In vivo hemostatic effect of the medicinal plant extract Ankaferd Blood Stopper® in rats pretreated with warfarin

Handan S. Cipil[1], Ali Kosar[1], Arif Kaya[1], Burak Uz[1], Ibrahim C. Haznedaroglu[2], Hakan Goker[2], Oktay Ozdemir[3], Mustafa Koroglu[1], Serafettin Kirazli[4], and Huseyin Cahit Firat[4]

[1]Departments of Hematology and Surgery, Faculty of Medicine, University of Fatih, Ankara/Turkey

[2] Department of Hematology, Faculty of Medicine, University of Hacettepe, Ankara/Turkey

[3] Department of Scientific Research, Yorum Konsultancy Inc., Istanbul/Turkey

[4] Department of Research&Development, Ankaferd Drug Inc., Istanbul/Turkey

Corresponding Author:

Ibrahim C. Haznedaroglu, MD

Department of Hematology,

Hacettepe University Medical School,

Sihhiye/ Ankara TR-06100

Turkey

e-mail: ichaznedaroglu@gmail.com

Running title: Hemostatic effects of *Ankaferd* BloodStopper®

Abstract

Aim: *Ankaferd* comprises a standardized mixture of plants *Thymus vulgaris, Glycyrrhiza glabra, Vitis vinifera, Alpinia officinarum* and *Urtica dioica.* Ankaferd Blood Stopper® (ABS) as a medicinal product has been approved in the management of external hemorrhage and dental surgery bleedings in Turkey. This study aimed to evaluate the *in vivo* hemostatic effect of ABS in rats pretreated with warfarin. **Materials and methods:** Wistar rats (210-270 g) of both sexes were used in this study. The rats were treated either with warfarin (2 mg/kg) or vehicle (0.9% NaCl) orally for 4 consecutive days before bilateral hind leg amputation. ABS was administered topically (a total of 4 mL [1 mL/puff x 4]) to one of the amputed legs. The duration of bleeding and the amount of bleeding were measured in order to evaluate the hemostatic effect of ABS. **Results:** Topical ABS administration to amputed leg shortened the duration of bleeding markedly in both untreated and warfarin-treated rats by 31.9% [1.42 min (95%CI: 0.35-2.49)] and 43.5% [5.12 min (95%CI: 2.16-8.07)] respectively. While the amount of bleeding in ABS-administered amputed leg showed a decrease by 53.8% in warfarin-treated group, the difference in the amount of bleeding was not significant in untreated group. **Conclusions:** ABS, a traditional folkloric medicinal plant extract, has *in vivo* hemostatic actions which may provide a therapeutic potential for the management of patients with deficient primary hemostasis in the clinical medicine.

Key words: *Ankaferd*; warfarin; bleeding; hemostasis; *in vivo*; rats

Introduction

Ankaferd is a medicinal plant extract, which has previously been used in Turkish traditional medicine as hemostatic agent (1). *Ankaferd* comprises a standardized mixture of plants *Thymus vulgaris, Glycyrrhiza glabra, Vitis vinifera, Alpinia officinarum* and *Urtica dioica*. Ankaferd Blood Stopper® (ABS) as a medicinal product has been approved in the management of external hemorrhage and dental surgery bleedings in Turkey (www.ankaferd.com). The safety and efficacy reports on the product have indicated its sterility and non-toxicity.

A very recent *in vitro* study by Goker et al. (1) has shown that exposure of ABS resulted in a very rapid formation of network within the plasma and serum. Routine hemostatis and biochemical tests revealed that the network formation due to ABS depended upon the interactions of the substance with the blood proteins, mainly fibrinogen and indicated that ABS could affect both fibrinogen and other proteins possibly via agglutination of these molecules. The network of ABS might cover the entire physiological hemostatic process without affecting any individual clotting factor (1). Thus, this unique mechanism of action provides ABS with the advantage over other hemostatically-active plant extracts and might, therefore, be effective both in subjects with normal hemostatic parameters and in those with primary and/or secondary hemostatic disorders.

In the light of the above-mentioned data, this study aimed to investigate the *in vivo* hemostatic effect of ABS in rats. Additionally, the hemostatic effect of ABS was also evaluated in rats which were pretreated with warfarin.

Materials and Methods

Animals

Fourteen Wistar albino rats (average weight 240±30 g) of both sexes were used in this study. The animals were kept in a room at a constant temperature of 22±1℃ with a 12 hours light and 12 hours darkness cycle and fed standard pellet chow and water which were available *ad libitum*. All experiments were carried out in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Fatih University Medical School Ethics Committee.

Experimental design

The rats were pre-treated with either warfarin dissolved in saline (2 mg/kg) or vehicle (0.9% NaCl) orally by a feeding catheter custom-made of silver for 4 consecutive days before bilateral hind leg amputation (see Table 1). Each group (warfarin pretreated group and control group) consisted of 7 animals.

Beforehand all operations, a list of randomization was developed by using RAND() function of Microsoft® Excel. RAND() function generates random real numbers between 0 and 1. A table of seven rows including seven cells, each representing an individual rat, was filled with RAND() function. For the rats, of which numbers generated were lower than 0.50, ABS was planned to be applied to right leg and saline to left leg. On the other hand, if the number generated was equal of higher than 0.50, then ABS would be applied to left leg and saline to right leg for those rats.

On the fifth day, the animals were anesthetized with ketamine (80 mg/kg). Both hind legs of the animals were prewarmed for 5 min in 10 mL of saline at 37°C in a water bath. Then, both legs were lifted from the saline and 5 mm leg segments amputed above the knees.

For each animal, one of the amputed legs was treated with topical ABS (a total of 4 mL [1 mL/puff x 4]) (Ankaferd Drug Inc, Istanbul, Turkey). The other leg was treated with the same volume (4 mL) of vehicle (0.9% NaCl) topically and served as the control.

Both ABS and saline were prepared in similar-looking dark-colored spray bottles before by a staff who worked in neither surgical operations, nor the evaluation of bleeding. The researchers and their assistants who had applied the study medication to the cut limbs and

who followed and evaluated the bleeding were not aware of the contents of the medication they applied, i.e. they work blinded to the medication they used.

Bleeding assay

Study parameters were duration of bleeding and amount of bleeding. Duration of bleeding was defined as the time passed after the start of bleeding (i.e. amputation or tail-cut) to cessation of bleeding. Bleeding start and stop times were measured by using a chronometer.

The amount of bleeding was measured by means of a blotting paper. The blood was collected on blotting paper, which was weighed before and after the procedure on a 0.1 g accurate scale. The difference in the weight of the blotting paper before and after the procedure indicated the amount of bleeding.

Statistical analysis

The data were presented as mean and 95% confidence limits. Median and interquartile ranges (IQR) were also given. Besides measured values of the duration of bleeding and the amount of bleeding, absolute differences and percent differences between right and left legs were calculated for each rat separately. These figures correspond to absolute and percent shortening of duration and lessening of amount of bleeding with ABS as compared to saline.

Since ABS and control groups were paired (limbs of same animals), statistical analyses appropriate for dependent samples were used. In the initial analysis, the data in each group was analyzed separately with Wilcoxon test, and also with paired Student's t test, in order to test the robustness of data with regards to parametric assumptions. Since two comparisons ((1) ABS vs control in warfarin-treated group and (2) ABS vs control in untreated group) were performed instead of a single comparison for testing the primary hypothesis of the study "whether the effect of ABS is significantly better than saline (*whatever the subjects are pretreated with*)", the type I error level was adjusted downward to 0.025 (0.05 divided by two) for the results of Wilcoxon tests and paired Student's t tests. Therefore *P* values less than 0.025 should be regarded as significant for these tests.

In addition to the univariate tests defined above, a repeated measures analysis of variance (RM-ANOVA) model was built. In the model, which was fully saturated, the between-subjects factor was the type of pretreatment (warfarin vs saline) and the within-

subjects factor was the type of solution applied to the bleeding limb (ABS vs saline). The dependent variables were the duration of bleeding and the amount of bleeding for the first and second models, respectively. The overall effects of ABS and warfarin and their interaction were analyzed by RM-ANOVA with Greenhouse-Geisser test. Overall effect of ABS correspond to effect of ABS independent of the type of pretreatment (whether warfarin-treated or not) and overall effect of warfarin correspond to effect of warfarin, whether treated with ABS or not). If ABS-warfarin interaction term is significant ($P<0.05$), it means that the effect of ABS is not similar between warfarin-treated and untreated groups. P values less than 0.05 were regarded as significant for RM-ANOVA.

All analyses were done using SPSS version 9 statistical analysis package.

Results

Duration of bleeding

In untreated group, the duration of bleeding following amputation of hind legs was shortened by 1.42 min (95%CI: 0.35-2.49) with ABS administration from 4.21 min (95%CI: 3.56-4.86) in the saline administered control subgroup to 2.79 min (95%CI: 1.97-3.61) (*P*=0.028). ABS shortened the duration of bleeding by 31.9% (95%CI: 7.9-55.8) in untreated group (Table 2, Figures 1&2).

In warfarin-treated group, duration of bleeding following amputation of hind legs was shortened by 5.12 min (95%CI: 2.16-8.07) with ABS administration from 12.05 min (95%CI: 10.44-13.67) in the saline administered control subgroup to 6.94 min (95%CI: 3.33-10.54) (*P*=0.018). ABS shortened the duration of bleeding by 43.5% (95%CI: 17.1-69.9) in warfarin-treated group (Table 2, Figures 1&2).

Analysis of variance revealed that the difference between ABS and saline subgroups (ABS effect) as well as the difference between warfarin-treated and untreated groups (warfarin effect) were both significant (ABS effect: F=25.957, *P*<0.001; warfarin effect: F=36.433, *P*<0.001). Moreover, it was established that the effect of ABS was not similar in the warfarin-treated and untreated groups (ABS-warfarin interaction: F=8.295, *P*=0.014). ABS is significantly more effective in shortening duration of bleeding as compared to control and its efficacy gets more pronounced in the warfarin-treated group than untreated group (Table 3).

Amount of bleeding

In untreated group, the amount of bleeding following amputation of hind legs was not different between ABS and saline groups (2.55 mL (95%CI: 1.62-3.48) and 3.24 mL (2.41-4.06, respectively) (*P*=0.25) (Table 2, Figures 1&2).

In warfarin-treated group, amount of bleeding following amputation of hind legs was decreased by 1.96 mL (95%CI: 0.63-3.29) with ABS administration from 3.60 mL (95%CI: 2.35-4.85) in the saline administered control subgroup to 1.64 mL (95%CI: 0.74-2.53) (*P*=0.018). ABS decreased the amount of bleeding by 53.8% (95%CI: 26.0-81.6) in warfarin-treated group (Table 2, Figures 1&2).

Analysis of variance revealed that while the difference between ABS and saline subgroups (ABS effect) was significant (ABS effect: F=14.488, *P*=0.003), the difference

between warfarin-treated and untreated groups (warfarin effect) was non-significant (warfarin effect: F=0.369, *P*=0.56). It was seen that the effect of ABS was similar in the warfarin-treated and untreated groups (ABS-warfarin interaction: F=3.395, *P*=0.09). In other words, ABS is significantly more effective in decreasing the amount of bleeding as compared to control, and its efficacy is similar in the warfarin-treated and untreated groups (Table 3).

Discussion

This *in vivo* study on rats demonstrated that topically administered ABS has a hemostatic effect on rats alone or in the presence of warfarin effect. Thus, the present data support the recent *in vitro* findings demonstrating the beneficial effect of ABS on hemostatic parameters (1). Taken together, ABS seems to be a promising therapeutic agent for the management of clinically evident coagulation disorders.

The previous i*n vitro* study clearly demonstrated that addition of ABS to plasma did not affect the individual coagulation factors II, V, VII, VIII, IX, X, XI and XIII (1). It decreased plasma fibrinogen activity concomitant with prolongation of the thrombin time (1). Additionally, total protein, albumin, and globulin levels showed significant decreases after addition of ABS. Thus, these results implied that the basic mechanism of ABS is the formation of an encapsulated protein network which provides focal points for aggregation of red blood cells. Since ABS seems to provide a protein-driven agglutination without affecting the individual coagulation factors, it is advantageous over other plant extracts with hemostatic activity.

In the present study, we observed that ABS was beneficial as a topical hemostatic agent in bleeding rats. ABS was found effective in shortening the duration of bleeding and decreasing the bleeding volume in amputed legs. Interestingly, ABS showed its hemostatic action not only in untreated animals, but also in the animals pretreated with warfarin.

Warfarin inhibits coagulation via different mechanisms. It inhibits the vitamin K-dependent synthesis of biologically active forms of calcium-dependent clotting factors II, VII, IX and X, as well as the regulatory factors protein C, protein S, and protein Z.

The data of the present study showed ABS was also effective in modulating hemostasis in rats pretreated with warfarin implying that application of ABS topically overcomes the actions of warfarin given systemically. The beneficial action of ABS did not seem to be attenuated in animals which pretreated with warfarin, as confirmed by statistical analysis. Although the previous *in vitro* study showed that ABS did not affect any of the individual clotting factors, we can not exclude this possibility under *in vivo* conditions. The duration of bleeding is known as an indicator of the effectiveness of platelet-thrombus formation and therefore, a prolonged duration of bleeding may show the presence of severe thrombocytopenia, platelet dysfunction syndromes, vascular defects and/or mixed abnormalities such as von Willebrand’s disease.

In the present study, shortening of the duration of bleeding by topical ABS suggests that the extract shows its hemostatic effect at least partly via modulation of the platelet functions. Other possible mechanisms of its action need further elucidation. Moreover, a very recent clinical case by Kurt et al (2) presented a 52 years old man who had undergone resection with the diagnosis of distal cholangiocarcinoma. He had signs of recent bleedings upon upper gastrointestinal endoscopic examination. Topical ABS (15 mL) showed effective control of bleeding from the biopsy site. A repeated endoscopy during the follow-up did not reveal any stigmata of bleeding as well.

Ankaferd Blood Stopper comprises a standardized mixture of five plants each having some hematological and vascular actions (3-8). *Glycyrrhiza glabra* has anti-inflammatory, anti-thrombin, anti-platelet, anti-oxidant, anti-atherosclerotic, and anti-tumor activities (6). It inhibits angiogenesis, decreases vascular endothelial growth factor production and cytokine-induced neovascularization (6). *Thymus vulgaris* has anti-oxidative actions, such as prevention of lipid peroxidation (4). *Vitis vinifera* exerts anti-tumor and anti-atherosclerotic effects (9, 10). *Alpinia officinarum* inhibits nitric oxide production by lipopolysaccharide-activated mouse peritoneal macrophages (3). *Urtica dioica* causes vasodilation via inducing nitric oxide production by endothelium (5). Thus, the mechanism(s) underlying the hemostatic control by ABS require further investigation.

As a conclusion, ABS, a traditional folkloric medicinal plant extract, may provide a therapeutic potential for the management of patients with deficient primary hemostasis in the clinical medicine with its *in vivo* hemostatic actions. In vitro data on the anti-infectivity of Ankaferd (11) and preliminary successful applications in mediastinal bleedings associated with cardiac surgery (12) represent novel clues for Ankaferd activity.

References

1. Goker H, Haznedaroglu IC, Ercetin S, et al. Haemostatic actions of the folkloric medicinal plant extract, Ankaferd blood stopper. J Int Med Res 2008; 36: 163-170.
2. Kurt M, Disibeyaz S, Akdogan M, Sasmaz N, Aksu S, Haznedaroglu IC. Endoscopic application of Anfaferd Blood Stopper as a novel experimental treatment modality for upper gastrointestinal bleeding: A case report. Am J Gastroenterol 2008 (In Press).
3. Matsuda H, Ando S, Morikawa T et al. Inhibitors from the rhizomes of Alpinia officinarum on production of nitric oxide in lipopolysaccharide-activated macrophages and the structural requirements of diarrylheptanoids for the activity. Bioorg Med Chem 2006; 14: 138-142.
4. Lee SJ, Umano K, Shibamoto T, et al. Identification of volatile components in basil (Ocimum basilicum L.) and thyme leaves (Thymus vulgaris L.) and their antioxidant properties. Food Chem 2007; 91: 131-137.
5. Testai L, Chericoni S, Calderone V, et al. Cardiovascular effects of Urtica dioica L. (Urticaceae) roots extracts: in vitro and in vivo pharmacological studies. J Ethnophramacol 2002; 81: 105-109.
6. Sheela ML, Ramakrishna MK, Salimath BP. Angiogenic and proliferative effects ıf the cytokine VEGF in Ehrlich ascites tumor cells is inhibited by Glycyrrhiza glabra. Int Immunopharmacol 2006; 6: 494-498.
7. Barka EA, Belarbi A, Hachet C, et al. Enhancement of in vitro growth and resistance to gray mould of Vitis vinifera co-cultured with plant growth-promoting rhizobacteria. FEMS Microbiol Lett 2000; 186: 91-95.
8. Barka E, Gognies S, Nowak J, et al. Inhibitory effect of endophyte bacteria on Botrytis cinerea and its influence to promote the grapevine growth. Biol Control 2002; 24: 135-142.
9. Zhao J, Wang J, Chen Y, et al. Anti-tumor-promoting activity of a polyphenolic fraction isolated from grape seeds in the mouse skin two-stage initiation-promotion protocol and identification of procyanidin B5-3’-gallate as the most effective antioxidant constituent. Carcinogenesid 1999; 20: 1737-1745.

10. Yamakoshi J, Kataoka S, Koga T, et al. Proanthocyanidin-rich extract from grape seede attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits. Atherosclerosis 1999; 142: 139-149.
11. Akkoc N, Akcelik M, Haznedaroglu I, Goker H, Aksu S, Kirazli S, Firat HC. In vitro anti-bacterial activities of Ankaferd blood stopper. Int J Lab Hematol. 2008;30:95.
12. Dogan OF, Ozyurda U, Uymaz OK, Ercetin S, Haznedaroglu IC. New anticoagulant agent for CABG surgery. Eur J Clin Invest. 2008;38:341.

Legends to the figures

Figure 1. (a) The effect of topically administered Ankaferd BloodStopper (ABS; 4 mL) on the duration of bleeding of amputed leg of rats pretreated with warfarin (2 mg/kg orally) or vehicle (0.9% NaCl orally) for 4 days. **(b)** The effect of topically administered Ankaferd BloodStopper (ABS; 4 mL) on the amount of bleeding of amputed leg of rats pretreated with warfarin (2 mg/kg orally) or vehicle (0.9% NaCl orally) for 4 days. *Each group consisted of 7 animals. Error bars correspond to 95%CI. *P <0.05 vs. control.*

Figure 2. The absolute and percent differences of the duration of bleeding and the amount of bleeding of amputed leg of rats, between topically administered Ankaferd BloodStopper (ABS; 4 mL) and saline in rats pretreated with warfarin (2 mg/kg orally) or vehicle (0.9% NaCl orally) for 4 days. *Each group consisted of 7 animals. Error bars correspond to 95%CI. *P <0.05 vs. control.*

Table 1. Description of study groups.

Group	Medication	Daily dose (mg/kg)	Route of admin.	Duration	Solution applied to bleeding area
Warfarin-treated group	Warfarin	2	PO	4 days	ABS for one limb and 0.9% NaCl for the other
Untreated group	Saline	-	-	4 days	ABS for one limb and 0.9% NaCl for the other

Table 2. The effect of topically administered Ankaferd BloodStopper (ABS; 4 mL) on the duration of bleeding of amputed legs of rats pretreated with warfarin (2 mg/kg orally) or vehicle (0.9% NaCl orally) for 4 days. *Data are given as mean (95% confidence interval) and median with IQR.*

	ABS	Control	ABS vs Control	ABS vs Control (%)	Statistics*	
Duration of bleeding (min)						
Warfarin-treated	6.94 (3.33-10.54) (Med: 7.55, IQR: 7.00)	12.05 (10.44-13.67) (Med: 12.10, IQR: 3.10)	5.12 (2.16-8.07) (Med: 4.30, IQR: 6.00)	43.5 (17.1-69.9) (Med: 30.1, IQR: 53.9)	Z=2.366 *P*=0.018[a]	t=4.241 *P*=0.005[b]
Untreated	2.79 (1.97-3.61) (Med: 2.30, IQR: 1.51)	4.21 (3.56-4.86) (Med: 4.20, IQR: 0.75)	1.42 (0.35-2.49) (Med: 1.90, IQR: 1.60)	31.9 (7.9-55.8) (Med: 47.0, IQR: 41.5)	Z=2.197 *P*=0.028[a]	t=3.250 *P*=0.017[b]
Amount of bleeding (mL)						
Warfarin-treated	1.64 (0.74-2.53) (Med: 1.90, IQR: 1.75)	3.60 (2.35-4.85) (Med: 3.50, IQR: 1.50)	1.96 (0.63-3.29) (Med: 1.80, IQR: 2.00)	53.8 (26.0-81.6) (Med: 45.3, IQR: 50.7)	Z=2.366 *P*=0.018[a]	t=3.613 *P*=0.011[b]
Untreated	2.55 (1.62-3.48) (Med: 22.0, IQR: 1.71)	3.24 (2.41-4.06) (Med: 3.40, IQR: 1.60)	0.68 (<0.00-1.74) (Med: 0.10, IQR: 2.40)	17.2 (<0.0-45.2) (Med: 2.4, IQR: 58.9)	Z=1.153 *P*=0.25[a]	t=1.574 *P*=0.17[b]

[a] *Wilcoxon test,* [b]*Student's paired t test, IQR: Interquartile range*

** Statistical significance is set as p values less than 0.025, due to multiple pairwise comparisons (see Statistical Analysis sub-section in Materials and Methods section)*

Table 3. The results of repeated measures ANOVA with Ankaferd BloodStopper effect and warfarin effect as independent factors and duration of bleeding and the amount of bleeding as dependent variables.

	Duration of bleeding		**Amount of bleeding**	
Term in ANOVA	**F**[a]	***P***	**F**	***P***
ABS effect (ABS vs control)	25.957	0.001	14.488	0.003
Warfarin effect (warfarin-treated vs untreated)	36.344	0.001	0.369	0.56
ABS vs warfarin interaction	8.295	0.014	3.395	0.09

[a] *Repeated measures analysis of variance, Greenhouse-Geisser test*

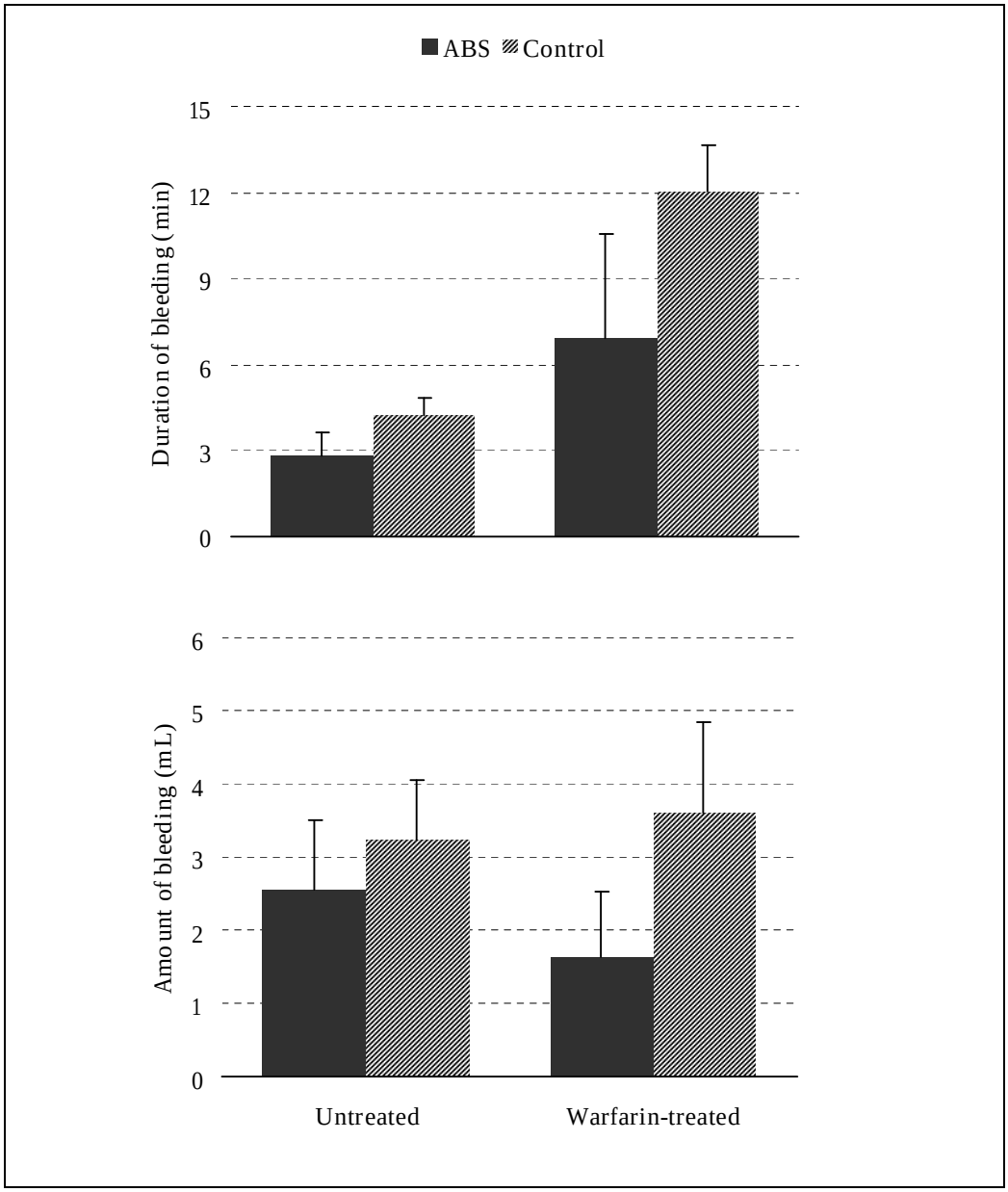
ABS
Control
Duration of bleeding (min)
15
12
9
6
3
0
Amount of bleeding (mL)
6
5
4
3
2
1
0
Untreated
Warfarin-treated

Figure 1.

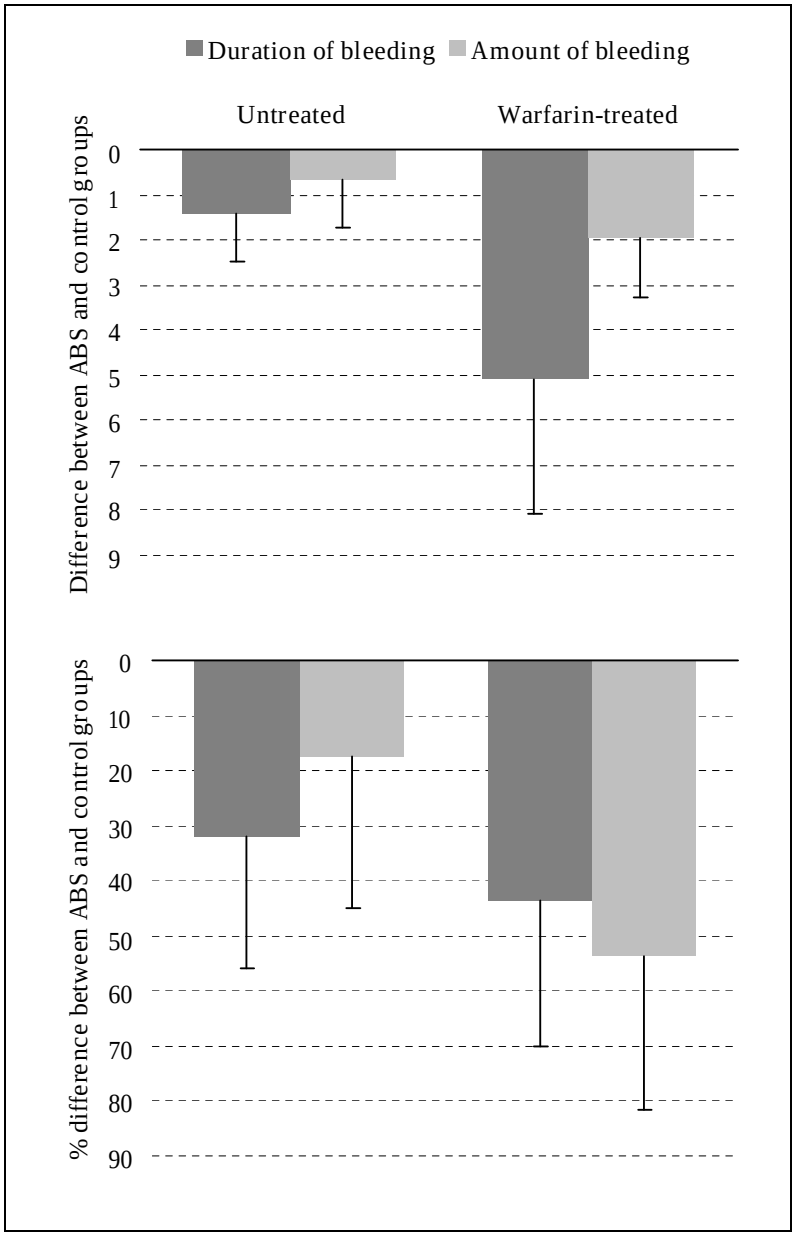
Duration of bleeding
Amount of bleeding
Untreated
Warfarin-treated
Difference between ABS and control groups
0
1
2
3
4
5
6
7
8
9
% difference between ABS and control groups
0
10
20
30
40
50
60
70
80
90

Figure 2.