Cutaneous and Systemic Effects of Varying Doses of Brown Recluse Spider Venom in a Rabbit Model

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OBJECTIVE: To ascertain whether a dose response exists between the dose of brown recluse spider venom (BRSV) and the cutaneous and coagulation effects in a rabbit model. Cutaneous necrosis is a serious complication of brown recluse spider envenomation (spider bite with venom). Disseminated intravascular coagulation (DIC) is a dreaded complication of brown recluse envenomation in humans. New Zealand white (NZW) rabbits have proved to be a model for the study of therapeutic regimens to prevent skin necrosis after spider bites. We studied the venom's effects on the skin and the coagulation mechanism in this rabbit model to determine if a clear dose-response relationship could be established. Establishment of a dose-response relationship is an important first step in determining if the NZW rabbit is a suitable model to study both cutaneous and systemic effects of the venom.

DESIGN: Thirty-six NZW rabbits were divided into three groups. One group received a saline injection, and the other two groups received a 4.0µg or a 10.0µg dose of purified BRSV intradermally into the skin on the dorsum of the back.

METHODS: Blood was collected at baseline, 24, 48, and 72 hours. Tissue specimens were obtained after seven days during the animal necropsy and gross and microscopic pathology examination was conducted to assess tissue damage. Measurements included complete blood count (CBC); platelets; PT; activated partial thromboplastin time (APTT); fibrinogen (clottable, immunological); coagulation factors II, V, VII, VIII, IX, X, XI, XII; anti-thrombin (AT); alpha-2 antiplasmin (AP); Protein C (PC); mixing studies; lupus anticoagulant screening; plasminogen; thrombin-anti-thrombin; fibrin degradation products (FDP); d-dimer; and thrombin time.

RESULTS: Gross pathology results were consistent with previous studies that used higher doses of BRSV. The WBC and platelet counts decreased at 24 hours in the two groups receiving the BRSV ($p<0.05$). BRSV produced a dose related prolongation in the APTT ($p<0.05$). Levels of fibrinogen as well as factors V, VII, VIII, IX, X, AT, and AP ($p<0.05$) were increased in response to the BRSV. Protein C decreased at 24 hours ($p<0.05$) and remained low in other time points. Mixing studies corrected the prolonged APTTs to normal ranges. Factor II XI and XII showed no significant alteration in response to the BRSV.

CONCLUSIONS: In the model, both the size and depth of the eschar were dose-related. We also observed a dose related elevation in the APTT that corrected with mixing studies. The dose-response relationship suggests direct interference by a component of the venom, rather than an idiosyncratic response. We did not detect a deficiency of commonly measured coagulation factors or evidence of a lupus anticoagulant. Protein C demonstrated a decrease. Although DIC did not occur in this rabbit model, a dose-related elevation in APTT was noted. The finding that the elevation corrected with mixing studies suggests that a plasma factor is essential in the coagulopathy associated with brown recluse envenomation. Further studies to identify this factor could shed light on human coagulopathy following envenomation.

ABBREVIATIONS: AP = alpha-2 antiplasmin; APTT = activated partial thromboplastin time; AT = anti-thrombin; BL = baseline; BRSV = brown recluse spider venom; DIC = disseminated intravascular coagulation; FDP = fibrin degradation products; LA = lupus anticoagulant; NZW = New Zealand white; PC = Protein C.

INDEX TERMS: brown recluse spider, coagulation; eschar; rabbit; skin necrosis.

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Previous studies at our institution have been performed to evaluate the effect of the brown recluse spider venom (BRSV) on human citrated plasma in vitro. Those studies found that when a purified extract of the BRSV was added to human plasma in vitro certain coagulation assays looked as if a lupus anticoagulant (LA) was present.[1,2] These findings consisted of a prolonged activated partial thromboplastin time (APTT), abnormal 1:1 mixing studies that did not correct into the normal range of the laboratory reagent/instrument combination, falsely decreased coagulation factors that corrected upon dilution, a positive dilute Russell's Viper venom assay, and a positive platelet neutralization assay. That study contradicts a previous study published in 1986 that stated that the BRSV depeleted clotting factors VIII, IX, XI, and XII by an average of 44.0%.[3] In humans, BRS bites can produce disseminated intravascular coagulation (DIC) as well as other coagulation deficiencies.[3-11] The New Zealand white (NZW) rabbit has proved to be a suitable model to determine the histologic changes of BRSV-induced necrosis and to evaluate therapeutic agents.[12-13] In prior studies each rabbit received an intradermal injection of 20µg of venom given between the layers of the skin over the back. An unexpected finding was a prolonged APTT, marked elevation of fibrinogen, and elevated coagulation factors at 72 hours post dosing with BRSV. In those prior studies, there was no remaining blood for further analysis. The dose of toxin used in the rabbits in previous studies caused extensive damage to the skin and surrounding muscle. In humans, DIC is often associated with lesser degrees of necrosis. By studying lower dose envenomation (mimicking a brown recluse spider bite), we hoped to develop a model suitable for the study of both skin necrosis and the coagulation effects of BRSV venom, thereby leading to better methods of treatment of this spider bite.

MATERIALS AND METHODS

This research was performed at an AALAC-accredited facility and in accordance with the Animal Welfare Act, the Public Health Services Policy, the Guide for the Care and Use of Laboratory Animals, and all other applicable laws, regulations and guidelines. This protocol was supported by the Office of the Surgeon General of the United States Air Force. Thirty-six NZW rabbits were divided into three groups of twelve animals. A purified BRSV toxin extract was used that was prepared by a method previously described.[14] Two groups of rabbits received doses of purified BRSV (4.0µg and 10µg respectively) compared with a group receiving only saline to see if we could reproduce the blood clotting abnormalities and the severe necrotic lesions (eschars) seen in previous studies at our institution. The injections were all given intradermally in 0.2 ml (mixed with sterile saline) amounts into the dorsal area of the back of each subject. Baseline blood specimens were collected in vacutainer tubes containing EDTA or 3.2% sodium citrate by venipuncture from the central ear artery using a 20 gauge needle. After envenomation, blood specimens were obtained at 24, 48, and 72 hours. The rabbits were held and observed for a period of seven days following envenomation. At the end of the seven-day period the rabbits were euthanized and the skin and muscles in the area of the injection site were harvested for gross and microscopic examination to assess tissue damage.

The EDTA blood was used for complete blood counts (CBC) with platelets. The CBC and platelets analyses were performed on an Abbot Cell-Dyne, 3700 (Abbott Park IL). The citrated blood was separated by centrifugation (2500g X 15 minutes) to obtain platelet-poor plasma. We then performed PT; activated partial thromboplastin time (APTT); fibrinogen (clottable and immunological); d-dimer; reptilase time; thrombin times; coagulation factors II, V, VII, VIII, IX, X, XI, XII; anti-thrombin (AT); alpha-2 antiplasmin (AP); protein C (PC); mixing studies (using rabbit normal plasma); lupus anticoagulant screening and confirmatory testing (dilute Russell's viper venom [American Diagnostica, Greenwich CT], STACLOT-LA, [Diagnostica-Stago, Inc.,

Parsippany NJ]); and plasminogen using reagents from Diagnostica-Stago, Inc. All of the assays performed using Diagnostica-Stago, Inc. reagents were performed on an STA automated coagulation analyzer (Parsippany NJ). The thrombin-anti-thrombin assay was performed using an ELISA assay from Dade-Behring Inc. (Deerfield IL). The fibrin degradation products (FDP) assays were performed using an ELISA assay from Biomerieux (Durham NC). The antigenic fibrinogen assays were performed under the supervision of Dr. Marjory Brooks at Cornell University. We also performed coagulation assays with rabbit platelet-poor citrated plasma in a manner described in two previous studies.[1,2] Chemistry assays to check for renal and liver function were performed by the Wilford Hall Medical Center, Core Element Laboratory on the Roche Modular P-800 (Indianapolis IN). The gross pathology observations in each subject were made by a veterinary pathologist for size of the eschar in centimeters. The inflammation and vasculitis and necrosis depth severity were based on an overall semi-quantitative score of zero to four from no effect to a severe response to the BRSV on the injected site. The pathologists were blinded to the dose used. The hematology and coagulation results were analyzed using descriptive statistics and the analysis of variance (ANOVA) with a statistical significance described as $p<0.05$.

RESULTS

The gross pathology results were as follows:

- The 10.0µg group had a mean depth of necrosis score of 3.75 (0-4). The mean depth was estimated by the level of necrotic material and the indentation of the lesion in comparison to the control saline group with no lesion present. The mean measurement of the area of necrosis in this group was 12.7 cm. The mean of the eshcar width was 6.0 cm. The overall severity of the inflammation and vasculitis ranged from three to four on a semi-quantitative four point visual analog scale in all 12 rabbits in this group.

- The 4.0µg group had a mean depth of necrosis of 2.83. The mean area of necrosis for this dose was 8.25 cm. The mean of the eschar width was 4.7 cm. The severity of inflammation and vasculitis ranged from two (one rabbit) to four (one rabbit) with a mean and median of three (ten rabbits). None of the saline injected group had any evidence of a lesion or area of necrosis.

The CBC and platelet results are displayed in Table 1. The 10.0µg dose group demonstrated a fall in WBC counts from a mean at baseline of $6.9x10^9$L to $3.4x10^9$L at 24 hours. The platelet count in the same group fell from $341x10^9$L to $61.0x10^9$L. The WBC counts gradually rose to $19.8x10^9$L at 72 hours. The platelet counts recovered to $344x10^9$L after 72 hours, which was not statistically different from baseline levels. The 4.0µg dose showed a lesser response in the WBC and platelet counts. The WBC counts showed a mild decrease, dropping from $7.0x10^9$L at baseline to $5.3x10^9$L at 24 hours and rising to $14.7x10^9$L at 72 hours. The platelet counts stayed within normal limits throughout the 72 hour monitoring period. The saline group was virtually unaffected. All of the animals showed a drop in the RBC ($x10^{12}$L), HGB(g/

Table 1. Complete blood counts and platelet results

Sample	WBC (10^9L)	RBC (10^9L)	HGB (g/dl) (10^{12}L)	HCT (%)	Platelets
Saline BL	6.9	6.0	13.1	41.1	331
Saline 24	7.8	5.2	11.5	35.7	305
Saline 48	7.6	4.9	10.9	34.2	328
Saline 72	8.0	4.5	10.1	31.5	347
4.0µg dose BL	7.0	6.1	13.2	41	391
4.0µg dose 24	5.3	6.0	12.8	40.2	*255
4.0µg dose 48	10	4.9	10.6	33.1	264
4.0µg dose 72	*14.7	4.4	9.4	29.6	398
10.0µg dose BL	6.9	6.3	13.9	43.6	341
10.0µg dose 24	3.4	5.7	12.5	39.3	*61
10.0µg dose 48	7.7	5.1	12	36.0	149
10.0µg dose 72	*19.8	4.0	9.2	28.0	344

BL = baseline; 24, 48, 72 = hours collection. Saline group is a negative control.
*WBC values were statistically significant from baseline at 72 hours ($p = 0.004$)
*Platelet values were statistically significant at 24 hours ($p = 0.001$)

dl), and HCT(%) levels. This was probably due to the daily venipuncture that took approximately 14 ml of blood for testing. However, there were no significant differences between the groups.

The coagulation factors testing revealed significant differences between the three groups of animals. See Tables 2a and 2b. The PT levels were virtually the same for all groups at baseline and each 24 hour time point. The APTTs increased significantly at 24 hours and stayed elevated in both of the groups receiving the BRSV. The 10.0µg dose group APTT increased from a baseline of 51.9 seconds to 79.8 seconds at 24 hours and increased to a high of 84.2 seconds at 48 hours. The 4.0µg dose group APTT mean at baseline was 52.8 seconds and was 79.8 seconds at 24 hours. It was virtually unchanged for the 72 hour monitoring period. We performed a 1:1 mixing study for the prolonged APTTs using normal pooled rabbit plasma and the prolonged APTT corrected from a mean elevated time of 100.3 seconds using a LA-sensitive APTT reagent called PTT-LS reagent to 58.9 seconds mean time in the 10.0µg group. This result is usually indicative of a factor deficiency. However we did not find any deficiencies of known clotting factors. Factors V, VII, VIII, IX, X, (% activity) and fibrinogen levels (mg/dl) (clottable and immunological) were statistically and clinically elevated at 48 hours (p<0.05) in both groups receiving the venom. Factors II and XII were virtually unchanged. Factor XI rose in all three groups including the saline group but was not statistically significant (p=0.275). This may be related to the repeated blood draws and not the effect of the BRSV. Antithrombin and alpha-2 antiplasmin levels were increased in test subjects receiving the BRSV(p<0.05). Protein C was decreased at 24 hours in animals in the high dose group and remained decreased for the 72 hours of monitoring (p<0.05). No lupus anticoagulant was detected with screening and confirmatory assays. See Table 3. Thrombin and reptilase times were negative for the presence of heparin. No heparin was used anywhere in the protocol but we performed the assays because of the prolonged APTT. We also assayed for plasminogen, thrombin-anti-thrombin, FDP and d-dimers but the assays did not work with rabbit plasma probably due to lack of cross-reactivity in assays using monoclonal antibodies. In the case of plasminogen, the substrate for the chromogenic assay with human calibration plasma did not yield results on the rabbit plasma. Renal and liver functions were unremarkable in the three groups of test animals.

Table 2A. Coagulation factor testing

Sample	PT	APTT	Fibrinogen (clottable)	Fibrinogen (immunological)	Factor II	Factor V	Factor VII
Saline BL	7.5	50.5	330.3	400	114.8	3529.4	315.3
Saline 24	7.3	51.3	369.3	421.7	115.7	3672.3	341.3
Saline 48	7.1	55.8	449	565.3	135.4	4512.9	408.8
Saline 72	7.1	50.5	540.8	631.2	151.0	5153.5	458.3
4.0µg dose BL	7.4	52.8	327.5	379.8	106.0	3433.3	347.1
4.0µg dose 24	7.2	79.8	786	981.5	110.8	3435.7	289.8
4.0µg dose 48	6.9	76.2	*1072.9	*1458.7	165.0	*5889.0	*645.2
4.0µg dose 72	7.0	75.8	*1089.6	*1358.0	188.7	*7384.9	*764.0
10.0µg dose BL	7.4	51.9	327.6	409.8	106.8	3424.1	340.8
10.0µg dose 24	7.5	71.2	607.6	722.2	94.7	2922.3	242.4
10.0µg dose 48	7.0	84.2	*1175.2	*1438	161.3	*5205.8	*615.4
10.0µg dose 72	7.0	78.8	*1289	*1683	222.6	*7470.4	*787.6

PT and APTT results are expressed in seconds. Coagulation factor results are expressed in % activity. Fibrinogen levels are expressed in mg/dl. *APTT; Fibrinogen levels (clottable and immunological); FV; and FVII were statistically significant at 48 hour to 72 hours at both the 4.0 and 10.0µg doses (p = 0.001)

DISCUSSION

In vitro studies are inadequate to study the pathogenesis and treatment of BRS envenomation, and suitable animal models are necessary. NZW rabbits have been shown to be a suitable model for the study of treatments to prevent cutaneous necrosis. The present study extended these observations by demonstrating dose dependent size and depth of necrosis. In humans, mild to fulminating intravascular hemolysis and DIC have been described following BRS bites, and these systemic effects remain the most feared complications of envenomation.[2-11] Similar systemic effects were reproduced in a rabbit model using venom from another type of brown recluse spider not native to the United States known as the *Loxoceles laeta.* That spider, the Chilean brown recluse, is reputedly the most toxic species of *Loxosceles*, which have necrotizing venom, and it has been implicated in a few deaths in South America. In that study the baseline platelet count decreased 80.0% after 12 hours with a complete recovery 54 hours after injection. Fibrinogen levels decreased 50.0% at 12 hours post-venom injection, then increased to above normal values for 60 hours. FDPs increased four-fold over the baseline values at 12 hours after the venom injection[13]. That study used a staphylococcal-clumping FDP assay that is no longer available. The thrombocytopenia, an initial development of hypofibrinogenemia, and an increase of FDP products over 24 hours are typical of DIC[13].

The results seen in our study do not completely parallel the coagulation picture seen in humans following BRS envenomation. Despite similarities in the low platelet count at 24 hours, DIC with lowered fibrinogen levels were not demonstrated at 24 hours. Even in humans, there may be elevation of fibrinogen or d-dimers in some stages of response to the venom.[14] FDPs could not be assayed because the ELISA technique in our study used rabbit anti-human antibodies. When BRSV was introduced to human plasma in vitro in two previous studies, a LA-like affect was witnessed in the coagulation assays[1,2]. We could not reproduce this affect in the in vitro setting with platelet-poor rabbit plasma used for coagulation assays. The lower dose of 10.0µg of BRSV used in this study did cause a similar response in the coagulation testing witnessed in two previous animal studies which used a 20µg concentration of venom.[12-13] The elevation of a number of coagulation factors confirmed findings of two previous

Table 2B. Additional coagulation factor testing

Sample	Factor VIII	Factor IX	Factor X	Factor XI	Factor XII	AP	AT	PC
Saline BL	553.8	146.3	169	264.9	107.3	109.5	112.5	92.2
Saline 24	578.5	177.2	172.5	330.0	105.8	110.0	115.5	88.9
Saline 48	668.3	213.5	223.8	448.0	109.3	121.6	118.8	93.3
Saline 72	617.3	347.8	271.3	653.8	115.4	133.9	125.8	96.8
4.0µg dose BL	506.8	163.5	176.3	306.9	105.8	102.4	106.6	91.1
4.0µg dose 24	589	174.6	164.8	195.2	100.8	145.1	112.7	*71.3
4.0µg dose 48	*889.3	*331.8	*412.4	499.2	109.7	*216.0	*142.0	*73.8
4.0µg dose 72	*882.9	*491.7	*468.4	815.2	114.6	*221.3	*165.4	*81.3
10.0µg dose BL	511.7	150.3	134.3	243	98.0	103.2	106.9	96.8
10.0µg dose 24	531.4	137.7	156.8	130.1	90.3	125.4	97.4	*63.9
10.0µg dose 48	*674.8	*206	*447.8	*341.8	103.7	*231.2	*127.4	*57.3
10.0µg dose 72	*942.3	*469.6	*540.9	*666.9	107.4	*247.7	*183.6	*68.5

All results in this table are expressed in % activity.

*Factors VIII, IX, and X were statistically significant at 48-72 hours with both venom doses (p = 0.09, 0.04, and 0.001 respectively). No significant difference was seen in factor XII

*AP and *AT levels were statistically significant (p = 0.001 and 0.01 respectively) at both the 4.0 and 10.0µg doses

*Protein C level was statistically significant (0.01) starting at 24 hours in both venom doses from baseline levels

studies.[12-13] A prolonged APTT with a 1:1 correction to normal limits is usually indicative of a factor deficiency. None of the rabbit coagulation factors we assayed were decreased. Coagulation factors V, VII, VIII, IX, X, and fibrinogen (clottable and antigenic) were significantly elevated. AP and AT levels were extremely elevated. In some reports of BRS bites in humans the anti-thrombin levels have been decreased and required anti-thrombin concentrates to correct the deficiency.[11] In the animal model, protein C levels were decreased at 48 hours in both dose groups and remained low throughout the monitoring of the subjects. In humans a decreased Protein C level is commonly related to thrombotic disorders.[14] Elevation of coagulation factors such as fibrinogen, factors VII, VIII, IX, and XI and factor VIII have also been cited as risk factors for venous thrombosis in humans.[15-20]

CONCLUSIONS

The rabbit may be a good model for examining altered coagulation factors and potential therapy for the systemic effects of BRS envenomation, but commercial assays that rely on rabbit anti-human antibodies may be problematic. We reproduced the effect of elevated coagulation factors seen in our own prior studies of high dose envenomation. Using a lower dose of BRSV, dose related skin necrosis and formation of an eschar were demonstrated. Using lower doses of BRSV we did not have the profound physical depression previously witnessed in high dose protocols at our institution and the survival rate of the subjects was significantly higher. Because the low dose venom/NZW rabbit model demonstrated a clear dose-response relationship to the venom, it may prove suitable to study therapeutic agents and systemic effects of BRS venom.

Table 3. Inhibitor screening results for the presence of a lupus anticoagulant

Assay	Baseline results (seconds)	72 hour results (seconds)
PTT-LS	58.9	100.3
PTT-LS 1:1 mix	-	58.5
DVVT	55.3	66.8
DVVT (1:1)	-	47.0
DVVT ratio	-	1.02 (ratio only)
STACLOT-LA 1	-	62.7
STACLOT-LA 2	-	52.4
STACLOT-LA difference	-	10.5

PTT-LS = APTT lupus sensitive reagent
DVVT = dilute Russell's viper venom time; DVVT ratio of greater than 1.3 is indicative of an LA
1:1= mixing studies with rabbit normal pooled plasma
STACLOT-LA = confirmatory high phospholipid assay
STACLOT-LA difference is amount of correction. Evidence of an LA presence is >10.0 seconds in humans.

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