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January 31, 1996

Scientific Officer
Naval Medical Research and Development Command
Naval Medical Command
National Capital Region
Bethesda, MD 20814-5055

Attn: CDR Lyn Yaffe
Re: Contract N00014-89-C-0024

Dear Commander Yaffe:

Enclosed is the Final Report pursuant to Clin. 0002 of the subject contract.

Please return a copy of the signed DD250 to the undersigned.

Very truly yours,

Jean M. Lentsch

Jean M. Lentsch
Associate Contract Representative





JML/mlb
Enclosure

cc: Lon Fowler, DCMAO Twin Cities
(under separate letter)

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# Hemostatic Activity of Chitosan in Wound Management

Contract No. N00014-89-C-0024

FINAL REPORT

The hemostatic activity of chitosan was first reported by Malette and Quigley.[1] Olsen et al completed initial preclinical safety and efficacy studies on several physical forms of various chitosan salts[2] and also elegant experiments which defined a possible mechanism for coagulum formation.[3] Based on this work, it appeared that an ionic interaction between the positively charged chitosan polymer and the negatively charged cell membrane of the red blood cell was responsible for coagulum formation. This mechanism can operate independently of the normal blood coagulation cascade which results in fibrin formation; that is, chitosan can form a stable coagulum with blood in the absence of fibrin. In vitro experiments have demonstrated that blood treated with heparin, which inhibits fibrin formation, forms a stable coagulum when aqueous solutions of chitosan salts are added. Addition of solid chitosan salts had no effect on heparinized blood under the same conditions. Details of these in vitro experiments may be found in Tables 1-3.

The observation that stable coagula could be formed with heparinized blood and chitosan salt solutions generated considerable interest in the possibility of using chitosan as a clinical hemostatic agent. Since all commercially available hemostat agents depend ultimately on the formation of fibrin, chitosan offered the opportunity for a truly differentiated product. A hemostatic agent which functioned independently of the normal blood clotting cascade would be useful in cases where fibrin formation was inhibited pharmaceutically (heparin or other anticoagulation therapy) or due to some other coagulopathy. When used in conjunction with a material which initiated the normal cascade sequence at some point, it was possible that chitosan would work even better than other commercially available materials in those cases where fibrin formation was not inhibited since two independent coagulation events could occur simultaneously.

This report summarizes the experimentation in animals which further defined the efficacy of various chitosan compositions. These compositions include chitosan glutamate lyophilized films, chitosan glutamate/collagen lyophilized pads, and chitosan glutamate aqueous solutions delivered with a variety of vehicles. In the course of this study, commercially available hemostatic agents including crosslinked and non-crosslinked collagen pads, oxidized cellulose woven pads, and aqueous thrombin solutions were also evaluated for the sake of comparison. Preclinical studies which evaluated the in vivo tissue response to chitosan and a number of other hemostatic agents have been reported elsewhere.[4]

## Materials and Methods

Most experiments used chitosan glutamate obtained from Protan, Inc., Protasan LV, lot number 808-572-01. This material is a highly purified grade which has undergone both analytical[5]

and preliminary cytotoxicity[6] testing. Some tests were completed using sterile-filled chitosan glutamate (MS-SLP-2905 lot 2001). Details relating to the composition and bench-top testing of chitosan glutamate or chitosan glutamate/collagen lyophilized pads may be found in the technical notebook references. Commercially available hemostatic agents were used as received, lot numbers are recorded in the surgical records.

The animal models included both rabbits and dogs. Fifteen New Zealand white rabbits, 2.5-3.5 kg each, were used in this study. The rabbits were prepared for surgery according to the Surgical Research Manual. The abdomen was shaved. Anesthesia consisted of ketamine-HCl (40mg/kg IM) and isoflurane/oxygen inhalation. An IV drip of lactated Ringer's solution was administered during surgery. The intraperitoneal cavity was exposed through a midline abdominal incision. Only those experiments in which the spleen was injured with an air-powered diamond burr (3 mm diameter) set at a fixed depth (1-2 mm) are reported here. Incision or burr wounds on the liver were evaluated and determined to be unacceptable due to greater wound to wound variance and lack of profuse bleeding. Incision wounds on the spleen were also unacceptable due to high bleeding variability. The rabbit spleen is approximately 7 cm long, 5-10 cm wide, and 3 mm thick. Generally, 4-6 wounds were made on each spleen. After the evaluations of the test samples were completed, the rabbits were euthanized (0.3 ml/kg T-61 solution IV) while under anesthesia.

Thirteen dogs (fox hounds and beagles) were also used in this study. The dogs were prepared for surgery according to the Surgical Research Manual. Anesthesia consisted of acepromazine (0.1 mg/kg), atropine sulfate (0.05 mg/kg), and Biotal thiamyal sodium (35 mg/kg) The animals were maintained on isofluorane/oxygen inhalation. An IV drip of lactated Ringer's solution was administered during surgery. The intraperitoneal cavity was exposed through a midline, abdominal incision. Wounds were made on the spleen with an air-powered diamond burr (7 mm diameter) set at a fixed depth (2-4 mm). The dog spleen is large enough to make 10-14 wounds. The dogs were euthanized (0.3 ml/kg T-61 solution IV) while under anesthesia upon completion of the sample evaluations.

Hemostat evaluations were also completed on rabbits and dogs which had been on either aspirin therapy for one week or had been given heparin after the spleen was exposed. The effect of aspirin on platelet aggregation is well known in humans, but aspirin therapy (40 mg/day for rabbits, 325 mg/day for dogs) did not seem to have a reproducible effect on the clotting systems of rabbits and dogs as measured by Lee-White clot times and observed hemostatic activity of various materials compared to normal animals. Administration of aspirin did not seem to inhibit the normal clotting mechanisms sufficiently in dogs or rabbits for any differentiation to be possible between hemostats. Administration of heparin IV in doses of 100-400 units/kg inhibited both dog and rabbit blood coagulation.

A typical hemostatic agent evaluation consisted of wounding the spleen, applying the test material with pressure for 3-5 minutes, and monitoring for further bleeding either through, underneath or around the sample after pressure had been removed. In some cases the agent was removed from the wound to test the stability of the clot which had formed. Wounds made with a diamond burr were fairly consistent in terms of resultant bleeding, but some variation was observed. When direct comparisons were made between two different agents, treatments and wound positions were alternated to compensate for wound to wound variation.

The Surgical Research Experiment Numbers for the work presented in this report are: 0S89AD0014; 0S90AB0001; and 0S90AD0009. D.M. Grussing was the surgeon in all experiments.

## Results and Discussion

### Lyophilized Pads

Samples of this type included lyophilized pads containing a mixture of both chitosan glutamate and collagen. These mixtures were of interest because chitosan salt solutions lyophilized alone result in pads which have very poor physical properties. The collagen/chitosan glutamate pads were fabricated by Semex Medical and contained 10-40 weight percent chitosan glutamate. These pads had excellent physical properties, comparable to the best collagen pads currently available. The collagen pads which were tested included partially crosslinked samples (Collastat and Helistat) and uncrosslinked samples (Lyostypt, Novacol, Avitene, and samples from Semex Medical). Gelfoam, a lightly crosslinked gelatin foam, was also evaluated.

In normal animals, the chitosan/collagen blends performed as well as the uncrosslinked collagen pads and better than the crosslinked collagen or gelatin materials. In animals which had been given heparin, no pad material was effective in controlling hemorrhage. This included all samples which contained chitosan glutamate and also a freeze-dried sample which was prepared with 100% chitosan glutamate. These experiments, in conjunction with the in vitro studies which showed that solid chitosan glutamate had no effect on heparinized blood (see Table 3), suggested that a solution form of chitosan would be a better candidate for treatment of animals with compromised clotting systems.

### Chitosan Glutamate Solutions

Chitosan glutamate solutions (1-2% solids, 200-400cps) may be easily prepared and sterilized by filtration methods. The solution may be delivered to the bleeding site with any appropriate vehicle; most experiments reported here utilized Gelfoam. Gelfoam is frequently used for delivery of Thrombostat, the commercially available thrombin hemostatic agent. When used

in conjunction with Gelfoam, chitosan glutamate solutions function as effective hemostatic agents in normal animals. One distinction, however, is that the chitosan loaded gelfoam pads are extremely slippery and do not adhere to the bleeding site. The slippery nature of the pads also makes it difficult to hold them in place at the bleeding site.

In order to directly assess the effectiveness of chitosan glutamate solution, it was evaluated with both Thrombostat and Surgicel in the dog model. Both Thrombostat and chitosan glutamate solutions were applied to the wound with Gelfoam for one minute intervals with pressure. Thrombostat achieved hemostasis in 5/6 wounds with one application. Chitosan required two applications to achieve hemostasis in 3/6 wounds and three applications in 3/6 wounds. In this experiment, Thrombostat proved to be more effective than chitosan glutamate. It should be noted that upon removal of the Thrombostat treatments, vigorous bleeding re-occurred. The chitosan treated wounds resulted in a stable clot formation on treatment removal. In a similar experiment with Surgicel, the agents were applied for three minute intervals until hemostasis was achieved. Both agents controlled hemorrhage with one application and were apparently equivalent as hemostatic agents. Chitosan glutamate solution did not show superior hemostatic activity compared to either Thrombostat or Surgicel in wounds made in normal dogs.

In animals treated with heparin, application of chitosan glutamate solution with sponge vehicles did not result in consistent control of hemorrhage.

**Conclusion**

Even though the results of these experiments have not been analyzed by statistical methods, it appears that chitosan salts do not offer a highly differentiated hemostatic agent. In a lyophilized pad form with collagen, chitosan exhibits similar activity to commercially available agents. In solution form, although easily made available in a commercial form and more stable (not a protein) and easier to use (no mixing) than Thrombostat, chitosan does not appear to offer any efficacy advantages. Chitosan glutamate solution has the disadvantage of being extremely slippery; which made initial placement difficult and adherence to wounds unlikely. Chitosan glutamate did not show any activity in heparinized animal models which would differentiate it from commercially available materials. This is similar to the conclusion drawn by other investigators who evaluated chitosan in a rat model.[7] Based on the efficacy described in this report, chitosan glutamate does not appear to offer significant advantages as a new hemostatic agent.

TWLewis

## References

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2. See the following 3M Technical Reports. Spence GL: Evaluation of Chitosan as a Hemostatic Agent, April-August, 1985; Steward GL: Chitosan, December, 1985 - March, 1986; Steward GL: Pilot Studies of Chitosan Hemostat, July, 1986 - January, 1987; and Steward GL: In Vivo and In Vitro Testing of Hemostasis and Clot Formation, January - July, 1988.

3. Olsen RA and Spence GL: First Trimester Report, July, 1985.

4. See the following 3M Technical Reports. Olsen RA: Preliminary Tissue Response to Gelfoam and Chitosan Solutions, April - September, 1988; Lewis TW and Lamprecht EG: Chitosan Glutamate Tissue Compatibility - Preliminary Study 1 and Preliminary Study 2, December, 1989 - June, 1990; Comeau PE: Growth Factor Research, June - December, 1989: and Johnson RS and Lamprecht: Chitosan Glutamate Tissue Compatibility - Preliminary Study 3, July, 1990.

5. Lewis TW: Hemostatic Activity of Chitosan in Wound Management. Technical Progress Reports No. 1 and 2, March and June, 1989, prepared for government contract number N00014-89-C-0024.

6. See MRFE 04974 and Olsen RA and Potter DT: Pyrogenicity Testing of Chitosan, 3M Technical Report, January - May, 1988.

7. Kind GM, Bines SD, Staren ED, Templeton AJ, and Economou SG: Chitosan: Evaluation of a New Hemostatic Agent. Current Surg Jan-Feb:37, 1990.

TABLE 1.

## IN VITRO COAGULATION OF BLOOD USING CHITOSAN GLUTAMATE SOLUTION

HEPARIN DOSE • 100 UNITS/KG DOG WT.

| SOURCE | AMOUNT | RESULT |
|---|---|---|
| 1% Chitosan Glutamate Protan/Sterling Drug | 0.2ml/ml Blood | 10 min stable clot |
| 2% Chitosan Glutamate Protan (808-572-01 SU) | 0.1ml/ml Blood | 11 min stable clot |
| 2% Chitosan Glutamate Protan (808-572-01 SU) | 0.2ml/ml Blood | 9 min stable clot |
| 2% Chitosan Glutamate Protan (808-572-01 SU) | 0.3ml/ml Blood | 6 min stable clot |

CONTROL DID NOT CLOT DURING EXPERIMENT

HEPARINIZED DOG BLOOD

TABLE 2.

## IN VITRO COAGULATION OF BLOOD USING CHITOSAN GLUTAMATE SOLUTION

HEPARIN DOSE • 400 UNITS/KG DOG WT.

| SOURCE | AMOUNT | RESULT |
|---|---|---|
| 2% Chitosan Glutamate Protan (808-572-01 SU) | 0.1ml/ml Blood | 10 min thicken, no stable clot |
| 2% Chitosan Glutamate Protan (808-572-01 SU) | 0.2ml/ml Blood | 10 min stable clot |
| 2% Chitosan Glutamate Protan (808-572-01 SU) | 0.3ml/ml Blood | 5 min stable clot |
| 2% Chitosan Glutamate Protan (808-572-01 SU) | 0.4ml/ml Blood | 8 min stable clot |

CONTROL DID NOT CLOT DURING EXPERIMENT

HEPARINIZED DOG BLOOD

TABLE 3.

## IN VITRO COAGULATION OF BLOOD COMPARING CHITOSAN GLUTAMATE: SOLUTION VS SOLID

HEPARIN DOSE • 143 units per 8ml of human blood.

| SOURCE | AMOUNT | RESULT |
|---|---|---|
| 1% Chitosan Glutamate Protan (808-572-01 SU) | 0.2ml/ml Blood | 25 min stable clot |
| 2% Chitosan Glutamate Protan (808-572-01 SU) | 0.2ml/ml Blood | 20 min stable clot |
| Chitosan Glutamate Protan (808-572-01 SU) | 2 mg/ml Blood (solid) | 30 min. no clot |
| Chitosan Glutamate Protan (808-572-01 SU) | 4 mg/ml Blood (solid) | 30 min no clot |
| Chitosan Glutamate Protan (808-572-01 SU) | 20 mg/ml Blood (solid) | 30 min no clot |

CONTROL DID NOT CLOT DURING EXPERIMENT

HEPARINIZED HUMAN BLOOD