The Effect of *Yunnan Baiyao* on Platelet Activation, Buccal Mucosal Bleeding Time, Prothrombin Time, Activated Partial Thromboplastin Time, and Thromboelastography in Healthy Dogs: A Randomized, Controlled, Blinded Study

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ABSTRACT

The purpose of this randomized, controlled, blinded, crossover study was to determine the effects of orally administered *Yunnan Baiyao* (100 mg/kg body weight) on tests of coagulation in dogs. Buccal mucosal bleeding time, prothrombin time, activated partial thromboplastin time, clotting parameters obtained from thromboelastography, and indices of platelet activation were determined before and after *Yunnan Baiyao* administration. Eight, adult dogs were randomly given either lactose or *Yunnan Baiyao* filled capsules. Jugular venous sampling and buccal mucosal bleeding time were performed before treatment (T=0), and then at 1, 3, and 12 hours after. Three weeks later the procedure was repeated to complete the crossover. Statistical analysis was performed using a mixed model ANOVA ($P \leq 0.05$). In addition, percentage change from baseline was calculated for each parameter to evaluate trends that might be present in the small number of animals used in this study. Statistical results demonstrated none of the measured parameters were significantly different within groups or between groups at any time. Comparison of percentage change between placebo and *Yunnan Baiyao* groups demonstrated a trend at some sampling test points in the *Yunnan Baiyao* group towards decreased buccal mucosal bleeding times, increases in platelet number and plateletcrit along with TEG parameter changes (quicker clot intiation and rate of formation). In conclusion, under the conditions of this study, *Yunnan Baiyao* demonstrated non-significant differences between pre- and post-administration buccal mucosal bleeding times, platelet numbers and some TEG values along with no evidence of induction of hypercoagulability in healthy, non-bleeding dogs.

Key words: activated partial thromboplastin time, clotting, coagulation, dog, hemostasis, platelets, thromboelastography, thromboplastin time, *Yunnan Baiyao*

ABBREVIATIONS

α angle	Alpha angle (rate of clot formation)
aPTT	Activated partial thromboplastin time
BMBT	Buccal mucosal bleeding time
G	Clot firmness
Hb	Hemoglobin concentration
HCT	Hematocrit
K	Time to complete clot formation
MA	Maximum amplitude (clot strength)
MPC	Mean platelet component concentration
MPV	Mean platelet volume
PDW	Platelet distribution width
PCT	Plateletcrit
PLT	Platelet number
PT	Prothrombin time
R	Time to clot initiation
TEG	Thromboelastography
YB	*Yunnan Baiyao*

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Hemorrhage can occur secondary to toxins, trauma, certain diseases, surgical and diagnostic procedures, and can result in the need for blood transfusions. Red cell transfusions add to the risk of complications and can increase morbidity and mortality, as well as the cost of care. Additionally, some clinical situations do not allow for red cell transfusion, as blood products are not readily available. *Yunnan Baiyao* (YB) is a well-known Chinese herbal medicine used to stop internal and external

bleeding [1,2] *Yunnan Baiyao* was first formulated in 1902 in the Yunnan province of China, is approved by the Chinese State Food and Drug Administration, and is a nationally protected traditional Chinese medicine.[3,4]

Yunnan Baiyao was initially used as a topical hemostatic agent but was found to be a multipurpose remedy to 'invigorate blood circulation', speed elimination of bruises, and stop bleeding when used topically or orally.[1,5,6] Mixing YB with human or rabbit blood *in vitro* significantly shortened clotting times.[7] Topical application has been shown to reduce bleeding in rats after tail tip amputation and liver lobe excision and oral administration reduced bleeding times in rats and rabbits[7-9]. In humans it has been administered per os as a treatment for various types of internal bleeding including gastrointestinal and preoperatively to reduce bleeding and subsequent transfusion during surgical procedures associated with a high incidence of hemorrhage.[4,10,11] *Yunnan Baiyao* was not found to decrease the severity of exercise-induced pulmonary hemorrhage in Thoroughbred horses, but did shorten the template bleeding time in halothane-anesthetized ponies[12,13] Information on the effects of YB in dogs is limited. One study in two dogs, published only as an abstract, found non-significant differences between pre- and post-administration thromboelastography (TEG) values, and a study of 19 dogs suggested that YB might have a place in conservative treatment of open-cervix pyometra, but there was no control group for comparison.[14,15] In a recent study, published only as an abstract, YB was found to be procoagulant *in vitro*, with the effect limited to the common pathway at the level of the prothrombinase complex.[16] No adverse effects have been reported after oral or topical administration.

Coagulation is divided into procoagulant and anticoagulant components.[17,18] The procoagulant system involves primary and secondary hemostasis. Primary hemostasis is initiated when circulating platelets interact with the vascular endothelium resulting in platelet activation, release of von Willebrand's factor, and platelet adhesion. Secondary hemostasis is a process in which coagulation factors are activated, fibrin is formed, and the clot is stabilized. The anticoagulant system relies on natural anticoagulants for clot prevention (e.g., antithrombin and activated protein C) and fibrinolysis for clot dissolution. Fibrinolysis removes clots through the action of plasmin on fibrin, forming fibrin fragments.

Laboratory assessment of primary and secondary hemostasis includes measurement of buccal mucosal bleeding time (BMBT), platelet number (PLT), plateletcrit (PCT), indices of platelet activation [mean platelet volume (MPV), platelet distribution width (PDW), mean platelet component concentration (MPC)], prothrombin time (PT), activated partial thromboplastin time (aPTT), and TEG.[19-26] Prothrombin time is thought to reflect the extrinsic and common pathway, and aPTT is thought to represent the intrinsic and common pathway. Both may provide information about hypocoagulability, but are not reliable predictors of hypercoagulability.[22]

Thromboelastography is used to assess both primary and secondary hemostasis, thus provides a good assessment of global hemostatic function. The TEG curve includes measurements of reaction time or time to first detectable fibrin clot formation (time to clot initiation) (R), time to complete clot formation (K), the rate of clot formation (α angle), clot strength (MA), and global clot strength (G), which is derived from MA. It has been evaluated in a number of animals, including dogs. It correctly identifies both hyper- and hypocoagulable states and is thought to be more sensitive than PT and aPTT, and more specific than activated clotting time, in recognizing coagulation changes in dogs.[22,26-29]

The mechanisms by which YB affects coagulation are unknown; it may induce hemostasis by platelet activation, activation of the clotting cascade, or both. It could also promote thrombus formation by inhibiting fibrinolysis. The objective of this study was to determine the effect of orally administered YB on indices of platelet activation, BMBT, PT, aPTT, and TEG in healthy dogs. It was hypothesized that administration of YB to dogs would result in signs of platelet activation and increased coagulability.

MATERIALS AND METHODS

This was a prospective, randomized, blinded, crossover study with a three-week washout. Eight adult (4-5 years), mixed breed, purpose bred, sexually intact dogs (14-22 kg; 6 male and 2 female), determined to be healthy based upon physical examination, serum chemistry, complete blood count, platelet count, and urinalysis. The dogs were randomly assigned to groups by using a random number table and treatment was conducted by oral administration of lactose filled gel capsules (placebo group) or the encapsulated YB Chinese herbal medicine (test article group). Dogs were fasted for 12 hours prior to each experimental day, but allowed free access to water. Jugular venous blood sampling was performed before administration (T=0), and then at 1 (T1), 3 (T3), and 12 (T12) hours after administration of YB. The dogs were monitored continuously for 3 hours, then every 8 hours for 48 hours for evidence of emesis, diarrhea, constipation, and inappetance after receiving either treatment. Dogs were fed at the end of the 12-hour sampling period and returned to normal feeding the following day. Three weeks later the procedure was repeated to complete the crossover. The study was approved by the University of Tennessee Institutional Animal Care and Use Committee (IACUC Protocol number 1926).

Yunnan Baiyao[a], packaged into 250 mg gel capsules, was used as the test article in this study. Dogs were given 4 capsules per 10 kg body weight, resulting in a dose of approximately 1 g/10 kg (100 mg/kg) body weight. Placebo consisted of 250 mg of lactose[b] in gel capsules, also dosed at 4 capsules per 10 kg body weight. Testing began at the same time every day to limit diurnal variation.

A blood sample (10 mL) was atraumatically collected from the jugular vein prior to administration of YB or placebo (T=0), and then again at T1, T3, and T12. All blood samples were drawn from the jugular vein into sterile plastic syringes using 20-gauge hypodermic needles. The blood was immediately placed into three vacutainer tubes[c], two with buffered sodium citrate and one with ethylenediaminetetraacetic acid (EDTA). The number of needle sticks required to obtain the blood sample was recorded. A BMBT was performed immediately after blood sampling and prior to administration of YB or placebo. A standard technique was used when performing the BMBT and a disposable, single-use, spring-loaded lancet[d] was used.[30] The same individual (DW) performed all BMBT measurements and was blinded to treatment.

Blood was stored at room temperature and, within 30 minutes of collection, complete blood counts, including platelet number and indices of platelet size and granularity, were determined on whole blood in EDTA with an automated hematology analyzer[e], using the methods recommended by the manufacturer. Clotting times (PT and aPTT) were measured on plasma within 30 minutes of collection with an automated coagulometric analyzer[f], using the methods recommended by the manufacturer. The same individual, blinded to treatment, performed all complete blood counts and PT and aPTT analysis.

Kaolin-activated TEG analysis was performed in duplicate with recalcified citrated whole blood using a TEG5000 analyzer.[g] The citrated whole blood specimens were allowed to rest 1 hour at room temperature after sample acquisition. After that, a 1-mL aliquot of the citrated blood sample was placed into a kaolin tube and inverted 5 times to ensure adequate mixing of sample and kaolin. Three hundred forty microliters of the kaolin-treated sample were then added to a warmed (37°C) TEG cup containing 20 μL of 0.2 M calcium chloride, and the assay was run according to the manufacturer's recommendations. Assayed variables included K, R, α angle, and MA. The G value was calculated from the MA value as follows: G=(5000 X MA)/92-MA.[31] Duplicate samples were run simultaneously and the obtained values were averaged for analysis. Laboratory technicians performing the TEG assays were also blinded.

The average of two measurements was used to determine R, K, α angle, MA and G. A mixed model ANOVA [PROC MIXED][h] was used to assess the effect of treatment (*Yunnan Baiyao* versus placebo) on the dependent variables BMBT, Hb HCT, PLT, MPV, PDW, PCT, MPC, PT, aPTT, R, K, α angle, MA, and G. Dog, week, time and treatment were included as class variables in the model. Independent variables included treatment, time (0, 1, 3, or 12 hours post treatment), treatment X time interaction, and number of needle sticks to obtain sample. Week, dog, and the week X dog X treatment interactions were included as random factors and time as a repeated measure with week X dog X treatment interaction as the subject. The fit of each model to the data was measured with the -2 log likelihood ratio. The assumption that the residuals of all models approximated a normal distribution was evaluated with the test statistic of Shapiro-Wilk. When necessary, data were transformed first using a log distribution and, if unsuccessful, a rank procedure [PROC RANK]. Results are reported as least squares means (LSM) $\pm$ 1 standard error of the mean (SEM) unless dependent variables were transformed. In this case data are reported as the median and range. Where 3 or more levels of the independent variable existed, the method of Tukey was used to adjust for multiple comparisons. A p-value of ≤ 0.05 was used as the cut-point for statistical significance in all tests.

In addition to statistical evaluation, percentage change from baseline was estimated for each parameter at each time period using the following calculation:

$$\% \text{ mean change from baseline} = \frac{\text{Mean baseline value} - \text{mean treatment value}}{\text{Mean baseline value}}$$

Reporting a percentage change from baseline presents the results of a randomized trial with small numbers of test subjects in clinically relevant terms for evaluation of trends.

RESULTS

The results of whole blood parameters, BMBT, PT, aPTT, and TEG are shown in Tables 1 and 2, and are expressed as the least square mean (LSM) ± 1 standard error of the mean (SEM) with the exception of the PT data, which is expressed as median and range. Published normal values for platelet parameters, as well as values determined in activated platelets, are shown in Table 1.[23,25] Published normal values for BMBT, aPTT, PTT, and TEG are shown in Table 2.[30-32] There was no statistically significant change ($P \leq 0.05$) for any variable tested within or between treatment groups over time and values, with the exception of PT, were within published normal limits (Tables 1 and 2). Overall, statistically significant changes were observed over time (T1, T3, T12) for both groups for PLT (P=0.004), BMBT (P=0.0130), α angle (P=0.0052) and MA (P=0.0011). Time had an overall significant effect on PLT in both groups with PLT at T0 and T1 significantly different from T3 (P=0.048 and P=0.034, respectively). Overall, the rate of clot formation (α) at T1 and T3 was significantly faster than at T0 (P = 0.004 and 0.009, respectively) and at T12 (P = 0.0175 and 0.0482, respectively). Clot strength changed significantly over time in both treatment groups (P=0.0011) and overall, the MA at T1 was significantly greater than the MA at T0 (0.0022) for both groups. The number of venipuncture attempts did not significantly affect any of the measured parameters with the exception of Hb (P = 0.0010) and HCT (P=0.0024).

When evaluating trends in percentage change from baseline values for PCT (Figure 1), the PCT at T1 was unchanged from T0 in the placebo group, while it increased by almost 7% in the YB group. The PCT at T3 and T12 decreased by 3.1 and 9.4% in the placebo group while it

Table 1: Comparison of whole blood parameters measured in healthy dogs receiving *Yunnan Baiyao*[a] (100 mg/kg) versus placebo. Data presented as LSM ± SEM

Parameter	Placebo group				*Yunnan Baiyao*[a] group				Normal	Activated
	T0	T1	T3	T12	T0	T1	T3	T12		
[Hb]	17.32 ± 0.47[a,1]	17.18 ± 0.48[a,1]	17.00± 0.44[a,1]	17.85 ± 0.52[a,1]	17.48 ± 0.47[a,1]	17.33 ± 0.49[a,1]	16.84 ± 0.44[a,1]	16.97 ± 0.51[a,1]	14.7-21.6[#]	N/A
HCT	51.83 ± 1.42[a,1]	50.85 ± 1.42[a,1]	50.65 ± 1.42[a,1]	52.69 ± 1.45[a,1]	52.26 ± 1.36[a,1]	50.92 ± 1.37[a,1]	49.34 ± 136[a,1]	51.12 ± 1.36[a,1]	41-60[#]	N/A
PLT (x 10^9/L)	342.60± 21.90[a,1]	323.13± 19.95[a,1]	311.63± 21.23[a,1]	309.48± 22.34[a,1]	324.57 ± 21.88[a,1]	333.16 ±20.00[a,1]	308.03 ± 21.22[a,1]	310.41 ± 22.08[a,1]	204-398[25] 147-423[23]	128-285[25]
PCT	0.32 ± 0.03[a,1]	0.32 ± 0.04[a,1]	0.31 ± 0.03[a,1]	0.29 ± 0.03[a,1]	0.30 ± 0.03[a,1]	0.32 ± 0.04[a,1]	0.30 ± 0.03[a,1]	0.31 ± 0.03[a,1]	0.33[@]	N/A
MPV (fL)	9.35 ± 0.35[a,1]	9.83 ± 0.53[a,1]	9.95 ± 0.44[a,1]	9.02 ± 0.63[a,1]	9.28 ± 0.35[a,1]	9.67 ± 0.53[a,1]	9.66 ± 0.44[a,1]	9.02 ± 0.61[a,1]	8.4-11.5[25] 12.6[23]	9.4-28.9[25]
PDW (%)	58.57 ± 1.44[a,1]	58.98 ± 1.35[a,1]	59.97 ± 1.68[a,1]	57.05 ± 1.73[a,1]	58.76 ± 1.44[a,1]	59.02 ± 1.36[a,1]	59.45 ± 1.68[a,1]	56.93 ± 1.82[a,1]	54.4-60[25] 61.6[23]	62.2-72[25]
MPC (g/dL)	23.21 ± 0.42[a,1]	23.02 ± 0.63[a,1]	22.78 ± 0.72[a,1]	23.84 ± 0.73[a,1]	23.37 ± 0.42[a,1]	23.31 ± 0.63[a,1]	23.46 ± 0.72[a,1]	23.54 ± 0.79[a,1]	20.9-22.5[25] 19.6-24.2[23]	15.2-20.8[25]

Hb = hemoglobin concentration; HCT = hematocrit; PCT = plateletcrit; MPV = mean platelet volume; PDW = platelet distribution width; MPC = mean platelet component concentration

T0 = immediately prior to treatment; T1, T3, T12 = 1, 3, and 12 hours after treatment, respectively

Normal = published normal values in dogs; N/A = not available

Activated = activated platelets in dogs with inflammatory disease[23] or after lipopolysaccharide injection[25]

[#] = University of Tennessee normal values

[a] = Statistical comparisons within treatment groups; values with the same letter are not statistically different (P>0.05)

[1] = Statistical comparisons between treatment groups; values with the same number are not statistically different (P>0.05)

Table 2: Clotting times (PT, aPTT), buccal mucosal bleeding times (BMBT), and thromboelastrography (TEG) parameters (R, K, MA, G values, α angles) measured in healthy dogs receiving *Yunnan Baiyao*[a] (100 mg/kg) versus placebo. Data presented as LSM ± SEM with the exception of PT data, which is presented as median and range

Parameter	Placebo group				*Yunnan Baiyao*[a] group				Normal Values
	T0	T1	T3	T12	T0	T1	T3	T12	
PT (sec)	8.2 (7.7-9.9)[a,1]	8.1 (6.7-9.0)[a,1]	8.4 (6.7-9.2)[a,1]	8.3 (7.0-8.8)[a,1]	8.2 (6.8-8.8)[a,1]	8.2 (6.6-8.5)[a,1]	8.3 (6.8-8.8)[a,1]	8.2 (7.0-13.7)[a,1]	9-25 sec[#]
aPTT (sec)	26.39 ± 5.26 [a,1]	24.43± 5.25 [a,1]	31.59 ± 5.25 [a,1]	20.73 ± 5.31 [a,1]	23.62 ± 4.63 [a,1]	25.95 ± 4.65 [a,1]	27.36 ± 4.64 [a,1]	27.09 ± 4.64 [a,1]	5-30 sec[#]
BMBT (sec)	72.62 ± 12.12 [a,1]	86.40 ± 12.75[a,1]	68.88 ± 13.56 [a,1]	72.85 ± 12.86[a,1]	79.76 ± 12.1 [a,1]	81.60 ±12.81[a,]	56.51 ± 13.55 [a,1]	59.26 ± 12.48 [a,]	<180 sec[30]
R (min)	6.22 ± 1.15 [a,1]	5.57 ± 0.91 [a,1]	6.19 ± 1.12 [a,1]	7.25 ± 1.58 [a,1]	7.24 ± 1.15 [a,1]	6.0 ± 0.92 [a,1]	6.46 ± 1.12 [a,1]	8.31 ± 1.53 [a,1]	1.8-8.6[31] 1.7-6.1[32]
K (min)	2.62 ± 0.32[a,1]	2.13 ± 0.24[a,1]	2.28 ± 0.26[a,1]	3.08 ± 0.73[a,1]	2.60 ± 0.32[a,1]	2.11 ± 0.24[a,1]	2.26 ± 0.26[a,1]	3.74 ± 0.71[a,1]	1.3-5.7[31] 0.9-3.1[32]
α angle (°)	51.77 ± 3.66[a,1]	58.21 ± 2.94[a,1]	58.06 ± 3.53[a,1]	48.94 ± 6.53[a,1]	50.73 ± 3.64[a,1]	59.87 ± 3.00[a,1]	58.81 ± 3.52[a,1]	47.49 ± 6.36[a,1]	37-75[31] 49-75[32]
MA (mm)	54.94 ± 2.68[a,1]	59.06 ± 2.27[a,1]	59.17 ± 2.50[a,1]	57.36 ± 2.83[a,1]	55.74 ± 2.68[a,1]	59.64 ± 2.28[a,1]	58.29 ± 2.50[a,1]	55.64 ± 2.78[a,1]	43-68[31] 46-64[32]
G (dyn/cm^2)	6634 ± 823[a,1]	7493 ± 1062[a1]	7700± 812[a,1]	6788 ± 797[a,1]	6549 ± 822[a,1]	7687 ± 1064[a,1]	7202 ± 814[a,1]	6789± 797[a,1]	3200-9800[31] 4200-8000[32]

PT = prothrombin time; aPTT = activated partial thromboplastin time; BMBT = buccal mucosal bleeding time; R = reaction time (time to clot initiation); K = time to clot formation; α angle = rate of clot formation; MA = clot strength; G = clot firmness

T0 = immediately prior to treatment; T1, T3, T12 = 1, 3, and 12 hours after treatment

[#] = University of Tennessee Laboratory normal values; Published values[30,31,32]

[a] = Statistical comparisons within treatment groups; values with the same letter are not statistically different (P>0.05)

[1] = Statistical comparisons between treatment groups; values with the same number are not statistically different (P>0.05)

remained unchanged from baseline at T3 and increased by 3.3% at T12 in the YB group. Platelet number (Figure 2) showed a similar trend with a decrease of 5% at T1 in the placebo group while the treated group increased by 2.6%. Additionally, the trend continued with the platelet numbers remaining stable at T3 for the treatment group while placebo decreased by 9%. This same trend continued at T12 where the YB group had a 3% increase while placebo decreased 9.7%.

Percentage change for BMBT (Figure 3) had an increase in the placebo group of 19% at T1 while the YB group increased by only 2.3%. At T3, there was a large shift in the YB group with a decrease of 29.1%, which remained stable through the T12 time point at 25.7%. The placebo group decreased 5.2% from baseline at T3 and increased 0.3% at T12. Reaction time (R) (Figure 4) decreased at T1 and T3 compared with T0 and T12 in both the YB and placebo groups but decreased more profoundly in the YB group at 17.1% and 10.8%, respectively versus placebo at 10.5% and 0.5%, respectively. Percentage change at T1 and T3 for α angle (Figure 5) again had more profound changes in the YB group, with an increase of 18.0 and

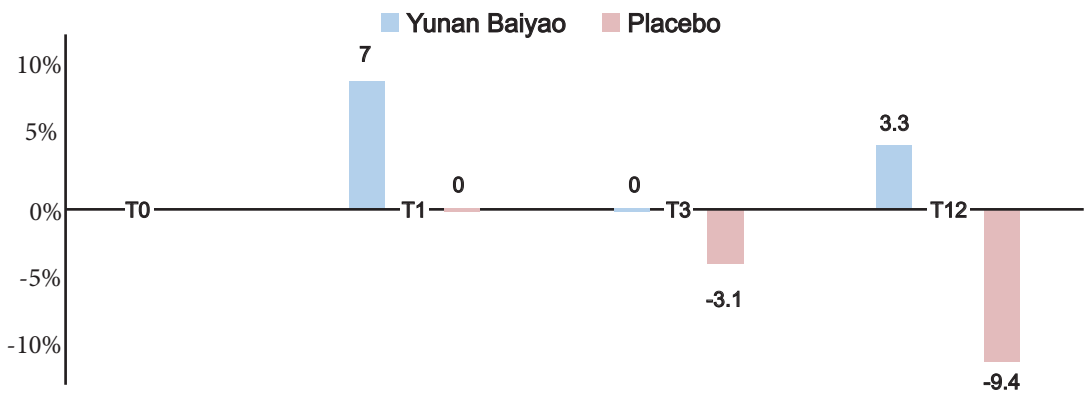

Figure 1: Plateletcrit, percent change from baseline at each study sampling time point.

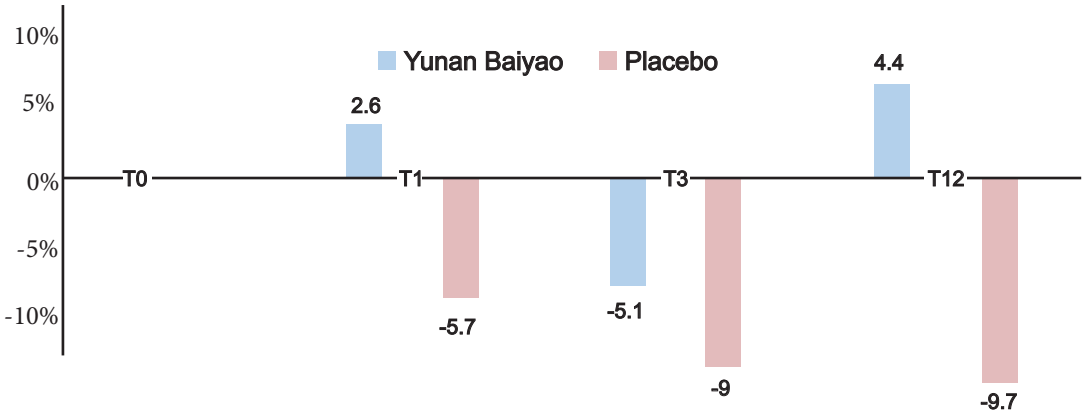

Figure 2: Platelet number, percent change from baseline at each study sampling time point.

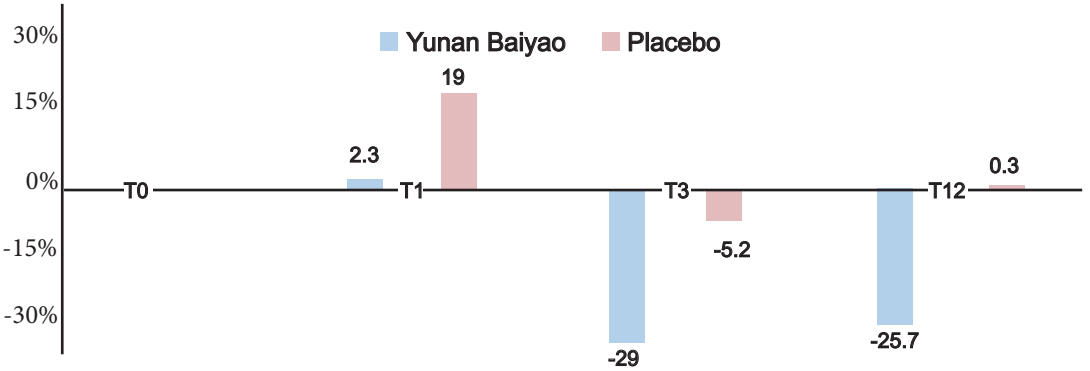

Figure 3: Buccal mucosal bleeding time, percent change from baseline at each study sampling time point.

15.9% respectively versus 12.4 and 12.1%, respectively, in the placebo group. All other study variable percentage changes were either similar between groups, demonstrated wide variable fluctuations or did not demonstrate any significant biological trends. No adverse effects were seen in any study animal during the conduct of the study and all animals completed all phases of the study.

DISCUSSION

In this study, there was no statistically significant change ($P \leq 0.05$) for any variable tested within or between treatment groups over time and values, with the exception of PT, were within published normal limits (Tables 1 and 2). Overall statistically significant changes were observed over time (T1, T3, T12) for both groups for PLT (P=0.004), BMBT (P=0.0130), α angle (P=0.0052) and MA (P=0.0011). Although similar trends were seen in both groups for some parameters, for others the direction or degree of change from baseline was different and worthy of note. For example, the BMBT was decreased 29.1 and 25.7% from baseline at T3 and T12 in the YB group, while it was decreased only 5.2% or unchanged in the placebo group at those times (Figure 3). Reaction time decreased by 10-17% in the YB group, compared to 0.5-10% in the placebo group (Figure 4), and α angle increased as much as 18% in the YB group compared to 12% in the placebo group (Figure 5). These differences may suggest an effect of YB on a functional coagulopathy test such as BMBT and on some TEG parameters. The increasing PLT and PCT at early study time points is interesting and may suggest an effect on the splenic pool of platelets which exists in dynamic equilibrium with the axial blood platelet pool.

A number of factors affect primary and secondary hemostasis including clotting factors, Hb, HCT, PLT, PCT, and platelet function.[22] Several tests are used in clinical patients to evaluate primary and secondary hemostasis, including BMBT, PT, aPTT, TEG, and indices of platelet activation.[19,22,29,33,34] The BMBT is affected by platelet number and function, integrity of the vascular endothelium, and the release of von Willebrand's factor.[30] The BMBT, along with PLT, is used to describe primary hemostasis, and a shortened BMBT may be expected with platelet activation. One previous study, published only as an abstract, found that YB significantly decreased template-bleeding time in 6 halothane-anesthetized ponies.[13] In the current study, BMBT was decreased by 20-25 seconds at T3 and T12 compared to T0 and T1 in the YB group and the BMBT at T3 and T12 in the YB group was almost 30 seconds faster than the BMBT in the placebo group at T1.[30]

The most widely used test to evaluate activated platelets is the detection of platelet membrane P-selectin

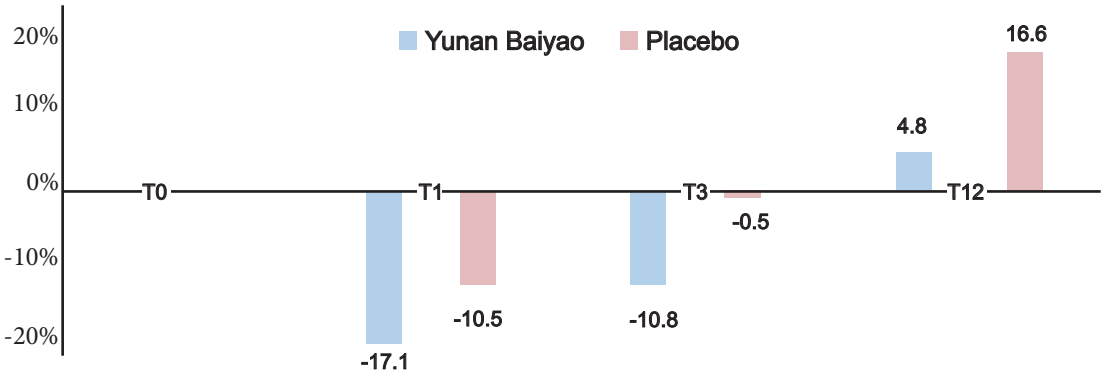

Figure 4: Reaction time (time to clot initiation), percent change from baseline at each study sampling time point.

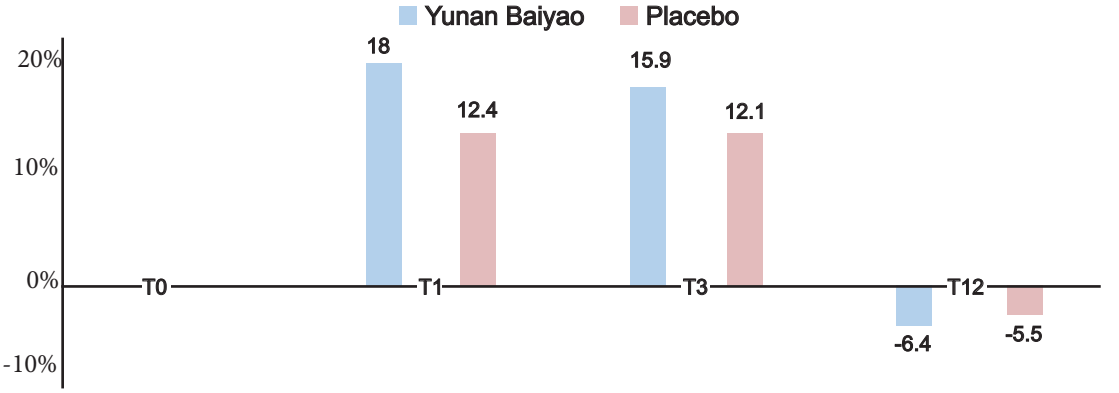

Figure 5: Alpha angle (rate of clot formation), percent change from baseline at each study sampling time point.

with flow cytometry, as P-selectin is a component of alpha granules and is expressed on the platelet surface after alpha granule secretion.[33] There are, however, a number of measured platelet parameters that reflect platelet activation, including MPV, PDW, and MPC, a measure of platelet alpha granule content derived from the refractive index of platelets.[19-21,23-25,35] Platelet activation is associated with expression of P-selectin on the platelet surface, a decrease in MPC and number of circulating platelets, and an increase in MPV and PDW.[21,23-25] The MPV increases and MPC decreases due to degranulation and uptake of fluid by the platelet. Moritz et al. investigated the changes in MPC, MPV, and PDW and their correlation with P-selectin expression during platelet activation in dogs with septic and non-septic inflammatory disease.[23] They found that platelet number and MPC decreased, while MPV and PDW increased, with platelet activation in that inflammatory model. Correlation with the expression of P-selectin on the platelet surface was best with MPC (r=0.62) and much lower with MPV (r=0.2) and PDW (0.16). In humans, the correlation of MPC with P-selectin expression is as high as 0.87, and MPC is considered an excellent marker for activated platelets.[19-21,36] There were no statistically significant or percentage change trends noted for MPV and PDW when comparing the placebo and YB groups in this study. Some platelet parameters, such as MPV and MPC, show time-dependent changes in EDTA within the first 15 minutes after collection; MPC increases and MPV decreases, but both stabilize by 15-20 minutes.[24,37] As storage time in EDTA increases, the MPC decreases and the MPV increases, and results are unreliable by 4 hours. In the present study our samples were analyzed within 30 minutes of collection, and time related changes should be comparable in both groups. Although YB was found to cause release of platelet components in an *in vitro* electron microscopy platelet study, platelet activation and degranulation, as indicated by a decrease in MPC, was not observed in the current study.[38,39]

Defects in secondary hemostasis are divided into abnormalities of the intrinsic, extrinsic, or common pathways. This separation of the coagulation cascade into intrinsic, extrinsic, and common pathways is now thought to be an *in vitro* phenomenon. Tissue factor released from damaged endothelial cells combines with factor VIIIa and also activates factor X; thus, the intrinsic and extrinsic pathways are not isolated entities *in vivo*.[17] Tests used to assess coagulation status include the PT, aPTT, and TEG. The PT provides information about the common and extrinsic coagulation pathways and the aPTT provides information about the common and intrinsic coagulation pathways; neither provides completely reliable information about *in vivo* coagulation status, particularly in patients that are hypercoagulable.[22] One would expect both PT and aPTT to be shortened in a hypercoagulable state but in this study they did not differ between groups or within groups over time, and this suggests that increased coagulability did not occur with the administration of YB.[26,28,29]

Thromboelastography is used to assess primary and secondary hemostasis, including platelet number and function, clotting factor activity, clot strength, speed of clot formation, and fibrinolysis. Thromboelastography assesses the cellular components of clotting (e.g., platelet function) in addition to the proteins associated with the formation of fibrin (e.g., clotting factors), can identify both hypo- and hypercoagulable states, and may more closely reflect the *in vivo* coagulation state.[22,26-29,31,40-42] Five standard values are used in interpretation of TEG tracings. The reaction time (R), or precoagulation time, is the distance in millimeters from the start of the tracing to the point where the lines have diverged 1 mm. Reaction time is influenced by factors VIII, IX, XI, and XII, and inhibitory antithrombin, protein C, and protein S activity, and is shortened in hypercoagulable states. The clot formation time (K) is the distance in millimeters between the end of the R and the point at which the distance between the 2 branches reaches 20 mm. This point corresponds to the maximal divergence obtained and is a measurement of the rapidity of clot development from the beginning of the visible phase of coagulation to a defined level of clot strength. The combined R and K values reflect the coagulation time from its beginning to a predetermined clot strength. The K value is influenced by factor II and VIII, platelet number and function, thrombin formation, fibrin precipitation, fibrinogen concentration, and HCT, and platelet hyperfunction will shorten K.[31] The α angle is the angle between the midline and the tangent to the curve drawn from the 1 mm wide point, describes the rate of clot formation, is affected by the same factors as K, and is increased with increased platelet function.[22,31,41] The maximal amplitude (MA) is the maximal distance in millimeters between the two diverging branches and is a reflection of final clot strength. It is affected by fibrin and fibrinogen concentration, platelet count and function, thrombin concentration, factor XII, and HCT, and should increase with increased coagulability. Global clot strength (G) is derived from MA, is affected by the same parameters, and has been used to identify dogs as hypocoagulable (decreased G), normal, or hypercoagulable (increased G) in some studies.[26,28,29] Therefore, a hypercoagulable state would be indicated by a decrease in R and K values, and an increase in the α angle, MA, and G values.

Patterson, in a study published only as an abstract, found that treatment with YB had no significant effect on TEG, although only two dogs were studied.[14] Some TEG parameter results in this study are worth noting although none reached statistical significance. The R in the placebo group decreased between 0.5-10.5%, while it decreased by 10.8-17.1 percent in the YB group. The α angle, MA, and G tended to increase with time in both groups but the α angle in the placebo group increased around 12% while the YB group had an increase that was as high as 18%.[26] Differences in venipuncture technique, sample handling and individuals performing the TEG assay, as well as high

variability both within and between a small number of dogs, may account for the lack of statistical differences in TEG parameters between the two groups. In a study published after the current study was performed it was shown that direct jugular venipuncture using a needle and syringe resulted in indications of increased coagulability on TEG when compared with samples obtained using the evacuated tube method.[43] The major disadvantage of direct blood aspiration with a syringe is that the first few milliliters of sample could not be discarded, resulting in increased tissue factor in the sample and premature activation of coagulation within the sample. Although the number of needle sticks did not significantly affect any TEG parameters, repeated venipuncture or repositioning of the needle could lead to release of tissue factor and activation of coagulation *in vitro*, resulting in evidence of increased coagulation in both the YB and the placebo group.

Yunnan Baiyao has been used for over 100 years to stop bleeding, and there is plenty of anecdotal evidence that it is effective when administered topically and parentally. It is formulated as a powdered combination of herbs in a starch base and most formulations contain Notoginseng. Until recently, the exact ingredients of the formulation were unknown. However, international demand for quality control and product analysis resulted in a list of the drug's major components (per 0.5 g sample): 200 mg *Tienchi ginseng* root (Panax notogingseng), 85 mg *Diels* plant (Ajuga forrestii), 66.5 mg Chinese yam root (Dioscorea opposita), 57.5 mg Makino root, (Dioscoreae nipponica), 36 mg Erodium stephanianum and Geranium wilfordii plant, 30 mg Dioscoreae parvilora ting root, and 25 mg *Inula cappa* plant.[3,44,45] Although the pharmacology of YB has not been completely determined, many of its constituents are suspected to have anti-cancer, anti-inflammatory, hemostatic, wound healing, and analgesic properties.[7,43-46]

Notoginseng, a component of YB, is known to have hemostatic properties and contains a number of saponins that affect hemostasis.[8,48-51] Panaxadiol and various other saponins extracted from YB have antimicrobial and anti-inflammatory properties, and YB may have anti-inflammatory effects through regulating arachidonic acid metabolism in osteoblasts.[4,5,15,46] A recent study found that YB caused dose and time dependent hemangiosarcoma cell death *in vitro* using canine hemangiosarcoma cell lines.[43] Interestingly, nanofibers have been identified in the YB formulation and it has been proposed that nanofibers play a role in platelet activation and aggregation, leading to clotting and the sealing of wounds.[52,53].

Objective, scientific evidence of the efficacy of YB for hemostasis is beginning to accumulate. Oral administration of 0.25 g of YB four times daily for 3 days prior to bilateral maxillary orthognathic surgery significantly reduced intraoperative bleeding in human patients.[4] Men taking 500 mg of YB four times daily for 3 days prior to transurethral resection of the prostate had significantly less bleeding than those taking placebo and patients undergoing cervical open-door laminoplasty had significantly less bleeding intraoperatively after taking 500 mg of YB three times daily for 5 days preoperatively.[10,11]

Studies of the effects of YB on blood clotting in animals are sparse. A recent study in the author's laboratory showed that topical YB significantly shortened bleeding times in dogs using an incisional model (unpublished data). In rats, topical application significantly shortened gross bleeding times after tail tip amputation and topical application and oral administration shortened bleeding time after liver lobe excision in rats.[7-9] Clotting times in rabbits were significantly reduced by mixing blood with YB *in vitro* and after oral administration of YB.[7,9]. Another study in dogs and rabbits showed non-significant differences in TEG parameters after administration of YB, but, as the study was published only as an abstract and does not include a description of the methodology, it is difficult to compare these studies.[14]

In this study, no statistically significant differences were noted in measurements of platelet activation, clotting factor activity, or TEG between groups; however, there were some noteworthy trends. There were more profound decreases of BMBT and R and increases of α angle when the YB group and placebo group were compared to baseline. In addition, when compared to baseline, the PLT and PCT demonstrated increases in the YB group when compared to the placebo group. Time had a significant affect on BMBT, PLT, α angle, K, and MA values, but this was true of both treatment groups. There are a number of possibilities for the lack of statistically significant changes in measured parameters. Tests such as BMBT, PT, aPTT and TEG can be fraught with technical difficulties and there is a wide range of normal results.[39] While the same individuals performed the BMBT, PT, aPTT, and platelet indices tests, slight variability in technique and times to run samples may have been a factor. Variability in oral bioavailability of YB may also have contributed to the lack of significant differences and it is also possible that the dose, frequency, and duration of administration were not adequate, as the pharmacokinetics of this Chinese herbal medicine are unknown in dogs. Additionally, individual patient variability was high and there were only 8 dogs in this study, so Type II error was likely significant. Finally, the mechanism of action of YB has not yet been elucidated and it is possible that the anticoagulant effects of YB may only be obvious when there is active bleeding in the patient. This may explain the studies in humans that showed a reduction in intraoperative hemorrhage with the preoperative use of YB.[10,11]

In conclusion, the oral administration of YB (100 mg/kg) to healthy dogs in the present study did not demonstrate the development of a hypercoagulable state or statistically significant changes in indices of coagulation. Comparison of percentage change between placebo and *Yunnan Baiyao* groups demonstrated a trend at some sampling test points in the *Yunnan Baiyao* group towards decreased buccal

mucosal bleeding times, increases in platelet number and plateletcrit along with TEG parameter changes (quicker clot initiation and rate of formation). No adverse effects were noted in any of the dogs during any study phase and all dogs completed the study.

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FOOTNOTES

a. Yunnan Baiyao, Kumming, Inc., Yunnan, P.R., China, Jing-Tang Herbal, Reddick, FL, USA
b. Lactose NF, Humco, Inc., Texarkana, TX, USA
c. BD Vacutainer; BD Corporation, Franklin Lakes, NJ, USA
d. Surgicutt Junior, Jorgen Laboratories Inc., Loveland, CO, USA
e. Bayer ADVIA™120, Siemens Diagnostics, Tarrytown, NY, USA
f. ACL 9000, Beckman Coulter, Inc., Bree, CA, USA
g. TEG5000 Thromboelastograph, Haemonetics Corporation, Braintree, Mass, USA
h. SAS version 9.4, SAS Institute, Cary NC, USA

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