Cutaneous and Systemic Effects of Varying Doses of Brown Recluse Spider (Loxoceles reclusa) Venom in a Rabbit Model (Oryctolagus cunniculus)

David L. McGlasson, MS; Hugh H. Harroff, DVM; Jackie Sutton; Edward Dick, DVM; Dirk M. Elston, MD

Correspondence: David L. McGlasson, MS, CLS/NCA, davemcglasson@hotmail.com

Abstract

Objective: To ascertain the cutaneous and systemic effects of a single variable dose of Brown Recluse Spider venom (BRSV) in a rabbit model. Cutaneous necrosis is a serious complication of brown spider envenomation. Disseminated intravascular coagulation (DIC) remains the most dreaded complication of brown recluse envenomation in humans. New Zealand white (NZW) rabbits have proved to be a suitable 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 the NZW rabbit is a suitable model to study both cutaneous and systemic effects of the venom.

Design and Setting: Thirty-six New Zealand white rabbits were divided into three groups. One group received a saline injection, and the other two groups received a 4.0µg/ml or a 10.0µg/ml dose of purified BRSV intradermally into the skin on the dorsum of the back. Samples were collected at baseline, 24, 48 and 72 hours. Tissue specimens were obtained at 72 hours. Measurements included CBC with platelets, PT, APTT, fibrinogen (clottable and immunological), coagulation factors II, V, VII, VIII, IX, X, XI, XII, antithrombin, alpha-2 antiplasmin, protein C, mixing studies, lupus anticoagulant screening, plasminogen, thrombin-anti-thrombin, FDP, d-dimer, and thrombin time.

Interventions: None

Results: The WBC and platelet counts decreased dramatically at 24 hours in the two groups receiving the BRSV. BRSV produced a dose related increase in the APTT. Levels of fibrinogen as well as Factors V, VII, VIII, IX, X, anti-thrombin III, and alpha-2 antiplasmin were greatly increased in response to the BRSV. Protein C decreased at 24 hours and remained low in other time points. Mixing studies corrected the prolonged APTT's to normal ranges. Factor II, FXI and FXII 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. We did not detect a factor deficiency or evidence of a lupus anticoagulant. The assayed coagulation factors were elevated or normal following the BRS envenomation, while Protein C demonstrated a sustained decrease. Although DIC did not occur in the rabbit model, alterations in coagulation factors were evident that could shed light on a human coagulopathy following envenomation. Gross pathology results were consistent with previous studies that used higher doses of BRSV.

Background

Previous studies at our institution have been performed to evaluate the effect of the BRSV venom 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 an elevated APTT, abnormal 1:1 mixing studies, falsely decreased coagulation factors that corrected upon dilution, a positive dilute Russell Viper venom assay and a positive platelet neutralization assay. In humans, BRS spider bites can produce disseminated intravascular coagulation (DIC) as well as other coagulation deficiencies. [3-11] Our findings in of an LA-like inhibitor in the in vitro setting led to control testing for the coagulation reagents used to test for the presence of an LA.[2] The NZW rabbit has proved to be a suitable model to determine the histologic changes of BRS-induced necrosis and to evaluate therapeutic agents.[12-13] In these studies each rabbit received an intradermal injection of 20µg/ml of venom given between the layers of the skin over the back. An unexpected finding was a prolonged APTT, marked elevation of fibrinogen and dramatically increased coagulation factors at 72 hours post dosing with BRSV. Unfortunately, there was not enough blood taken from each animal during those studies for further analysis. The dose of toxin given to the rabbits in the previous study 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 we hoped to develop a model suitable for the study of skin necrosis as well as the coagulation effects of BRSV venom, thereby leading to better methods of treatment of this spider bite.

Methods

This research was performed at an AAALAC 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. Two groups of rabbits received doses of purified BRSV (4.0µg/ml and 10µg/ml 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 1.0 ml 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 7 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 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-Dyn, 3700 (Abbott Park, IL). The citrated blood was separated by centrifugation (2500g X 15 minutes) to obtain platelet-poor plasma. We then performed PT, APTT, Fibrinogen (clottable and immunological), d-dimer, Reptilase time, thrombin times.

coagulation factors II, V, VII, VIII, IX, X, XI, XII, anti-thrombin III, alpha-2 antiplasmin, Protein C, mixing studies (using rabbit normal plasma), lupus anticoagulant screening and confirmatory testing (dilute Russell Viper Venom (American Diagnostica, Greenwich, CT), (STACLOT-LA, Diagnostica-Stago, Inc.), plasminogen using reagents from Diagnostica-Stago, Inc. (Parsippany,

NJ). 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 (Ithaca, NY). We also "spiked" invitro some rabbit platelet-poor citrated plasma that was used for the other coagulation assays in a manner described in tow previous studies. 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 width of the area of necrosis and the size of the eschar in centimeters. The inflammation and vasculitis and necrosis depth severity were based on a overall score of 0-4 from no effect to a severe response to the BRSV on the injected site.

Results

The gross pathology results were as follows: The 10.0µg/ml group had a depth of necrosis mean score of 3.75 (0-4). The mean measurement of the area of necrosis in this group was 12.7 centimeters. The mean of the eshcar width was 6.0 centimeters. The overall severity of the inflammation and vasculitis were all 3 to 4 in the overall score. The 4.0µg/ml subjects had a depth of necrosis estimation of 2.83. The area of necrosis mean for this dose was 8.25 centimeters. The mean of the eschar width was 4.7 centimeters. The severity of inflammation and vasculitis had one rabbit with a score of 4, one with a 2 score and the other rabbits were all 3. None of the saline injected group had any evidence of a lesion or area of necrosis.

The CBC and platelet level results are displayed in Table 1. The 10.0µg/ml dose group dramatically dropped the WBC counts from a mean of $6.9x10^9$L to $3.4x10^9$L at 24 hours. The platelet count in the same group also dropped dramatically from $341x10^9$L to $61.0x10^9$L. The WBC counts gradually rose to $19.8x10^9$L at 72 hours, which may be indicative of the effect and inflammation caused by the BRSV injection. The platelet counts recovered to $344x10^9$L after 72 hours, which was virtually the same as the baseline levels. The 4.0µg/ml dose showed a lesser response in the WBC and platelet counts. The decrease was minimal compared to the higher dose. The saline group was virtually unaffected. All of the animals showed a drop in the RBC ($x10^{12}$l), HGB(g/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 factor testing revealed some interesting and dramatic differences between the three groups of animals. See Table 2. The PT levels were virtually the same for all groups at baseline and each 24 hour time point. The APTT's increased dramatically at 24 hours and stayed elevated in both of the groups receiving the BRSV. We performed a 1:1 mixing study for the prolonged APTT's using normal pooled rabbit plasma and the prolonged APTT corrected from a mean elevated time of 100.3 seconds using PTT-LS reagent to 58.9 seconds mean time at baseline in the 10.0µg/ml group. This result is usually indicative of a factor deficiency. However we did not find any factor deficiencies. All of the coagulation factors V, VII, VIII, IX, X (% activity) and fibrinogen levels (mg/dl) (clottable and immunological) were statistically and clinically elevated at 48 hours. Factors II and XII were virtually unchanged. Factor XI did rise in all three groups including the saline group. This finding may be caused by the repeated blood draws and not

apparently attributable to the effect of the BRSV. No evidence of lupus anticoagulants was evident 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. Anti-thrombin III and alpha-2 antiplasmin levels were greatly increased in test subjects receiving the BRSV. Protein C results were decreased at 24 hours in animals in the high dose group and stayed decreased for the 72 hours of monitoring. See Table 4. We also assayed for plasminogen, thrombin-anti-thrombin, FDP and d-dimers but the results did not work well with rabbit plasma probably due to lack of cross-reactivity in assays using a monoclonal antibody. Renal and liver functions were unremarkable in the three groups of test animals.

Conclusions

In vitro studies are inadequate to study the chain of events and treatment options for severe BRS envenomation, and suitable animal models are necessary. NZ white 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 with and without DIC have been described following BRS bites, and these systemic effects remain the most feared complications.[2-11] Similar systemic effects have not previously been described in an animal model. The results seen in this study do not completely parallel the coagulation picture seen in humans following BRS envenomation. Specifically, DIC with lowered fibrinogen levels was not demonstrated in the rabbits. Even in humans, there may be elevation of fibrinogen or d-dimers in some stages of response to the venom.[14] When BRSV was introduced to human plasma in vitro in two previous studies a LA-like affect was witnessed in the coagulation assays. We could not reproduce this affect in the in vitro setting with platelet-poor rabbit plasma used for the coagulation assays in this study. The lower dose of 10.0µg.ml of BRSV did cause a similar response in the coagulation testing witnessed in two previous animal studies.[12-13] The elevation of a number of coagulation factors confirmed findings of two previous 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. Alpha-2 antiplasmin, anti-thrombin III were extremely elevated. In some reports of BRS bites in humans the anti-thrombin levels have been decreased and required anti-thrombin III concentrates to correct the deficiency.[11] In our 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 predictors of venous thrombosis in humans.[15-20]

The rabbit may be a good model for examining altered coagulation factors and potential therapy for the systemic effects of BRS envenomation. We reproduced the effect of elevated coagulation factors seen in our prior studies that had used high dose envenomation. Using a lower dose of BRSV, dose related skin necrosis and formation of an eschar were demonstrated. At the lower dose of BRSV rabbits did not have the profound physical depression previously witnessed in high dose protocols at our institution and the survival rate of the subjects were significantly higher. The low dose venom / NZ white rabbit model may prove suitable to study therapeutic agents for the systemic effects of BRS venom.

Disclaimer

The views expressed are those of the authors and are not to be construed as official or as reflecting those of the Department of Defense. Several authors were employees of the US federal government when this work was investigated and prepared for publication; therefore, its is not protected by the Copyright Act and there is no copyright of which the ownership can be transferred.

Acknowledgements: This research was performed under the authority of the Department of Defense and the Wilford Hall Medical Center, 59th Clinical Research Squadron, Lackland AFB, TX, Institutional Animal Care and Use Committee and Institutional Review Board. We would also like acknowledge Dr. William Van Stoecker, M.D., Rolla, MO, and Dr. Gary Wasserman, D.O., Chief of Medical Toxicology, Division of Pediatric Pharmacology and Medical Toxicology, Professor at University of Missouri, Kansas City School of Medicine for their encouragement and assistance.

References

1. McGlasson DL, Babcock JL, Patterson WR, Haun DE, Triplett DA. Evaluation of the lupus anticoagulant effects of brown recluse spider venom on normal plasma. J Mil Medicine. 1990;18:95-98.
2. McGlasson DL, Babcock JL, Berg L, Triplett DA. ARACHNase: An Evaluation of a positive control for platelet neutralization procedure testing with seven commercial activated partial thromboplastin time reagents. AJCP. 1993;100:576-578.
3. Vorse H, Seccareccio P, woodruff K et al: Disseminated intravascular coagulopathy following fatal brown spider bite (necrotic arachnidism). J Pediatr. 1972;80:1035-1037.
4. Eichner ER. Spider Bite Hemolytic Anemia: Positive Coombs' Test, Erythrophagocytosis, and Leukoerythroblastic Smear. AJCP. 1984;81:683-687.
5. Futrell JM. Loxoscelism. Am J Med Sci. 1992;304:261-267.
6. Leung LK, Davis R. Life-threating hemolysis following a brown recluse spider bite. J Tenn Med Assoc. 1995;88:396-397.
7. Williams ST, Khare VK, Johnston GA, Blackall DP. Severe intravascular Hemolysis Associated with brown recluse spider envenomation: A report of two cases and review of the literature. AJCP. 1995;104:463-467.
8. Wasserman G, Garola R, Marshall et al: Death of a 7 year old y presumptive brown Recluse spider bite. J Toxicol Clin Tocxicol. 1999;104:614-615.
9. Elston DM. What's Eating You? Loxoceles recluse (Brown Recluse Spider). Cutis. 2002; 69:91-95.
10. Lane DR, Youse JS. Coombs-positive hemolytic anemia secondary;y to brown recluse spider bite: A review of the literature and discussion of treatment. Cutis. 2004;74341-347.
11. Wasserman G. Critical Care Toxicology-diagnosis and management of the critically poisoned patient. Brent J et al: Elsevier Mosby. 2005;112:1195-1203.
12. Elston DM, Miller SD, Young III RJ, Eggers J, McGlasson DL, Schmidt WH, Bush A. Comparison of Colchicine, Dapsone, Triamcinolone, and Diphenydramine Therapy for the Treatment of Brown Recluse Spider Envenomation. Arch Dermatology. 2005;141:595-597.
13. Elston DM, Eggers JS, Schmidt WE, Storrow AB, Doe RH, McGlasson DL, Fischer JR. Histological findings following brown recluse envenomenation. Amer J of Dermatopathology. 2000;22:242-246.
14. Fritsma GA. Thrombosis risk testing. In Rodak B. WB Saunders Co. 2002;44:646-681.

15. Koster T et al.: "Role of clotting factor VIII in effect of von Willebrand factor on occurrence of DVT". Lancet 1995;345:152-5.

16. Kamphuisen PW et al.: "High factor VIII antigen levels increase the risk of venous thrombosis but are not associated with polymorphisms in the von Willebrand factor and factor VIII gene". British Journal of Haematology 2001;115:156-8.

17. Koster T et al.: "Factor VII and fibrinogen levels as risk factors for venous thrombosis". Thrombosis and Haemostasis 1994;71:719-22.

18. van Hylckama VA et al.: High levels of factor IX increase the risk of venous thrombosi. Blood 2000;95:3678-82.

19. Meijers JCM et al.: "High levels of coagulation factor XI as a risk factor for venous thrombosis". New England Journal of Medicine 2000;342:696-701.

20. Wen-Harn P, Chyi-Huey B, Jiunn-Rong C, Hou-Chang C. Associations between carotid atherosclerosis and high factor VIII activity, dyslipidemia and hypertension. Stroke. 1997;28:88-94.

Table 1: Complete blood count results/Platelets

SAMPLE	WBC	RBC	HGB	HCT	PLT
HEMQCBL	6.9	6	13.1	41.1	331
HEMQC24	7.8	5.2	11.5	35.7	305
HEMQC48	7.6	4.9	10.9	34.2	328
HEMQC72	8	4.5	10.1	31.5	347
HEM4BL	7	6.1	13.2	41	391
HEM424	5.3	6	12.8	40.2	255
HEM448	10	4.9	10.6	33.1	264
HEM472	14.7	4.4	9.4	29.6	398
HEM10BL	6.9	6.3	13.9	43.6	341
HEM1024	3.4	5.7	12.5	39.3	61
HEM1048	7.7	5.1	12	36	149
HEM1072	19.8	4	9.2	28	344

Table 2: Coagulation factor testing. Results in % activity. Fibrinogen levels in mg/dl.

SAMPLE	PT	APTT	FIB CLOT	II	V	VII	X	VIII	IX	X
SALINE-BL	7.5	50.5	330.3	114.8	3529.4	315.3	169	553.8	146.3	
SALINE-24	7.28	51.3	369.3	115.7	3672.3	341.3	172.5	578.5	177.2	
SALINE-48	7.14	55.8	449	135.4	4512.9	408.8	223.8	668.3	213.5	
SALINE-72	7.12	50.5	540.8	151	5153.5	458.3	271.3	617.3	347.8	
4.0-BL	7.4	52.8	327.5	106	3433.3	347.1	176.3	506.8	163.5	
4.0-24	7.2	79.8	786	110.8	3435.7	289.8	164.8	589	174.6	
4.0-48	6.93	76.2	1072.9	165	5889	645.2	412.4	889.3	331.8	
4.0-72	7	75.8	1089.6	188.7	7384.9	764	468.4	882.9	491.7	
10.0-BL	7.4	51.9	327.6	106.8	3424.1	340.8	134.3	511.7	150.3	
10.0-24	7.5	71.2	607.6	94.7	2922.3	242.4	156.8	531.4	137.7	
10.0-48	7.03	84.2	1175.2	161.3	5205.8	615.4	447.8	674.8	206	
10.0-72	7.01	78.8	1289	222.6	7470.4	787.6	540.9	942.3	469.6	

SALINE-BL
SALINE -24
SALINE-48
SALINE 72
4.0 –BL
4.0-24
4.0-48
4.0-72

10.0-BL
10.0-24
10.0-48
10.0-72

Table 3: Inhibitor screening for the presence of 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
STACLOT-LA1		62.7
STACLOT-LA2		52.4
STACLOT-LA DIFFERENCE		10.5

Table 4

SAMPLE	ALPHA-2 ANTIPLASMIN	ANTI-THROMBIN III	PROTEIN-C
SALINE-BL	109.5	112.5	92.2
SALINE -24	110	115.5	88.9
SALINE-48	121.6	118.8	93.3
SALINE 72	133.9	125.8	96.8
4.0 –BL	102.4	106.6	91.1
4.0-24	145.1	112.7	71.3
4.0-48	216	142	73.8
4.0-72	221.3	165.4	81.3
10.0-BL	103.2	106.9	96.8
10.0-24	125.4	97.4	63.9
10.0-48	231.2	127.4	57.3
10.0-72	247.7	183.6	68.5