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HEMOSTATIC PROPERTIES OF A VENOMIC PROTEIN IN RAT ORGAN TRAUMA

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Abstract

Previous in vitro work characterized the protease Q8009 isolated from the venom of the Australian brown snake *Pseudonaja textilis textilis* with Factor Xa-like activity and hemostatic properties. The purpose of the work described here characterizes the in vivo hemostatic properties in a rat model of parenchymatous organ injury. The key parameters of activity included reduction in time-to-hemostasis and total volume of blood loss in spleen, liver and kidney wound models in rats. The surgical protocols involved exposure of the organs via a midline abdominal laparotomy. Using a clean metal template with 6, 6.5 9 mm holes for spleen, liver and kidney, respectively, a predetermined volume of the organ was gently extruded through the template hole and excised with a razor blade. About 50 to 75 μ L of collagen matrix with the different test solutions was applied to the wounds. Blood was collected and at the end of the procedure animals were humanely sacrificed with an anesthetic overdose. Determination of blood was performed using the hematin assay using a standard curve. Blood loss per minute and total blood loss were calculated. Results from the studies demonstrated that the application of Q8009 and collagen matrix to surgical wounds significantly reduced the total amount of blood loss and the time-to-hemostasis. In the spleen wound model, Q8009 at 100, 250 and 1000 μ g/ml) significantly reduced ($p < 0.001$) the total volume of blood lost relative to thrombin and reduced the time-to-hemostasis by 25–50%, as compared to 7% by thrombin. In the liver wound model, Q8009 at 250 and 1000 μ g/ml significantly reduced ($p < 0.001$) the total volume of blood lost relative to thrombin and reduced the time-to-hemostasis from 10.5 minutes by thrombin to 5.6 minutes with Q8009. In the kidney wound model, Q8009 at 250 μ g/ml significantly reduced ($p < 0.05$) the total volume of blood lost and reduced the time-to-hemostasis by 25% when compared to thrombin. The hemostasis levels were consistent with previous findings in skin wound rat models where Q8009 consistently reduced the total volume of blood lost and shortened time-to-hemostasis. Application of Q8009 plus collagen matrix significantly reduced the volume of total blood loss and time-to-hemostasis in rat surgical organ wound models induced bleeding, as compared to a commercially available hemostat device. The protein Q8009 has greater capacity to reduce blood

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Authors Contribution:

Roscoe L. Warner: Designed research, performed research, data analysis, manuscript preparation

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The authors do not have a financial interest in the potential product that was studied in the present work.

loss and shorten time-to-hemostasis; highly desirable properties where rapid hemostasis is needed in surgical wounds in parenchymatous organs.

Keywords

Time-to-hemostasis; Blood Loss; Q8009; Venomic Protease; Thrombin

INTRODUCTION

Reduction of blood loss and quick hemostasis are important elements in the surgical setting. Hemorrhagic shock on account of significant blood loss has been studied in several animal models (Toth et al., 2004; Vallejo et al., 2005; Zakaria et al., 2005), and in human traumatic injury, multiple organ failure and hemorrhagic shock after resuscitation (Fruchterman et al., 1998; Geppert et al., 2002; Jarrar et al., 1999). Complications include depression of myocardial contractility and sepsis (Horton, 1989; Rollwagen et al., 1997). Prolonged periods of hemorrhage decrease immune function facilitating bacterial migration across the gut, leading to sepsis (Jarrar et al., 1999). Replenishment of circulating volume with subsequent reperfusion, results in activated neutrophils and efflux of cytokines from the gut (Detch et al., 1994; Upperman et al., 1998). The production of oxygen-derived free radicals and tumor necrosis factor alpha (TNF α) cause myocardial dysfunction as well (Bolli et al., 1989; McCord, 1985; Oral et al., 1995). Included in the array of post hemorrhagic shock symptoms is a stimulation of the general inflammatory response (Gonzalez et al., 2001), with induction of nitric oxide synthase (iNOS; Hierholzer et al., 1998), cyclooxygenase (COX-2), cytokines, chemokines and complement cascade (Ayala et al., 1991; Geppert et al., 2002; Hamano et al., 1998; Hierholzer et al., 1998; Hierholzer et al., 1999; Jarrar et al., 2004; Meng et al., 2000; Meng et al., 2001; Patrick et al., 1996; Zingarelli et al., 1994). Additionally, hemorrhagic shock can lead to activation or suppression of a number of complement components in the coagulation cascade (Riedemann et al., 2004; Spain et al., 1999; Szebni et al., 2003), leading to impairment of coagulation (Laudes et al., 2002), contributing to continued sustained loss of blood. Therefore development of compounds to improve hemostasis in the surgical setting is of paramount medical importance.

Numerous approaches have been attempted to decrease time-to-hemostasis in surgical models and the Q8009 protease was effective in dermal injury (Warner et al., 2007). Natural products such as sponges impregnated with diatomaceous earth or raw cotton materials were used to reduce blood flow with varying degrees of success in minor to severe battle field wounds. Isolated human fibrin was used during surgery to stop bleeding (Bergel, 1909) and fibrin sheets were used in World War I (Grey, 1915) and in civilian hospitals (Harvey, 1916) for traumatic surgeries, with limited success. Increased use of thrombin and fibrinogen from human sources were developed over time (Cronkite et al., 1944). Although these procedures were successful in reducing blood loss (Webster and Slansky, 1968), they caused immunological and infectious complications (Bove, 1978). More recently, different matrices capable of absorbing blood and decreasing clotting times *in situ* became available. Hemostatic agents include gelatin sponges which have no intrinsic hemostatic properties, but the meshwork retains platelets with subsequent clot formation. Gelfoam, a sponge from isolated porcine gelatin became available in 1945 (Pfizer, Groton, CT). Oxidized cellulose, a fabric-like material, is produced by oxidation of cotton gauze or other cellulose material with nitrous oxide. The low pH of the carboxyl groups on cellulosic acid is caustic and hemostasis is achieved by denaturation of blood proteins. Oxycel (Becton Dickinson, Franklin Lakes, NJ) became available in 1945 followed by Surgicel (Johnson & Johnson Medical Inc, Arlington TX) in 1960. Surgicel is similar to Oxycel in that it is extruded as continuous uniform fibers which are then oxidized with the resulting material more uniformly absorbed. Microfibrillar bovine collagen is water-

insoluble with a microcrystalline structure. Platelets attach to specific sites on the coarse fibers and subsequently degranulate initiating the clotting cascade and Helitene (Integra LifeSciences Inc., Plainsboro, NJ) was introduced in 1985. Medical devices now contain hemostatic agents and utilize specialized delivery systems to allow placement of material in highly localized vasculature sites. The advent of medical materials manufactured from collagen and cellulose in addition to the use of bovine, porcine or human components, either alone or in combination with collagen and cellulose, greatly increased survival of patients during surgery.

Recent studies indicate that unique proteins isolated from snake venom have hemostatic properties (Marsh et al., 1997; Monterio and Zingali, 2000; Rao and Kini, 2002; Rao et al., 2004; Speijer et al., 1986; St Pierre et al., 2008). Several of these novel serine proteases have factor Xa or Prothrombin activation properties. This report describes studies from our laboratory employing Q8009, a protein isolated from the venom of the Australian brown snake, *Pseudonaja textilis textilis* (Masci et al., 1988) whose activity we have shown previously to reduce blood loss when applied topically (Warner et al., 2007). This factor Xa-like protein significantly reduced time-to-hemostasis and blood loss in several surgical rat wound models of parenchymal organs.

MATERIALS and METHODS

Reagents and Materials

All materials were reagent grade or higher and were purchased from Sigma Chemical Company (St. Louis, MO) except where noted.

Collagen Matrix

Baxter FloSeal Hemostatic Matrix Kits (Baxter Healthcare Corp., Fremont, CA) a preparation for surgical use, containing the following: 1) Collagen Matrix, a proprietary preparation composed of cross linked granules of bovine-derived gelatin; 2) Thrombin as lyophilized recombinant bovine thrombin (GenTrac Inc, Middleton, WI); 3) Sterile saline, Sodium Chloride USP 0.9%. In the treatments described below, 800 μ L of reconstituted thrombin and 75 mg of matrix were mixed for 30 seconds in a sterile 1.5 mL micro centrifuge tube and allowed to stand for one minute before use.

Laboratory animals

Sprague Dawley rats utilized in these studies (male, 275–300 grams) were obtained from Charles River Laboratories (Wilmington, DE). Animals were maintained under pathogen-free conditions in 12 hrs diurnal cycles, with water and food *ad libitum*. Animal rooms were kept at $21 \pm 3^\circ\text{C}$ with several changes of air per hour. All husbandry and animal procedures were in accordance of humane animal handling practices under the guidance of the Unit for laboratory Animal Medicine at the University of Michigan. Rats used in the different surgical procedures were anesthetized with 60 mg/kg body weight Ketamine (Fort Dodge Labs, Fort Dodge, IA) and 10 mg/kg body weight Rompun (Lloyd Laboratories, Shenandoah, IA). This procedure was adequate for providing adequate anesthesia during surgery. At the end of each procedure, all animals were humanely sacrificed by ketamine administration. All surgery-model specific protocols were approved by the Animals Care Committee of the University of Michigan and in compliance with AAALAC guidelines.

Factor Xa assay

Test solutions were prepared 10 minutes prior to use by mixing 800 μ L of test reagents (thrombin or Q8009) with 75 mg of collagen matrix such that the resulting matrix would rehydrate to a final volume of 1.0 mL. Each collagen matrix preparation of either thrombin or

Q8009 was used within 2 hrs. Q8009, a factor Xa-like protease was supplied by QRxPharma Pty Ltd, Brisbane, Australia. The properties of this sample (lot no. Q 70040-12-03-17) were stated in the Analytical Certificate supplied and in the laboratory file. Stability was assayed monthly by using the Prothrombin Cleavage Activity Assay. Factor Xa activity was assessed in the Q8009 sample utilizing the synthetic substrate S-222 (Diapharma Group Inc.). Briefly, Bovine Factor Xa standard (Sigma, Chemical Co.), Q8009 sample and S-222 substrate, were diluted in Tris-HCl buffer (pH 7.0) and 100 μ l of each reagent were placed in a 96-well microtiter plate and incubated at 37° C for 15 minutes. One hundred μ L of Q8009 solution or thrombin standards were transferred to plates containing S-222 substrate. Plates were incubated at 37° C for 5 minutes and the reaction was read at 405 nm in a plate reader (SpectraMax 190, Molecular Devices, Union City, CA). Factor Xa activity of Q8009 sample was determined from a standard curve, with activity varying $\pm$ 8% over the duration of experiment.

Hemoglobin Assay

Hemoglobin was measured in each of the tubes collected at one minute intervals and the assay was performed using a modification of a previously published protocol (Shaw et al., 1972). Briefly, glass tubes containing NaOH and blood soaked Whatman #4 filter paper chads were placed in a rotary shaker overnight at room temperature to elute hematin. Samples were serially diluted in NaOH and read at 550 nm on a plate reader (SpectraMax 190, Molecular Devices). Hematin concentration was determined against a standard curve containing known volumes of blood in NaOH and reported as μ L of blood lost per minute.

Time-to-Hemostasis

Time-to-hemostasis was defined as the interval between the initiation of the organ injury to the time where less than 1 μ L of blood was detected in the Hematin assay. The average time-to-hemostasis was determined by dividing the sum of the Time-to-hemostasis values in the group by the number of animals.

Spleen Wound Model

The spleen wound model was based on a previous protocol (Lindblom et al., 1990). Anesthetized rats had the abdominal hair clipped, and the skin wiped with 70% ethanol. A midline laparotomy incision was made to expose the spleen. The anterior tip of the spleen was gently lifted and wrapped with a saline-moistened sterile gauze pad. Using a metal template with a 6 mm perforation, the anterior tip of the spleen was extruded through so 4 to 5 mm of the spleen tip extended above the template orifice and cut with a blade. Immediately, 50 microliters of Baxter Collagen Matrix (previously mixed with test reagents) was applied to the wound surface using a pipette. Blood was collected onto filter paper chads (Whatman #4 filter paper) at one-minute intervals and placed into glass test tubes containing 2 mL of 10% NaOH. Blood was collected for 12 one-minute intervals or until no visible blood could be seen on chads. At the end of the procedure the spleen was removed, weighed (total and portion excised) and the weight was recorded.

Liver Wound Model

The liver wound model was based on a previously protocol (Matsuoka et al., 1995). The surgical approach was the same as in the spleen model. The right lateral hepatic lobe was gently lifted to expose the tip and wrapped with saline-moistened sterile gauze extruded through the 6.5 mm port of the metal plate until 4 to 5 mm protruded above the template and the tissue was dissected using a sterile razor blade. The collagen matrix was applied to the wound surface and the blood collection method was the same as in the spleen model. At the end of the procedure the remainder of the right lateral lobe was removed, weighed and recorded.

Kidney Wound Model

The kidney trauma injury model was based on a previously protocol (Tuthill et al., 2001). The rats were surgically prepared in the same fashion as described for the spleen wound model. Following the midline incision and removal of the perirenal fat, the kidney was wrapped with saline-moistened sterile gauze and the caudal pole of the kidney was extruded through metal plate with a 9 mm hole so that about 3 to 4 mm protruded above the plate and the tissue was severed with a blade. Collagen matrix was applied to the wound surface and the blood collection method was the same as in the spleen model. At the end of the procedure the kidney was removed, weighed (total and portion excised) and weights were recorded.

Statistics

Statistical comparisons were performed by means of the computer program GraphPad Prism 4.0 (GraphPad Software Inc, San Diego, CA. Comparisons of Total Blood Loss between groups was first tested by the Kruskal-Wallis Test ($p < 0.001$) for non parametric median distribution followed by a One-Way ANOVA with individual group significance determined by a Dunn's Multiple Comparison test. The percent of animals at established intervals was plotted using the Kaplan-Meyer design.

RESULTS

Spleen wound model

In the spleen wound model, $12.40\% \pm 0.15\%$ of the spleen was removed producing a wound surface area of $18.85 \pm 0.95 \text{ mm}^2$ (data not shown) onto which $50 \mu\text{L}$ of the collagen matrix was applied. Blood loss levels for animals not receiving any intervention (None treatment group) bled for 12 minutes and lost $59.44 \pm 25.21 \mu\text{L}$ of blood (Table 1). Animals receiving saline mixed with collagen matrix bled for 12 minutes and lost $109.37 \pm 43.17 \mu\text{L}$ of blood. Thrombin plus collagen matrix group did not decrease the amount of blood lost at $78.95 \pm 44.45 \mu\text{L}$ (Table 1), and there was no significant reduction of the time-to-hemostasis at 8.71 ± 2.92 minutes (Table 4). Q8009 at $100 \mu\text{g/mL}$ plus collagen matrix significantly reduced (65%, $p < 0.001$) the amount of blood lost at $27.18 \pm 18.72 \mu\text{L}$ (Table 1), relative to the thrombin plus matrix group and decreased the mean time-to-hemostasis to 6.45 ± 3.63 minutes post injury (Table 4). Q8009 at $250 \mu\text{g/mL}$ plus collagen matrix stopped the bleeding at 5 minutes following the initiation of injury and significantly reduced (67%, $p < 0.001$) blood lost at $25.97 \pm 13.25 \mu\text{L}$, relative to the thrombin group. Collagen matrix plus Q8009 at $1,000 \mu\text{g/mL}$ also stopped bleeding at 7 minutes following injury and significantly reduced (56%, $p < 0.001$) blood lost at $34.53 \pm 19.46 \mu\text{L}$, relative to thrombin treatment. The application of Q8009 plus collagen matrix at 100, 250 and $1,000 \mu\text{g/mL}$, significantly reduced (65%, 67%, 56%, $p < 0.001$) the amount of blood lost at minutes 5–7, relative to thrombin treatment. In addition only the use of Q8009 reduced blood loss relative to untreated group (Table 1). More importantly, while both the negative and saline control groups had 50% of all animals still bleeding at 10–11 minute interval and thrombin at 6–8 minutes, all three concentrations of Q8009 had 50% of the animals reaching hemostasis by the 3–5 minute interval (Figure 1A).

Liver wound model

In the liver wound model, we removed $20.32\% \pm 2.48\%$ of the right lateral lobe of the liver, with a resulting wound surface area of $23.30 \pm 2.54 \text{ mm}^2$ (data not shown) onto which $50 \mu\text{L}$ of collagen matrix was applied. Animals not receiving any intervention (None treatment group) continued to bleed for 12 minutes and lost a total of $357.57 \pm 267.31 \mu\text{L}$ of blood (Table 2) with 50% of the animals still bleeding at the end of the experimental period (Figure 1B). Animals receiving saline plus collagen matrix also bled for 12 minutes and lost $283.42 \pm 178.30 \mu\text{L}$ of blood. The thrombin ($1,000 \text{ U/mL}$) plus matrix group decreased the amount of blood

lost at $105.43 \pm 33.25 \mu\text{L}$ (Table 2), relative to the no treatment (70%) and saline (63%) plus matrix groups. However, with thrombin treatment the mean time-to-hemostasis increased from 9.91 (None treatment group) to 10.50 minutes when thrombin plus collagen matrix was applied (Table 4) and 40% of all animals were still bleeding by the 12th minute post injury (Figure 1B). Q8009 at 100 $\mu\text{g/mL}$ plus collagen matrix did not significantly reduce the amount of blood lost at $138.57 \pm 90.94 \mu\text{L}$ (Table 2), relative to the thrombin plus matrix group or significantly decreased the mean time-to-hemostasis (Table 4) following treatment. Q8009 at 250 $\mu\text{g/mL}$ plus collagen matrix stopped bleeding 6 minutes following the initiation of injury and treatment significantly reduced (62%, $p < 0.001$) blood loss ($38.05 \pm 20.95 \mu\text{L}$), relative to thrombin. Q8009 at 1,000 $\mu\text{g/mL}$ plus collagen matrix also stopped bleeding 6 minutes following injury and significantly reduced (50%, $p < 0.01$) blood loss ($52.34 \pm 25.81 \mu\text{L}$), relative to thrombin (Table 2). Application of Q8009 plus collagen matrix at 250 and 1,000 $\mu\text{g/mL}$ significantly reduced ($p < 0.001$ and $p < 0.01$, 62% and 50%, respectively) the amount of blood lost at 6 minutes, relative to thrombin. In addition, while 50% of the animals were still bleeding in the thrombin treatment group at the 10–11 minute interval, this same percentage of hemostasis (50%) occurred earlier (i.e. the 3–5 minute time interval) in both Q8009 250 $\mu\text{g/mL}$ and 1,000 $\mu\text{g/mL}$ groups (Figure 1B). Moreover, the mean time-to-hemostasis for thrombin treatment was 10.5 ± 2.04 minutes while for Q8009 at 250 $\mu\text{g/mL}$ and 1,000 $\mu\text{g/mL}$ was reduced to 5.10 ± 1.67 and 6.20 ± 1.96 minutes, respectively (Table 4).

Kidney wound model

In the kidney wound model, we removed $5.26\% \pm 1.37\%$ of the kidney resulting in a wound surface area of $35.62 \pm 2.99 \text{ mm}^2$ (data not shown) onto which 75 μL of the test agent plus collagen matrix was applied. Animals not receiving any intervention (None treatment group) bled for 12 minutes and lost $629.28 \pm 195.65 \mu\text{L}$ of blood (Table 3) with 50% of the animals still bleeding by the 10th–11th minutes interval (Figure 1C). Animals receiving saline plus collagen matrix bled for 12 minutes and lost $448.64 \pm 202.75 \mu\text{L}$ of blood. Thrombin (1,000 U/mL) plus matrix group significantly decreased (46%, $p < 0.001$) the amount of blood lost at $244.18 \pm 87.17 \mu\text{L}$ (Table 3), relative to saline plus matrix group and the mean time-to-hemostasis was marginally reduced from 8.20 ± 2.99 minutes (Saline treatment group) to 7.45 ± 3.14 minutes by the application of thrombin (Table 4). In addition in the thrombin treatment group, 85% of the animals reached hemostasis by the 11–12 minute interval (Figure 1C). Q8009 at 100 $\mu\text{g/mL}$ plus collagen matrix did not significantly reduce the amount of blood lost at $366.70 \pm 182.12 \mu\text{L}$ (Table 3), relative to thrombin plus matrix group but decreased the time-to-hemostasis to 10 minutes post injury. Q8009 at 250 $\mu\text{g/mL}$ plus collagen matrix significantly reduced (40%, $p < 0.05$) the total blood lost to $145.22 \pm 52.25 \mu\text{L}$, relative to thrombin treatment and 50% hemostasis was achieved by the 5th minute (Figure 1C). The mean time-to-hemostasis was reduced from 7.45 ± 3.14 minutes with thrombin treatment to 5.60 ± 1.60 with Q8009 at 250 $\mu\text{g/mL}$ (Table 4). While Q8009 at 1,000 $\mu\text{g/mL}$ plus collagen matrix stopped bleeding by the 6th minute, it did not significantly reduce total blood loss ($203.81 \pm 137.16 \mu\text{L}$), relative to thrombin treatment. In addition, Q8009 at 1,000 $\mu\text{g/mL}$ reduced the overall blood loss by an additional 15% less than thrombin ($244.18 \pm 87.17 \mu\text{L}$) and reduced the time at which 50% of the animals reached hemostasis to the 5th minute, while 50% of the animals in the thrombin treated group were still bleeding by the 8–9 minute interval (Figure 1C).

DISCUSSION

The group of studies herein reports the *in vivo* procoagulant effects of a venom-derived protein with Xa-like activity. The choice of animal models utilized in this work was based on current literature in which several modifications were implemented to improve reproducibility and accuracy. Evaluations of existing techniques for determining blood loss were imprecise and highly variable. Historically, blood loss determinations in models of liver, spleen or kidney

wound were performed using a preweighed aluminum cup (Bengmark et al., 1980; Kullendorff et al., 1984; Kullendorff and Zoucas, 1985; Lindblom et al., 1990; Lindfeldt et al., 1987; Vagianos et al., 1987; Zoucas et al., 1984a; Zoucas et al., 1984b;) or a stack of gauze pads (Holcomb et al., 2000; Tuthill et al., 2001) and determining final blood loss by weight. A more extensive search of the literature provided a protocol for hematin determination (Masci et al., 1988) and coupled with the use of Whatman filter paper “chads” this provided the desired accuracy. A standard curve made by spiking a series of tubes with a known volume of blood proved to be highly reproducible and able to detect as little as 1 μL /minute blood lost.

A review of the literature demonstrates that bleeding-time models of spleen, liver and kidney lacked reproducibility and resulted in too much individual variability. Resection varied from approximately 2.5–65% of the liver (Alwmark et al., 1986; Bengmark et al., 1980; Kullendorff et al., 1984; Kullendorff and Zoucas, 1985; Lindfeldt et al., 1987; Vagianos et al., 1987; Zoucas et al., 1982), 6–10% of kidney (Jackson et al., 1998; Kullendorff and Zoucas, 1985; Raccuia et al., 1992) or 1% of the caudal pole of the spleen (Lindblom et al., 1990). Most papers stated that resection followed a standardized technique, although only one article employed the use of a template to resect a standardized 10% of the kidney, thereby producing an actively bleeding area of 1 $\times$ 5 cm (Raccuia et al., 1992). In an effort to standardize the amount of tissue removed and generate more reproducible wound surface areas exposed to test material a metal plate with various sized holes of known diameters was employed. For the spleen a 6 mm diameter hole, for the liver a 6.5 mm diameter hole and the kidney a 9 mm diameter hole was used. Using these standardized size holes, the percentage of intact organ resected was consistent for the spleen, right lateral lobe of the liver and kidney for all animals in the experiments. Thus by utilizing a metal plate, we consistently produced tissue wound surfaces of known area for the kidney (32.95–39.59 mm^2), liver (21.00–25.14 mm^2) and spleen (18.54–20.40 mm^2) that were not statistically different between the different treatment groups.

We found similarities of the models used in this work to those in the literature addressing blood loss and bleeding times. Blood loss in the spleen wound model totaled $59.44 \pm 25.21 \mu\text{L}$ and continued for the duration of the 12 minute test period. This is different from the other findings (Lindblom et al., 1990), which demonstrated that in a splenic injury model 200 μL of blood was lost and hemostasis occurred within 3 minutes. These differences are possibly due to the fact that 1% of the spleen was resected and in our study, the extent of the wound was standardized. Blood loss in the liver injury model with no treatment was $357.57 \pm 267.31 \mu\text{L}$ and lasted the entire experimental period. These findings are similar to those in the literature when 2.5% to 4% of the liver was resected and bleeding lasted between 5–9 minutes with 1.0 to 3.4 mL of blood lost (Alwmark et al., 1986; Bengmark et al., 1980; Bengmark et al., 1981; Holcomb et al., 2000; Kullendorff et al., 1984; Lindfeldt et al., 1987; Tanaka et al., 1985; Zoucas et al., 1982; Zoucas et al., 1984a; Zoucas et al., 1984b;). Additionally, Holcomb *et al.* (2000) demonstrated a reduction in blood loss by 52% using fibrin glue in a model of median hepatic lobe injury in rats. Blood loss in the kidney wound model with no treatment totaled $629.28 \pm 195.65 \mu\text{L}$ and lasted 12 minutes of the experimental period. This is similar to Tuthill *et al.* (2001) who demonstrated that in survival bleeding studies, rats lost $5.4 \pm 2.1 \text{ mL}$ of blood over a 50 minute period and heparinized animals lost $8.0 \pm 1.3 \text{ mL}$ of blood before the animals died 20 minutes after the sagittal heminephrectomy injury. The amounts of blood loss in these studies were consistent with compromised survival.

These findings demonstrate that treatment with the procoagulant Q8009 increased efficiency in reducing blood loss from actively bleeding wounds, and more efficacious than the thrombin standard. Blood loss from internal organ wounds with no treatment averaged $348.76 \pm 285.02 \mu\text{L}$. Thrombin plus collagen matrix treated internal organs wounds reduced the overall blood loss by 44% (Spleen 0%, Liver 70% and Kidney 61%). Treatment of wounds on internal organs with Q8009 at 100 $\mu\text{g/mL}$ plus collagen matrix reduced the overall blood loss by 52% (Spleen

54%, Liver 61% and Kidney 42%). These findings are similar to those of Tuthill *et al.* (2001) at reducing blood loss (7%, 45% and 49%) in rat kidney wounds with, sprayed fibrinogen, Gelfoam + thrombin and fibrin sealant, respectively. When the concentration of Q8009 was increased to 250 µg/mL, the overall blood loss was reduced by 75% (Spleen 56%, Liver 90% and Kidney 77%) from internal organ wounds. Zoucas *et al.* (1984a) demonstrated in a rat liver injury model, a reduced blood loss of between 50%–75% with the application of gelatin foam, oxidized cellulose, collagen fleece or microcrystalline collagen. At a concentration of Q8009 at 1,000 µg/mL plus collagen matrix the overall blood loss was reduced by 65% (Spleen 42%, Liver 85% and Kidney 68%) from wounds on internal organs. This is similar to Coln *et al.* (1983), who demonstrated in a rabbit spleen laceration model that Gelfoam, Avitene, Surgicel and Collastat reduced blood loss (43%, 70%, 82% and 85%, respectively) when compared to control animals. This degree of injury caused 100% mortality in the control group within 2.5 hours and 15% mortality in all other animals. Additionally, Jackson *et al.* (1998) demonstrated that a combination of Tachotop, fibrinogen and thrombin reduced blood loss by 60% in 30 minutes in a kidney wound model in rats. When the Spleen, Liver and Kidney models were averaged together, thrombin reduced the overall blood loss by 44%, while Q8009 at 100 µg/mL, 250 µg/mL and 1,000 µg/mL reduced blood loss by 61%, 81% and 73%, respectively.

These data further demonstrate that treatment with the procoagulant Q8009 causes a reduction in the mean time-to-hemostasis from actively bleeding organ surface wounds, relative to thrombin treatment. Mean time-to-hemostasis for internal organ wounds with no treatment averaged 9.60 ± 0.29 minutes. Thrombin plus collagen matrix treatment of wounds on internal organs reduced the mean time-to-hemostasis by 10% (Spleen 7%, Liver 0% and Kidney 22%). Treatment of wounds on internal organs with Q8009 at 100 µg/mL plus collagen matrix reduced the mean time-to-hemostasis by 22% (Spleen 31%, Liver 19% and Kidney 16%). When the concentration of Q8009 was increased to 250 µg/mL, the overall reduction in mean time-to-hemostasis for wounds on internal organs was 47% (Spleen 50%, Liver 49% and Kidney 41%). At a concentration of Q8009 at 1,000 µg/mL plus collagen matrix the overall reduction in mean time-to-hemostasis for wounds on internal organs was 34% (Spleen 27%, Liver 37% and Kidney 37%). These findings are similar to those of Raccuia *et al.* (1992) who demonstrated in a rat kidney resection model a reduction in the time-to-hemostasis with Surgicel, Avitene and Superstat was 2%, 7% and 30%, respectively. When the Spleen, Liver and Kidney models are averaged together thrombin reduced the overall mean time-to-hemostasis by 20%, while Q8009 at 100 µg/mL, 250 µg/mL and 1,000 µg/mL reduced blood loss by 33%, 52% and 41%, respectively.

Three rat surgical models where the hemostatic protein Q8009, significantly reduced the amount of blood loss and produced a shorter time-to-hemostasis support the notion that the procoagulant protein is more efficacious than thrombin or some of its device combinations. This is supported by the fact that the extent and severity of the wounds were standardized between the different groups and known amounts of procoagulant materials were administered, therefore eliminated sampling or procedural bias. Under the condition of these experiments, the use of the procoagulant protein Q8009 offers the potential to reduce blood loss and the time-to-hemostasis under conditions of catastrophic blood loss thereby eliminating one of the main causes of mortality during surgery.

CONCLUSIONS

The native protein isolated from the Australian snake *Pseudonaja textilis textilis* possesses procoagulant properties which in the overall assessment of results, has more potency and efficacy than thrombin preparations. The wound models studied included here for liver, spleen and kidney plus skin from a previous publication confirm the Factor Xa activity with shortened

time to hemostasis as well as reducing the volume of blood lost. The model used is representative of significant blood loss that can compromise survival should the protein not be as effective as found. Further work will be necessary to translate this procoagulant protein into a pharmacologic entity acceptable for human use. We know of no other work or agent in the literature reporting the degree of efficacy and potency observed.

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Figure 1.

Percent of animals bleeding at each 1 minute interval, number of animals bleeding at defined time interval divided by the number of animals in the group. (Untreated ■, Saline ▲, Thrombin ▼, Q8009 100 µg/ml ◆, Q8009 250 µg/ml ◈, Q8009 1,000 µg/ml ●

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Blood Loss in the Spleen Wound Model

Time (minutes)	Treatments			
	Untreated	Saline	Thrombin 1,000 U/mL	Q8009 µg/ml
			100	250
0-1	18.52 ± 7.42	32.71 ± 22.70	24.43 ± 18.27	13.93 ± 7.18
1-2	11.13 ± 6.19	18.78 ± 9.49	17.11 ± 14.26	5.98 ± 3.52
2-3	6.27 ± 3.98	12.46 ± 6.33	10.70 ± 7.70	3.62 ± 3.53
3-4	4.19 ± 3.21	8.17 ± 5.16	7.80 ± 5.92	1.03 ± 1.37
4-5	2.69 ± 2.24	5.79 ± 4.21	4.17 ± 3.19	0.59 ± 0.82
5-6	2.37 ± 2.93	5.39 ± 4.00	3.30 ± 4.98	0.43 ± 0.73
6-7	2.21 ± 3.00	4.14 ± 3.97	2.09 ± 2.07	0.14 ± 0.33
7-8	2.57 ± 3.92	4.41 ± 5.38	1.83 ± 2.46	0.09 ± 0.28
8-9	2.33 ± 3.10	4.00 ± 4.70	1.66 ± 2.33	0.07 ± 0.32
9-10	2.38 ± 3.47	3.82 ± 4.01	1.83 ± 3.00	0.04 ± 0.13
10-11	2.16 ± 3.32	4.94 ± 7.16	1.75 ± 2.65	0.03 ± 0.09
11-12	2.64 ± 3.95	4.75 ± 6.50	2.29 ± 3.97	0.02 ± 0.11
Blood Loss [*] Reduction [#]	59.44 ± 25.21	109.37 ± 43.17 (0%)	78.95 ± 44.45 (0%)	25.97 ± 13.25 ^{**} (56%)
			27.18 ± 18.72 ^{**} (55%)	34.53 ± 19.46 ^{**} (42%)

Minimum of 20 rats per treatment were used in this work

* Data represent µL blood loss, means ± SD;

Percent reduction in blood loss relative to untreated group;

** Significance p<0.001

Table 2

Blood Loss in the Liver Wound Model

Time (minutes)	Treatments			
	Untreated	Saline	Thrombin 1,000 U/mL	Q8009 μ g/ml
			100	250
			1,000	
0-1	117.11 $\pm$ 47.54	113.69 $\pm$ 58.90	60.50 $\pm$ 36.53	18.08 $\pm$ 7.52
1-2	62.25 $\pm$ 34.15	55.06 $\pm$ 38.35	31.54 $\pm$ 26.28	10.16 $\pm$ 8.63
2-3	43.61 $\pm$ 39.21	32.28 $\pm$ 28.81	21.65 $\pm$ 25.02	5.24 $\pm$ 5.12
3-4	27.58 $\pm$ 47.19	18.42 $\pm$ 21.70	9.17 $\pm$ 11.28	2.12 $\pm$ 1.88
4-5	18.67 $\pm$ 26.91	14.80 $\pm$ 22.96	4.19 $\pm$ 3.66	1.20 $\pm$ 1.29
5-6	12.95 $\pm$ 22.53	9.25 $\pm$ 18.91	2.69 $\pm$ 2.32	0.48 $\pm$ 0.63
6-7	14.57 $\pm$ 25.07	8.25 $\pm$ 18.20	1.48 $\pm$ 1.31	0.36 $\pm$ 0.58
7-8	11.43 $\pm$ 23.35	7.93 $\pm$ 17.93	1.33 $\pm$ 1.27	0.19 $\pm$ 0.46
8-9	13.18 $\pm$ 24.97	7.62 $\pm$ 17.50	1.09 $\pm$ 1.04	0.14 $\pm$ 0.41
9-10	13.28 $\pm$ 28.07	6.86 $\pm$ 17.56	0.82 $\pm$ 1.52	0.05 $\pm$ 0.11
10-11	11.34 $\pm$ 19.26	4.93 $\pm$ 14.78	1.71 $\pm$ 5.56	0.02 $\pm$ 0.06
11-12	11.61 $\pm$ 23.57	4.35 $\pm$ 12.49	2.39 $\pm$ 7.34	0.01 $\pm$ 0.05
Blood loss* Reduction#	357.57 $\pm$ 267.31	283.42 $\pm$ 178.30 (21%)	105.43 $\pm$ 33.25 (70%)	38.05 $\pm$ 20.95*** (90%)
			138.57 $\pm$ 90.94 (61%)	52.34 $\pm$ 25.81** (85%)

Minimum of 20 rats per treatment were used in this work

* Data represent μ L blood loss, means $\pm$ SD;

Percent reduction in blood loss relative to untreated group;

** Significance $p < 0.01$,

*** $p < 0.001$

Table 3

Blood Loss in the Kidney Wound Model

Time (minutes)	Treatments			
	Untreated	Saline	Thrombin 1,000 U/mL	Q8009 µg/mL
			100	250 1,000
0-1	241.41 ± 42.87	183.48 ± 62.48	124.56 ± 33.48	87.67 ± 26.08
1-2	191.83 ± 73.09	118.53 ± 64.31	63.38 ± 31.22	41.02 ± 22.06
2-3	102.75 ± 68.38	71.10 ± 53.97	23.90 ± 18.04	11.72 ± 10.40
3-4	47.57 ± 54.28	40.32 ± 42.59	11.83 ± 9.74	2.44 ± 3.39
4-5	20.30 ± 21.26	16.56 ± 20.01	6.09 ± 5.71	0.95 ± 1.03
5-6	7.39 ± 8.63	7.47 ± 9.23	3.08 ± 3.49	0.40 ± 0.82
6-7	7.05 ± 7.34	4.05 ± 6.13	3.71 ± 4.51	0.40 ± 0.61
7-8	3.22 ± 3.53	2.35 ± 3.97	2.62 ± 3.10	0.27 ± 0.56
8-9	1.88 ± 2.30	1.88 ± 3.89	1.73 ± 2.18	0.13 ± 0.34
9-10	1.42 ± 2.13	0.99 ± 2.06	1.55 ± 2.19	0.15 ± 0.34
10-11	2.07 ± 2.44	0.86 ± 2.75	1.02 ± 1.53	0.03 ± 0.09
11-12	2.38 ± 4.16	1.04 ± 5.33	0.70 ± 1.65	0.04 ± 0.19
Blood Loss* Reduction#	629.28 ± 195.65	448.64 ± 202.75 (29%)	244.18 ± 87.17 (61%)	145.22 ± 52.25*** (77%) 203.81 ± 137.16 (68%)

Minimum of 20 rats per treatment were used in this work

* Data represent µL blood loss, means ± SD;

Percent reduction in blood loss relative to untreated group;

*** Significance p<0.001

Table 4

Mean Time-to-Hemostasis (minutes)

Treatments	Surgical Models		
	Kidney Wound	Liver Wound	Spleen Wound
	Mean $\pm$ SD		
Untreated	9.57 $\pm$ 2.91	9.91 $\pm$ 2.48	9.33 $\pm$ 3.18
Saline	8.20 $\pm$ 2.99	9.97 $\pm$ 2.37	10.10 $\pm$ 2.40
Thrombin 1,000 U/mL	7.45 $\pm$ 3.14	10.50 $\pm$ 2.04	8.71 $\pm$ 2.92
Q8009 μ g/ml			
100	8.05 $\pm$ 2.24	8.03 $\pm$ 2.36	6.45 $\pm$ 3.63
250	5.60 $\pm$ 1.60	5.10 $\pm$ 1.67	4.62 $\pm$ 2.01
1000	6.00 $\pm$ 2.73	6.20 $\pm$ 1.96	6.85 $\pm$ 3.25

* Time-to-hemostasis: average time all animals in each group stopped bleeding.