly. ABS also up-regulates the GATA/FOG transcription system affecting erythroid functions [13,15]. Additionally, ABS affects the levels of various critical transcription factors active in the erythrocyte protein profile [15].

ABS also mediates endothelial cells. ABS has dual diverse dynamic reversible actions on EPCR and PAI-1 inside the vascular endothelial cells. Based on these findings, sudden anti-hemorrhagic efficacy of ABS has been attributed to its immediate enhancement of expression of pro-hemostatic PAI-1 and down-regulated anti-coagulant EPCR, resulting in the unique hemostatic effects of ABS [19]. That mechanism could also be important to elucidate ABS effects in a given tissue. Karabıyık et al.[19] presented in this study, the effect of Ankaferd, related to dose and time, on EPCR and PAI-1. It has been shown that Ankaferd exerts a similar effect on cells after LPS treatment and it becomes more effective as dose increases and it loses its effect by time.

There are experimental studies in literature, showing the effects of ABS on arterial wall and tissue. In the experimental arterial model developed by Ulus et al.[20] they showed that topical ABS treatment decreased bleeding time and bleeding amount at normal and high arterial pressures. In the experimental epistaxis model developed by Iynen et al.[21] they determined that topical ABS ensures more effective homeostasis than saline irrigation and shortens the bleeding time.

Partial splenectomy model was selected for the efficacy of topical hemostatic agent in our study. No study was found which examined efficacy of Ankaferd in partial splenectomy model previously. Efficacy of ankaferd in bleeding was evaluated by developing an experimantal partial splenectomy model. The effect of ankaferd on intra-abdominal adhesion and spleen histology were evaluated also.

When hematocrit values obtained from each rat before operation and after operation were examined, it was determined that blood loss of fibrin glue and Ankaferd groups were statistically lower than the control and suture groups not using hemostatic agent. However when Fibrin Glue and Ankaferd groups were compared, there was no significant difference determined. The findings obtained from our study showed that Ankaferd provided efficient hemostasis and at the same time this efficacy was strong as Fibrin Glue. When bleeding times were evaluated it was detemined that Ankaferd was as effective as fibrin glue and that bleeding times were significantly lower than

control and suture groups.

When the side effects (intraabdominal adhesiveness, coagulation necrosis and fibrosis) of hemostatic methods used in our study were considered, intraabdominal adhesiveness in Sham group was significantly lower than the other groups because only laparatomy was done in Sham group. While adhesion was significantly higher in Suture group when compared among the group forming bleeding model we could not determine any significant difference between Ankaferd, Fibrin Glue and Control groups. These findings asserted that Ankaferd had no serious side effects. Similarly all the pathological variances on tissue was rarer as expected in Sham group and we only detected that widespread coagulation necrosis and widespread fibrosis was significantly higher in Fibrin glue group among bleeding model groups. When these are considered, we proved side effect potential of Ankaferd is lower than the potential of Fibrin Glue ($P<0.01$).

In summary, ABS is as efficient as Fibrin Glue, with respect to bleeding control and more advantageous, with respect to side effect potential and cost. Besides it is east to use in case of emergecy. Thus, ABS can be used sefely in bleeding control.

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