Development of a Standard Swine Hemorrhage Model for Efficacy Assessment of Topical Hemostatic Agents

Bijan S. Kheirabadi, PhD, Françoise Arnaud, PhD, Richard McCarron, PhD, Alan D. Murdock, MD, Douglas L. Hodge, MS, Brandi Ritter, MS, Michael A. Dubick, PhD, and Lorne H. Blackbourne, MD

Background: The diverse information of efficacy of hemostatic products, obtained from different military laboratories using different models, has made it difficult to ascertain the true benefit of new hemostatic agents in military medicine. The aim of this study was to recommend a standard hemorrhage model for efficacy testing acceptable by most investigators in the field and avoid contradictory and duplicative efforts by different laboratories.

Methods: The swine femoral artery injury model (6-mm arteriotomy) with some modifications was tested to standardize the model. The suggested modifications included no splenectomy, one-time treatment, 30 seconds free bleeding, and 5 L limit for fluid resuscitation. The model was tested with all or some of these modifications in four experimental conditions (n = 5–6 pigs per condition) using Combat Gauze (CG) as control agent.

Results: The primary end points including blood pressure, blood loss, and survival rates were modestly changed in the four conditions. The second experimental condition in which bleeding was treated with a single CG with 3-minute compression produced the most suitable results. The average blood loss was 99 mL/kg, and hemostasis was achieved in one-third of the pigs, which led to matching survival rate.

Conclusion: A rigorous hemorrhage model was developed for future evaluation of new hemostatic agents and comparison with CG, the current standard of care. This model may not be suitable for testing every agent and some modifications may be necessary for specific applications. Furthermore, laboratory studies using this or similar models must be accompanied by operational testing in the field to confirm the efficacy and practical utility of selected agents when used on the battlefield.

Key Words: Hemorrhage model, Hemostatic agent, Combat gauze, Efficacy, Swine.

(J Trauma. 2011;71: S139–S146)

The U.S. Department of Defense (DoD) on reviewing combat-preventable deaths following Operation Gothic Serpent/Code Irene, known as the First Battle of Mogadishu or “Day of the Rangers,” recognized that more research was needed to improve wound care for “junctional” injuries or some compressible wounds that could not be treated with tourniquets. Development of different dressings and hemostatic agents began in earnest after that. Over the past 10 years, an extensive amount of research and development on hemostatic agents has been accomplished. A meta-analysis of 17 different studies illustrated the disparity of research parameters in the different hemostatic agent studies (unpublished data). The diverse nature of each research protocol for testing these agents has made accurate comparison of the different products very difficult. Therefore, DoD medical experts met on June 30, 2009, to discuss future evaluations of the efficacy and safety of new hemostatic agents. This meeting concentrated on achieving a consensus on animal model test parameters and determining how to avoid contradictory and duplicative efforts by different military laboratories. Although the goal was to standardize parameters for testing, it is widely recognized that there are clear differences between standard efficacy testing in the laboratory and user testing in the field, known as operational testing. As newly developed hemostatic dressings are becoming more efficacious than in the previous generations, new test models with increased severity are needed for a more rational selection of new hemostatic agents. The wound model discussion in this article applies only to laboratory efficacy testing of new agents.

After reviewing and discussing historic hemorrhage models1-4 used by Navy and Army scientists, which validated the former products (e.g., QuikClot, Z-Medica Corp, Wallingford, CT and HemCon Bandage, HemCon Inc, Portland, OR), a femoral artery injury model described in recent publications5,6 was selected as the basic injury/hemorrhage model for future efficacy testing of new hemostatic agents. To standardize the procedures among different military laboratories, several modifications to the original femoral injury model of the US Army Institute of Surgical Research (ISR) were recommended. These modifications were based on the parameters of other hemorrhage models that have been used successfully to evaluate various hemostatic products.8-11 In addition, it was agreed that Combat Gauze (CG), a kaolin-coated surgical gauze that is currently used as the standard dressing in military, would be used as the control agent for future studies. An ideal hemorrhage model for future efficacy studies is expected to produce 30% to 50% hemostasis with matching survival rates in pigs when the bleeding is treated with CG as the control agent. This article illustrates the efficacy portion of this discussion and the
1. REPORT DATE
MAR 2011

2. REPORT TYPE

3. DATES COVERED
00-00-2011 to 00-00-2011

4. TITLE AND SUBTITLE
Development of a Standard Swine Hemorrhage Model for Efficacy Assessment of T apical Hemostatic Agents

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

6. AUTHOR(S)

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Naval Medical Research Center,Neuro Trauma Department,503 Robert Grant Avenue,Silver Spring,MD,20910

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR’S ACRONYM(S)

11. SPONSOR/MONITOR’S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:

<table>
<thead>
<tr>
<th>a. REPORT</th>
<th>b. ABSTRACT</th>
<th>c. THIS PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>unclassified</td>
<td>unclassified</td>
<td>unclassified</td>
</tr>
</tbody>
</table>

17. LIMITATION OF ABSTRACT
Same as Report (SAR)

18. NUMBER OF PAGES
8

19a. NAME OF RESPONSIBLE PERSON

Standard Form 298 (Rev. 8-98)
Prepared by ANSI Z39-18
### TABLE 1. Hemorrhage Treatment Conditions

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>Laparotomy and Cystostomy</th>
<th>Splenectomy</th>
<th>6-mm Femoral Artery Punch</th>
<th>Free Bleeding (sec)</th>
<th>No. CG Applied</th>
<th>Application Time (min)</th>
<th>Compression* Time (min)</th>
<th>Fluid Resuscitation (L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 n = 5†</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>30</td>
<td>1</td>
<td>~1</td>
<td>2</td>
<td>Hextend (0.5) + LR (5)</td>
</tr>
<tr>
<td>2 n = 6</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>45</td>
<td>1</td>
<td>~1</td>
<td>3</td>
<td>Hextend (0.5) + LR (10)</td>
</tr>
<tr>
<td>3 n = 6</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>45</td>
<td>2</td>
<td>1.5-2</td>
<td>2</td>
<td>Hextend (0.5) + LR (10)</td>
</tr>
<tr>
<td>4 n = 6</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>45</td>
<td>2</td>
<td>1.5-2</td>
<td>3</td>
<td>Hextend (0.5) + LR (10)</td>
</tr>
</tbody>
</table>

* To perform compression, a laparotomy sponge (18 in × 18 in) was folded, placed in the wound over the CG, and manually compressed to stop the bleeding. After compression, sponge was left in the wound for the entire experiment (up to 150 min).

† Three additional experiments were performed in this group, but the pigs were excluded. The reasons for exclusions were the use of new CG (z-fold samples), which may not have the active agent.

The following specific changes were recommended to the ISR’s basic femoral artery hemorrhage model:

- Avoid splenectomy and fluid replacement because these procedures alter the native coagulation response and may add additional variability to the outcomes.
- Allow pretreatment bleeding from the femoral artery injury only for 30 seconds instead of 45 seconds to avoid significant hypotension before hemostatic treatment.
- Conduct treatment with each specific agent only once regardless of outcome.
- Spend as long as 1 minute to pack the wound with the test agent, cover it with a laparotomy sponge (gauze), and compress manually for 2 minutes. Compression pressure should be sufficient to completely stop bleeding and oozing during this period.
- Leave the laparotomy sponge on the wound during the monitoring period.
- After compression, infuse the pigs with 500 mL of warm (32–37°C) Hextend fluid at 50 mL/min or less to raise the mean arterial pressure (MAP) to 65 mmHg (close to baseline pressure and consistent with hypotensive resuscitation strategy). Once Hextend infusion is complete, administer additional fluid resuscitation as needed (up to 5 L of warm lactated Ringer’s [LR] solution at 100 mL/min) in an attempt to maintain the MAP between 60 mmHg and 65 mmHg for the rest of the experiment.
- Monitor the pigs for up to 2.5 hour (150 minutes) from the time of injury.

These recommended changes were tested under four experimental conditions, which included all or some of the changes to determine whether these changes were appropriate and whether they constituted an increase in bleeding severity over the original model to identify more efficacious hemostatic products in future.

### METHODS

This study was approved by the ISR’s Animal Care and Use Committee. All animals received care and were used in strict compliance with the Guide for the Care and Use of Laboratory Animals.

To test and validate the consensus model, we used CG (the roll form without X-ray detectable stripe) to treat the arterial hemorrhage in the pigs under various conditions, which incorporated all (experimental condition 1) or some of the recommended changes to the original model (experimental conditions 2–4, Table 1). Briefly, the femoral artery was isolated and treated with lidocaine for optimum dilation (Fig. 1, A). The vascular injury (6-mm punched hole in the femoral artery), as shown in Figure 1, B, was kept constant for all the experiments. Finally, the hemostatic treatment was applied (Fig. 1, C). All other treatments and monitoring procedures were similar to those described in previous studies except for some changes, which are listed in Table 1. The methodology is described in detail in the Appendix. The primary end points measured include time to achieve stable hemostasis (no sign of bleeding through the dressing), posttreatment blood loss, and survival outcomes.

Data are expressed as mean ± standard deviation and analyzed by paired and unpaired t tests, Fisher’s exact, and log-rank tests for statistical comparison. The nonparametric data were analyzed by using the Newman-Keuls multiple

---

Figure 1. Pictures of preparation of femoral artery injury model in swine: (A) femoral artery vessel was isolated and treated with lidocaine for optimum dilatation; (B) vessel was clamped distally and proximally and arteriotomy made using 6-mm diameter round vascular punch; note the oval shape appearance of the injury (arrow) because of the vessel longitudinal tension; (C) groin wound appearance after a successful hemostatic treatment; note the coverage of test dressing with a lap sponge for additional absorbency and compression.
comparison test, and the big posttest was done using Dunnett's test. A \( p < 0.05 \) was considered statistically significant.

**RESULTS**

In the first group of experiments (Table 1, experimental condition 1), all recommended changes were incorporated into the protocol. The results showed a very severe bleeding condition with only a 20% (or less) success for CG to stop the bleeding and prevent death. In one failed experiment, in which CG did not produce hemostasis and bleeding continued for an extended period (>1 hour), the pig survived the 2.5-hour observation period at very low blood pressure (MAP \( \leq 25 \) mm Hg). This seemed to be the result of limited fluid resuscitation volume (5 L) that was infused during the first 50 minutes (100 mL/min) and stopped afterward. To overcome this inconsistency (hemostatic failure but survival outcome), we increased the limit of resuscitation fluid (LR) to 10 L as in the original protocol to extend the infusion time, maintain higher blood pressure, and further challenge the efficacy of hemostatic agents. Other changes were also made in the subsequent experiments to reduce the early severity of bleeding and compare the results. These changes include the following:

- Increase of free bleeding time to 45 seconds (to reduce initial blood pressure at time of treatment).
- Increase of compression time to 3 minutes.
- Use of two CGs instead of one to pack the wound and treat the hemorrhage.

These changes are listed in Table 1 as experimental conditions 2, 3, and 4. Each was tested in six pigs, and the results were compared with experimental condition 1.

Baseline values of complete blood count (CBC), coagulation, and blood gas measurements for all pigs were within the normal range and met the inclusion criteria (Table 2, Appendix). There were no differences in these measurements among experimental groups (experimental conditions, 1–4), thus the data were combined. The averages of the baseline data for all the subjects are shown in Table 2. As expected, these values changed significantly at the conclusion of experiments (final) with larger changes in nonsurvivors than in survivors (Table 2). Because there were no differences in the final measurements of different groups, the data from all survivors (\( n = 9 \)) and all nonsurvivors (\( n = 14 \)) were combined, the averaged values are shown in Table 2. The 30% to 40% reduction in hemoglobin, platelet count, and fibrinogen concentration measured in surviving animals was partly because of the pretreatment (30- to 45-second initial free bleeding) and posttreatment blood losses and partly because of the hemodilution caused by fluid resuscitation, which was necessary even in successful experiments. CG rarely provided immediate hemostasis after application (in two cases only); the wounds often bled for 10 minutes to 20 minutes before hemostasis was achieved. These blood losses and subsequent fluid resuscitation (hemodilution) reduced the clotting capacity of blood, as measured by significant prolongation of activated partial thromboplastin time (aPTT) even in surviving animals (Table 2). This decrease was also evident when the blood clotting activity was analyzed by the thrombelastography (TEG) method. Although the initial reaction time and clotting rate remained unchanged, the maximum clot strength was significantly reduced in survivors (Table 2). The blood from the nonsurviving pigs could not be tested by the TEG method because of extreme hemodilution and inability to form a clot. The PT and aPTT, however, were determined in most samples and found to be increased fourfold to fivefold (Table 2).

The hemodynamic and hemostatic findings of testing CG under four slightly different conditions (experimental conditions, 1–4) are shown in Table 4. The survival rates ranged from 20% to 66% with no significant difference in survival time. Other parameters also measured were not significantly different among groups. The higher MAP after free bleeding and lower pretreatment blood loss in experimental condition 1 (\( p < 0.05 \)) were because of the shorter free bleeding time (30 sec) in that group. In experimental conditions 2–4, the incidence of survival generally corresponded to the number of successful experiments (hemostasis achieved) except in one animal in group 4, where the pig

---

**TABLE 2. Baseline and Final Physiological and Hematological Measurements of the Pigs**

<table>
<thead>
<tr>
<th>Groups (Models)</th>
<th>Baseline All Groups (n = 23)</th>
<th>Final Survivors (n = 9)</th>
<th>Final Nonsurvivors (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>37.7 ± 0.4</td>
<td>38.0 ± 0.3</td>
<td>37.1 ± 0.5</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>80.5 ± 13.7</td>
<td>57.3 ± 14.5*</td>
<td>17.5 ± 2.1*</td>
</tr>
<tr>
<td>HGB (g/dL)</td>
<td>9.7 ± 0.6</td>
<td>5.6 ± 1.2*</td>
<td>1.6 ± 0.8*</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>29.6 ± 2.1</td>
<td>16.9 ± 3.6*</td>
<td>5.4 ± 2.1*</td>
</tr>
<tr>
<td>Platelets (1,000/µL)</td>
<td>378 ± 109</td>
<td>229 ± 98*</td>
<td>53.5 ± 14.6*</td>
</tr>
<tr>
<td>PT (sec)</td>
<td>11.7 ± 0.6</td>
<td>12.9 ± 2.2</td>
<td>49 ± 23.5*</td>
</tr>
<tr>
<td>aPTT (sec)</td>
<td>14.2 ± 0.9</td>
<td>17.8 ± 4.8*</td>
<td>63.5 ± 41*</td>
</tr>
<tr>
<td>Fibrinogen (mg/dL)</td>
<td>240 ± 30</td>
<td>176 ± 51*</td>
<td>N/A</td>
</tr>
<tr>
<td>pH</td>
<td>7.4 ± 0.4</td>
<td>7.4 ± 0.4</td>
<td>7.5 ± 0.1*</td>
</tr>
<tr>
<td>Lactate (mM)</td>
<td>1.8 ± 1</td>
<td>3.6 ± 4.1</td>
<td>16.7 ± 2.6*</td>
</tr>
<tr>
<td>Base excess (mM)</td>
<td>6.3 ± 2.1</td>
<td>6.9 ± 5.3</td>
<td>-4.5 ± 4.5*</td>
</tr>
</tbody>
</table>

* \( p < 0.01 \) vs. corresponding baseline values.  
\( 1 \) \( p < 0.01 \) vs. survivor group.

Data represent mean ± SD. Blood was collected at baseline (before operation) and at the conclusion of experiments (final). N/A, unable to measure because of excessive hemodilution.

---

**TABLE 3. Coagulation Analysis of Pigs' Blood by Thrombelastography (TEG) Method**

<table>
<thead>
<tr>
<th>TEG Parameter</th>
<th>Baseline (Preinjury), n = 23</th>
<th>Final, All Survivors, n = 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-time (min)</td>
<td>5.9 ± 1.1</td>
<td>6.1 ± 2.3</td>
</tr>
<tr>
<td>K-time (min)</td>
<td>2.3 ± 0.8</td>
<td>2.9 ± 1.9</td>
</tr>
<tr>
<td>Angle (°)</td>
<td>61.6 ± 8.2</td>
<td>56.9 ± 14.5</td>
</tr>
<tr>
<td>MA (mm)</td>
<td>73 ± 4.5</td>
<td>61.5 ± 10.3*</td>
</tr>
</tbody>
</table>

* \( p < 0.01 \) vs. corresponding baseline values.  

Data represent mean ± SD. The baseline data include values of all pigs. Blood collected at baseline (before injury) and at the conclusion of experiments (final) for survivor pigs. The final blood from nonsurvivor pigs could not be analyzed by TEG methods because of excessive hemodilution.
survived despite hemostatic failure and continued bleeding during the entire observation period. This outcome seemed to be caused by an excessive tamponade effect that was produced by packing two CGs in the wound.

**DISCUSSION**

On June 30, 2009, a panel of DoD medical experts convened at the ISR to discuss future evaluations of the efficacy and safety of new hemostatic agents. To unify this effort and deploy more effective products to the field, the panel devoted the first part of the discussion to characterize an ideal dressing for tactical use. The result is summarized in Table 5. The panel also recognized that some of these criteria (i.e., efficacy and acute safety) should be investigated by experimental studies in large animals under controlled laboratory conditions. Others, such as readiness, ease of use, and wound coverage, require operational testing by care providers in a simulated tactical environment for validation. Most hemostatic agents indicated for treating external wounds are considered to be class I medical devices and rapidly marketed by receiving Food and Drug Administration clearance without entering clinical trials for safety or efficacy evaluation. Safety evaluation of new hemostatic agents in laboratory animal models is critically important before those agents are placed in the hand of patients or first responders for treating injured tissues. A prime example was the case of smectite granules (WoundStat, TraumaCure, Bethesda, MD) that was discovered to have significant toxicity effect toward endothelial cells\(^1\) causing local intravascular thrombosis and embolism of distant organs when tested in large animals.\(^2\) The result of this study and confirmatory findings by another laboratory\(^3\) led to permanent suspension of this effective and yet unsafe agent in military medicine. Currently, no hemostatic product is available that meets all these criteria. CG stops arterial bleeding only after significant blood loss, and of course, this product is only suitable for temporary treatment of external wounds. A recent study has also shown that CG is significantly less effective under coagulopathic conditions.\(^4\)

**TABLE 5. Ideal Hemostatic Dressing for Tactical Application**

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Is approved or cleared by the U.S. Food and Drug Administration</td>
<td></td>
</tr>
<tr>
<td>Stops severe arterial and/or venous in ≤2 min</td>
<td></td>
</tr>
<tr>
<td>Has no toxicity or side effect (see Refs. 12–14)</td>
<td></td>
</tr>
<tr>
<td>Causes no pain or thermal injury</td>
<td></td>
</tr>
<tr>
<td>Poses no risk to medics</td>
<td></td>
</tr>
<tr>
<td>Is ready to use and requires little or no training</td>
<td></td>
</tr>
<tr>
<td>Is durable and lightweight</td>
<td></td>
</tr>
<tr>
<td>Is flexible enough to fit complex wounds and easily removed without leaving residues</td>
<td></td>
</tr>
<tr>
<td>Is stable and functional at extreme temperatures (−10°C to +40°C) for at least 2 wk</td>
<td></td>
</tr>
<tr>
<td>Is practical and easy to use under austere conditions (low visibility, rain, wind, etc.)</td>
<td></td>
</tr>
<tr>
<td>Is effective on junctional wounds not amendable with tourniquet</td>
<td></td>
</tr>
<tr>
<td>Has a long shelf life, &gt;2 yr</td>
<td></td>
</tr>
<tr>
<td>Is inexpensive and cost-effective</td>
<td></td>
</tr>
<tr>
<td>Is biodegradable and bioabsorbable</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Refs. 12–14

\(^2\) Authors acknowledge the contributions of Dr. S. Brown (W.M.D., Bethesda, MD) for the expert dissection of the cases. Dr. Brown's dissection was kindly facilitated by a grant from WoundStat, TraumaCure, Bethesda, MD.
The second part of the discussion was devoted to selection of a hemorrhage model that would be suitable for testing topical agents and identifying more efficacious agents in future studies. With the exception of the aortotomy model, the majority of large-animal (swine) hemorrhage models that have been developed in the past for validating hemostatic products were potentially treatable with gauze. For example, bleeding from a grade V liver injury or from a groin wound with transected femoral artery and vein was controlled with standard gauze packing, which resulted in survival of nearly 50% of pigs without coagulopathy.\textsuperscript{1,2} The aortotomy (4.4-mm punch hole) model was shown to be a lethal injury and not treatable with gauze,\textsuperscript{3} but it does not represent an extremity wound accessible to topical hemostatic agent treatment in the field. The arteriotomy injuries (4-mm or 6-mm punch holes on the femoral artery) in the groin area also produce severe bleeding that is difficult to control with standard gauze dressing and is more suitable for testing topical hemostatic agents.\textsuperscript{7,9} For these reasons, the committee selected the more severe femoral artery injury model that was originally developed at the ISR\textsuperscript{7} with some modifications as the standard model. This model mimics a severe injury to the groin area with a partial destruction of the femoral artery, causing a life-threatening hemorrhage that cannot be controlled with gauze and that is not amenable to tourniquet application. The injury is produced by a 6-mm punch hole in an exposed and dilated femoral artery, which is highly reproducible and simulates a near transection of the vessel. Leaving the posterior wall of the artery intact prevents retraction of the vessel that can cause spontaneous hemostasis. This model allows for testing the true efficacy of hemostatic products under relatively normotensive conditions (MAP $\geq$ 60 mm Hg) and free of confounding physical or vascular reactions. However, like any other model, this model also has its own limitations and cannot be considered "gold standard." The easily accessible superficial groin wound with focal arterial injury does not resemble the most complex injuries seen in combat. This model may also give unfair advantage to tissue sealant agents that require access to bleeding sites over true hemostatic products that may be effective in any circumstance. Application of lidocaine to dilate the vessel is not clinically relevant; however, it is a necessary step in this model due to various degree of vasoconstriction of femoral artery following isolation of the vessel. For the hemostatic agents with strong vasoconstrictor effect, this step perhaps should be avoided.

To further refine the experimental conditions for performing efficacy studies, the panel recommended several modifications to the original (basic) model. These changes were incorporated into the model and tested under four experimental conditions. The primary end points measured (Table 4) were not significantly different among these experimental conditions (possibly because of the small number of tests in each group). However, based on the overall findings, experimental condition 2 in which one CG and 3-minute compression were used to treat the wound after 45 seconds free bleeding with 33% survival rate seemed to offer the best circumstances for future efficacy studies. Experimental condition 1 was rejected because of poor survival rate, even with a reduced fluid resuscitation protocol. Experimental condition 3 was not acceptable because of longer application time and possible tamponade effect when two CGs are used to pack the wound. Experimental condition 4 was rejected because of mismatch between incidence of hemostasis and survival rate caused possibly by the tamponade effect of using two CGs. Statistically, proving the advantages of the experimental condition 2 over other conditions required testing of a much larger number of animals in each group and seemed unnecessary given the above observations.

The limits of fluid resuscitation (500 mL Hextend and up to 10 L of LR solution) in the model seem to be very high, but this amount seems necessary to maintain the blood pressure closer to normal level and continuously challenge hemostatic function of test agents. The average fluid resuscitation in this study was around 6.5 L, which was rather high; but it represented mostly the fluid volume infused to the nonsurviving pigs (8.1 L) rather than to the surviving pigs (3.1 L). This volume was also consistent with the findings in another study in which a group of pigs that were treated with placebo gauze and had a similar survival rate.\textsuperscript{6}

Experimental condition 2, used for treatment of arterial injury with CG, resulted in 33% hemostasis (2 of 6) and a matching 33% survival rate with an average of 99 mL/kg posttreatment blood loss and 13.4 minutes to achieve hemostasis in survivors. Although this hemorrhage model seems to be rather rigorous, it provides a suitable condition for future comparative studies to identify more effective hemostatic agents than CG, the current standard-of-care agent in the military. However, adjustment to this model or perhaps development of an entirely new wound model may be necessary to prove the efficacy of some agents, particularly those developed for a specific application. The overall 4-minute treatment with CG (1-minute packing and 3-minute compression) may be considered too long for the battlefield situation; however, this duration seemed necessary for CG to achieve hemostasis, at least in one-third of the experiments in this model. In the future, if other agents become available that can function in a shorter time, the treatment time in the study can be shortened accordingly (shorter application or compression time), whereas other parameters are kept constant demonstrating speed advantages of the new agent over CG.

In summary, to unify the military effort in evaluating new hemostatic agents, an experimental hemorrhage model was selected and tested under four different conditions. The best condition with desirable outcomes was recommended for future efficacy studies using CG as control agent. We recognize that this model is not a gold standard preclinical model suitable for all hemostatic studies. Adjustments to this model or development of an entirely new model may be necessary to demonstrate the specific advantages of some agents for treating more complex wounds. Nevertheless, we think that this model will provide a good foundation for evaluating the efficacy of most topical agents and avoid duplicative and sometimes contradictory findings reported by different military laboratories.
APPENDIX: DESCRIPTION OF SELECTED HEMORRHAGE MODEL

Goal
In general, the aim of a typical efficacy study is to prove that a test agent is significantly more effective in controlling hemorrhage than the control (standard of care) product without any apparent side effect. A 50% reduction in posttreatment blood loss is considered to be clinically significant. This measure is used to calculate (power analysis) the number of test animals that are required in each group for reaching statistical significance ($p < 0.05$).

End Points
The primary end points measured are posttreatment blood loss, bleeding/hemostasis time (time period necessary for bleeding to stop), MAP, survival time, and percentage survival. The secondary end points include hemoglobin, hematocrit, platelet counts, pH, lactate, base deficit, and coagulation values (PT, aPTT, fibrinogen, and TEG parameters).

The details of the revised hemorrhage model for laboratory testing of new hemostatic agents are described below. A video of the surgical procedure has been made and can be made available to other investigators.

The inclusion and exclusion criteria for this model are as follows:

<table>
<thead>
<tr>
<th>Inclusion Criteria</th>
<th>Exclusion Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit: 27%-40%</td>
<td>Unexpected death due to anesthesia or technical error</td>
</tr>
<tr>
<td>Platelet: $\geq200$ K/mm$^3$</td>
<td>Persistent low MAP ($&lt;55$ mm Hg) at the baseline</td>
</tr>
<tr>
<td>PT: $\leq14$ s</td>
<td>Significant blood loss ($&gt;300$ mL) because of surgical complication or error before femoral injury</td>
</tr>
<tr>
<td>PTT: $\leq25$ s</td>
<td>Pretreatment blood loss (during 45 s of free bleeding) of $&lt;10$ mL/kg or $&gt;25$ mL/kg</td>
</tr>
<tr>
<td>Fibrinogen: $\geq100$ mg/dL</td>
<td>Persistent hypotension and unresponsive to fluid resuscitation despite no bleeding</td>
</tr>
<tr>
<td>Body weight: 34-44 kg</td>
<td></td>
</tr>
<tr>
<td>Gender: male</td>
<td></td>
</tr>
</tbody>
</table>

General Procedures
1. Purchase Yorkshire cross-bred castrated male pigs weighing 34 kg to 44 kg from a clean supplier facility (e.g., Midwest Research Swine Inc., Gibbon, MN).
2. House the pigs in an approved facility for at least 4 days for proper acclimation before surgical experiments.
3. At some time before the surgery, collect venous blood samples from the cephalic vein (percutaneous needle insertion) and perform CBC and standard clotting tests (PT, aPTT, and fibrinogen) to ensure that these measures are within the normal range and that the pigs are sufficiently healthy to endure surgical procedures.
4. Fast the pigs for 12 hours to 18 hours before surgery with free access to water.
5. On the day of surgery, premedicate the pigs with buprenorphine (0.025 mg/kg, intramuscularly [IM]) for analgesia and glycopyrrolate (0.01 mg/kg, IM) or atropine sulfate 0.05 mg/kg IM or other agents to reduce saliva secretion and block vagally mediated bradycardia during the surgical procedure. Induce the animals with an injection of tiletamine-zolazepam (Telazol, 4-6 mg/kg, IM) or ketamine 20 mg/kg to 33 mg/kg and initially anesthetized with 5% isoflurane in oxygen via face mask.
6. Then intubate the pigs (8 mm or adapted size trachéal tube) and connect them to a mechanical ventilator to assist them with respiration using 100% oxygen. Adjust the tidal volume (8-10 mL/kg) and ventilation rate (12-16 times/min) to maintain an end tidal $CO_2$ of 40 mm Hg 2 mm Hg. Maintain anesthesia with 1% to 2% isoflurane added to oxygen by the ventilator.
7. Place a Teflon catheter (21 G X 1.5 in) in the ear vein of the pig and administer maintenance fluid, LR solution, at 5 mL/kg/h to 10 mL/kg/h. Hydrate the pigs with at least 10 mL/kg LR solution for up to 500 mL during the surgical procedure.

Surgical Procedures
1. Cannulate the right carotid artery (Tygon tubing, 20 cm long, inside diameter of 1.3 mm, outside diameter of 2.3 mm, or 14-18 gauge introducer) for blood withdrawal and connect it to a pressure transducer for continuous recording of blood pressure (systolic, diastolic, and mean) and heart rate throughout the experiment. Display the data on the monitors in the operating room and record it by a computer, if possible, for future analysis.
2. Catheterize the right jugular vein (8.5 Fr catheter) for administering resuscitation fluid during hemorrhage and wound treatment.
3. Perform a midline laparotomy (avoiding the penile region); and grossly examine the internal organs, including liver, kidneys, and GI tract, for normal appearance. The laparotomy is inevitable for the cystostomy procedure and adds an element of tissue trauma to the protocol.
4. Then perform a cystostomy and place a Foley catheter (18 Fr) in the bladder to aid in the drainage of urine and measurement of urine output during surgery.
5. Close the abdomen by suturing with Vicryl (size 1) sutures and staple the skin.
6. Next, make an incision of $\sim$10 cm on the skin in the groin area parallel and close to the femoral artery; excise and remove the thin adductor muscle that directly overlies the femoral canal by using electrocautery to expose the femoral artery. A retractor may be used for better wound exposure and during isolation of the vessel but must be removed before injury and hemorrhage.
7. Dissect $\sim$5 cm of the artery free from surrounding tissues with cautery and ligation (using 7-0 Prolene) of small arterial branches. Completely clean the vessel wall, and remove a protective sheet surrounding the adventitia. Avoid injury to the surrounding tissues, including the adjacent femoral vein and nerve.
8. To measure wound temperature (if necessary), suture a microelectrode to the muscle adjacent the vessel but at
least 1 inch away from the artery so that it does not interfere with the hemostatic treatment. Perform this procedure only in cases of suspected exothermic agents.

9. Then cover the artery with a small piece of gauze and bathe with a few milliliters of 2% lidocaine to relax vasospasm and dilate the artery to its normal diameter (fully dilated).

10. Next, clamp the artery proximally and distally and make a 6-mm-diameter arteriotomy on the anterior surface of the vessel about 2 cm to 3 cm from the bottom of groin using a 6-mm vascular punch (International Biophysics Corp., Austin, TX).

11. Collect preinjury (baseline) blood samples from the arterial line for CBC, coagulation, and arterial blood gas (ABG).

12. Next, clamp the artery proximally and distally and make a 6-mm-diameter arteriotomy on the anterior surface of the vessel about 2 cm to 3 cm from the bottom of groin using a 6-mm vascular punch (International Biophysics Corp., Austin, TX).

13. Release the clamps and allow unrestricted (free) bleeding for 45 seconds. Collect the shed blood by suction, weigh, and record as pretreatment blood loss.

Wound Treatment and Resuscitation

In general, surgeons are blinded to the identity of test materials until the time of agent application. To the extent possible, the products are applied according to the manufacturer’s instruction (longer compression time or extra application will not be considered). The following steps are taken to treat the hemorrhage and provide fluid resuscitation:

1. Immediately after the free bleeding and while bleeding continues, open a package of each product and pack the material in the wound in about 1 minute.

2. Cover the material immediately with a folded laparotomy sponge (18 in × 18 in., Kendall Curity) or equivalent gauze and manually press for 3 minutes against the wound with sufficient and constant pressure (200 mm Hg if measurement is possible) to occlude the artery and stop the bleeding. Record any change in wound temperature if needed.

3. Pull the skin flaps over the laparotomy sponge or equivalent gauze without clamping or creating additional pressure on the test materials.

4. Start fluid resuscitation once the 3-minute compression is completed (~5 minutes after injury). Infuse 500 mL of Hextend (6% HES in balanced electrolyte solution + glucose) via the jugular vein catheter at 33 mL/min (for ~15 minutes) to raise and maintain the MAP between 60 mm Hg and 65 mm Hg. After completion of the Hextend infusion, continue the fluid resuscitation with LR solution at 100 mL/min as needed to maintain the MAP at the same level. A maximum of 10 L of LR solution may be infused.

5. After compression, slowly release the pressure by lifting the hand, and observe hemostasis for 3 minutes without disturbing the dressings. If no bleeding is apparent during this period, it is considered that initial hemostasis is achieved. However, if bleeding occurs after compression release or at any other time during the experiment, collect the shed blood continuously by suctioning without disturbing the wound. Record the time period needed for the bleeding to stop following compression as the bleeding/hemostasis time (min). Weigh the shed blood during this period; calculate the volume of blood loss (assuming 1 g/mL to be the specific gravity of blood diluted with fluid) and report as posttreatment blood loss.

6. Monitor the pigs up to 2.5 hours or until death, which is pronounced when their end tidal Pco₂ and MAP fall below 15 mm Hg and 20 mm Hg (respectively) and remain at these levels for at least 2 minutes. Record the survival time, and collect final blood samples (arterial) for CBC, coagulation, and ABG measurements.

7. Scan the surviving pigs with a CT (if available) and obtain images of arterial blood flow and vascular structures of legs.

8. Next, flex and stretch the treated legs of anesthetized, surviving pigs 5 times to simulate walking to test the stability of the hemostasis produced by the test agent.

9. At this point, slowly remove the hemostatic product from the wound, and examine the status of hemostatic clots and the patency of the vessel.

10. Euthanize the animals with an intravenous injection of euthanasia solutions (e.g., Fatal Plus, Vortech Ltd, Dearborn, MI or Euthasol, Virbac Corp, Forthworth, TX); and collect tissue samples, including the treated artery, adjacent femoral vein, femoral nerve, and muscle tissues, for histologic examination. Perform gross necropsy to examine the vital organs.

11. Prepare histologic slides according to standard procedure and stain them with hematoxylin and eosin. Code the slides and have them examined by a veterinary pathologist or other qualified individual who is initially blinded to the treatment group. Once the examination of individual slides is completed, break the codes and categorize the results under each specific group and report them.

Note: This recommended protocol will be further validated in future studies, and some deviations from this protocol are permissible.

ACKNOWLEDGMENTS

We thank LTC John Devlin for his review of the manuscript and his insightful comments. We thank Ms. Inasema Terrazaas, Ashley Cox, and Krystal Valdez for their excellent technical assistance. We also appreciate the staff of our Veterinary Support Division for their assistance and technical support in conducting these experiments.

REFERENCES


