NAVAL MEDICAL RESEARCH UNIT SAN ANTONIO

Testing a Chitosan-derived Hydrogel as a Drug Vehicle to Control Bleeding and Promote Wound Healing

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DECLARATION OF INTEREST

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ABBREVIATIONS

ACS	American Chemical Society
AMR	Antimicrobial resistance
API	Active pharmaceutical ingredient
CoTCCC	Committee on Tactical Combat Casualty Care
DoD	Department of Defense
FDA	Federal Drug Administration
G	Clot strength
GAA	Gallic acid
IFI	Invasive fungal infection
MA	Maximum amplitude
MATTERs	Military Application of Tranexamic Acid in Trauma Emergency Resuscitation
POI	Point of injury
PXRD	Powder X-ray diffraction
TXA	Tranexamic acid
XRD	X-ray diffraction

EXECUTIVE SUMMARY

Background: Controlling bleeding is a serious concern for clinicians in the field and in a hospital setting. Traumatic hemorrhage remains the leading cause of preventable deaths of warfighters. In the field, wound dressings that initiate coagulation and a systemic dose of tranexamic acid are the preferred method to control bleeding until a casualty can be transported to a hospital. Due to foreseen peer or near-peer threats, a 72-hour timeline for point-of-injury care has been established as the new goal for field stabilization. For this reason, there is a need to support long-term coagulation and prevent sepsis. In this project, we investigated a chitosan-derived hydrogel as a vehicle for delivering a novel coagulation therapeutic cocrystal.

Objective: The objective of the proposed research is to develop a hemostatic, antibacterial wound healing medicament consisting of cocrystals suspended in a field deployable chitosan-based hydrogel. While others have attempted to develop a chitosan-based wound dressing with varying success, to our knowledge, this is the first attempt to generate a wound dressing that utilizes pharmaceutical cocrystals consisting of FDA-approved hemostasis compounds.

Methods: Hydrogel and cocrystals were generated based on previously established research. Cocrystal synthesis was verified using X-ray diffraction. The hydrogel and crystals effect on coagulation and clot formation was determined using a TEG 5000 hemostasis analyzer with swine blood.

Results: Successful generation of hydrogel and cocrystals was achieved, and crystal formation was verified with x-ray diffraction. However, the hydrogel demonstrated a negative effect on coagulation. Clot strength was reduced in the hydrogel sample compared to the control (hydrogel: 6.4 ± 1.9 dynes/cm^2, control: 14 ± 6.3 dynes/cm^2, $p<0.005$), as was the maximum amplitude of tracing (hydrogel: 71.2 ± 9.3 mm, control: 54.6 ± 9.6 mm, $p<0.005$).

Conclusions: The hydrogel we tested reduced clot strength and increased the time to form the clot. For these reasons, we determine that it would not make a suitable vehicle for drug delivery to support hemostasis, especially in a trauma situation. The tranexamic acid-gallic acid cocrystal did show potential benefits and requires further examination.

INTRODUCTION

Hemorrhaging wounds, received through trauma or surgery, have always been among the leading causes of death for warfighters during conflicts [1]. Even if hemorrhaging is halted, these injuries are highly susceptible to bacterial and fungal infections [2]. It is of the utmost importance that the best tools are provided to medics at the point-of-injury (POI) to stop hemorrhaging, minimize recovery times, and reduce secondary complications. The long-term goal of this project is to test the ability of a specific hydrogel to deliver necessary drugs more efficiently to a hemorrhagic wound in the form of suspended crystalline solids that potentially provide hemostasis, wound healing, and infection prevention.

Two main types of hemostatic dressings are currently recommended for use by the United States military: kaolin-based bandages and chitosan-based wound dressings. QuikClot, the cutting-edge hemostatic dressing used by the United States military, is a battle gauze mixed with powdered kaolin (chemical formula: $Al_2Si_2O_5(OH)_4$), an aluminosilicate mineral that promotes blood clotting [3]. The product consists of a non-woven gauze coated in kaolin that can be applied to the traumatic injury without causing an exothermic reaction. While generally regarded as safe, kaolin often forms silica nanoparticles that are toxic byproducts due to the high reactivity of Si-O bonds with cellular membranes in human tissue [4]. Besides QuikClot, Celox gauze [5], and ChitoGauze [6], chitosan-based wound dressings, are considered effective in stopping bleeding in the majority of cases involving significant bleeding [7, 8]. Celox Gauze hemostat has been approved by the Committee on Tactical Combat Casualty Care (CoTCCC) for the Department of Defense (DoD). ChitoGauze was also recommended by the CoTCCC. Chitosan is a nontoxic, biodegradable, and biocompatible biopolymer [9], and known to have procoagulant and antimicrobial effects in wound healing [10]. QuikClot, Celox gauze, and ChitoGauze all have a risk of introducing hemostatic dressing residues into the systemic circulation and increasing the risk of embolus formation when used for longer than 24 hours. Due to these adverse effects, these hemostatic dressings are not recommended for long-term use [11].

Hydrogels do not have these same adverse effects as many wound dressings and the cocrystals are water soluble such that they do not form precipitates in the body. We wanted to test their ability to safely deliver necessary drugs to a wound in the event of prolonged care, as is common in forward-deployed, austere combat casualty conditions. A hydrogel-based delivery system allows for increased drug stability, tunability of crystalline nanoparticle size to control and

enhance dissolution rates, and modifiable bioavailability of active pharmaceutical ingredients (APIs) [12]. These attributes result in gradual and prolonged delivery of vital drugs directly to the wound site. Tranexamic acid (TXA) has shown to be extremely beneficial in treating trauma patients due to its antifibrinolytic activity. A meta-analysis of 1,333 patients [13] showed that TXA significantly reduced the requirement for blood transfusions and total blood loss compared to the control group. Another study of over 20,000 adult trauma patients concluded that a loading dose of 1 gram over ten minutes, followed by infusion of 1 gram over eight hours administered within three hours of initial injury had a significantly reduced risk of mortality due to bleeding [14]. The hemostatic effect of TXA was already studied and confirmed under Military Application of Tranexamic Acid in Trauma Emergency Resuscitation (MATTERs) Study at a Role 3 Echelon surgical hospital in Southern Afghanistan in 2012 [15]. In this study, among 896 combat casualties who received transfusions, TXA-injected casualties had a 27% lower mortality rate than those who did not. In the subgroup that received massive transfusions, the TXA group had nearly half the mortality rate compared to the control group. Although TXA does not induce new clot formation, it effectively stabilizes existing clots, a crucial action in achieving hemostasis in bleeding casualties. Tranexamic acid competitively (and reversibly) inhibits the conversion of plasminogen to plasmin, the major pathway that leads to lysis and degradation of fibrin clots. In high concentrations, it acts as a noncompetitive inhibitor of plasmin. Plasmin also leads to the cleavage of high-molecular-weight kininogen to bradykinin [16]. Bradykinin has been shown to produce vasodilation and increased vascular permeability in the blood-brain barrier [17]. It is suggested that an antifibrinolytic like TXA could inhibit this cascade and decrease capillary vessel bleeding [18]. Due to both the short biological half-life (two hours) of TXA and possible side effects [19, 20], a small dose is generally administered systemically to support other hemostatic interventions. The cocrystallization approach was considered as part of several efforts to further enhance the advantages of TXA, mentioned above, in a controlled manner over prolonged periods of time. Crystalline forms of drugs can confer many benefits due to their tendency to afford highly pure products with high reproducibility and scalability [21]. Cocrystals are formed from two or more components where at least one component (coformer) is molecular, a solid at room temperature, and forms a supramolecular synthon (crystal structure). Cocrystallization of various drugs offers an opportunity for the development of new drug products with superior physicochemical properties such as solubility, stability, and bioavailability, while preserving the pharmacological properties.

TXA has the ability to cocrystallize with multiple coformers [22]. One identified coformer is gallic acid (GAA). In combat, immediate threats to the warfighter's life such as hemorrhage may be addressed but prevention of infection tends to be given a lower priority than hemostasis. However, infection prevention is a factor that greatly affects the patient's future prognosis, and therefore should be considered as an important medical factor in treating the injured. Trauma-associated fungal infections generally occurred in blast injuries with penetrating wounds contaminated by environmental debris [23]. When uncontrolled hemorrhaging from puncture wounds occurs in combat, there is a high risk of infection. Gallic acid is a phenolic compound found in various fruits and herbs and is known to have antimicrobial activity [24] and to promote wound healing [25, 26]. Regarding clinical settings, GAA has been shown to inhibit the generation of antimicrobial resistance (AMR) in pathogens commonly found in hospitals, further supporting its effectiveness for prolonged infection control [27]. In an *in vivo* study, intraperitoneal injection of GAA (80 mg/kg/day) significantly enhanced the cure rate in a mouse infection model of systemic fungal infection [24]. At the same time, the toxicity of GAA has been studied in multiple animal models, including zebrafish, rats, and rabbits [28, 29]. In zebrafish, a 96-h lethal dose was above 100 mg/L, while in rats they found the lethal dose to be 119 and 128 mg/kg/day in male and female rats respectively. These numbers are generally considered non-toxic in humans. The addition of GAA in a hemostatic wound dressing would be extremely beneficial for the prevention of invasive fungal infections (IFIs), which are of the most serious concerns associated with combat-related penetrating wounds, and reduced occurrence of AMR pathogens during prolonged infection treatment.

When engaged with peer or near-peer enemies in multi-domain operations, the assumption that wounded soldiers will be rapidly evacuated to the rear medical facility is highly unlikely. Therefore, wounded soldiers may not be able to receive early treatment from clinic-based providers equipped with sufficient medical supplies and equipment, and in some cases may have to endure considerable time being treated by combat medics with limited medical supplies. In addition to the limitation of long-term use of these existing commercialized products, recurrence of bleeding is observed in most of these products [30, 31]. Constraints associated with long-term use and rebleeding when definitive surgical care is postponed are key issues that present hemostatic dressings must overcome. In this study we attempted to develop a chitosan-based hydrogel as an active drug carrier for the TXA:GAA cocrystal for use in hemorrhagic wounds. We

established protocols to synthesize the TXA:GAA cocrystal and verified successful synthesis using powdered X-ray diffraction (PXRD). A chitosan-based hydrogel was synthesized and effects on coagulation were assessed using thromboelastography.

MATERIALS AND METHODS

Cocrystal Synthesis

Crystal synthesis was accomplished following a previously established protocol [32]. Briefly, a controlled evaporation technique was used to prepare a molar ratio of one-to-one TXA:GAA cocrystal. A mass of 602.04 mg (3.83 mM) of TXA (98% purity, Oakwood Chemicals, SC) was dissolved in 16.5 mL of deionized water and any residual TXA in the weigh boat was rinsed into solution (2.5 mL additional DI water). The TXA was dissolved via swirling motion. A mass of 717.56 mg (4.22 mM) of GAA (99% ACS grade, Oakwood Chemicals, SC) was added to the solution and residual GAA was rinsed from the weigh boat into solution (3 mL additional DI water). An additional 15 mL DI water was added to solution and the GAA was dissolved via swirling motion at 30°C. Once both components were fully dissolved, the solution beaker was partly closed off to slow the evaporative process. Full evaporation occurred after 7 days. On the fourth day, the solvent experienced a color change to a yellow tint consistent with the initial formation of TXA:GAA cocrystal. Cocrystals of 1:1 TXA:GAA solidified as the solvent evaporated. Once the solvent had completely evaporated, the cocrystal was collected and weighed. From this, a total of 1.336 g cocrystal was retrieved (101.2% returned or 4.031 moles). A minor contamination existed within the beaker which explains the discrepancy in final weight.

Chitosan Hydrogel

Synthesis of our hydrogel followed previously established methods [33], with slight modifications. A stock solution of pentasodium tripolyphosphate (Sigma-Aldrich, MA) was prepared by dissolving 200 mg in 20 mL of 10% HCl. Vial 1: 600 µL acrylamide (99% purity, Oakwood Chemicals, SC, 50% w/v in DI water), 100 µL bis-acrylamide (99% purity, Oakwood Chemicals, SC, 1% w/v in DI water), and 250 µL tetramethylethylenediamine (TMEDA, 99.5% purity, Oakwood Chemicals, SC, 8% w/v in DI water) were mixed via vortexing. Vial 2: 1000 µL chitosan (Creative Biomart Inc, NY, 3% w/v in DI water), 50 µL glacial acetic acid (> 95% purity,

Thermo Fisher, MA), and 50 μL TMEDA (26% v/v in DI water) were mixed via vortexing. A total of 10.7 mg of ammonium persulfate was added to vial 1 and mixed via vortexing. Vial 1 was added to vial 2. A mass of 40.1 mg sodium bicarbonate was added, and the resulting solution was vortexed for 15 minutes. The gel was transferred to the 20 mL stock solution of pentasodium tripolyphosphate. Dropwise sodium hydroxide (2N, Thermo Fisher, MA) was used to increase the pH until optimal polymerization of the gel occurred (pH = 7.6).

Crystal Analysis

Samples were analyzed using a Rigaku MiniFlex 600 (Rigaku Corporation, Matsubara-cho, Akishima-shi, Tokyo, Japan) Experimental conditions: Cu Kα radiation (λ = 1.5418 Å); 40 kV, 15 mA; scan range 10 – 80° 2θ. X-ray powder diffraction data were collected at room temperature using a Rigaku scintillation counter (SC-70S).

Coagulation Assay

A TEG 5000 hemostasis analyzer (Haemonetics, Braintree, MA) was used to measure coagulation profiles of all samples. All blood samples were ordered citrated (Animal Technologies, TX). Samples were analyzed following Haemonetics TEG 5000 protocol. Five sample groups were prepared: 1) blood control, 2) blood mixed with 5 mg TXA, 3) blood mixed with 5 mg GAA, 4) blood mixed with 5 mg cocrystal, and 5) blood mixed with 10 mg chitosan hydrogel. A high dose of TXA was selected based on previous work [34]. All sample groups used 500 μL of swine blood. Only fresh blood was used; all experiments involving blood were performed within 48 hours of the blood being drawn from the animals. Coagulation assays were performed until 6 replicates were received per group.

Statistical Analysis

The results received from the coagulation assay were analyzed using statistical analysis system (SAS version 9.4, SAS Institute, Cary, NC) software. One-tailed Dunnett t-tests were used to compare TEG-5000 output variables received from the control to those received from TXA, GAA, the cocrystal, and the hydrogel. Before running these tests, all values were ranked. P-values at or below 0.05 are interpreted as statistically significant.

RESULTS

Chitosan Hydrogel

The chitosan hydrogel's transparency would allow for wound healing to be observed without the need for removing and redressing the wound. Figure 1 shows an image of the chitosan hydrogel which was prepared without pH balancing that would cause polymerization and shows the opacity and consistency of the hydrogel. The chitosan hydrogel has a light-yellow coloring to it.

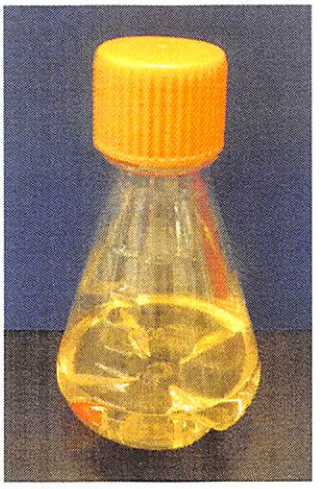

Figure 1: Chitosan-derived hydrogel. Photographic image of the prepared chitosan hydrogel immediately following synthesis. The hydrogel demonstrated a high degree of transparency and increased viscosity.

PXRD analysis of TXA:GAA Cocrystal

TXA:GAA cocrystal structure and purity analyses are required after its synthesis. A commonly employed method to accomplish this is the use of PXRD. The PXRD data show that relative to the theoretically calculated diffraction pattern, the newly synthesized TXA:GAA cocrystal structure is of a pure crystal composition and was successfully synthesized. PXRD pattern analysis was done by our collaborators at the University of Iowa, Chemistry Department (Figure 2A). It was confirmed that the TXA:GAA cocrystal demonstrated a high degree of crystallinity in the correct 1:1 molar ratio. Crystals were not generated at a uniform size (Figure 2B) but were uniform in color.

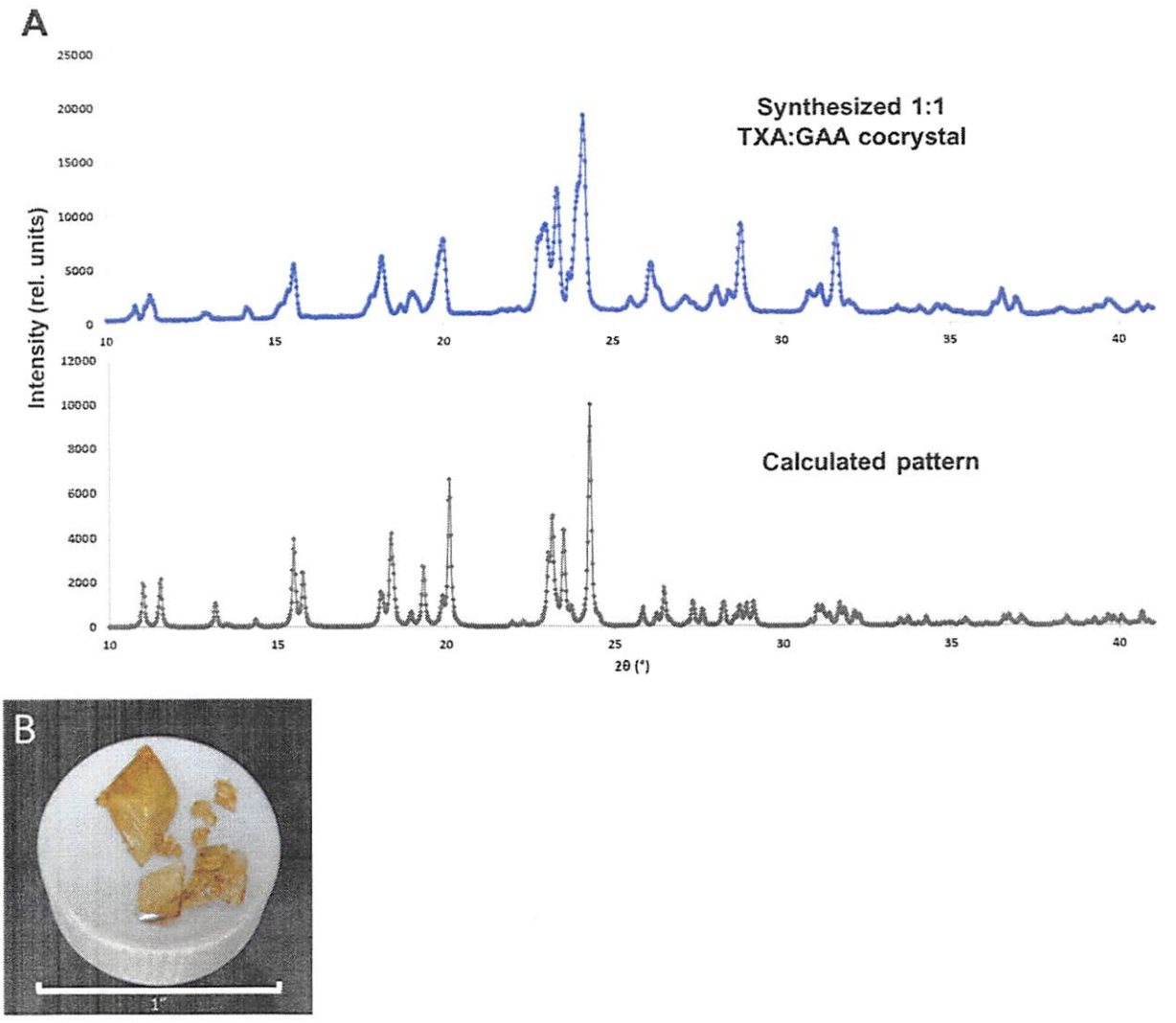

Figure 2. Cocrystal analysis. A) A comparison between the observed powder X-ray diffraction pattern of TXA:GAA cocrystal synthesized in the laboratory and its theoretical pattern. B) Picture taken of synthesized cocrystal to document color, shape, and differing sizes.

Combination of TXA:GAA and Chitosan Hydrogel

Cocrystal was ground with a small mortar and pestle until a uniform powder was left with no visibly large crystals. When our cocrystal was combined in the hydrogel a color change occurred from light yellow to a darker yellow as the crystals partially dissolved. Samples prepared were still translucent and consistency did not seem to be noticeably altered.

Figure 3: Chitosan-derived hydrogel and cocrystal. Photographic images of the prepared chitosan hydrogel and cocrystal combination.

Coagulation

To characterize the effects of the hydrogel as a potential drug carrier in hemorrhagic wounds, we analyzed clot formation of swine blood using a coagulation assay and a TEG 5000 (mo. number: 05L1-1581-LBB) (Figure 4). One control replicate was omitted from data analysis after the TEG-5000 returned an error message with the results being indiscernible. Five replicates from the control group could be compared to the 6 replicates from each experimental group.

Adding chitosan hydrogel to the sample decreased clot strength and increased clot formation time compared to the control. Clot strength (G) was reduced in the hydrogel sample to 6.4 ± 1.9 dynes/cm^2 compared to the control 14 ± 6.3 dynes/cm^2 ($p = 0.0052$). Maximum amplitude of tracing (MA), a measure of the maximum clot strength, was reduced from 71.2 ± 9.3 mm (control) to 54.6 ± 9.6 mm ($p = 0.0052$). Alpha angle (angle), a measure of the maximal speed of clot formation, was reduced from $72.9 \pm 9.9^{\circ}$ (control) to $60.3 \pm 14.3^{\circ}$ which trends towards significance ($p = 0.0603$).

The results also revealed the hydrogel offered no significant improvements from the control in the kinetic time for fibrin cross linkage to reach a specified level of clot strength (K-time) and the reaction time to initial fibrin formation (R-time). The control had a K-time of 0.98 ± 0.18 minutes and an R-time of 2.6 ± 0.76 minutes respectively, but when the chitosan hydrogel was added, these times showed no statistical difference in their K-time of 2.4 ± 1.3 minutes and an R-time of 3.2 ± 0.57 minutes (K-time: $p = 0.1122$; R-time: $p = 0.1571$).

Our two coformers were tested separately, as well as together in a cocrystal to measure their effects on coagulation. Tranexamic Acid did not affect the coagulation cascade in any

significant way, while GAA did extend the time from the test start to the initiation of the clot (R-time: p = 0.0097).

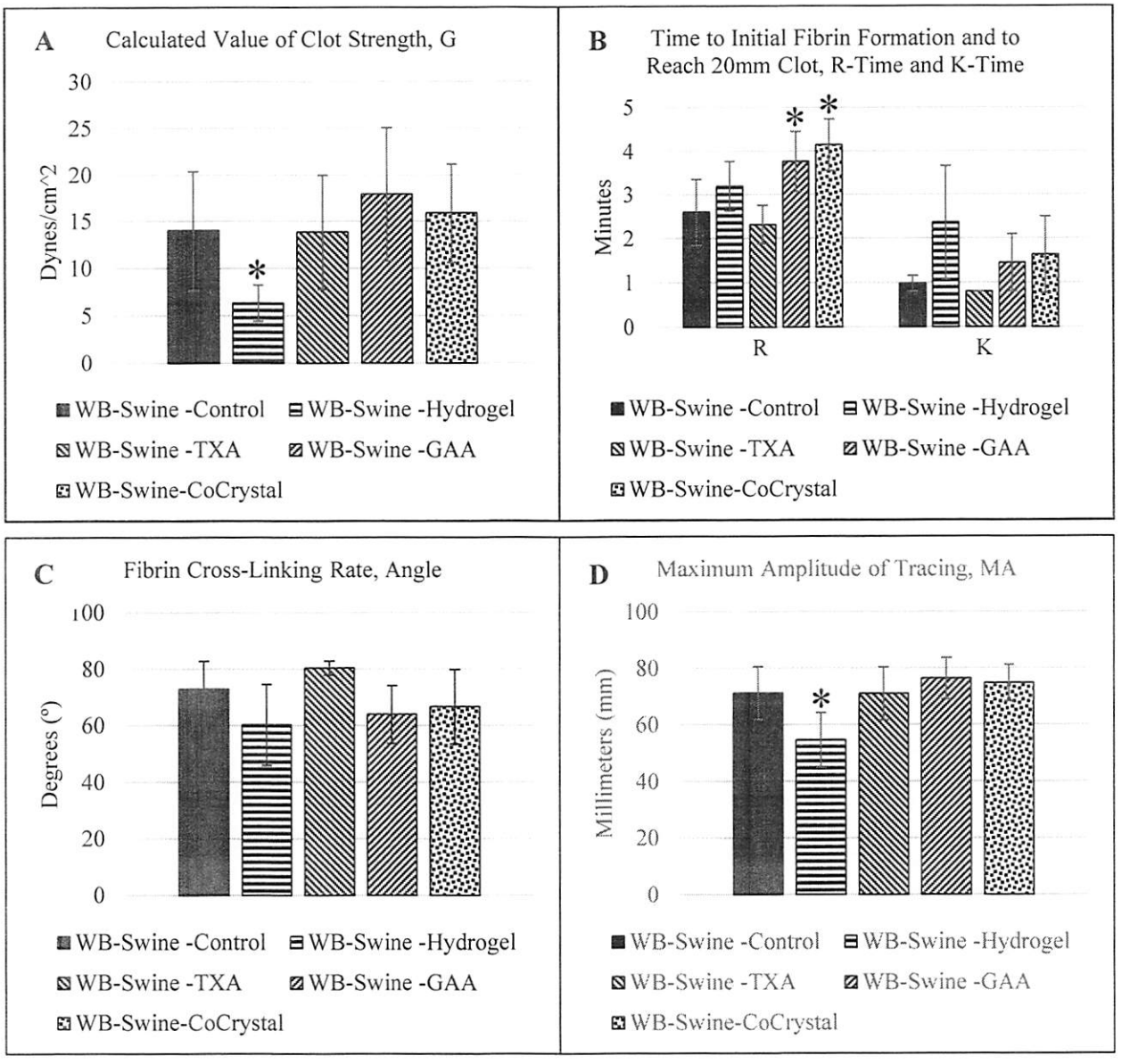

Figure 4. Coagulation assay results for swine blood tested with a TEG 5000. A) Clot strength. G= Calculated value of clot strength. Hydrogel alone significantly reduced the overall clot strength (p = 0.0052). B) Clot properties. (Left) R= Reaction time to initial fibrin formation, (right) K= Kinetic time for fibrin cross linkage to reach 20 mm clot strength. C) Clot formation time. Angle= angle from baseline to slope of tracing that represents clot formation. D) Maximum strength of the

clot before fibrinolysis starts. MA= maximum amplitude of tracing. Hydrogel alone significantly reduced the maximum amplitude ($p = 0.0052$).

Discussion

Hydrogel dressings are ideal in many ways for wound dressings. They provide necessary moisture to potentially dry, sloughing, or necrotic wounds compared to dry gauze dressings [35, 36]. Hydrogels are generally translucent, which would allow for wound healing to be observed without the need for removing and redressing the wound [36]. Another major advantage is that hydrogel viscosities are reduced when heated (i.e., applied to a warm body), meaning they can flow in hard-to-reach crevices and wounds that require medical intervention [37]. Due to these benefits, hydrogels remain attractive as a vehicle to deliver pharmaceuticals to battlefield wounds.

We wanted to understand the possibility of one chitosan-based hydrogel to be used as a drug carrier in hemorrhagic wounds. We tested the hydrogel and pharmaceuticals separately to better understand each component's role in overall effect on coagulation formation and stability. Swine models are the current relevant animal model for prolonged field care of non-compressible hemorrhaging [38]. As a result, our coagulation test was performed on swine blood for direct comparison with possible future studies. Our data indicate that our chitosan-based hydrogel in its current form was not effective in supporting coagulation. In fact, we observed a statistically significant reduction in overall clot strength and increase in the clot formation time. In emergency situations, even a small decrease in overall clot strength and an increase in clot formation time can have a significant impact on survival rates. For the pharmaceutical coformers, TXA did not seem to have any effect on clot formation or strength while GAA exhibited a minor increase in R time. Further studies will need to investigate this further and determine if this is due to the concentration of GAA or an inherit property.

Hemorrhagic wounds are not limited to the military, as they also affect civilians. The military responds to mass casualty incidents, such as natural disasters and terrorist attacks, which frequently involve penetrating trauma. Equipping first responders with a device that can deliver the appropriate pharmaceutical treatment to stabilize the injury and prevent sepsis until hospital intervention would lead to an overall increase in readiness and survival rate of casualties.

Future studies should aim to verify and quantify the effects of the individual coformers when a cocrystal is formed. With the event of prolonged field care (> 72 h), the risk of infection

and sepsis increases drastically, so the addition of bactericidal/fungicidal GAA to the wound site would be advantageous for the subsequent treatment. Therefore, this novel treatment option could aid the Role I provider in the field, who is largely responsible for POI, in treating patients successfully while decreasing the risk of infection. There is a need to assess the cocrystals biocompatibility, antimicrobial properties, wound healing, and effect on clot duration. A different vehicle for drug delivery that is neutral or actively supports coagulation will also need to be tested.

MILITARY SIGNIFICANCE

Nearly 25% of all battlefield deaths are potentially survivable, with a staggering 90.9% of these total deaths resulting from uncontrolled hemorrhage [39]. By reducing the dependency on excessive blood transfusions, medications, and extended recovery, we can significantly decrease the strain on medical resources, ultimately saving even more lives indirectly. Beyond sustaining a clot for more than 72 hours, sepsis is a severe risk. Current hemostatic dressings do not address this issue and focus solely on hemostasis. This is dangerous for a warfighter that must rely on that dressing for prolonged time at early Echelons of care, or in a mass casualty situation where hospitals and surgical rooms are overwhelmed. Once the synthesis and drug delivery vehicle are optimized, the GAA:TXA cocrystal has the potential to increase a casualty's survivability and injury stability. Additionally, this cocrystal can be carried with service members in the field, which could allow for immediate application to a wound to mitigate hemorrhagic shock and inhibit infection. Hydrogels are attractive drug vehicles, but the hydrogel we tested in this study should not be used for hemorrhage control. There are many types of hydrogels that are used in pharmaceuticals that can be investigated, as well as medical grade foams and gauzes that work as suitable drug carriers for the cocrystal and which also aid in coagulation.

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14. ABSTRACT

Traumatic hemorrhage remains the leading cause of preventable deaths of warfighters. In the field, wound dressings that initiate coagulation and a systemic dose of tranexamic acid are the preferred method to control bleeding until a casualty can be transported to a hospital. Due to foreseen peer or near-peer threats, a 72-hour timeline for point-of-injury care has been established as the new goal for field stabilization. For this reason, there is a need to support long-term coagulation and prevent sepsis. In this project, we investigated a chitosan-derived hydrogel as a vehicle for delivering a novel coagulation therapeutic cocrystal. The hydrogel we tested reduced clot strength and increased the time to form the clot. For these reasons, we determine that it would not make a suitable vehicle for drug delivery to support hemostasis, especially in a trauma situation. The tranexamic acid-gallic acid cocrystal did show potential benefits and requires further examination.

15. SUBJECT TERMS

Hemorrhage, Tranexamic Acid, Hydrogel, Hemostasis

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