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# Expanding Hydrophobically Modified Chitosan Foam for Internal Surgical Hemostasis: Safety Evaluation in a Murine Model

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ABSTRACT

*Background:* A novel injectable expanding foam based on hydrophobically modified chitosan (HM-CS) was developed to improve hemostasis during surgeries. HM-CS is an amphiphilic derivative of the natural biopolymer chitosan (CS); HM-CS has been shown to improve the natural hemostatic characteristics of CS, but its internal safety has not been systematically evaluated. The goal of this study was to compare the long-term *in vivo* safety of HM-CS relative to a commonly used fibrin sealant (FS), TISSEEL (Baxter).

*Methods:* Sixty-four Sprague–Dawley rats (275-325 g obtained from Charles River Laboratories) were randomly assigned to control ($n = 16$) or experimental ($n = 48$) groups. Samples of the test materials (HM-CS [$n = 16$], CS [$n = 16$], and FS [$n = 16$]) applied to a nonlethal liver excision (0.4 ± 0.3 g of the medial lobe) in rats were left inside the abdomen to degrade. Animals were observed daily for signs of morbidity and mortality. Surviving animals were sacrificed at 1 and 6 wk; the explanted injury sites were microscopically assessed.

*Results:* All animals (64/64) survived both the 1- and 6-wk time points without signs of morbidity. Histological examination showed a comparable pattern of degradation for the various test materials. FS remnants and significant adhesions to neighboring tissues were observed at 6 wk. Residual CS and HM-CS were observed at the 6 wk with fatty deposits at the site of injury. Minimal adhesions were observed for CS and HM-CS.

*Conclusions:* The internal safety observed in the HM-CS test group after abdominal implantation indicates that injectable HM-CS expanding foam may be an appropriate internal use hemostatic candidate.



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## Introduction

In surgical procedures, topical hemostatic agents are used to control local active bleeding.[1] These agents are typically indicated for use as adjuncts to hemostasis when cautery or suture is either ineffective or impractical. Often, these agents are used in cases where bleeding from parenchymal soft tissue needs to be controlled, as cautery results in permanent tissue damage and risk of infection, whereas suturing is usually impractical, given the nature of wounds in parenchymal tissue.[2] Many of the topical hemostatic agents widely used in the hospital setting are members of the fibrin sealant family. These agents include FLOSEAL and SURGIFLO and only contain thrombin as the active hemostatic agent. Alternately, similar hemostatic products, such as TISSEEL or EVICEL, can contain both fibrinogen and thrombin, which polymerize into robust fibrin networks on mixing. Fibrinogen is extracted from human blood; thrombin is typically sourced from either human or bovine blood. To manufacture these products, plasma must be obtained, traced, and processed in a highly controlled process for proper safety precaution in product design.[3] The regulatory requirements for clinical use and the need for stringent production controls increases the expense of these key components, resulting in the costs for fibrinogen typically about $400/g[4] and thrombin at about $90-$100/g[5] for clinical use. Finally, fibrin sealants generally require cold storage[6] and are provided in multicomponent kits, which require significant assembly and preparation steps before use.[7]

Hydrophobically modified chitosan (HM-CS) is an amphiphilic derivation of the biopolymer chitosan (CS). HM-CS has been shown in prior studies to amplify the natural hemostatic capability of CS.[8-11] CS is a polysaccharide obtained from the exoskeleton of crabs, shrimps, mollusks, and insects[12,13] and is the second-most abundant biopolymer on earth next to cellulose.[14] It has long been of interest in wound treatment due to unique inherent qualities: (1) hemostatic effect,[15,16] (2) antimicrobial characteristics,[17,18] (3) scar reduction,[19] (4) wound healing acceleration,[20] and (5) durability.[21] Due to these advantageous properties, several, CS-based products have been cleared by the FDA for external hemostatic applications for bleeding from minor cuts and scrapes to severe, life-threatening hemorrhage.[22] Although biocompatibility for external applications has been established, CS-based products have not been approved for internal use as an absorbable hemostatic agent in surgical applications. However, several studies have demonstrated CS to be safe for use *in vivo* as an internal, absorbable hemostatic agent.[23-26]

Hydrophobic modifications of CS allow the biopolymer to be used in a solution format, an advantage over unmodified CS. CS-based external-use hemostats historically have all been solid, textile-based compression devices. In the solid state, CS acid salts adhere to bleeding sites due to the mucoadhesive properties of the biopolymer backbone.[24] However, in solution, CS does not staunch blood flow, although it electrostatically interacts with blood cells. In contrast, HM-CS allows for the formation of a 3-dimensional network with blood cells,[8] thus forming a physical barrier that is able to control bleeding without compression. The hydrogelating characteristics of HM-CS results in the polymer acting more like a fibrin sealant in the presence of blood.[8-11] Due to this unique property, HM-CS represents a useful platform to create fibrin sealant mimics, which are ready-for-use at room temperature. In the present study, HM-CS is delivered as an injectable foam, which expands due to the creation of $CO_2$ gas on mixing with sodium bicarbonate in the tip of a double-barrel syringe. Our goal was to define an important safety endpoint: survival in an *in vivo* model for HM-CS hemostatic agents. Thus far, we have studied the material in many acute instances of bleeding, with short observation period of 1 to 3 h, mimicking the transit time of a patient to the hospital after traumatic injury.[8-11] However, the material has not yet been evaluated internally in a survival study. Here, we apply the material on a nonlethal liver excision in rats for characterizing properties after 1 and 6 wk of exposure. HM-CS was compared with native CS, formulated in the same double-barrel syringe configuration and a commercial FS gel product for effects on survival, morbidity/mortality, visual degradation of product, and a microscopic analysis of the injury site. Our overall hypothesis was that HM-CS would have no significant differences in morbidity and mortality relative to CS and FS at the time points studied.

## Materials and methods

### Materials

CS of medium molecular weight (190-310 K) and Brookfield viscosity of 286 cps was purchased from Primex (ChitoClear hqg400). The reported degree of deacetylation was about 95%. Acetic acid and sodium bicarbonate were purchased from Sigma. Palmitic anhydride was purchased from TCI. TISSEEL fibrin sealant was purchased from Baxter.

### Synthesis

HM-CS was synthesized by attaching *n*-palmitoyl tails to the CS backbone via reaction with palmitic anhydride. The procedure is similar to that reported in our earlier work,[8-11] and it also follows those described in the literature.[25] The degree of hydrophobic substitution follows the reaction stoichiometry, and here, it was fixed at 1.5 mol% of the available amine groups.

### Solution preparation

HM-CS or CS was dissolved in 0.2 M acetic acid solution at 2 wt % (w/v). Sodium bicarbonate was dissolved in water a 0.3 M.

### Preparation of foams

First, the double-barrel syringe chamber was capped to prevent leakage of fluid. 2 mL of polymer solution, either CS or HM-CS, at 2.0 wt% was added to one chamber of a double-barrel syringe (purchased from J. Dedoes), and the other chamber was filled with 2 mL of sodium bicarbonate solution. The syringe's plungers were inserted into the barrels and

pushed until resistance was felt due to hydrostatic pressure of the fluid. Finally, the cap was removed, and the mixing tip was fastened onto the chamber port. A schematic illustration of the HM-CS double-barrel syringe is shown in Figure 1.

### *Rat injury model (safety)*

All animal studies were approved by the University of Georgia Institutional Animal Care and Use Committee, and all protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health. Sixty-four eight-week-old Sprague–Dawley rats (275-325 g obtained from Charles River Laboratories) were randomly assigned to control or experimental groups. Sixteen animals served as controls, receiving a midline laparotomy and partial hepatectomy but no applied hemostatic agent. The remaining 48 animals were randomized across three experimental groups that received one of three possible hemostatic agents: a fibrin-based agent (TISSEEL), an unmodified CS agent or an HM-CS. Half of the animals were euthanized 1 wk after surgery, and the remainder were euthanized at 6 wk after surgery. All rats were weighed before operation and anesthetized using isoflurane gas (induced at 4% and maintained at 2%). The animals were placed on a heated pad to maintain a body temperature of 37°C, with the abdomen exposed underneath a sterile surgical drape. The surgical site was aseptically prepared three times using alternating chlorhexidine and ethanol swabs, and lidocaine was administered along the incision site on the abdomen. A ventral midline approach to the abdominal cavity was performed, and the medial liver lobe was identified. A sterile ruler was used to mark the medial liver lobe 0.5 cm from the distal end of the lobe, and the distal end was sharply excised according to that marking.

Sixty seconds after the partial hepatectomy was performed, animals in the experimental groups received the respective hemostatic agent delivered directly to the injury site via syringe applicator, and the rat was left under anesthesia for 1 h with the abdominal cavity open to allow for formation of a stable clot. Sterile 4 × 4 gauzes were weighed before surgery and then used to collect hemorrhage from the abdomen and reweighed to determine the amount of blood loss that occurred. Figure 2 shows the excision area treated with each agent immediately after application. After 1 h, the abdominal cavity was closed using a simple continuous pattern of absorbable suture in the abdominal body wall and an intradermal pattern of absorbable suture for skin closure. Triple antibiotic cream was layered on top of the sutured skin. Buprenorphine (0.05 mg/kg) was injected subcutaneously before animals were removed from anesthesia, and individual rats were placed into new, clean cage under a heat lamp to recover. Animals were returned to the animal facility after recovery, housed with free access to food and water and under regulated conditions of temperature (here), relative humidity (here), and illumination (12 h light/dark cycles).

### *Animal observation and euthanasia*

Animals were observed every 12 h for the first 72 h after surgery and once a day over the following 2 wk. One wk and 6 wk after surgery, animals were anesthetized using 5% isoflurane gas and transcardially perfused with 200 mL saline rinse (with 10 units/mL heparin, pH 7.4) followed by 200 mL of 4% paraformaldehyde in PBS. In the ISO standard for implantation testing of medical devices, time points of 1, 4, and 13 wk are evaluated. Here, in this initial survival safety study, we observe 1- and 6-wk time points because they provide screening confidence on the outcomes of (1) mortality, (2) morbidity, (3) tissue reactivity, and (4) adhesions. Although we ultimately care about safety over the period of years/decades, for a degradable implantable material, we will understand critical outcomes in these four areas in a fairly short period of time. The 1- and 6-wk time points give us a screening framework to quickly understand the utility of a material for implantable biosurgical purposes. The injury site of the medial lobe was removed, along with a section of an untouched lobe.

### *Adhesions*

Upon euthanasia, and the subsequent reopening of the abdomen, adhesions between the liver and surrounding tissues were noted. Adhesions could be categorized as filmy, thick or dense, and the strength of adhesions would be semiquantitatively scored from 0 to 4, according to the following separation conditions using surgical tweezers (0 = no adhesion, 1 = mild adhesion, easy to separate, 2 = moderate adhesion, requires light force to separate, 3 = marked adhesion, requires significant force to separate, 4 = severe adhesion, cannot be separated without resection using scalpel blade). Only six animals from each cohort underwent adhesion analysis.

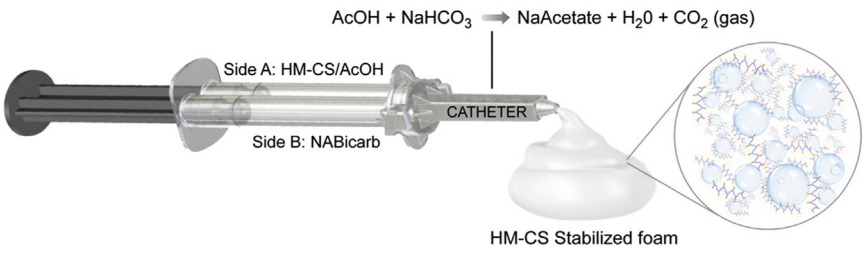


**Fig. 1 – Schematic of HM-CS foam delivered from double-barrel syringe. Side A contains HM-CS dissolved in dilute acetic acid. On mixing with sodium bicarbonate in side B via mixing tip, an expanding foam is generated due to stabilization of bubbles formed due to $CO_2$ gas generation. (Color version of figure is available online.)**

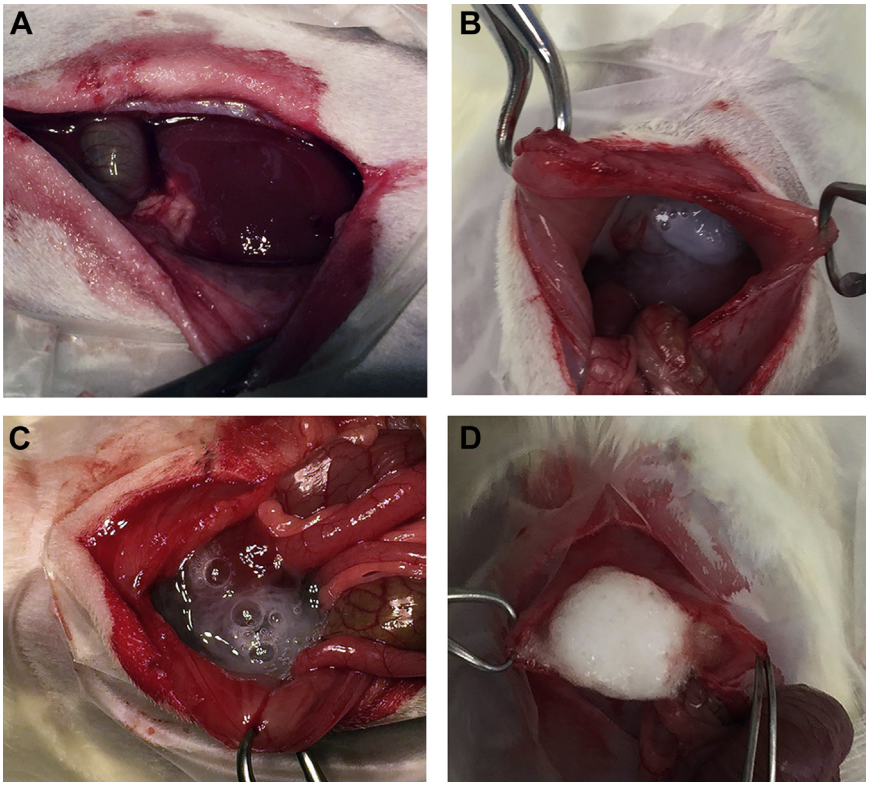


**Fig. 2 – Photographs of hemostatic agents immediately after treatment of hepatic injury. (A) No treatment control, (B) TISSEEL fibrin sealant (FS), (C) chitosan (CS), and (D) hm-chitosan (HM-CS). (Color version of figure is available online.)**

### *Tissue processing for histology*

Liver tissue samples, stored in 10% neutral buffered formalin after necropsies, were processed by standard paraffin embedding methods and sectioned using a microtome (Leica Biosystems) into 2- to 3-μm thick sections and placed onto glass slides. Paraffin-embedded sections were stained with standard hematoxylin and eosin solutions. Sections were observed under the microscope for white blood cells and macrophages on tissue boundaries, as these cell types indicate normal tissue reconstruction.

### *Procedure and statistics*

Animals were assigned to treatments according to a random number table. Treatment groups were designated as following: (1) HM-CS, (2) CS, and (3) FS. The CS foam was a placebo foam and differed from the HM-CS in that hydrophobes are grafted along the backbone of the CS. The investigators were blinded to treatment. The weight of the excised median lobe divided by the preinjury total body weight of the rat was used as a measure of the reproducibility of the injury. Blood loss was corrected for body weight (mL/kg). All measures are presented as mean ± standard deviation. For measures with differences between group means, direct comparisons of the HM-CS groups with the CS and FS groups were performed using a student's unpaired *t*-test. Statistical significance was assigned at a greater than 95% confidence level ($P < 0.05$).

## Results

### *Rodent hepatic injury model was reproducible*

There was no significant difference in amount of liver excised from rats between treatment groups. In Figure 3, the average amount of liver removed per test group is shown. Cumulatively, an average of 0.4 ± 0.3 g of liver was excised from the medial lobe of each rat. The standard deviation was high due

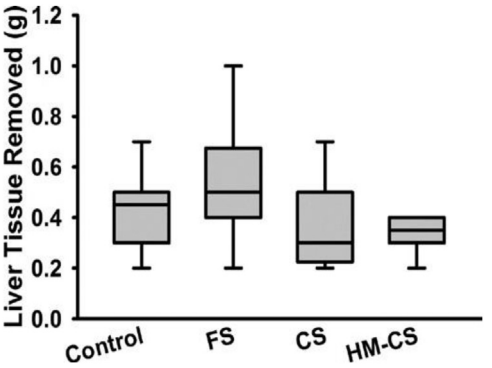


**Fig. 3 – Rodent liver excision weights per hemostatic agent test group. In our rodent model of hepatic injury, each excised portion of liver lobe was weighed immediately after excision. Means are displayed above, with error bars representing standard error ($n = 8$ per group).**

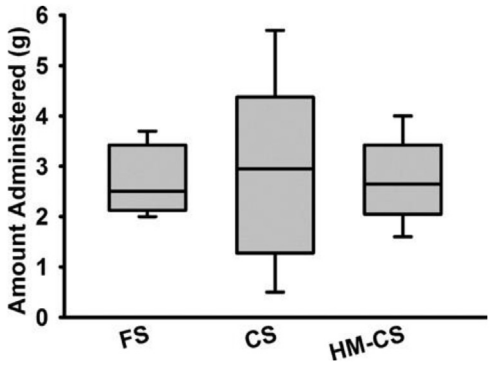


**Fig. 4 – Amount of hemostatic agent delivered per test group. Exactly 1 min after the excised liver lobe was removed, the hemostatic agent was applied to the injury site and left on the injury for 1 h to induce a stable clot. The entire volume of the prepackaged syringes of hemostatic agent was delivered, and difference in syringe mass was determined. Mean weights are displayed above, with error bars representing standard error ($n = 8$ per group).**

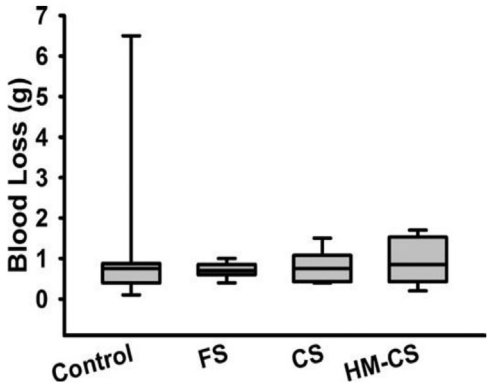


**Fig. 5 – Total blood loss per test group after hepatic injury. Blood loss was measured by weighing preweighed blood-soaked gauze pads immediately after the animal was closed. One control animal appeared to be coagulopathic and lost a significant amount more blood than any other animals in the study, but eventually, the bleeding stopped and it did not die or display any other abnormal symptoms during the 6-wk time period after the injury. Means are displayed above, with error bars representing standard error ($n = 8$ per group).**

to the small mass of liver that could be excised, taken relative to the whole liver, to ensure nonlethality of the injury. Despite this variability, the average weights per test group was consistent (0.45 g no treatment control, 0.49 g FS, 0.31 g CS, 0.39 g HM-CS; $P > 0.05$). Furthermore, as shown in Figure 4, consistent amounts of test material were applied to the injury (2.4 g FS, 2.7 g CS, 2.5 g HM-CS; $P > 0.05$), thus creating a relatively reliable framework to control for the amount of lobe excised relative to amount of hemostatic agent added.

### *All hemostatic agents successfully induce a blood clot after hepatic injury*

Bleeding after hepatic injury ceased within minutes of application of HM-CS, CS, or FS treatment. Bleeding was observed for up to 30 min after injury in the control animals, before slowing and stopping to form a stable clot. There were no significant differences observed in the amount of blood lost between animals among different treatment groups. Figure 5 shows that the amount of blood loss is consistent between test groups, with approximately 1 g of blood collected in the 60 s before application of the given hemostatic agent.

### *Hemostatic foam administration resulted in survival after hepatic injury*

All animals survived the hepatic injury and hemostat agent administration. As shown in Table 1, 100% of animals also survived the entire 6-wk time period after injury regardless of the hemostatic agent in the body cavity ($P > 0.05$). Furthermore, none of the animals showed signs of morbidity or mortality in either the 1-wk group or the 6-wk group.

### *Postoperative inspection of animals revealed no inflammation around the treatment sites*

In the cases of FS and HM-CS, no impaired healing was apparent. For CS, histology showed possible impaired healing due to significant space in the interstices of infiltration fibroblast cells.

Visual observation demonstrated the only residual material remained within the abdominal cavity at the 1-wk time point for HM-CS, CS, and FS. For the HM-CS and CS, there appeared to be fatty deposits at the site of injury. Figure 6

**Table 1 – Survival outcomes after treatment of a hepatic hemorrhage with different hemostatic agents in rodents.**

| Dressing type | 1 wk | | 6 wk | |
|---|---|---|---|---|
| | % survival- | Clinical signs of morbidity/mortality | % survival | Clinical signs of morbidity/mortality |
| No treatment | 100 (8/8) | None | 100 (8/8) | None |
| TISSEEL (FS) | 100 (8/8) | None | 100 (8/8) | None |
| Chitosan (CS) | 100 (8/8) | None | 100 (8/8) | None |
| Hm-chitosan (HM-CS) | 100 (8/8) | None | 100 (8/8) | None |

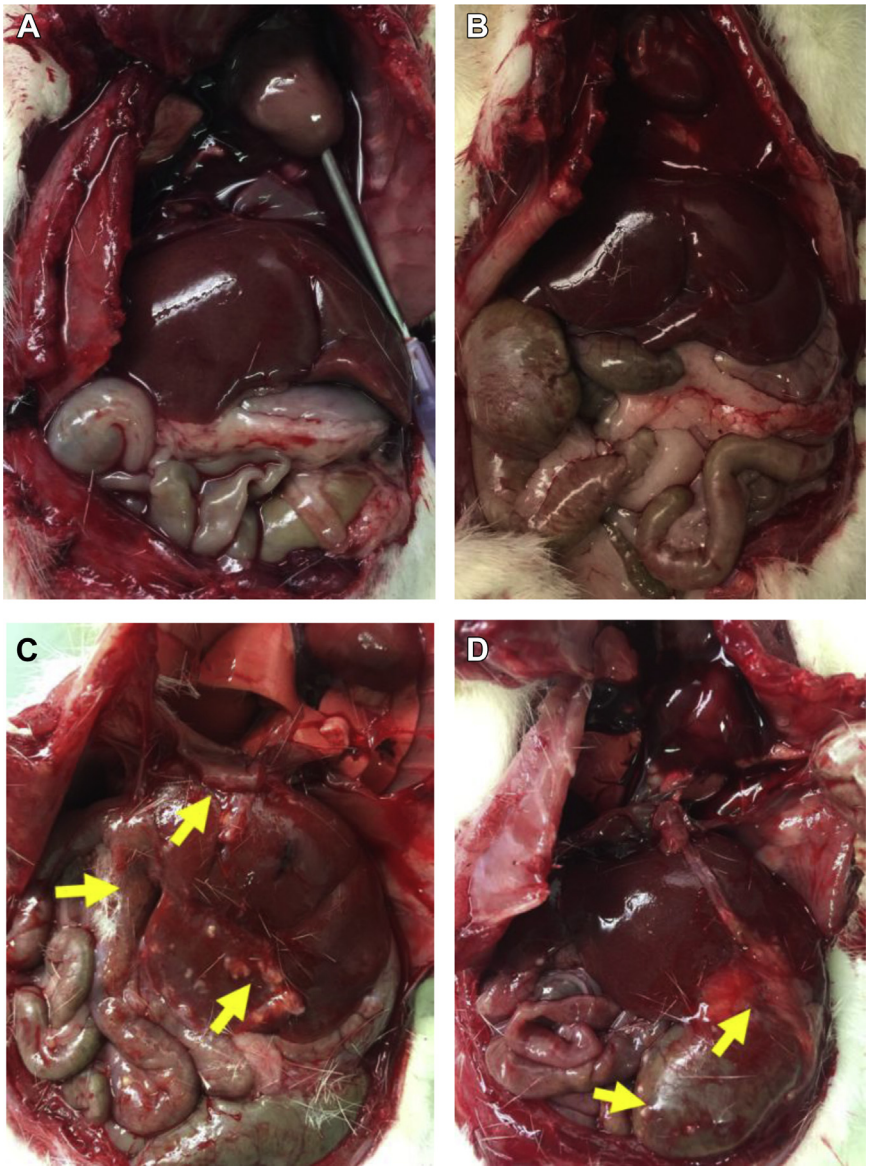


**Fig. 6 – Abdominal necropsy 6 wk after surgery for each hemostatic agent. In panel A, negative control shows normal healed lesion, no fat deposits on the liver, no discoloration. In panel B, TISSEEL treatment shows normal appearing lesion area. In panel C, CS treatment demonstrates small amounts of CS left in cavity, multiple fatty deposits around lesion area; yellow arrows point to fatty deposits. In panel D, HM-CS treatment shows residual amounts of HM chitosan left in the cavity around lesion and small fat deposits on intestines below; yellow arrow points to fatty deposits. (Color version of figure is available online.)**

shows postmortem livers before explantation. Negative controls showed a clean tissue presentation, except for some noticeable scarring at the injury site. FS-treated wounds showed no visual residual material. Both CS and HM-CS showed fatty deposits around the injury site, likely due to interaction with the material during the early stages of clot formation after application.

As shown in Table 2, at both 1-wk and 6-wk time points, the FS animals had significant (scores of $2.67 \pm 0.52$ and $2.50 \pm 0.55$, respectively) thick adhesions between the liver and colon. In contrast to FS, both CS and HM-CS produced filmy adhesions at 1 wk or 6 wk (adhesion scores of $\leq 2$). HM-CS in particular was nearly adhesion-free in all six of the animals in this test group at the 6-wk time point with an average score of $0.33 \pm 0.52$ ($P < 0.05$ relative to FS group).

Tissue histopathology images are shown in Figure 7. In the negative control (Fig. 7A), normal cell architecture is observed; white blood cells and macrophages can be seen on tissue boundaries, demonstrating healthy tissue reconstruction. In contrast, for FS (Fig. 7B), stunted tissue reconstruction with potential biliary retention at tissue boundaries. The CS group demonstrated a combination of normal white blood cell and macrophage infiltration, also with mild biliary retention at tissue boundaries (Fig. 7C).

**Table 2 – Adhesion outcomes after treatment of a hepatic hemorrhage with different hemostatic agents in rodents.**

| Dressing type | 1 wk | | 6 wk | |
|---|---|---|---|---|
| | Adhesion score | Adhesion type | Adhesion score | Adhesion type |
| No treatment | 0.33 ± 0.52 | Filmy | 0.17 ± 0.41 | Filmy |
| TISSEEL (FS) | 2.67 ± 0.52 | Thick | 2.50 ± 0.55 | Thick |
| Chitosan (CS) | 1.00 ± 0.63 | Filmy | 0.5 ± 0.55 | Filmy |
| Hm-chitosan (HM-CS) | 0.50 ± 0.55 | Filmy | 0.33 ± 0.52 | Filmy |

## Discussion

Surgical hemostatic agents are commonly used to stabilize diffuse, excessive bleeding of traumatic lesions by inducing blood coagulation. Commonly used hemostatic agents use concentrated coagulation or procoagulation factors to control bleeding. However, several limitations including poor biodegradability, difficulty with assembly or source of materials, and the necessity for cold storage and short shelf-life prevent their widespread use in hospital settings. Here, we demonstrate the utility of HM-CS as a hemostatic wound treatment with internal biocompatibility in a rodent model of hepatic injury. HM-CS was evaluated alongside unmodified CS and FS control treatments after partial hepatectomy, wherein HM-CS was observed to successfully induce a stable clot with minimal inflammation and adhesions observed in the lesion cavity 6 wk after application.

This histological observation of no impairment of healing in HM-CS–treated animals is encouraging, considering that previous studies have demonstrated CS-based dressings to result in significant capsular formation around the material.[27,28] However, in these cases, solid CS dressings applied to an internal hemorrhage resulted in significantly more dry weight of the applied material relative to the dry weight of the "foaming gels" used in the present study. When lysozymes infiltrate a solid polysaccharide network, held tightly together by a hydrogen-bonded crystal structure, the degradation profile is likely to be much more protracted, resulting in much greater risk of inflammation or capsule formation as fibroblasts and macrophages gather around the implant. In contrast, applying the polysaccharide in gel format allows for easier access of these critical enzymes to cleave the β 1→ 4 linkages along the CS backbone without significant steric hindrance.

The observation of fatty deposits on the liver surface for both CS and HM-CS is interesting in light of claims in the literature that CS is able to bind fat when used as a weight management supplement.[29] CS has been reported to bind fat inside the body,[30] likely the reason for our observed residual

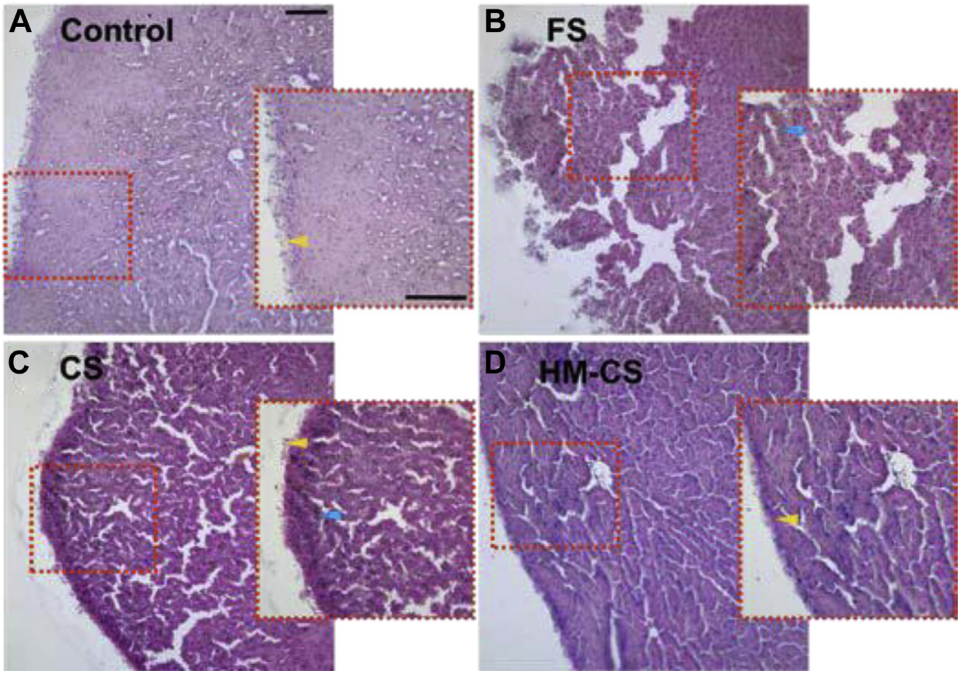


**Fig. 7 – Liver tissue histopathology at 6-wk time point. In panel A, no treatment control shows control tissue showing normal cell architecture; yellow arrow shows white blood cells and macrophages on end, demonstrating healthy tissue reconstruction. In panel B, TISSEEL-treated tissues have potential biliary retention. In panel C, CS-treated animals demonstrate both white cell/macrophages and biliary retention. In panel D, HM-CS treated animals show tissue repair phenotype similar to control. Scale bars = 100 μm. (Color version of figure is available online.)**

material in deposits around the treatment site. The longer term (> 6 wk) effect of fatty deposits on this material site is unknown, but this deposition is unlikely to cause significant risk to the treated subject, as the amount of fat deposited is quite small relative to the fat deposits in the rest of the body and did not enhance adhesion to other organs in the immediate vicinity of the lesion site. In addition, the withholding of fat in these regions posed no significant increased morbidity or mortality.

Fibrin clots are known to degrade inside the body within 3-4 d.[31] The results of the present study provide indirect evidence that both CS and HM-CS in a 2 wt% solution format can degrade in approximately the same timeframe as fibrin. This fast degradation observed relative to previously reported internal preclinical work with CS dressings is likely due to the steric availability of the CS backbone in a solubilized gel format. Adhesions in TISSEEL-treated animals are not surprising, given similar observations reported in several other studies.[32,33] These adhesions were particularly significant at the 6 wk time point and considerably enhanced binding to other organs in the peritoneal cavity of FS-treated rodents. Excessive adhesion formation was likely a result of using the human size–recommended dosage applied to our rodent injury model, but it is worth noting that adhesions to the intestine after liver injury are common and not generally a significant concern in clinical practice.

The observation of faster biodegradation of HM-CS relative to CS has been observed previously.[33] However, in these previous reports, the hydrophobic modification occurs simply through grafting of single carbon groups to the CS backbone through the reacetylation process. In the study by Huang *et al.*, CS with a significant portion of monosaccharide units along the backbone modified with N-acetyl groups appears to increase the speed to degradation relative to highly deacetylated or highly acetylated chitin variants.[33] It is important to note that above 50% acetylation of CS, the molecule becomes water insoluble and thus intractable as a flowable agent. In case of HM-CS, significant reacetylation relative to the CS molecule occurs; however, the reacetylation in this case results in the grafting of hydrocarbon groups rather than the traditional methyl group introduced by standard reacetylation with acetic anhydride. Thus, it is possible that hydrophobic modification of CS with significantly larger-sized hydrophobic grafts results in not only improved hemostatic effect but also faster degradation of the molecule due to increased relative ability of lysozymes to degrade the polymer.

### *Limitations*

With respect to the injury type used in the study, the excision of medial lobe was not lethal but represented a significant injury which could negatively impact both tissue necropsy and histopathology analyses. Selecting the injury type was both challenging and important because to prove internal safety via survival study, the untreated controls must remain alive. Otherwise, death could not necessarily be attributable to negative effects of the material on the body. On the other hand, we needed to create a reproducible injury to retain relevance toward ultimate clinical utility. No injury at all would eliminate applicability of the results to clinical utility as an internal hemostatic agent for biosurgery. Thus, we selected a mild-to-moderate injury type that did not result in death of the untreated controls.

Six-week survival was the primary endpoint for the present preclinical study, and given that all animal survived the intervention at this time point, both CS and hydrophobic CS derivatives demonstrated a basic utility as internal use absorbable materials. In light of earlier results in the literature,[23-26] it is not surprising that we achieved this result. Given that the material breaks down primarily by the activity of lysozymes in the body, the survival prognosis for the material is favorable. Of course, this points out a further limitation of the study in the time points selected. At least one time point in the range of 10-20 wk would have been a useful addition to the study, as it would have provided some projected assurance around the long-term safety of the material inside the body. Finally, the study is limited by lack of understanding of biodistribution and clearance from the body.

## Conclusions

Samples of HM-CS foam, CS foam, and FS were applied to a nonlethal liver excision in rats and left inside the abdominal cavity to degrade over time. None of the materials used in this study resulted in signs of morbidity or mortality at either the 1-wk or 6-wk time points. Residual FS was evident at 6 wk after application in the form of significant adhesions observed between the hepatic injury site and surrounding tissues. In contrast, minimal adhesions were observed in CS and HM-CS animals. The explanted injury sites that were subjected to histopathological assessments demonstrated a comparable pattern of degradation and wound healing for the three test materials. This is the first survival study demonstrating safety and resorption of HM-CS. In future work, we will systematically study the degradation of different HM-CS variants in both *in vitro* and *in vivo* models. Furthermore, we will characterize biodistribution and clearance by treating with radiolabeled CS molecules, allowing X-ray analysis to generate profiles of the digestion of the degraded pieces of CS molecules.

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Authors' contribution: M.T.L. was involved in all aspects of research including animal testing and article development; M.B.D. was involved in biomaterial synthesis, animal testing, data analysis, and article development; S.R.R. contributed to data analysis and article development; M.L.W. contributed to animal surgeries and data analysis; C.S. contributed to animal surgeries, protocol development, and data analysis; S.S. contributed to data analysis and article development; L.K.

contributed to study design, data analysis, and article development.

## Disclosure

M.B.D. is a 20% owner of the entity gel-e, Inc, a medical device company which has developed multiple chitosan-based products for bleeding control and wound treatment.

## REFERENCES


1. Neveleff DJ. Optimizing hemostatic practices:matching the appropriate hemostat to the clinical situation. *AORN J*. 2012;96:S1–S17.
2. Camp MA. Hemostatic agents: a guide to safe practice for perioperative nurses. *AORN J*. 2012;100:132–147.
3. Jackson MR. Fibrin sealants in surgical practice: an overview. *Am J Surg*. 2001;182:S1–S7.
4. Cheng CM, Meyer-Massetti C, Kayser SR. A review of three stand-alone topical thrombins for surgical hemostasis. *Clin Ther*. 2009;31:32–41.
5. Okerberg CK, Williams 3rd LA, Kilgore ML, et al. Cryoprecipitate AHF vs. fibrinogen concentrates for fibrinogen replacement in acquired bleeding patients - an economic evaluation. *Vox Sang*. 2016;111:292–298.
6. Mintz PD, Mayers L, Avey N, Flanagan HL, Burks SG, Sponitz WD. Fibrin sealant: clinical use and the development of the university of virginia tissue adhesive center. *Ann Clin Lab Sci*. 2001;31:108–111.
7. Burks SG, Sponitz WD. Safety and usability of hemostats, sealants, and adhesives. *AORN J*. 2014;100:160–176.
8. Dowling MB, Kumar R, Keibler MA, Hess JR, Bochicchio GV, Raghavan SR. A self-assembling hydrophobically modified chitosan capable of reversible hemostatic action. *Biomaterials*. 2011;32:3351–3357.
9. De Castro GP, Dowling MB, Kilbourne M, et al. Determination of efficacy of novel modified chitosan sponge dressing in a lethal arterial injury model in swine. *J Trauma Acute Care Surg*. 2012;72:899–907.
10. Dowling MB, Balogh P, Duggan MJ, et al. Hydrophobically-modified chitosan foam: description and hemostatic efficacy. *J Surg Res*. 2015;193:316–323.
11. Dowling MB, MacIntire IC, White JC, et al. Sprayable foams based on an amphiphilic biopolymer for control of hemorrhage without compression. *ACS Biomater Sci Eng*. 2015;1:440–447.
12. Muzzarelli RAA, Muzzarelli C. Chitosan chemistry: relevance to the biomedical sciences. *Adv Polym Sci*. 2005;186:151–209.
13. Elieh-Ali-Komi D, Hamblin MR. Chitin and chitosan: production and application of versatile biomedical nanomaterials. *Int J Adv Res (Indore)*. 2016;4:411–427.
14. Rao BS, Sharma CP. Use of chitosan as a biomaterial: studies on its safety and hemostatic efficacy. *J Biomater Res*. 1997;34:21–28.
15. Malette WG, Quigley HJ, Gaines RD, Johnson ND, Rainer WG. Chitosan: A new hemostatic. *Ann Thorac Surg*. 1983;36:55–58.
16. Brandenberg G, Leibrock LG, Shuman R, Malette MG, Quigley H. Chitosan: a new topical hemostatic agent for diffuse capillary bleeding in brain tissue. *Neurosurgery*. 1984;15:9–13.
17. Rabea EI, Badawy M, Stevens CV, Smagghe G, Steurbaut W. Chitosan as antimicrobial agent: applications and mode of action. *Biomacromolecules*. 2003;4:1457–1465.
18. Dai T, Tegos GP, Burkatovskaya M, Castano AP, Hamblin MR. Chitosan acetate bandage as a topical antimicrobial dressing for infected burns. *Antimicrob Agents Chemother*. 2009;53:393–400.
19. Azad AK, Sermsintham N, Chandrkrachang S, Stevens WF. Chitosan membrane as a wound-healing dressing: characterization and clinical application. *J Biomed Mater Res B Appl Biomater*. 2004;69:216–222.
20. Mo X, Cen J, Gibson E, Wang R, Percival SL. An open multicenter comparative randomized clinical study on chitosan. *Wound Repair Regen*. 2015;23:518–524.
21. Altiok D, Altiok E, Tihminlioglu F. Physical, antibacterial and antioxidant properties of chitosan films incorporated with thyme oil for potential wound healing applications. *J Mater Sci Med*. 2010;21:2227–2236.
22. Whang HS, Kirsch W, Zhu YH, Yang CZ, Hudson SM. Hemostatic agents derived from chitin an chitosan. *J Macromol Sci C: Polym Rev*. 2005;45:309–323.
23. Khora E, Limb LY. Implantable applications of chitin and chitosan. *Biomaterials*. 2003;24:2339–2349.
24. Zou J, Asukas J, Inha T, Toppila E, Kelloma M, Pyykko I. Biocompatibility of different biopolymers after being implanted into the rat cochlea. *Otol Neurotol*. 2008;29:714–719.
25. Tomihata K1, Ikada Y. In vitro and in vivo degradation of films of chitin and its deacetylated derivatives. *Biomaterials*. 1997;18:567–575.
26. Sun J, Jiang G, Wang Y, Ding F. Thermosensitive chitosan hydrogel for implantable drug delivery: blending pva to mitigate body response and promote bioavailability. *J Appl Polym Sci*. 2012;125:2092–2101.
27. Xie H, Lucchesi L, Teach JS, Virmani R. Long-term outcomes of a chitosan hemostatic dressing in laparoscopic partial nephrectomy. *J Biomed Mater Res B Appl Biomater*. 2012;100B:432–436.
28. Panith N, Wichaphon J, Lertsiri S, Niamsiri N. Effect of physical and physicochemical characteristics of chitosan on fat-binding capacities under in vitro gastrointestinal conditions. *LWT - Food Sci Technol*. 2016;71:25–32.
29. Kroez M, Lany W, Dickneite G. Wound healing and degradation of the fibrin sealant Beriplast_ P following partial liver resection in rabbits. *Wound Repair Regen*. 2005;13:318–323.
30. Walsh AM, Sweeney T, Bahar B, O'Doherty JV. Multi-functional roles of chitosan as a potential protective agent against obesity. *PLoS One*. 2013;8:e53828.
31. Hellebrekers BW, Trimbos-Kemper GC, van Blitterswijk CA, Bakkum EA, Trimbos JB. Effects of five different barrier materials on postsurgical adhesion formation in the rat. *Hum Reprod*. 2000;15:1358–1363.
32. Spotnitz WD. Fibrin sealant: the only approved hemostat, sealant, and adhesive—a laboratory and clinical perspective. *ISRN Surg*. 2014;2014:1–28.
33. Huang X, Sun Y, Nie J, et al. Using absorbable chitosan hemostatic sponges as a promising surgical dressing. *Int J Biol Macromol*. 2015;75:322–329.