A Keratin Biomaterial Gel Derived from Human Hair is Hemostatic in an Acute Liver Hemorrhage Pilot Study

Tamer Aboushwareb, MD*† taboushw@wfubmc.edu
Daniel Eberli, MD*† deberli@wfubmc.edu
Catherine Ward, BS† catward@wfubmc.edu
Christopher Broda, BS† cbroda@wfubmc.edu
John B. Holcomb, MS‡ john.holcomb@amedd.army.mil
Anthony Atala, MD‡ aatala@wfubmc.edu
Mark Van Dyke, PhD‡ mavandyk@wfubmc.edu

† The Wake Forest Institute for Regenerative Medicine
Wake Forest University School of Medicine
Medical Center Boulevard
Winston Salem, NC 27157

‡ U.S. Army Institute of Surgical Research
3400 Rawley E. Chambers Avenue
Fort Sam Houston, TX 78234–6315

Corresponding Author: Mark Van Dyke, PhD
Wake Forest Institute for Regenerative Medicine
Winston Salem, NC 27157

* Both authors contributed equally to this work
Running Title: Hemostatic keratin biomaterial

Funding support for this work was provided by the Wake Forest Institute for Regenerative Medicine
Abstract

Background: Tourniquets and new hemostatic pressure dressings effectively prevent exsanguination from most extremity wounds. The unmet need is effective control of truncal bleeding. Cessation of hemorrhage soon after injury is essential for survival in cases where vascular injury is severe and the coagulopathy of trauma is present. However, few products exist that are highly effective for solid organ damage. Hemostatic agents that can be used in the operating room are desirable.

Methods: In this proof of principle study, animals received a standard liver injury to the left central lobe. Four animals each were assigned to no treatment (negative control), QuickClot®, Hemcon® bandage, and keratin gel treatment groups. All treatments were administered without compression and within five minutes of injury. Hemodynamic variables and other data were recorded during surgery, blood samples for CBC were taken during and after surgery, and all surviving animals were sacrificed after 72 hours.

Results: 24 hour survival rates were 0%, 50%, 50%, and 100% for the no treatment, QuickClot, Hemcon, and keratin gel groups, respectively. The keratin and QuickClot groups showed a significant reduction in shed blood volume compared to the control (p < 0.05). The keratin gel group also demonstrated greater maintenance of mean arterial pressure and the lowest shock index (HR/MAP).

Conclusions: The keratin gel used in this preliminary study produced favorable outcomes and warrants additional investigation as a hemostatic agent. The keratin’s gelatinous consistency,
ease of application and efficacy without compression may make it amendable to use in many settings.

**Keywords:** Keratin, biomaterial, hemostasis, liver trauma, hemorrhage
Introduction

Rapid, voluminous hemorrhage instigates a cascade of events that are almost impossible to reverse without immediate and effective intervention. According to the Centers for Disease control, motor vehicle trauma is the leading cause of death for Americans under age 64 with more than 40,000 victims per year. The number of people who die from motor vehicle-related injuries has not changed for the past 10 years. Historically, more than half of those severely injured with concomitant hemorrhage die. While it has been shown that immediate intervention is the best method to limit patient mortality, the methods for controlling bleeding in the pre-hospital setting have not substantively changed for centuries. Thus, many new hemostatic products have been developed to try to meet this need. Tourniquets and recent innovations in hemostatic pressure dressings have been effective for treatment of surface and extremity wounds, but there are few options for head, neck, chest, and abdominal hemorrhage.

On the battlefield, seventy percent of ballistic injuries result in death within the first hour. This is due primarily to the massive blood loss associated with penetrating trauma. In Vietnam, five thousand deaths resulted from bleeding from the extremities. It was estimated that twenty percent of these casualties could have been avoided with better first aid. Surprisingly, the percent of wounded who survive the first hour has not changed since the US Civil War. In Iraq and Afghanistan, like in all previous wars, extremity wounds are the predominant injury. Deaths due to hemorrhage represents the majority of those killed in action in Iraq.

Many products exist that effectively treat hemorrhage from the extremities (e.g. tourniquets and hemostatic pressure dressings) as well as those that work systemically. Numerous large animal
preclinical and human clinical trials have been conducted and published, and many comparative investigations of hemostatic agents have been undertaken.\textsuperscript{7-9} Coagulation adjuvants include mineral-based granules (e.g. QuickClot®),\textsuperscript{10-13} numerous dressings,\textsuperscript{14-18} clotting factors,\textsuperscript{19,20} and surgical approaches.\textsuperscript{21,22} Results have been mixed and are dependent on the model used. The zeolite-based QuickClot material has done well in some large animal trials, as has the chitosan-based bandage, Hemcon. In one extensive comparative trial, fibrin dressings were clearly superior.\textsuperscript{7} However, each of these hemostats has its challenges and limitations. QuickClot is known to produce localized heating which has been shown to damage tissue, both Hemcon and QuickClot are not intended for internal injuries and must be removed from the wound site, and fibrin bandages are expensive and not yet approved by the Food and Drug Administration. None of these materials, with the exception of a fibrin foam product that is still in development, can be applied without clear access to the site of hemorrhage, a major goal of first responders.

In the present investigation, we have tested a novel biomaterial derived from human hair that has demonstrated interesting hemostatic properties. Hard keratins are the constitutive proteins found in the protective structure of vertebrates, most notably in hair fibers. Numerous methods exist to extract keratins from hair fibers and process the soluble proteins into films and gels.\textsuperscript{23} Numerous products utilize hydrolyzed keratins, most notably in personal care applications and drug formulations. In early experiments, we discovered that certain keratin preparations instigated excessive red blood cell aggregation when exposed to fresh whole blood. We have now developed a powerful hemostatic keratin gel and tested it in a rabbit liver hemorrhage model.
Materials and Methods

Keratin Biomaterial

The keratin used in this study was extracted from human hair according to modifications of a long-established method. Briefly, hair from a local barber shop was washed in mild detergent and degreased with organic solvent. The clean, dry, degreased hair was reduced to break cystine bonds that would otherwise render the keratin proteins insoluble. After chemical reduction, a crude fraction of kerateines was extracted using a denaturing solution. This solution was centrifuged, filtered, and dialyzed against deionized (DI) water. The dialyzate was titrated to neutral pH, concentrated, and loaded into syringes. Upon exposure to air, this so-called kerateine solution forms a crosslinked hydrogel. The gel was sterilized in the syringe using gamma irradiation prior to use.

Study outline

The purpose of this preliminary study was to evaluate the hemostatic potential of keratin gel in a modestly challenging animal model. Liver injuries are notoriously problematic as both the size of the liver and of the wound increase. While the rabbit model is not proposed to be indicative of the human situation, it can produce both profuse and lethal hemorrhage. We used controlled liver transection as a means to establish a consistent set of conditions that would result in exsanguination in the absence of treatment (negative control), yet provide for the recovery of test animals when a conventional hemostat was applied (positive control). It should be noted that the hemostats used as positive controls in this study are indicated for topical wounds and require concomitant pressure; they were applied without compression in this study. This was done to
Hemostatic keratin biomaterial

avoid the confounding contribution compression would add as it was not used with the keratin gel.

A total of 16 New Zealand rabbits (3.7 kg average) were used in this study. The animals received a standardized liver injury that consisted of transection of approximately one third of the left central lobe and were then randomized into one of four groups. Four animals served as negative controls and received no treatment, four animals received treatment with QuickClot, four animals were treated with Hemcon bandage, and four animals were treated with keratin gel. No resuscitation fluids were given and all animals were closely monitored during surgery. After one hour the surgical wound was closed and the animals transferred to the housing facility. All surviving animals were sacrificed after 72 hours. At the time of sacrifice, liver tissue was retrieved for histological analysis.

Surgeries and Postoperative Treatment

All procedures were performed in accordance with Wake Forest University’s Animal Care and Use Committee guidelines, which encompass regulatory and accreditation agencies’ guidelines. The animals were weighed immediately before surgery. All animals were sedated using a combination of Ketamine 10 mg/kg and Xylazine 4 mg/kg through an intramuscular injection, intubated and maintained on 2-3% Isoflurane for the remainder of the procedure. The animals were then placed in a supine position, shaved and connected to the monitoring devices. All animals were connected to ECG leads, pulse oximeter cuff on the tail, and an intra-esophageal probe for temperature monitoring. After sterile prepping and draping, the abdominal incision was performed and the liver exposed. Prior to the liver injury, the abdominal aorta of the
animals was exposed and canulated using a 23 gauge needle connected to a pressure transducer (Lab-stat, ADInstruments Pty. Ltd. Castle Hill, Australia) which in turn was connected to a PowerLab® (ADInstruments) system for data acquisition. The mean arterial pressure (MAP) was recorded continuously throughout the procedure. All animals were monitored for several minutes and assured to be in a stable state prior to liver injury.

The median lobe of the liver was used for the injury due to its ample size and easy accessibility. Preliminary data during model development showed that a consistent liver injury cross sectional area could be created that resulted in death when left untreated, but that when treated with a control material could rescue the animal. A 2.0 cm² surface area ring was used to inflict a consistent sized injury to the liver by pulling the left central lobe through the ring and cutting immediately adjacent to the ring with a surgical blade (Figure 1). The MAP, temperature, heart rate, O₂ saturation, and shed blood were recorded throughout the procedure at 30 seconds, 5, 15, 30, 45 and 60 minutes. Shed blood was measured at each time point using pre-weighed sterile surgical gauze which was placed under the liver injury. In addition, blood samples were taken for CBC through an ear vein.

All animals were randomized into the previously mentioned four experimental groups. The negative control group did not receive any treatment and the time of death was recorded in minutes after infliction of the injury. As for the other experimental groups, the treatment was administered at the 5 minute time point unless the MAP fell to half of the starting value. For standardization, the hemostatic materials applied were measured or weighed. The keratin gel does not require compression so no compression was used in any of the other treatment groups.
so as not to confound the results. In the Hemcon treatment group, a 4.5 x 2.5 cm piece of bandage that was placed on the bleeding surface of the liver throughout the procedure and was removed prior to closure. In the QuickClot treated group, 2.5 grams of autoclave sterilized material per animal was used. The material was spread on the bleeding surface and was left after closure in the surviving animals. In the case of the keratin treatment group, 2 ml of the gel was used per animal. Sterile keratin gel was applied to the bleeding surface through a 1 ml syringe. The keratin was also left in place after closure of the animals. These parameters were determined during initial model development based on complete coverage of the wound site.

For the surviving animals, the monitoring continued for 60 minutes, after which the animal was considered to have survived the initial trauma and the bleeding stopped. The animals that were treated with Hemcon had to undergo removal of the material since it could not be left intra-abdominally as indicated by the manufacturer. The aortic cannula was removed and hemostasis established at the insertion site. No aortic bleeding was observed in any animal at necropsy. The fascia and skin of the abdomen was closed, in two layers. After complete closure of the abdomen, the animals were allowed to recover and transported to the housing facility where they were monitored every 15 minutes until complete recovery from anesthesia, then three times per day thereafter for the following three days. Blood samples were taken from all surviving animals every day for CBC analysis. Upon sacrifice at the 72 hour time point, the liver of each animal was harvested for histological evaluation.
Mean Arterial Pressure

The mean arterial pressure (MAP) was recorded using a 23 gauge needle placed into the lower part of the abdominal aorta. The needle was connected to a PE 50 tube which in turn was connected to a pressure transducer (Lab-stat) that was connected to a PowerLab system for pressure recording. The MAP was continuously monitored during the entire course of the procedure or until the death of the animal. To further evaluate the significance of a change in MAP and heart rate, shock index was used. Shock index is a well established clinical scoring system for fast assessment of trauma patients. The modified shock index was calculated by dividing heart rate by MAP (mmHg).

Temperature

The central temperature was recorded with an esophageal probe connected to the surgery room monitor. The temperature of the animal was continuously monitored throughout the procedure and recorded at the previously mentioned time points.

ECG and Heart Rate

The ECG and heart rate were monitored using a three lead system connected to the surgery room monitor and was maintained throughout the entire procedure. Flat line or irregular electrical activity with electrical mechanical dissociation was used to define the time of death.

Shed blood

Shed blood was measured by weight after subtracting the pre-weighed gauze. Weights were recorded at each time point and fresh gauze placed under the liver injury. The shed blood was
Hemostatic keratin biomaterial represented as a percent of the original body weight for each animal. CBC was determined from samples taken from an ear vein on a HEMAVet® multi-species hematology system (Model 950FS, Drew Scientific, Dallas, TX).

**Histology**

A tissue sample including the damaged liver surface was removed from each animal within one hour of euthanasia. Each sample was placed in Tissue-Tek® O.C.T. Compound 4583 (Sakura®) and frozen in liquid nitrogen. The frozen blocks were sectioned into 8μm slices using a cryostat (Model CM 1850, Leica Microsystems, Bannockburn, IL) to include the transected portion of the liver and mounted onto microscope slides. The slides were fixed and stained with Hematoxylin and Eosin (H&E). Technical difficulties in sectioning arose with both the QuikClot and the Hemcon sections. The brittle QuickClot made level sectioning difficult and created voids in the sections. The Hemcon bandage was removed before abdominal closure and therefore the clotted blood was only partially visible. Digital images were taken (Zeiss Axio Imager M1 Microscope, Carl Zeiss, Thornwood, NY) at varying magnifications to observe the interactions between the hemostat and the damaged area of the liver. A magnification of 100X showed the overall response of the tissue, while magnifications of 200X and 400X were used to visualize the cellular response.

**Statistics**

All presented data is expressed as averages and the corresponding standard deviations. For statistical analysis SPSS v11 (SPSS Inc, Chicago, IL) was used. Outliers were defined as having a z-score larger then +3.0 or smaller then -3.0 using a modified z-score (median of the absolute
deviation). Data at all time points were analyzed by one-way analysis of variance (ANOVA). If significant F values were found, the groups were further analyzed by Fischer’s Least Significant Difference Test (LSD). An alpha of p<0.05 was considered significant. The probability of a Type I error was minimized by limiting comparisons; only negative control versus the 3 treatment groups were performed. In order to compensate for bias generated by early drop out of dead animals (i.e. animals that exsanguinated before the end of the 60 minute operative period), we used polynomial regression to known pathologic endpoints to estimate values during the first 60 minutes. For the percent blood loss graphical data (Figure 3) where statistical relevance was reached with some groups, values are expressed as means with their corresponding standard error.

**Results**

*Surgeries and Postoperative Treatment*

Negative control animals (i.e. no treatment), as expected, exsanguinated within the 60 minute operative period (31 ± 19 minutes). Two animals in the QuickClot group and one in the Hemcon group did not survive beyond the initial 60 minute operative period. Also in the Hemcon group, one animal was euthanized 24 hours post-op on the advice of the veterinary staff. This animal was not ambulatory and could not eat or drink. One animal in the keratin group was also sacrificed at 48 hours. Although the animal was moving freely in its cage, it was not eating or drinking. At necropsy, these animals showed no evidence of additional bleeding after the operative period. All other surviving animals recovered without incident, were freely moving in their cages within 24 hours, and had normal CBC by 72 hours (data not shown). A summary of the survival data is shown in Figure 2.
*Shed Blood*

Blood loss was measured by weighing the surgical gauze placed below the injured liver lobe. The blood loss was expressed as percentage of starting body weight. As expected in uncontrolled hemorrhage studies, all animals showed an initial phase of profuse bleeding followed by a linear phase with a lower bleeding rate, as MAP falls (Figure 3). A comparison of the keratin and QuickClot groups to the negative controls shows a significantly decreased amount of blood loss at the 30, 45, and 60 minute time points (p values for keratin vs. negative control were 0.018, 0.011 and 0.007; p values for QuickClot vs. negative control were 0.009, 0.005 and 0.004, respectively).

*Median Arterial Pressure*

The mean arterial pressure in the abdominal aorta was recorded for 60 minutes. Animals in the keratin and Hemcon group were able to achieve stable MAPs after 5 minutes at 75% of the starting value. The QuickClot and control groups failed to stabilize MAP and dropped to 45% of the starting value after 60 minutes (Figure 4). However, these data did not reach statistical significance between groups.

The shock index (SI), a predictive score grading system for the severity of blood loss, showed a beneficial outcome for the keratin group with low values throughout the first 60 minutes (Figure 5). The high values of QuickClot matched with two early deaths during the first 20 minutes of observation supporting the predictive nature of this measure. Although a trend was noted, these data did not reach statistical significance in the present study.
Temperature and Heart Rate

The liver damage model employed in this study represented severe trauma with significant, rapid blood loss. None of the animals were able to compensate for loss of blood volume with an increase in heart rate. All animals showed a comparable decrease from 263 bpm to 188 bpm after 30 minutes and 154 bpm after one hour. There were no statistically significance differences between the groups. However, the keratin group showed a trend toward compensation and recovery with an increase in heart rate in the second half of the surgical period from 30 min to 60 min. The temperature of all animals dropped in a similar fashion with a step drop of 0.8°C in the first 5 minutes and a total of 2.7°C over 60 minutes. There was no statistically significant difference between the experimental groups.

Histology

The transected liver surfaces were examined by light microscopy of H&E stained sections. The negative control group showed a clean cut with no tissue response or necrosis (Figure 6A). Moreover, no functional clotting was observed with little thrombus adhered to the surface. The Quick Clot samples were difficult to process due to the presence of this hard, granular zeolite in the clot. Histology revealed necrotic tissue mixed with blood clots (Figure 6B). The transparent areas represent QuickClot particles removed during processing. The Hemcon group showed some areas with clotted blood and adjacent cellular infiltration (Figure 6C). Since the Hemcon bandage was removed after 60 minutes, most of the liver surfaces had only a thin layer of blood clots. The keratin group showed a thick layer of biomaterial attached to the damaged liver surface (Figure 6D). Granulation-like tissue with cellular infiltration had formed in the pores of the keratin biomaterial gel (Figure 7).
Discussion

The present study was a preliminary investigation into the potential efficacy of human hair keratins as hemostatic hydrogels. The liver transection produced a lethal injury, typically involving one or two large vessels of approximately 1mm diameter and several in the 0.5 to 1.0 mm diameter ranges. The severity of the injury was such that untreated rabbits all exsanguinated within the 60 minute operative period. As one would expect, the survivability of the animals appeared to be dependent on the vascular anatomy at the injury site, which was not consistent from animal to animal even though the total surface area transected was controlled. When a single very large bleeder (> 1 mm), or multiple large bleeders (> 2 to 3 in the 1m size range) were encountered within the injury area, the animal’s chance of survival was negligible in the QuickClot and Hemcon groups. In the QuickClot group in particular, a single very large bleeder or an excess of 2 to 3 large bleeders would ensure lethality. It should be noted however, that when used according to manufacturer’s instructions with concomitant pressure, other studies have shown better survival rates using QuickClot\textsuperscript{11} and Hemcon.\textsuperscript{25} In all cases of treatment with keratin gel, which was also used without any compression, the animals survived for at least 24 hours, regardless of the size of the severed vessels. Although a small number of animals were used in all test groups (n=4), these outcomes are encouraging.

The keratin hemostatic gel consistently performed well by each outcome measure, particularly shed blood volume, MAP and importantly, survival. One particularly distinguishing outcome was shock index. In most cases of hemorrhage, cardiac output is increased to compensate for the drop in blood pressure. Once this mechanism takes over, the value of shock index increases
rapidly and survivability becomes doubtful. Remarkably, the shock index in the keratin treatment group remained the lowest of all the materials tested, consistent with early effective hemostasis.

The keratin hemostatic gel was adherent to the tissue and hydrophilic. When deposited onto the bleeding surface of the liver it was sufficiently adhesive to not be washed away, even in the presence of profuse bleeding. The gel absorbed fluid from the blood and became even more adherent within a few minutes of administration. Clotting and adherence was almost instantaneous with contact. Interestingly, the keratin gel formed a thick seal of granulation-like tissue over the wound site by 72 hours. Upon inspection of histological sections, 3 days after injury host cells could be seen infiltrating the gel. We believe that the keratin gel used in these experiments serves two purposes. First, contact of the gel with whole blood instigates thrombus formation, probably through platelet activation or concentration of clotting factors. Second, the adherent gel forms a physical seal of the wound site and provides a porous scaffold for cell infiltration and granulation-like tissue formation, much like clotted blood.

Limitations to this study include:

- Control products not used according to manufacturer’s instructions
- Small animal numbers
- New model
- No BP measurements after the first 60 minutes
Conclusions

In this pilot study we demonstrated the potential efficacy of a hemostatic keratin hydrogel. The gel was derived from keratins extracted from human hair, a plentiful and renewable resource. The process for producing the gels is relatively simple, non-inflammatory, absorbable, scalable, and efficient. The present study demonstrated initial efficacy in arresting hemorrhage and most importantly improving survivability in a rabbit model of liver injury. Future studies will address arterial or liver bleeding in larger models, including those with a coagulopathy.

Acknowledgements

The authors would like to express their appreciation to Mandy Lockard and Cindy Andrews for their assistance during the surgeries, and to Paul Sikoski for veterinary support. We also thank Cathy Mathis for assistance with histology. We would like to thank Drs. Michael Dubick and Bijan Kheirabadi from the US Army Institute of Surgical Research at Ft Sam Houston, TX for useful discussions. Finally, the funding support provided by the Wake Forest Institute for Regenerative Medicine is gratefully acknowledged.
References


**Figure Legends**

**Figure 1. Application of Keratin Gel:** A) the ring device is placed around the left central lobe of the liver. B) 5 minutes after generation of the standard liver trauma or drop to 50% of starting MAP the keratin gel was applied. C) Image taken 2 minutes after applying the keratin gel, showing some remnant bleeding. D) Image taken after 20 minutes showing hemostasis.

**Figure 2. Kaplan-Mayer survival graph:** Time is presented in minutes on a logarithmic scale. All animals in the control group died within 60 minutes. One animal from the keratin and one from the Hemcon group was sacrificed upon recommendation of the animal care staff. Overall, keratin outperformed the other groups with only one death compared with 2 deaths in the QuickClot and Hemcon groups.

**Figure 3. Shed blood:** Blood loss is normalized to body weight and expressed at percentage of body weight. Keratin and QuickClot group lost significantly (*) less blood than the control and Hemcon group.

**Figure 4. Mean Arterial Pressure (MAP):** Blood pressure is expressed in percentage of initial pressure. The negative control and Quick clot group showed a steep drop in pressure to 40% of initial map. Animals treated with keratin or Hemcon were able to stabilize the map around 80% of initial pressure. These differences were not statistically different compared to the control group.
Figure 5. **Shock Index (SI):** The modified shock index was calculated by dividing heart rate by MAP. This index is clinically used to assess the severity of a shock with low values being better. The animals in the keratin groups showed compensated low values over the entire study period while QuickClot and Hemcon had similar values as the negative control. There was no statistical significance between the groups.

Figure 6. **Histological Assessment:** Representative tissue sections stained with hematoxylin and eosin, 50x. A) The negative control group shows signs of poor perfusion with wide and empty sinusoids. The surface is lacking a functional blood clot. B) The surface of the QuickClot treated samples show area of necrosis (arrow) and clotting. Only minimal cellular infiltration and tissue regeneration is visible. The void areas represent the removed QuickClot granules. C) Tissue samples from Hemcon treated animals showed patchy areas of adherent clotted blood where there was a low level of cellular infiltration. D) Liver samples from animals treated with keratin show a thick layer of keratin biomaterial attached to the injured surface. There are signs of excellent biocompatibility with a high cellular activity and the formation of early granular tissue (large arrow) in the spaces between the keratin. Further, there is a high level of direct contact of hepatocytes with the keratin biomaterial (small arrow).

Figure 7: **Keratin treated group, high magnification:** A) Formation of early granulation-like tissue within the spaces of the keratin gel, 200x. B) Interface between keratin gel and liver tissue showing integration of the biomaterial and tissue, and early cellular infiltration, 400x.
Figures

Figure 1

Figure 2
Figure 3

Figure 4
Figure 5

Figure 6
Figure 7