COLLAGEN SPONGE HEMOSTAT
(Tests Results)

HYPRO-SORB™
What is the product
HYPRO-SORB™ collagen absorbable hemostat is a purified and lyophilised "freeze dried" bovine dermal collagen. The material, prepared as a sponge-like pad, is not cross-linked by any chemical substance, sterile non-pyrogenic "does not cause a fever", and absorbable. It combines the efficiency of collagen to control bleeding with the excellent handling properties of a sponge.

Why is it used
HYPRO-SORB™ Hemostat is indicated in surgical procedures "other than in neurosurgical urological and ophthalmological surgery", for use as an adjunct to hemostasis when control of bleeding by ligature or other conventional methods is ineffective or impractical. Neurosurgical and urological clinical studies are on-going.

How is it used and how fast will it stop bleeding
HYPRO-SORB™ Hemostat is applied directly to the bleeding surface with pressure. HYPRO-SORB™ can be cut to size. The amount needed and the period of time necessary to apply pressure will vary with the type and amount of bleeding to be controlled. Hemostasis time depends upon the type of surgery and degree of pretreatment bleeding. It usually occurs between two to five minutes with HYPRO-SORB™.

How should it be used
HYPRO-SORB™ maintains its integrity in the presence of blood and is not dispersed when wet. It is most effective when used dry. It is the only collagen hemostat that can be used dry or moistened with sterile saline depending on the preference of the surgeon.

Can it be left in the body and how fast is it absorbed
HYPRO-SORB™ may be left in situ whenever necessary. However, the surgeon, at his or her discretion, should remove any excess of HYPRO-SORB™ prior to wound closure. In studies most of HYPRO-SORB™ was found to be absorbed in 5-8 weeks after implantation.

HYPRO-SORB™ advantages
Clinical experience with HYPRO-SORB™ in 94 patients has indicated several distinct advantages of this product over other absorbable hemostats. HYPRO-SORB™ can be cut to the precise size needed to control bleeding at the surgical site. The pad maintains its shape but can be easily molded when moistened with saline. HYPRO-SORB™ adheres to bleeding surfaces when wet but does not stick to instruments, gloves, or sponges.
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PRIOR INVESTIGATIONS

BRIEF HISTORY AND PRESENT STATE OF TOPICAL HEMOSTASIS
INTRODUCTION

Investigations into methods of achieving hemostasis by utilizing and enhancing the natural coagulative mechanisms began in antiquity (see Table I). Hippocrates introduced thermal energy in his advocacy of high temperature cauterization, pressure was recommended by Celsus, and Galen suggested cloth bandages.

Cotton as gauze sponges or interlaced fibers has withstood the test of time in the control of oozing, but it cannot be left in situ and tends to pull the clot away as the sponge is removed. Cushing and Horsley reported success with patches of muscle (9,15). Other "autoplastic" sponges for which hemostatic ability has been claimed are subcutaneous fat (14), omentum (21), and fascia (4,19).

The ideal topical hemostatic material is one which embodies the following characteristics:

1. High hemostatic action
2. Minimal tissue reactivity
3. Non-antigenicity
4. In vivo biodegradability
5. Ease of sterilization
6. Low cost
7. Simulated tissue structure including:
   a. Small interstices
   b. Uniform thickness
8. Can be tailored to specific needs

In 1915, in search of such a hemostatic agent, Grey at Cushing's suggestion utilized washed, sterilized patches of sheep blood fibrin (12). Sheep fibrin was used because it was then readily available in Wasserman's laboratory at the Peter Bent Brigham Hospital. Harvey
later improved on Grey's concept by mincing fibrin and then compressing it into a thin sheet (13). In the 1940's Bering further improved on Grey's and Harvey's materials by developing a fibrin "foam" formed by fractionating and drying human plasma (2). This foam consisted of a brittle mass of interlaced fibrin fibers with multiple air spaces ranging in size from the microscopic to the macroscopic. Bering felt that there was no intrinsic hemostatic value to the fibrin foam but that its hemostatic activity depended on the addition of thrombin.

However, the more sophisticated studies of Bruck and others on the general phenomenon of the interaction of blood components with synthetic and natural substances showed that the process begins with the adsorption of plasma proteins onto the sponge (3). Enzymatic or conformational changes of these proteins then activate the coagulation factors and affect the formed blood elements, especially platelets, resulting in the extrusion of their granules and liberation of adenosine nucleotides, serotonin, and other coagulation activators. Since fibrin foams tend to vary in their hemostatic activity, they will vary in the level of their hemostatic activity. Olwin and Wahl have pointed out that while thrombin itself is a potent topical coagulatory agent, the pH of the matrix sponge to which it is added may inactivate the thrombin (18). Thus a clinical test of the mixture at the time of application is recommended, a somewhat cumbersome procedure under emergency circumstances.

Frantz and Lattes noted the unexpected hemostatic effect of the cellulosic acid which is formed by passing surgical cotton gauze over fuming nitric acid (11,16). In 1942 Yackel and Kenyon introduced the use of oxycellulose prepared in this process (22). Oxidized cellulose is a fabric which can be handled easily and tailored conveniently to fit any bleeding surface area. It is a physical matrix which presumably collects uncoagulated blood in its interstices, thus entrapping and concentrating blood coagulation factors. Although this material meets many of the criteria for a model topical hemostatic agent, it provides only a moderate level of hemostasis. Bailey has reported an increased number of adhesions following the use of oxidized cellulose (16).
In 1945 Correll and Wise introduced a tough, insoluble, rigid organic polymer prepared from gelatin whose interstices consist of a honeycomb of air bubbles (7,8). The sponge can be cut to size and takes on 50% of its weight in water and 45% of its weight in blood. This gelfoam presents the disadvantages of rigidity and is subject to crumbling during handling and application to a bleeding surface. To overcome this disadvantage, Jenkins advocated presoaking the gelatin foam in a 200 gm/ml solution of topical thrombin obtained from cattle (19). Prior admixture with thrombin enhances the hemostatic qualities of the material and provides both flexibility and an ability to accept light pressure. Unfortunately, the wet material is difficult to maintain in position, tends to disintegrate under pressure, cannot be sutured to the surface of a wound, and provides only a moderate degree of hemostasis.

In 1967 Battista introduced microcrystalline collagen powder prepared from bovine corium (Avitone®)(1). This material is a partial acid salt of specifically cross-linked collagen. It is water soluble, biodegradable, non-allergenic, and can be sterilized. When placed on a surface, particularly under light pressure, this collagen powder has proved to be the most effective topical hemostatic agent advanced thus far.

However, in addition to high cost, the principal disadvantages of Avitone® result from its powdered structure and high electrostatic charge. These make it difficult to pick up and apply to tissue surfaces as it tends to adhere to surgical instruments and gloves. It must be applied dry and will adhere to any moist surface. It cannot be resterilized.

In order to overcome these disadvantages Chvapil and Holusa suggested the use of a lyophilized collagen sponge as a hemostatic tampon (5). Recently, Eckmayer et al (6) developed a process for preparing pure collagen in long and thin fibers which could be further processed into an unwoven felt or fleece. This material embodies the high hemostatic properties of collagen powder and has an ease of handling similar to oxidized cellulose. It is now on the market under the name of Novacol® (Datascope Corp., NY) or Hemopad ® (Astra Pharm., NY).
The older reported studies indicating hemostatic effectiveness of noncross-linked collagen sponge matrix (Chvapil and Holusa) found the reduction to practical commercial products, such as Collastat® (U.S. Surgical Corp.) or Instat (J & J). In Europe and Japan other topical hemostatic agents based on collagen sponge matrix are sold.

During the last 8 years, we have had the opportunity to participate in the research and development of hemostatic agents based on film technology (Novacol, Datadscope Corp.) and sponge matrix (Hemosponge®, Bio-Products, Inc.). We learned that there are minimal differences in the hemostatic effectiveness of various products. The existing differences reflect mainly the different textures and are obvious in the handling. One of the common defects is fast gelling of the material in contact with blood. Some sponges are soaked too quickly with the blood, which oozes through the sponge. Some hemostatic material based on collagen is effective only at a certain magnitude of bleeding. With moderate or severe bleeding the product looses its effect and handling becomes difficult.

Having in mind all these aspects of safe and effect hemostatic topical collagen material in mind, we developed Hemosponge® to achieve a safe product with effective hemostasis and excellent handling under various bleeding conditions.

REFERENCES


TABLE 1
AVAILABLE HEMOSTATIC MATERIALS

<table>
<thead>
<tr>
<th>Type</th>
<th>Date</th>
<th>Material</th>
<th>Hemostatic Mode of Action</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgical cotton gauze</td>
<td>Antiquity</td>
<td>Woven gauze mesh</td>
<td>Concentrates blood and coagulation products by physical adsorption to its fibers, trapping them in its interstices.</td>
<td>Only moderate hemostatic action. Cotton gauze adheres to wound. Removal tends to remove clot.</td>
</tr>
<tr>
<td>Fibrin foam</td>
<td>1915</td>
<td>Mammalian fibrin with air interstices</td>
<td>Thought to concentrate clotting factors by physically adsorbing blood in its interstices.</td>
<td>Only moderate hemostatic action.</td>
</tr>
<tr>
<td>Gelatin foam</td>
<td>1945</td>
<td>Gelatin foam with air field interstices</td>
<td>Thought to concentrate clotting factors by physically adsorbing blood in its interstices.</td>
<td>Only moderate hemostatic action.</td>
</tr>
<tr>
<td>Microcrystalline collagen</td>
<td>1967</td>
<td>Collagen polymer powder</td>
<td>After contact, blood forms an insoluble gel fibrin mesh to which there is adhesion.</td>
<td>Handling</td>
</tr>
<tr>
<td>Fibrillar Collagen</td>
<td>1978</td>
<td>Collagen polymer in fibrillar form fabricated into unwoven fleece</td>
<td>After contact, blood forms an insoluble gel fibrin mesh to which there is adhesion.</td>
<td></td>
</tr>
</tbody>
</table>
SUNSET ENTERPRISE HEMOSTATIC COLLAGEN SPONGE

Specifications:

1. White color

2. Spongy texture consisting of continuous channels, with average pore size 90 microns

3. lipids - < 0.1 weight %

4. Density - 0.053 ± 0.005 g/cm

5. Fluid binding - 17.4 g H₂O/g

6. Tₛ - 41.0°C

7. pH - 5.14 ± 0.1

8. UV extractables in H₂O

   \[ \frac{A_{265-268}}{1 \text{ hr}} \approx 0.354 \pm 0.02 \quad \text{and} \quad \frac{A_{265-268}}{2 \text{ hr}} \approx 0.411 \pm 0.02 \]

9. Amino acid computation - corresponds to highly pure collagen

10. Tissue reaction - resorption

   7 d - minimal inflammatory response
   14 d - partial resorption

11. Hemostatic effectiveness:

   Rabbits - comparable to other CS on the market
   Pigs - comparable to other CS on the market
PURITY OF COLLAGEN SPONGE

A. Amino Acid Composition

Objective

To ascertain the purity of collagen sponge by the analysis of cysteine, tyrosine, proline, hydroxyproline and glycine content by complete amino acid analysis of the final product.

Identification of Test Article

Test article: Collagen sponge, supplied by Sunset Enterprises, Texas.

Testing Facilities

Section of Surgical Biology
Department of Surgery
University of Arizona
Health Sciences Center
Tucson, AZ 85724

Procedure

Aliquots of collagen samples were dried at 105°C for 16 hours. The known weight of the dry collagen was then hydrolyzed in screw capped test tubes in 6N HCl (1 ml per 100 mg powder) at 105°C for 18 hours. After hydrolysis, 50 mg of charcoal (Norite) was added to each test tube, shaken and filtered into an evaporation dish.

The solution of HCl with individual amino acids was evaporated on a boiling water bath to dryness. The dried residue was dissolved in 10 ml double distilled water and aliquots, corresponding to 200 µg was transferred to 10 ml all-glass tubes, evaporated to dryness and closed with a screw-cap. Each sample was analyzed by Arizona University Research Labs, Interdisciplinary Lab., Biological Sciences.

The amino acid analysis was run in the standard gradient manner using an Interaction 4 x 250 mm sterile steel column packed with cation exchange resin. Post-column detection of the amino acids were performed with ninhydrin in a stainless steel
reaction coil heated to 135°C in a silicone oil bath. The instrument was rigorously calibrated and found to produce analysis with 99.5% reproducibility.

Results

The most commonly used criteria for the purity of collagen is a complete amino acid analysis of the collagen, which shows a unique composition. A type I collagen molecule consists of three polypeptide (α) chains, each having about 1000 amino acid residues. The unique amino acid sequence with glycine as every third amount acid residue allows the triple helix configuration of the collagen molecule and about one third of total amino acid residues are glycine. High content of glycine, proline and hydroxyproline form almost two-thirds of all amino acid content. There are only 2-4 tyrosine residues per α chain of the collagen molecule. There should be a half of cysteine residue per α chain.

The analysis of individual amino acids of collagen hemostatic sponge was performed. The results in the enclosed table were expressed as the number of residues of amino acids per 1000 residues, which approximately corresponds to 1 α chain of the collagen triple helix. The averaged values of amino acid residues were compared with the amino acid composition of standard type I pure collagen performed by K. Piez et al in 1966. The differences between the results and Piez analysis were calculated and % deviations are shown for individual batches. The averaged values of the analyses were then compared with the results from FDA approved hemostatic agent, Avitene, and the amino acid composition of type I collagen reported by K. Piez (Table 2).

Glycine and hydroxyproline contents are in excellent agreement with the published data, showing an average of 324 glycine residues/1000 residues, 91 hydroxyproline residues/1000 residues and 131 proline residues/1000 residues.
No cysteine residue was identified in any of the collagen samples tested, although it should form 0.5 residue of cysteine/1000 residues of amino acids. No cysteine residue was detected from Avitene or in the results by Piez.

Various ranges of tyrosine contents have been reported by several investigators. Piez\(^1\) reported 2.8 tyrosine residues/1000 residues of amino acids in purified skin collagen. Nimni\(^2\) reported 1-4 tyrosine residues/1000 residues of amino acids and Oneson\(^3\) et al reported 1.9-3.5 tyrosine residues/1000 residues of amino acids for purified collagen.

An average of 3.5 tyrosine residues/1000 residues of amino acids was obtained from the collagen samples analyses and the results indicate that the collagen tested is highly pure collagen.

**Conclusions**

The collagen in the collagen sponges (provided by Sunset Enterprises, TX) is highly purified without any evidence of contamination by noncollagenous proteins.
### Amino Acid Analysis

**10/20/89**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Mol. Wt. (pmol/mg)</th>
<th>Theoretical Mole %</th>
<th>Actual Mole %</th>
<th>Theoretical Weight (ng)</th>
<th>Actual Weight (ng)</th>
<th>Residues per 1000 Theoretical</th>
<th>Residues per 1000 Actual</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteic Acid</td>
<td>169.14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6.540921</td>
<td>0</td>
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<tr>
<td>Aspartic Acid</td>
<td>132.12</td>
<td>0.5</td>
<td>4.3</td>
<td>3.5</td>
<td>5.476315</td>
<td>0.066533</td>
<td>1.8</td>
</tr>
<tr>
<td>Threonine</td>
<td>119.12</td>
<td>0.16</td>
<td>1.78</td>
<td>1.8</td>
<td>1.752464</td>
<td>0.019059</td>
<td>1.8</td>
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<tr>
<td>Serine</td>
<td>105.09</td>
<td>0.33</td>
<td>3.7</td>
<td>3.5</td>
<td>3.614457</td>
<td>0.034679</td>
<td>3.5</td>
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<tr>
<td>Glutamic Acid</td>
<td>147.13</td>
<td>1.42</td>
<td>7.3</td>
<td>9.2</td>
<td>15.88170</td>
<td>0.213333</td>
<td>9.2</td>
</tr>
<tr>
<td>Glycine</td>
<td>75.07</td>
<td>2.96</td>
<td>32.9</td>
<td>20.3</td>
<td>32.42059</td>
<td>0.222207</td>
<td>20.3</td>
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<tr>
<td>Alanine</td>
<td>89.09</td>
<td>1</td>
<td>11.6</td>
<td>7.9</td>
<td>10.95290</td>
<td>0.08909</td>
<td>7.9</td>
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<tr>
<td>Cystine</td>
<td>240.29</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Valine</td>
<td>117.15</td>
<td>0.35</td>
<td>1.84</td>
<td>2</td>
<td>3.833515</td>
<td>0.041002</td>
<td>2</td>
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<tr>
<td>Methionine</td>
<td>149.21</td>
<td>0.08</td>
<td>0.64</td>
<td>0</td>
<td>0.867232</td>
<td>0.011938</td>
<td>0.9</td>
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<tr>
<td>Isoleucine</td>
<td>131.17</td>
<td>0.06</td>
<td>0.32</td>
<td>1.3</td>
<td>0.657174</td>
<td>0.007870</td>
<td>1.3</td>
</tr>
<tr>
<td>Leucine</td>
<td>131.17</td>
<td>0.2</td>
<td>2.4</td>
<td>3</td>
<td>2.190580</td>
<td>0.026234</td>
<td>3</td>
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<tr>
<td>Tyrosine</td>
<td>181.19</td>
<td>0.05</td>
<td>0.2</td>
<td>0.8</td>
<td>0.547645</td>
<td>0.009059</td>
<td>0.8</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>163.19</td>
<td>0.15</td>
<td>1.21</td>
<td>2</td>
<td>1.642933</td>
<td>0.024778</td>
<td>2</td>
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<tr>
<td>Histidine</td>
<td>195.16</td>
<td>0.05</td>
<td>0.46</td>
<td>0.7</td>
<td>0.547645</td>
<td>0.007755</td>
<td>0.7</td>
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<tr>
<td>Hydroxylysine</td>
<td>198.16</td>
<td>0.87</td>
<td>2.6</td>
<td>1</td>
<td>2.519167</td>
<td>0.033623</td>
<td>3.4</td>
</tr>
<tr>
<td>Lysine</td>
<td>146.19</td>
<td>0.23</td>
<td>2.6</td>
<td>3</td>
<td>2.519167</td>
<td>0.033623</td>
<td>3.304484</td>
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<tr>
<td>Tryptophan</td>
<td>204.22</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Aminoa</td>
<td>174.21</td>
<td>0.52</td>
<td>4.9</td>
<td>8</td>
<td>5.695509</td>
<td>0.090589</td>
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<tr>
<td>Arginine</td>
<td>131.13</td>
<td>9.65</td>
<td>10.9</td>
<td>9</td>
<td>11.39101</td>
<td>0.119733</td>
<td>10.9</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>115.13</td>
<td>1.04</td>
<td>12.3</td>
<td>13.6</td>
<td>11.39101</td>
<td>0.119733</td>
<td>13.6</td>
</tr>
<tr>
<td>Proline</td>
<td>3094.79</td>
<td>9.13</td>
<td>99.9%</td>
<td>97.5</td>
<td>100</td>
<td>999.7</td>
<td>1000</td>
</tr>
</tbody>
</table>

**TOTAL**
B. Lipid Content in Raw Collagen

Objective

To determine the amount of lipids in hemostatic collagen sponge HYPROSORB.

Identification of Test Articles

Test Articles: One batch of collagen sponge Hyprosorb supplied by Sunset Enterprises, Texas.

Sponsor

Sunset Enterprises, Texas

Testing Facilities

Section of Surgical Biology
Department of Surgery
University of Arizona
Health Sciences Center
Tucson, AZ 85724

Procedure

The analyses were done in triplicates. To each sample 30 ml chloroform:methanol (2:1, Folch reagent) was added and mixed well for 30 seconds using a homogenizer. The homogenizer was rinsed with 10 ml of methanol. The sample mixture and washing were pulled up in a glass stoppered centrifuge tube, mixed thoroughly and allowed to stand for 16-20 hours. The supernatant was obtained by centrifugation for 15 minutes at 1000 rpm.

TABLE I. DRY WEIGHT OF DRY COLLAGEN SAMPLES

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Dry Weight (Grams)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1480</td>
</tr>
<tr>
<td>2</td>
<td>0.1110</td>
</tr>
<tr>
<td>3</td>
<td>0.1110</td>
</tr>
</tbody>
</table>

Five milliliters of Folch extract was placed in 15 ml screw cap conical centrifuge tubes and 200 μg of nonadecadienoic acid (C₁₉:₂) was added to each as the internal standard. The solution was evaporated to dryness at 70°C under a stream of N₂ gas. One-half milliliter of chloroform and 2 ml of 14% (w/v) boron trifluoride-methanol solution¹ were added to the dry residue and the solutions were mixed thoroughly. The reaction tubes were sealed with a Teflon lined screw cap and incubated in a 75°C water bath for 45 minutes. The tubes were cooled, 1 ml of distilled water and
2 ml of hexane were added and the methyl esters of the fatty acids were extracted by thoroughly mixing the tube contents. The hexane layer was removed, placed in a 15 ml glass stoppered centrifuge tube. The extraction was repeated with 2 ml of hexane. The combined hexane layer was evaporated to dryness in a water bath at 55°C using a N₂ stream to blow off the hexane. The dry fatty acid methyl ester residues were taken up in 0.1 ml of Folch reagent. Ten microliters of this solution was injected into the gas chromatographic column after mixing. Both the free and esterified fatty acids were detected using this method.

A Hewlett Packard Model 5708A with a 3390A integrator and model 7127A strip chart recorder was used. A six foot 80-120 mesh Gas Chrom Q column containing 10% silar scp was isothermally maintained at 220°C with the injection port at 250°C and the flame detector at 250°C. Helium gas was used to detect the various peaks eluted from the column. Good resolution of all of the peaks was achieved within 55 minutes of the time of injecting the sample onto the column.

Results

The average lipid content of the three samples tested was 2.94% of the dry residue of the collagen sponge. The composition of the extracted lipids were obtained by a comparison with standard fatty acid profiles. Palmitic acid and oleic acid are the most prominent components.

Bovine skin is used as the starting material to produce collagen slurry. This tissue contains variable amounts of lipids which are presumably removed during the purification process which should remove all the noncollagenous skin components was confirmed.

CONCLUSION:

The concentration of lipids expressed as a percentage of total collagen represents less than 3% of the dry residue, less than 0.1% of the collagen slurry used for manufacturing the collagen sponge. Characterization of lipids in raw collagen indicates that the most prominent components of the lipids are palmitic acid and oleic acid.
DETERMINATION OF STRUCTURAL STABILITY OF HEMOSTATIC
COLLAGEN SPONGE BY SHRINKAGE TEMPERATURE

Goal

One of the requirements of an adequate hemostatic collagen sponge is that it is resorbed in tissues within 6-8 weeks after implantation. The rate of biodegradation of collagen depends on its structural stability, which refers to degree of cross-linking, density of various intra- and mainly intramolecular cohesive forces holding the collagen units together. Such a structural stability can be determined by various methods (extractability, swelling, collagenase digestion, etc.). Determination of a temperature when collagen is transformed into amorphous gelatin is called shrinkage temperature ($T_s$) as this change is accompanied by contraction of the collagen chain due to the loss of collagen triplehelical crystalline structure.

Material and Method

Collagen sponge was provided by Sunset Enterprises, TX.

The method of $T_s$ determination is described in detail in the enclosed SOP # CC-02.

Results

The analysis was done in triplicates and showed change of anisotropy to isotropy under polarized light microscope at 41°C ± 0.05°C.

This degree of cross-linking clearly shows that

1. the collagen sponge is not cross-linked with any tanning agent
2. the collagen used in the reconstruction of the sponge was extensively chemically treated during the purification so that it was disassembled and chemically partially denatured
3. The $T_s$ is similar to that of other hemostatic collagen sponges presently on the market:

- Collastat (Marion Labs) $T_s = 44^\circ C$
- Instat (J & J) $T_s = 48^\circ C$
- Hemocol (Suwelack Co.) $T_s = 51^\circ C$

4. The degree of structural stability of the analyzed collagen sponge ($=41^\circ C$) suggests that the sponge will be resorbed within the animal (human) tissues within 6 weeks. This would be approximately two weeks faster resorption than has been found for the other commercially available hemostatic collagen sponges or even hemostatic fleece, sold under the name Novacol (Bioplex, Holland) or Hemopad (Astra, Pharm, USA). The only exception of the biodegradation time is Avitene, which results within 90 days. This bioresorption, in our experience, is too slow as it may produce chronic inflammation with fibrotic reactions (adhesions, encapsulation).
DETERMINATION OF SHRINKAGE TEMPERATURE \((T_s)\) OF COLLAGEN BY POLARIZED LIGHT MICROSCOPY AND DIRECT WATER BATH MEASUREMENT

1. **Purpose**

   This SOP outlines the basic procedure for measuring the shrinkage temperature of collagen, in either the fibrous form, or as a reconstituted collagen slurry, collagen sponge or collagen in biological membranes, including skin, tendon and ligament.

2. **Applicability**

   2.1. These methods shall be used for any preparation containing visible fragments or fibers of native or cross-linked collagen that can be brought to neutral pH; for the direct water bath method, the specimen needs to be of sufficient size to allow attachment within the clamp and direct visualization of the change in size. The direct method is more suitable for measurements of \(T_s\) of larger specimens; it allows measurements of as many as 12-15 samples at the same time.

   2.2. These methods cannot be used on soluble or denatured collagen.

3. **Definitions**

   3.1. Anisotropy - having unlike properties, being doubly refractive, indicates high degree of helicity-crystallinity.

   3.2. Isotropy - having like properties, being singly refractive, indicates the loss of helicity, refers to denaturation, loss of stereoconformation.

4. **References**


5. **Discussion**

   Native collagen exhibits anisotropy when viewed under polarized light. This anisotropy changes during denaturation to isotropy. The temperature at which this transition occurs is referred to as \(T_s\) (shrinkage temperature) and is directly related to the degree and amount of cross-links stabilizing the collagen structure. Additionally, for the direct measurement (water bath) method, the actual physical contraction of the specimen indicates the shrink temperature.
6. Responsibilities

6.1. All individuals using this method are responsible for keeping adequate and appropriate records and documentation.

6.2. The individual performing this procedure shall be responsible for making any revisions to this method, which must be approved by the director of research.

7. Materials and Equipment

7.1. Polarized light microscope method:

7.1.1. Polarized light microscope

7.1.2. Koffler heating block with thermometer

7.1.3. Capillary tubes, outside diameter not to exceed 1.5 mm

7.1.4. Phosphate buffered saline (PBS)(Prepared by dissolving 13.61 g $\text{KH}_2\text{PO}_4$, 9.46 g $\text{NaCl}$ and 3 g $\text{NaOH}$ in $\text{dH}_2\text{O}$ and bringing up to 1 L total volume. Adjust pH to 7.0 by conc. $\text{NaOH}$, pH 7.0 or other suitable neutral buffer (higher molar strength acceptable whenever needed to neutralize the sample).

7.1.5. Fine pointed forceps

7.1.6. Collagen containing samples of suitable size to fit into the capillary tube

7.2. Direct water bath method:

7.2.1. Circulating pump with heating capability

7.2.2. Auxiliary heating element, such as submersible heating coil, etc. (optional)

7.2.3. Rectangular, glass container of sufficient size to allow water circulation around the specimen without creating excessive turbulence.

7.2.4. Thermometer

7.2.5. Glass and polyethylene (or similar) tubing for connecting the pump and glass vessel

7.2.6. Plastic (or similar) cover for 7.2.3 vessel, with inlet bar containing clips for specimen attachment
7.2.7. $dH_2O$

7.2.8. The entire system is set up by connecting the pump (with the optimal heating element within the pump, to increase rate of heating) and the water bath vessel; a glass tube is used to syphon $dH_2O$ from the vessel back into the pump, while the pump keeps filling the vessel. Inlet (active) and outlet (passive syphon) lines should be located in the water bath away from the specimen, to minimize turbulences, but allow sufficient water circulation to ensure uniform heating of the entire system. A thermometer probe is placed within the system and heating elements turned on and adjusted to obtain approximately 2°C/min temperature rise.

8. Procedure

8.1. Polarized light microscope method:

8.1.1. Neutralize samples of sufficiently small size to fit into the lumen of the capillary tube by placing them for a minimum of one hour in PBS, pH 7.0.

8.1.2. Fill capillary tube with PBS, slowly, so as not to introduce bubbles.

8.1.3. Insert specimen (previously neutralized) into capillary tube.

8.1.4. Place heating block on microscope and center it.

8.1.5. Insert tube into Koffler heating block.

8.1.6. Insert glass thermometer into its slot (right hand side of heating block).

8.1.7. Focus on specimen.

8.1.8. Turn on power to heating block and adjust to increase by 2°C/min (setting 80 on the transformer).

8.1.9. Observe specimen; record temperature when anisotropy to isotropy transition begins and again when at least 75% of entire sample has undergone the change. This gives the shrinkage temperature range.

8.2. Direct water bath method:

8.2.1. Neutralize samples of sufficient size (1.5-2.0 cm length works very well) by lacing them for a minimum of one hour in PBS, pH 7.0.
8.2.2. Affect the neutralized specimen to the clips on the crossbar of the vessel cover.

8.2.3. Attach small counter weights to the lower end of each specimen (small alligator clips, 0.5-1.0 g work well).

8.2.4. Submerge specimen in the water bath containing dH₂O by placing the cover with the crossbar over the vessel.

8.2.5. Turn pump and heating coils on.

8.2.6. Place thermometer probe within the chamber (check for temperature variations by moving the probe; if present, readjust placement of inlet and outlet lines (see 7.2.8).

8.2.7. From a predetermined, fixed point of reference, align the specimen (visually) against the glass and place marks (magic marker) on the glass indicating zero time position of specimens.

8.2.8. Observe the specimen, until noticeable contraction begins to occur. Record this temperature, as well as the entire temperature range to achieve the total contraction of the specimen (~ 50% of the entire length).

9. Records:

9.1. The logbook for the shrinkage temperature apparatus shall be signed and dated at each use and retained indefinitely in room 5312.

9.2. All repairs and modifications to the instrument shall be recorded in its logbook. This shall include kind of repairs, replacement of parts, date performed and signature of technician performing the modifications or repairs.
REPORT COVER SHEET

CONFIDENTIAL

TITLE: MORPHOLOGICAL EVALUATION OF TISSUE REACTION AND THE RESORPTION OF THE COLLAGEN SPONGE IMPLANTED SUBCUTANEOUSLY IN ADULT RATS

ID NUMBER: R-8956

DATE: November 6, 1989

SPONSOR: John Namecek
Sunset Enterprises, TX

TESTING ORGANIZATION: Bio-Products, Inc.
Tucson, AZ

SITE OF INVESTIGATION: University of Arizona Health Sciences Center
Department of Surgery
Section of Surgical Biology
Tucson, AZ 85724

PROJECT DIRECTOR: Milos Chvapil, MD, PhD, DSc

STUDY DATES:
Initiation: October, 1989
Completion: November, 1989

This study was conducted according to approved protocols by the University of Arizona Laboratory Animal Care Committee (ULACC) at the Arizona Health Sciences Center and by established Standard Operating Procedures of the Section of Surgical Biology.

______________________________  ____________________
Project Director               Date

______________________________  ____________________
Sponsor                       Date

Revised October, 1987
1. TITLE

MORPHOLOGICAL EVALUATION OF TISSUE REACTION AND THE RESORPTION OF THE COLLAGEN SPONGE IMPLANTED SUBCUTANEOUSLY IN ADULT RATS

2. STATEMENT OF PURPOSE:

2.1. Background

Noncross-linked collagen sponges are prepared to function, besides other applications, as topical hemostatic materials. Although it is highly recommended that the surgeon remove the material after achieving the hemostasis, in some instances the course of the surgery dictates to leave the biodegradable material in situ. Therefore, it is important to know the rate at which the implant is resorbed. Too fast, as well as too slow resorption rates are not desirable. Fast degradation would promote more acute tissue reaction to chemotactic soluble collagens, while slow degradation may induce chronic fibrotic tissue reaction and adhesions between organs, etc. Based on our previous involvement in testing this aspect of hemostatic agent compatibility, we consider resorption within 2-4 weeks optimal.

2.2. Goals

2.1. Determine by morphological methods the rate of biodegradation of identical sizes of implanted collagen sponges into the subcutis.

2.2. Describe the tissue reaction to the implant at various times after the insertion of the sponge into the appropriate organ.

3. TEST SYSTEM:

3.1. 6 male Sprague Dawley rats, 300-325 g body weight

4. EXPERIMENTAL DESIGN:

4.1. Materials

4.1.1. Collagen sponge was supplied by Sunset Enterprises, October, 1989 (Noncross-linked hemostatic sponge)

4.2. Surgical Method

A total of 6 adult SD male rats, body weight 300 ± 30 g was used. Under Innovar Vet anesthesia, a 5 x 10 mm section of sponge, 1 mm thick, was implanted subcutaneously on the shaved dorsal aspect of the thorax. Two subcutaneous tunnels were made from the same incision, again the sponges were placed approximately 1.5 cm apart located in the subcutis. A single suture closed the
incision. An identical set of sponges was implanted through a second incision placed posterior to the first one. An additional rat was implanted as per protocol, but terminated 90 minutes later for time zero values. The site of implantation was grossly inspected for any abnormalities (swelling, adhesions, inflammatory changes and vessel infiltration). The subcutis with the implant was excised, fixed in 10% Baker's formalin and processed for histological evaluation by standard procedures. Mason's trichrome stain was used to identify the implant and its degradation stain. In trichrome, the intact implant stains blue-green, with advancing degradation turning it red (Chvapil et al, Identification of the Depth of Burn Injury by Collagen Stainability. Plastic and Recon. Surg. 73(3), 438, 1984). At least 2 sections were made per each implant; thus, at least 4 sections were evaluated per each rat for the subcutaneous implants.

4.3. MORPHOLOGICAL METHOD

In order to obtain sufficient insight into the morphology of the tissue reaction to the implanted collagenous material, 5 µ thick sections were stained by Hematoxylin and Eosin and by Masson's trichrome. In this method collagen stains blue-greenish. We noticed that when collagen is denatured by any physical means (temperature, in burns, also by tensions) or when collagen is infiltrated by inflammatory cells which degrade collagen by their collagenase system of lysosomes, the stainability of collagen changes from blue to red. We utilized this finding during our semi-quantitative attempt to document the kinetics of cell reaction and implant resorption of the two materials under the study.

Semiquantitation of Histological Observations

The following criteria and quantitation were used to evaluate the morphological reaction of collagen sponges implanted in the rat subcutis.

a. Inflammatory cell infiltration density
   1 - no reaction
   2 - minimal
   3 - moderate
   4 - severe

b. Capillary ingrowth or new collagen deposition into the implantation site
   1 - no
   2 - minimal
   3 - moderate
   4 - severe

c. Magnitude of resorption
   1 - completely resorbed no traces left
   2 - traces, infiltrated by cells
   3 - present with limited cell infiltration
   4 - intact deposit, cells only around the collagen
5. RESULTS:

5.1. Surgery

All subcutaneous implants were inserted without any complications. In several instances, the moderate amount of bleeding from the defect washed out the sponge, which was then immediately replaced by a new implant. Once the sponges were securely in their respective lesions, the abdomen was closed in 2 layers, with 4-0 chromic and 4-0 proline, respectively.

5.2. Gross observations at time of harvestings

5.2.1. Time zero - all samples were easily identified and collected with the surrounding tissues for histology.

5.2.2. 7 days - (2 rats terminated). All subcutaneous samples were easily identified with more than 50% of the original sponges remaining. No inflammation was observed in any of the 12 implants.

5.2.3. 14 days - (2 rats terminated).

5.2.4. 21 days - (2 rats terminated). All implants were identified but only with great difficulty.

5.3. Histological Observations

0-Time - Refers to the situation 1.5 hours after the implantation of the sponges. By this design we aimed to get the size and texture of the implants not yet affected by the degradative processes. Figure 1 documents by trichrome and hematoxylin and eosin stains the actual nonresorbed size of the implant. It has to be kept in mind that the actual magnification on the picture is 35x the original implant size.

7 days - The implants showed definite changes in texture and size. There was, however, a difference in the tissue reaction of noncross-linked and thermally cross-linked sponge, the latter inducing more cellular infiltration in the subcutis. This finding was also observed for this type of the sponge at later sampling periods. The collagen of the sponge, now filled with mixed cell population consisting mainly of granulocytes, macrophages. No inflammatory infiltrate around the implant was seen.

At 14 days - The overall size of the sponge was reduced by approximately 30-40% of the original size. Sponge is now infiltrated by cells with participation of fusiform size cells, possibly fibroblasts. Only minimal inflammatory reaction around the sponge implant was found.
At 21 days - The pictures show progressive resorption by the invaded cells. The noncross-linked sponges did not induce inflammatory reaction, although substantially resorbed by 80-90%.

6. CONCLUSIONS

The results of this study already indicate that noncross-linked collagen sponges are resorbed within 4 weeks from the subcutis of young adult rats. Several interesting observations were made:

6.1. The resorption is not a continuous process. The major changes in the size and texture of the implant occurred between 3 and 4 weeks.

6.2. The overall inflammatory reaction to the sponge implants was minimal. This finding was quite surprising in view of the mechanisms of the biodegradation resorption of collagen implants. (For details see Chvapil, M. Fate of Natural Tissue Prostheses. IN: Fundamental Aspects of Biocompatibility. CRC Series in Biocompatibility. Volume 1. David F. Williams (ed.) CRC Press, Boca Raton, Florida, pp 87-104, 1981.) The collagen implant is resorbed by enzymes (collagenases, gelatinases) present in lysosomes of granulocytes and macrophages, although other cells also have this complex system of enzymes degrading specifically collagen. Thus, the presence of inflammatory cells is a necessity for biodegradation of the implanted collagen. The lack of cellular inflammatory reaction to this type of collagen sponge suggests that other cells (hepatocytes, Ito cells) have the same collagenolytic capacity as typical inflammatory cells, represented mainly by granulocytes with the higher density of collagenases. As documented by our previous study (Chvapil, et al, Identification of the Depth of Burn Injury by Collagen Stainability, Plastic & Recon. Surg., 1984) the expected change in stainability of denatured collagen from green-blue to red-pink was not observed in this study. This may be related to the low degree of inflammation induced by the implanted sponges.

We conclude that the implanted noncross-linked collagen sponges, are resorbed from the subcutis within 4 weeks. Such a rate of resorption is similar to that reported for collagen hemostatic fleece, [(Novacol-Hemopad, Dataspope) (Silverstein et al, "Experimental and Clinical Experiences with Collagen Fleece as a Hemostatic Agent" J. of Trauma, 1981)] and is substantially shorter than the time of 84 days of resorption of Avitene microcrystalline collagen (Alcon, TX).

7. RECORDS:

All raw data and documentation will be maintained in accordance with SOP #AA-02, Rev. 0. All protocols and final reports will be maintained in the files of the Section of Surgical Biology, Department of Surgery, University of Arizona Health Sciences Center, Tucson, Arizona.
TISSUE REACTION OF THE SUBCUTIS TO IMPLANTED HEMOSTATIC COLLAGEN SPONGE

Sponge matrix with cell infiltrate at 250x magnification. Cells are granulocytes and macrophages. No giant cells are identified. (H & E, 250x magnification)

In specific stain for collagen (blue) the cells infiltrating the sponge are shown.
TISSUE REACTION OF THE SUBCUTIS TO IMPLANTED HEMOSTATIC COLLAGEN SPONGE

Sponge infiltrated by cells with indication of significant inflammatory reaction (H & E, 25x magnification)

Same as above at 125x magnification. Note the cells invading the sponge matrix.
HYPROSORB COLLAGEN SPONGE (SUNSET ENTERPRISES, TEXAS)

Appearance of the intrahepatic implant 90 min after insertion into the tissue

Note the blood and blood derived cells filling the sponge matrix (Trichrome stain, 36x magnification)
HYPROSORB COLLAGEN SPONGE (SUNSET ENTERPRISES, TEXAS)

Morphology of subcutaneous implant 7 days after insertion into the tissue

Trichrome, 36x magnification

Note minimal cellular infiltrate around the implant

Trichrome, 250x magnification

Sponge matrix is infiltrated by few cells which contribute to the digestion of the implant
REPORT COVER SHEET

CONFIDENTIAL

TITLE: TESTING OF HEMOSTATIC EFFECTIVENESS AND HANDLING CHARACTERISTICS OF HYPROSORB. COMPARISON WITH OTHER COLLAGEN BASED TOPICAL HEMOSTATIC MATERIALS

ID NUMBER: R-8956

DATE: October, 1989

SPONSOR: Sunset Enterprises, Inc. Texas

SPONSOR MONITOR: John Nemecek

TESTING ORGANIZATION: Bio-Products, Inc. Tucson, AZ

SITE OF INVESTIGATION: University of Arizona Health Sciences Center Department of Surgery Section of Surgical Biology Tucson, AZ 87524

PROJECT DIRECTOR: Milos Chvapil, MD, PhD, DSc

Project Director Date

Sponsor Date

Revised October, 1987
1. **TITLE:** TESTING OF HEMOSTATIC EFFECTIVENESS AND HANDLING CHARACTERISTICS OF HYPROSORB. COMPARISON WITH OTHER COLLAGEN BASED TOPICAL HEMOSTATIC MATERIALS

2. **STATEMENT OF PURPOSE:**

Evaluate in a dog model, with bleeding skin donor site and spleen lesion, the effectiveness of hemostatic collagen-based materials.

3. **TEST SYSTEM:**

3.1. **Animal Model**

Two male greyhound dogs, 25 kg body weight, ID# 25F207 and #112E3131.

3.2. **Description of Diet, Reagents**

Diet and housing for animals were in accordance with the policy of the University Animal Care at the University of Arizona Health Sciences Center. No special dietary requirements were needed for this protocol.

3.3. **Materials Tested**

3.3.1. Hemopad (Astra Pharmaceuticals, Inc.)
3.3.2. Noncross-linked sponge 1.5 mm (Dr. O. Suwelack Co., Batch #68,948, sent 4-14-87, our code #S-10)
3.3.3. Noncross-linked sponge 3 mm (Sunset Enterprises, TX, Hyprosorb)

4. **EXPERIMENTAL DESIGN**

4.1. The method as described in our original publication was used (enclosed). In principle, each dog was pre-anesthetized with 1 ml (2 mg/ml) xylazine and anesthesia was induced with sodium pentobarbital, 1M to effect. A 6-inch hand keratome was used to excise *split thickness donor sites*. All materials (pre-weighed) were of comparable size (~1 x 2 cm). Larger pieces were used for photographic documentation.

4.2. After laparotomy the *spleen* was visualized. Several 3 cm wide and 0.5 cm deep excisions of spleen margin tissue were made and treated with the tested materials. The time was observed when the bleeding stopped.

4.3. **Testing of handling characteristics:**

The arbitrary score for handling characteristics was made, ranging from 1-unacceptable, worst handling to 10 - ideal handling.
The aspects of handling involved:
- ease of placing dry material on the wound
- stickiness of the dry material to surgical instruments or gloves
- ease of removing from the bleeding site
- quantitative aspects of removal
- brittleness, disintegration of the material when soaked with blood
- rebleeding after removal of the material from the wound

In principle, a powderish hemostatic material with an electrostatic charge, which adheres to instruments and is difficult to administer to the wound, was considered unacceptable with a score of 1. It also was difficult to remove from the wound without time consuming scraping maneuvers. Collagen sponges, in general, have excellent handling characteristics due to their structured matrix.

5. RESULTS

5.1. Skin donor site bleeding model

One aspect of hemostatic effectiveness is shown in Table 1. It refers to the affinity of the material to soak with the blood. Although, there is no direct statistically proven relations of blood binding by the material and hemostasis, blood must soak into the tested material to induce platelet aggregation, which is the main mechanism of hemostasis induced by the collagenous material. In other words, platelets must be exposed to the matrix of collagen sponge.

The three tested collagen sponges (#1, #2, #3) absorb blood at the same rate. Hemostasis was achieved at the same time with either collagen sponge type tested. All the tested materials stopped the bleeding at the same time.

5.2. Spleen avulsion bleeding sites

We measured the time when the bleeding from freshly inflicted spleen avulsion (3 cm wide x 0.5 deep excision of spleen edge) was completely or almost completely under control. The results are shown in Table 2. The data show that spontaneous hemostasis was achieved around 24 minutes. By using any one of the tested collagen-based hemostatic materials the bleeding time was significantly reduced to approximately 5 minutes. No statistical differences in the hemostatic effectiveness among the individual products were found.

There were no noticeable differences in the handling. All the sponges were easy to handle. It became clear that a 1.3 mm thick sponge layer was inadequate and that 3 mm was the right thickness (Hyprosorb) to control the bleeding. Surprisingly and contrary to our previous experience with Hemopad (Astra Pharmaceuticals), the compressed collagen fleece was too stiff and difficult to keep on the wound. also, it did not soak the blood well.
6. CONCLUSIONS

6.1. Hyprosorb noncross-linked hemostatic sponge was as effective as noncross-linked sponge used by Suwelack Co.

6.2. Among the three tested collagen hemostatic materials, the two sponges performed as well as the others. In fact their effectiveness, evaluated subjectively by three surgical assistants, was slightly better than the commercially available Hemopad (Astra Pharmaceuticals, USA). The hemostatic collagen sponge shown in 3.3.3 (noncross-linked) corresponds to the final product selected for commercialization.
EVALUATION OF THE HEMOSTATIC EFFECTIVENESS OF THREE COLLAGENOUS MATERIALS (FLEECE AND SPONGES) IN A STANDARDIZED MODEL IN DOGS

<table>
<thead>
<tr>
<th>Material Tested</th>
<th>No. of Tests</th>
<th>Dry Weight (mg)</th>
<th>Weight After 2 min (mg)</th>
<th>Increase in Weight (multiple)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Hemopad (Astra Pharm.)</td>
<td>3</td>
<td>73.3 ± 1.53</td>
<td>159.7 ± 6.6</td>
<td>2.17 ± 0.065</td>
<td>Too stiff, poor adherence, floated</td>
</tr>
<tr>
<td>2. Noncross-linked sponge 1.5 mm (Suwelack)</td>
<td>6</td>
<td>22.2 ± 3.00</td>
<td>91.0 ± 22.8</td>
<td>4.10 ± 0.590</td>
<td>very effective, too thin a layer, excellent handling</td>
</tr>
<tr>
<td>3. Noncross-linked sponge 4 mm (Sunset Enterprises)</td>
<td>5</td>
<td>27.4 ± 5.13</td>
<td>92.6 ± 16.9</td>
<td>3.44 ± 0.540</td>
<td>more effective than #4, excellent handling</td>
</tr>
</tbody>
</table>

The volume (weight) of blood retained within the hemostatic material within 120 seconds administration over the bleeding site was measured. Variability is given as X ± SD.
### TABLE 2

**HEMOSTATIC EFFECTIVENESS OF FOUR COLLAGEN-BASED MATERIALS IN THE SPLEEN AVULSION BLEEDING MODEL IN DOGS**

<table>
<thead>
<tr>
<th>Material Tested</th>
<th>No. of Tests</th>
<th>Hemostasis time (min)</th>
<th>Handling Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Hemopad (Astra Pharm.)</td>
<td>6</td>
<td>5.00 ± 1.10</td>
<td>4</td>
</tr>
<tr>
<td>2. Noncross-linked sponge 3 mm (Suwelack)</td>
<td>5</td>
<td>4.56 ± 0.80</td>
<td>6</td>
</tr>
<tr>
<td>3. Noncross-linked Control - no treatment</td>
<td>6</td>
<td>4.53 ± 0.038</td>
<td>9</td>
</tr>
<tr>
<td>4. Control - no treatment</td>
<td>2</td>
<td>24.80 ± 3.53</td>
<td>N</td>
</tr>
</tbody>
</table>

All treated wounds had significantly reduced bleeding time when compared to controls.

No statistically significant differences exist among treatment methods.

Handling score: 10 = best  
1 = worst

Sample #2 existed as 1.3 mm sponge layer; too thin to be effective, was folded several times.