

## Enclosure 1

Wayne State University  
**DMC** Children's Hospital  
of Michigan

July 11, 2002

Dr. A. Bart Flick, M.D.  
Agaricus Research  
36 Lake Park Road  
Lawrence, GA 30242

Dear Dr. Flick,

Here are the results of the Pall Thickness Wound Healing Study done in domestic swine. The affiliations and areas of expertise of the scientists that did the work are as follows:

Dr. Gay Fluck-Maryshak, Ph.D.	Children's Hospital of Michigan	Study Director
Dr. Janice Lee, M.D.	Honey Ford Hospital	Research Resident (Surgery)
Dr. Raju Rahak, M.D.	Children's Hospital of Michigan	Chief of Pathology
Dr. David E. Marx, Ph.D.	Scoutman University	Chair/Associate Professor of Chemistry
Dr. Marc Colten, M.D.	Children's Hospital of Michigan	Director of Burn Unit

Sincerely,



Dr. Gay Fluck-Maryshak, Ph.D.  
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## Full Thickness Wound Healing Study in Domestic Swine

### Specific Aims:

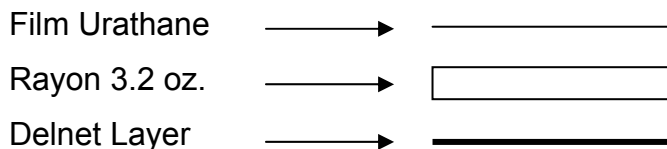
- To characterize the healing process of full thickness wound treated with multi laminate wound dressings with Silverlon® and without Silverlon®.
- To evaluate the presence and depth of penetration of silver released from Silverlon® at the wound site histologically.
- To quantify the silver absorbed into the blood after each Silverlon® dressing change.

### Material and Methods

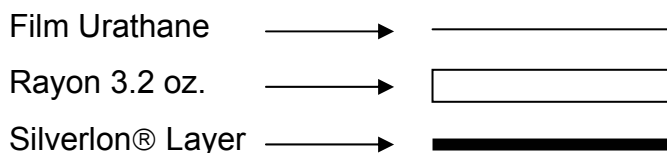
#### Dressings:

The dressing were composite wound dressings as described below:

#### Control Dressing:



#### Test Dressing:



#### Pre-operative Care:

Domestic swine weighing 20-25 kg were housed in a climate controlled animal facility with a 12:12 hour light and dark cycle. The animals were given at least 7 days to acclimatize to the new environment. They were fed standard laboratory pig chow and given water *ad libitum*. During the initial 5-7 days the pig were place in a panapinto sling to acclimatize them to this procedure. The swine also had a fentanyl patch (50 mcg/day) placed at the back of the neck or low on the flank 24 hours before surgery all food was removed from the kennel the night before surgery.

### Surgical Procedure:

Body weight was recorded prior to surgery. General anesthesia was induced with an IM injection of ketamine (22mg/kg) and acepromazine maleate (1.1mg/kg). An IV injection of thiopental 10-20mg/kg was administered prior to intubation. The animals were placed on isoflurane inhalant anesthetic (2.5-3.0%) for continued general anesthesia. An ophthalmic ointment was placed in each eye and a heating pad was placed under the animal to maintain the animal body temperature. A preoperative dose of Ancef (20-30 mg/kg, IV) and morphine sulfate (1mg/kg, IM) were also given.

The hair on the ventral neck and behind the right ear was closely clipped with electric clippers. The operative sites were scrubbed with a providone/iodine scrub and draped in a routine fashion. A pocket between the dermis and muscle was made behind the right ear to hold a blood donor port. The catheter was tunneled under the skin to the ventral neck. The right jugular vein was isolated and the catheter was inserted into the vein and advanced to the aortic arch. The muscle was closed in layers and the skin was closed with subcuticular stitches.

The hair on the dorsal back area was closely clipped with electric clippers and then shaved with a safety razor and shaving cream. The operative sites were scrubbed with a chlorhexidine soap and rinsed with sterile water. The surgical sites were draped in a routine fashion.

A circular dermal punch, 2 cm in diameter, was used to create uniform circular wounds through epidermis and dermis (6mm deep) on the pig's back. Twelve wounds total were made. Six on each side lateral to the spine. Pressure was placed over the wounds until homeostasis was achieved. Immediately after surgery digital photographs with measurement and identification markers were taken. The wounds were irrigated with sterile water and composite laminate dressings (3cm in diameter) were dampened with 1 ml sterile water and placed

over the wounds. The periphery of the wounds outside the 3 cm diameter bandage was painted with tincture of benzoin and the dressings were secured with surgical tape and a cotton stockinet t-shirt. The number of wounds treated with Silverlon® varied between pigs. Pig 1 had 9 control dressings and 3 Silverlon® dressings. Pig 2 had 6 control dressings and 6 Silverlon® dressings. And pig 3 had 3 control dressings and 9 Silverlon® dressings.

#### Postoperative Care:

Following the procedure, the pigs were closely monitored in the panipinto sling until they showed signs of recovery from the anesthetic. When they could walk, the pigs were walked back to animal housing and once again food and water was allowed *ad libitum*. Every four hours for the first twenty-four hours after surgery the pigs received 1mg/kg morphine. The animals were observed daily for general health and state of the bandages evidence of pain. On day 2 after the surgery a new 50 mcg fentanyl was applied. More fentanyl patches were applied every 72 hours if the pig seemed to have pain. Clariton(10 mg) was given daily for itching.

The pigs were placed in the panipinto sling for all blood draws and bandage changes. The dressings were changed every day for the first three days and then every third day. The wound dressings, if not removed, were moistened daily with sterile water. Depending on the amount of drainage, 1-3 ml of water was used. The rayon pad was visually moistened to the edges. During dressing changes, the pigs were anesthetized with isoflurane using mask induction. The old bandages were removed and weighed for fluid content. The wounds were flushed with sterile water and gently wiped with sterile gauze. This was necessary for accurate clinical assessment. The wound were evaluated and photographed. A fresh dressing of the correct type was moisten and placed over the wound.

### Clinical Observations:

Clinical observations were made by an individual blinded to the wound dressing placement. At the time of dressing changes, the wounds were examined for wound drainage, edema, redness and color, presence of granulation tissue, eschar formation, odor, and any abnormal characteristics.

### Blood Draws:

Blood was drawn 24 hours prior to the surgery. Blood was also drawn at 2 hours, 4 hours, 8 hours and 24 hours after the dressing change on days 1,3,6,9,12, and 15. A final blood draw was taken just prior to euthanasia.

The wounds were followed to complete closure. One week after closure, the pig were euthanized and skin samples taken from the wound area. Half of the sample was sent for histological evaluation and half were sent for atomic absorption spectroscopy

### Atomic Absorption Spectrometry:

The tissue samples were weighed and dissolved in nitric acid. They were then heated to complete the dissolution process and drive off any nitrogen oxides formed in the process.

The resulting solution were analyzed with a Perkin-Elmer Analyst 300 Atomic Absorption Spectrometer with an HGA 850 Graphite Furnace with autosampler. The silver levels were analyzed at the 328.1 nm line with .70nm slit settings. The detection limit for this instrument is 0.10 ppb which would correspond to 0.05 ppm Ag in the tissues considering the dilutions used.

The blood samples were measured directly with the graphite furnace technique under manual conditions using the same instrumental conditions listed above. Calibration and reproducible detection levels were 2ppb in this study.

#### Pathology:

Tissue samples were fixed and sectioned. Sections of each sample were stained with hematoxylin and eosin (H & E) or by the rhodanine method for silver. The sample were then evaluated by a pathologist for presence of silver, the amount of foreign material present and the degree of inflammation.

#### **Results:**

The data was analyzed using SigmaStat statistical software. The Mann-Whitney Rank Sum Test was used with a P value  $\leq 0.05$  considered significant.

A surgeon, blinded to the treatment modality, evaluated the wounds for infection, odor, edema, color, drainage, and the presence of granulation tissue and echar. The wound diameter was also measured. There was no significant difference between the Silverlon® treated wounds and the control wounds on infection, odor, edema, color or drainage.

None of the wounds were infected or had an odor. The wound edema was greatest post-op day 2 and tapered to no edema by post-op day 21 (fig. 1) and the wound color varied from pink to red. Amount of drainage visible on the surface of the wound was greatest days 3 and 6 post-op and tapered down to a moist wound bed by post-op day 18 (fig. 2). When the amount of drainage present in the bandages was measured, the Silverlon® treated wounds had significantly more drainage post-op days 1 through 9 and significantly less drainage post-op days 15 through 24 (fig.3).

The wounds reached a peak amount of granulation tissue between post-op days 6 and 9 followed by a fall to nothing by day 24 (fig. 4). Though a trend was

apparent by day 12 that the wounds treated with the control bandages had significantly more granulation tissue; only at post-op days 18 and 21. The wounds treated with Silverlon® formed an eschar earlier than the control wounds and had significantly more eschar post-op days 12, 15, and 18 (fig. 5).

The wound diameter was at maximum post-op days 1,2, and 3 and was healed by post-op day 24. Silverlon® treated wound diameter was significantly smaller than control wound diameter post-op days 12 through 21 (fig. 6). The wound area, from digital photos of the wounds taken at the time of bandage changes, was measured with the ImageTool computer software using calibrated pixel analysis. Using this technique the wound area of the Silverlon® treated wounds post-op days 12 through 24 was significantly smaller (fig. 7).

Tissue samples of the healed wound areas were examined by a pathologist for the presence of silver, foreign material, and tissue inflammation. The rhodanine method for silver did not detect any silver present in any tissue samples. There was no significant difference between control and Silverlon® treated wounds in inflammation and foreign material (fig. 8). The tissue samples were also analyzed by atomic absorption spectrometry for the presence of silver. Both control and Silverlon® treated tissue samples had detectable silver (>0.05 ppm), however, many samples in each group had no silver detected at all (fig. 9). There was no significant difference between the two groups. None of the blood samples had silver concentrations greater than 2 ppb the detection limit of the instrument.

### **Conclusions:**

The wounds treated with Silverlon® healed slightly faster than the control wounds (fig. 10). Post-op days 1 through 3 the wounds were amorphous, the skin would stretch and flex changing the wound area. The skin surrounding the

Silverlon® treated wounds seemed to stabilize sooner. The wounds granulated at about the same rate though the Silverlon® treated wounds tended to have less granulation tissue after post-op day 9. The amount of wound fluid in the bandages decreased as the amount of eschar formed increased. The eschar could provide a barrier to the absorption of wound fluids.

Histology showed no difference between the skin of Silverlon® treated wounds and control wounds. No silver was found by silver staining. Atomic absorption spectrometry found silver present in both control and Silverlon® treated wounds. Perhaps this is due to the nap of the Silverlon® bandages where small fibers may fall off the bandages while being placed. These surface fibers may have been washed free during fixing of the tissue samples for histology. No silver was found in the blood samples by atomic absorption spectrometry.

### Wound Edema (blinded)

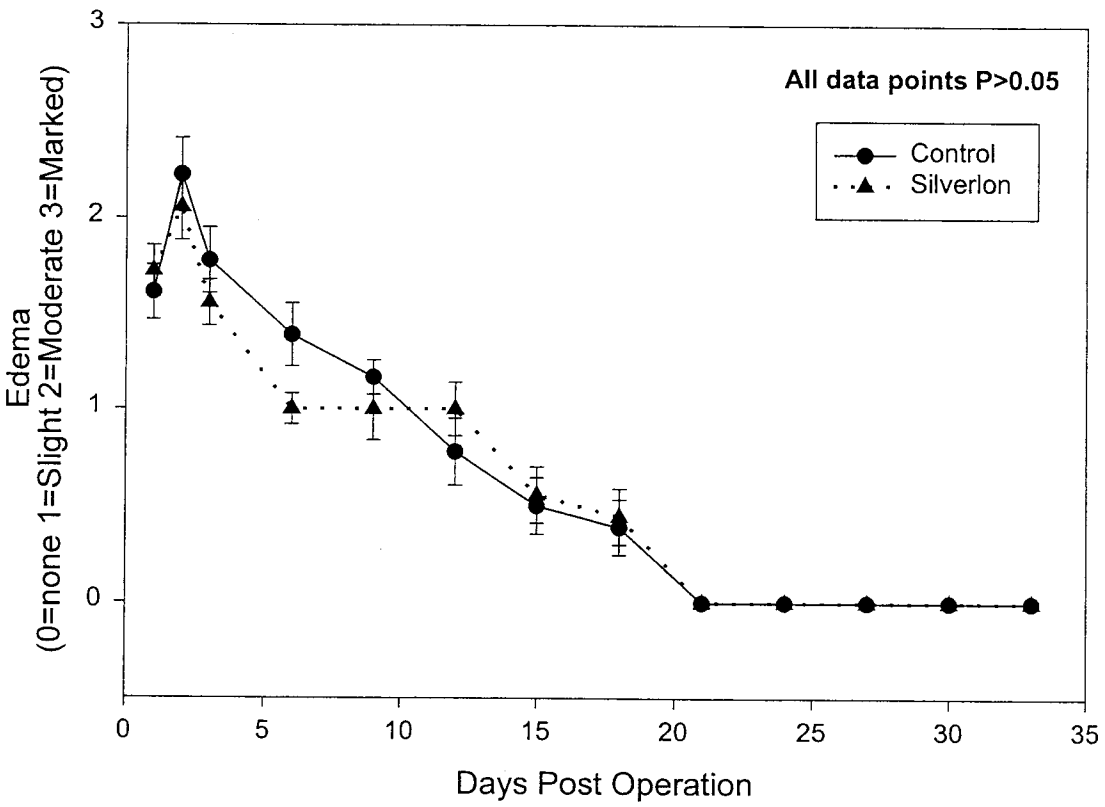


Figure 1

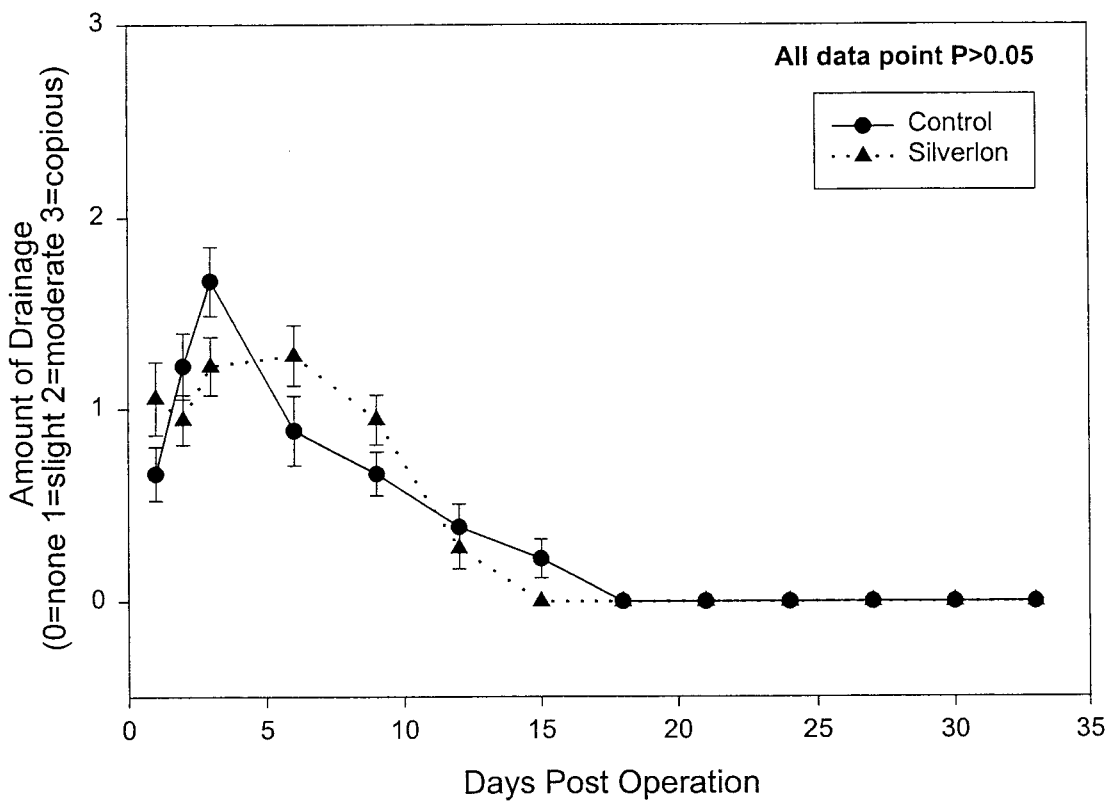
Wound Drainage  
(blinded)

Figure 2

## Wound Fluid Weight in Bandage

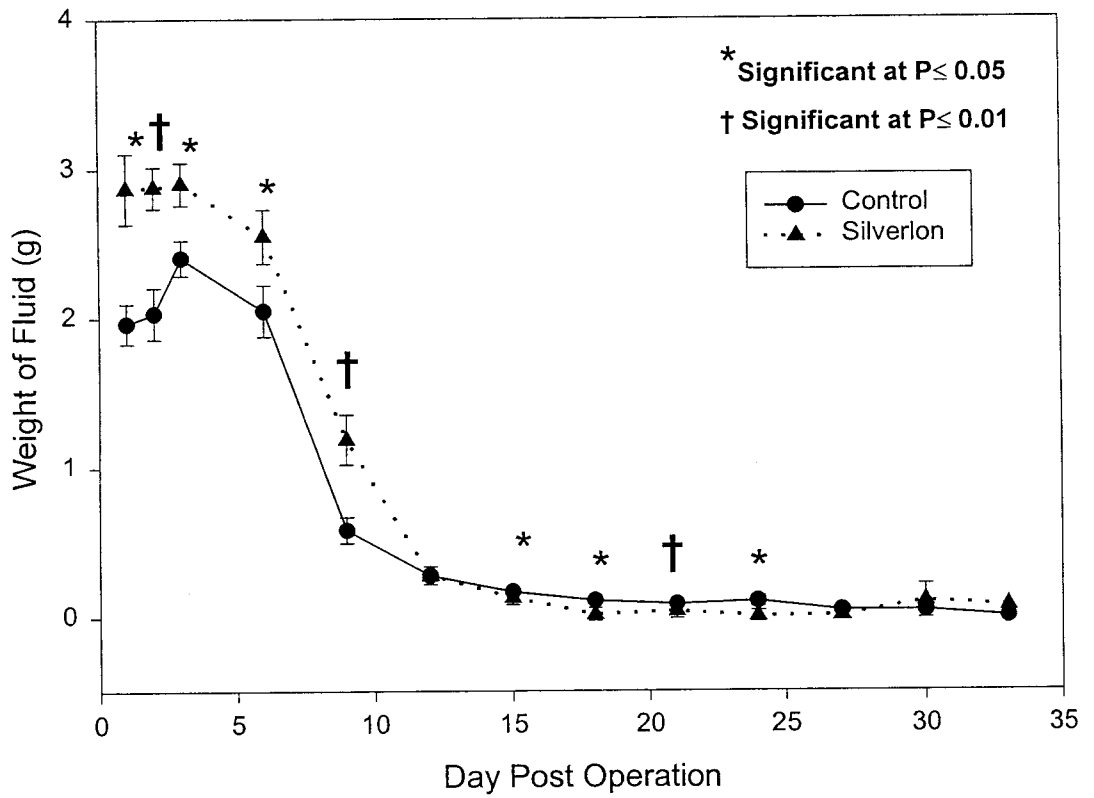


Figure 3

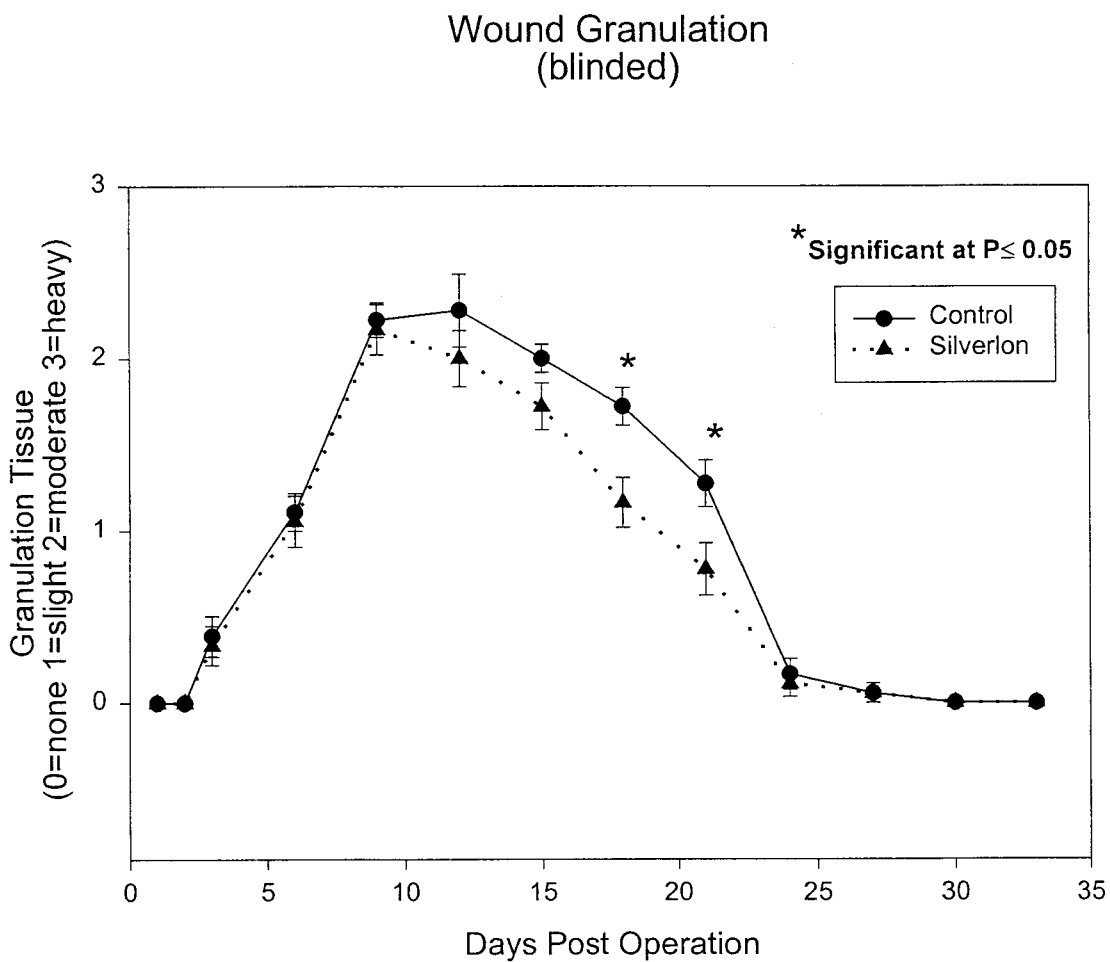


Figure 4

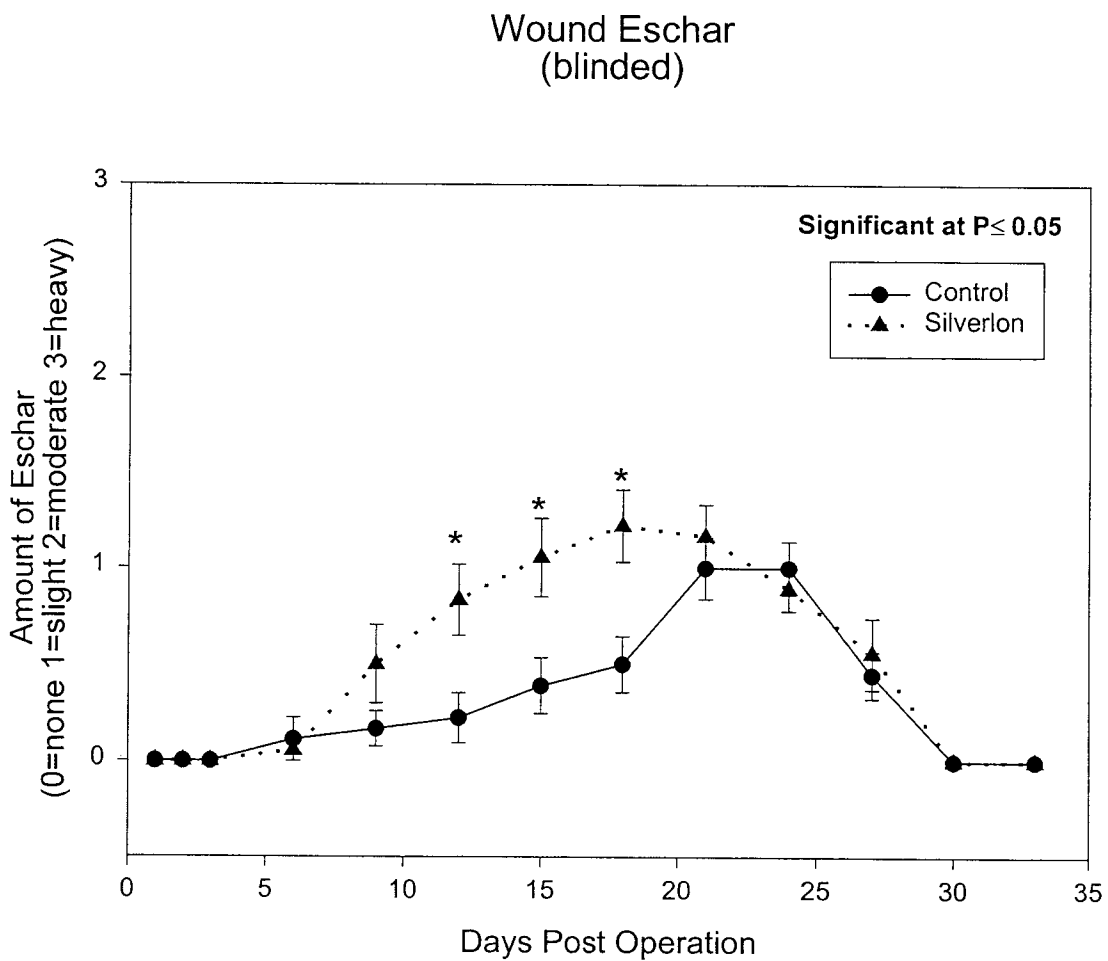


Figure 5

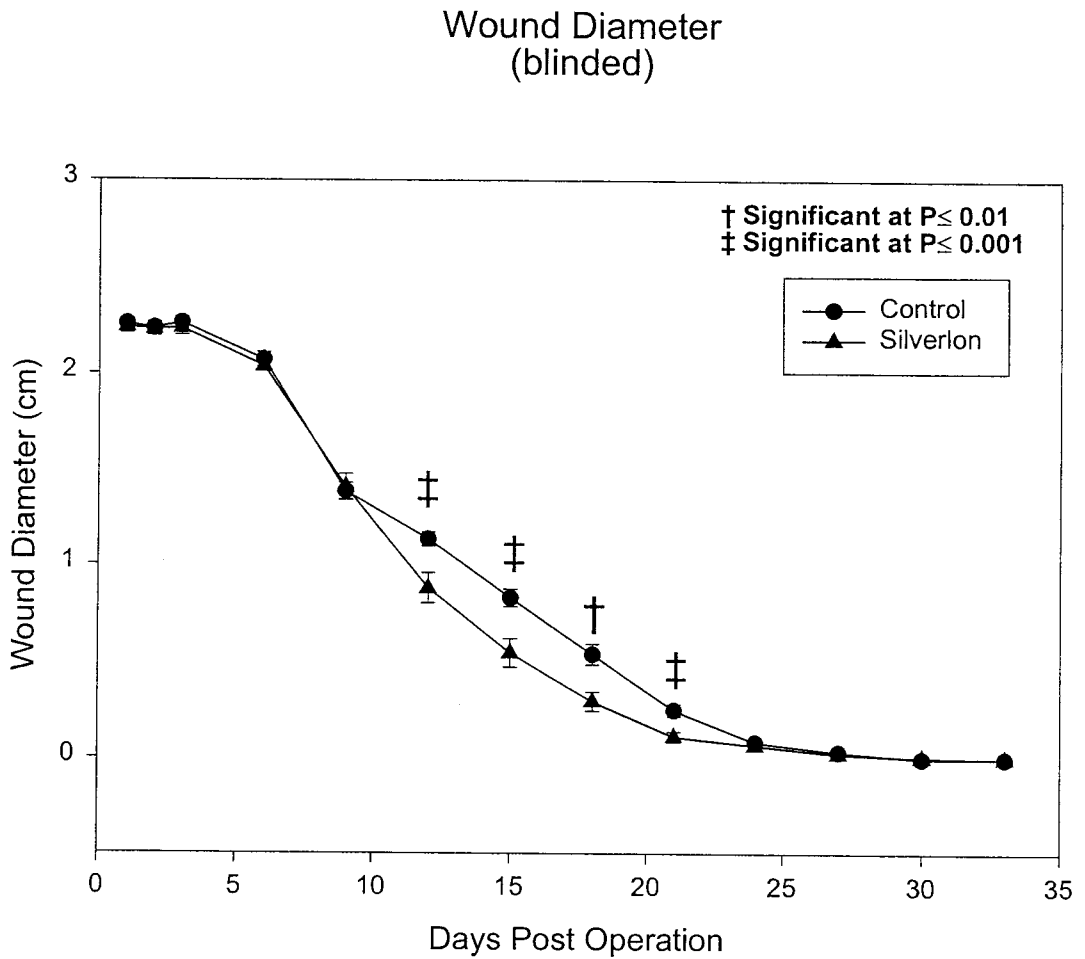


Figure 6

## Wound Area from Digital Photos

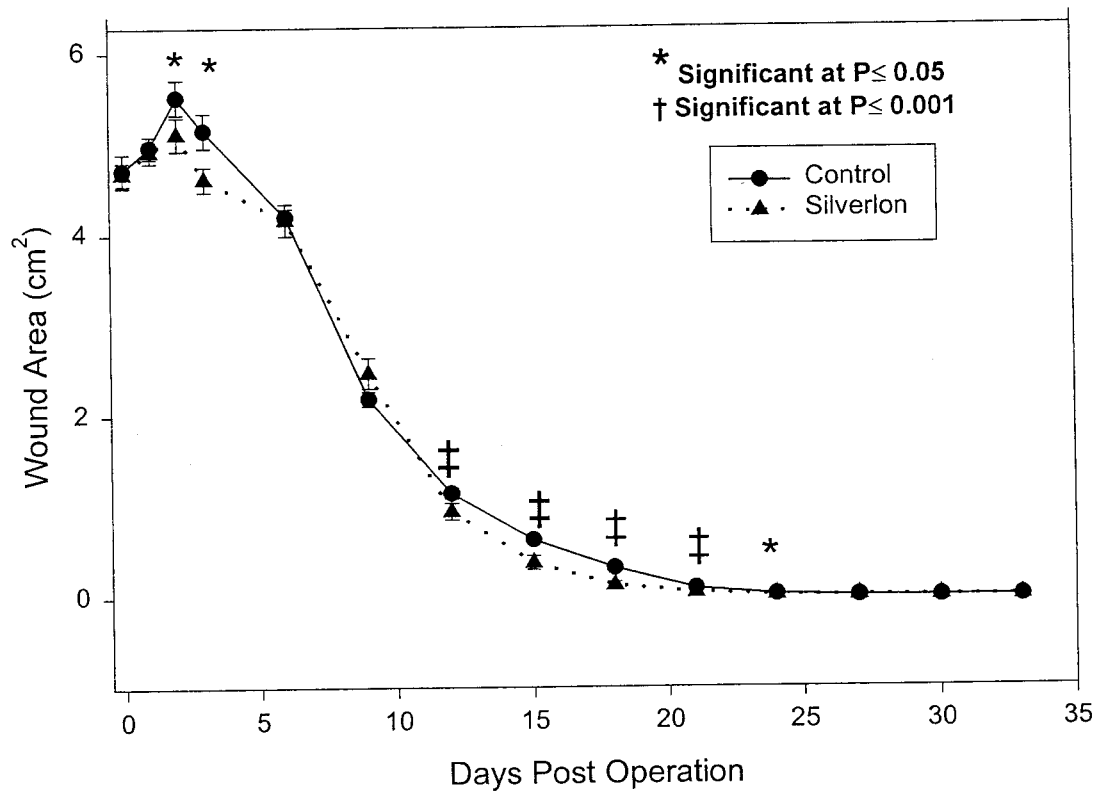


Figure 7

## Histology of Tissue Samples

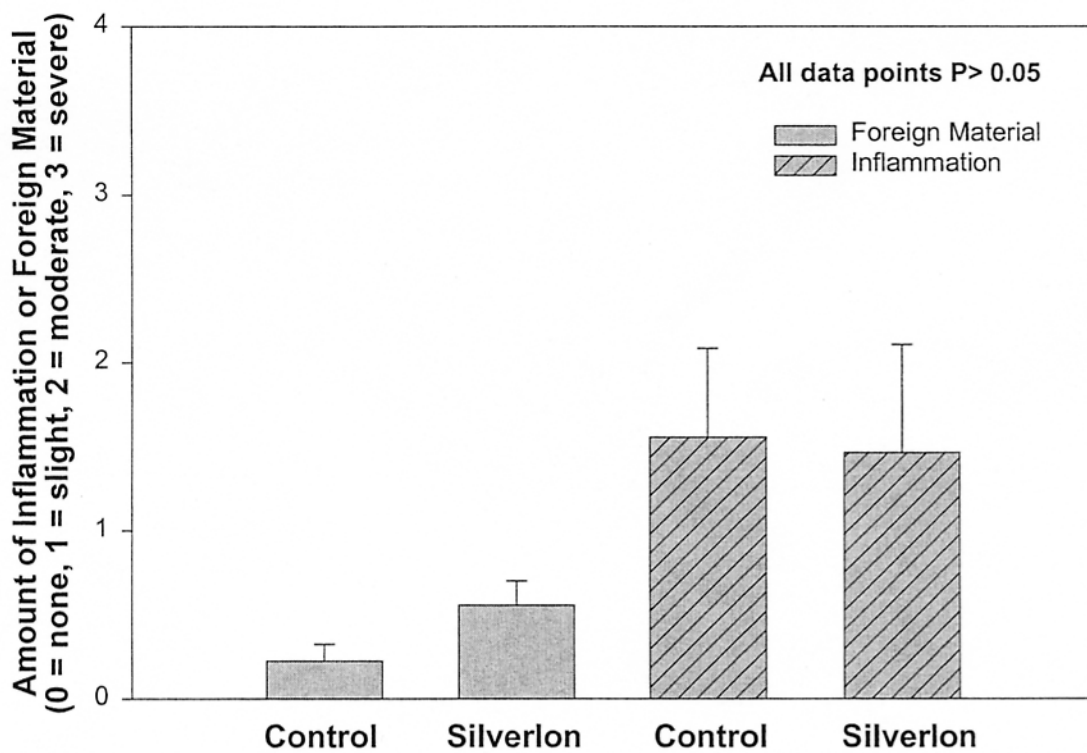


Figure 8

### Amount of Silver Present in Tissue (Atomic Absorption)

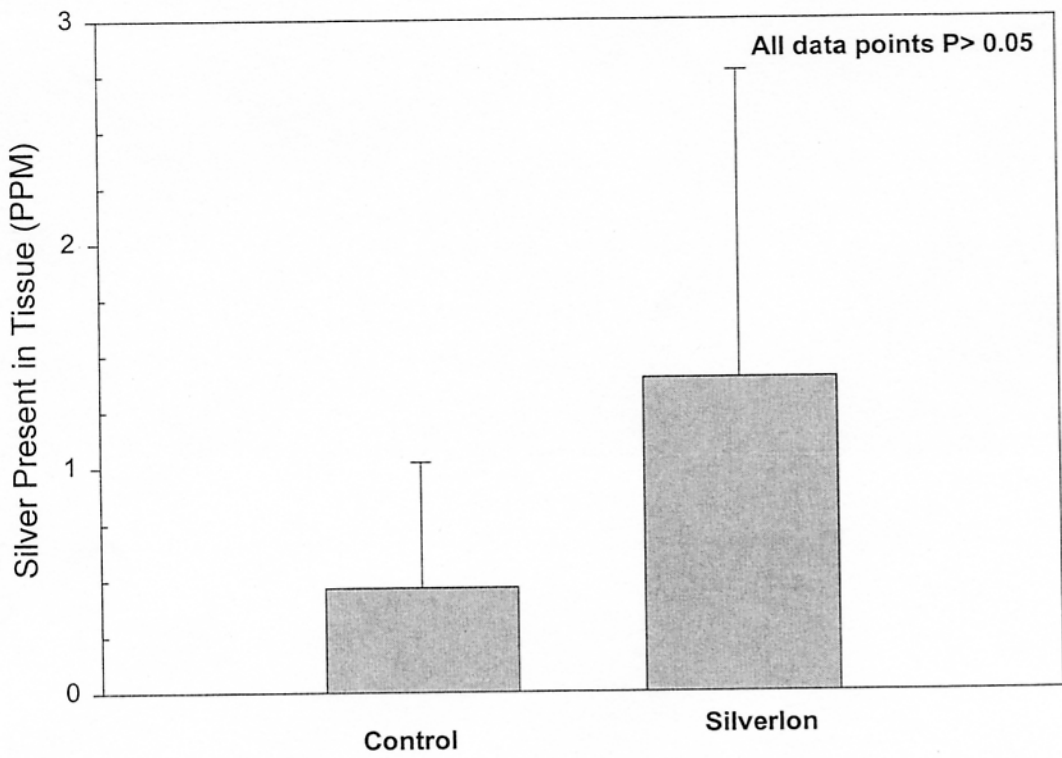


Figure 9

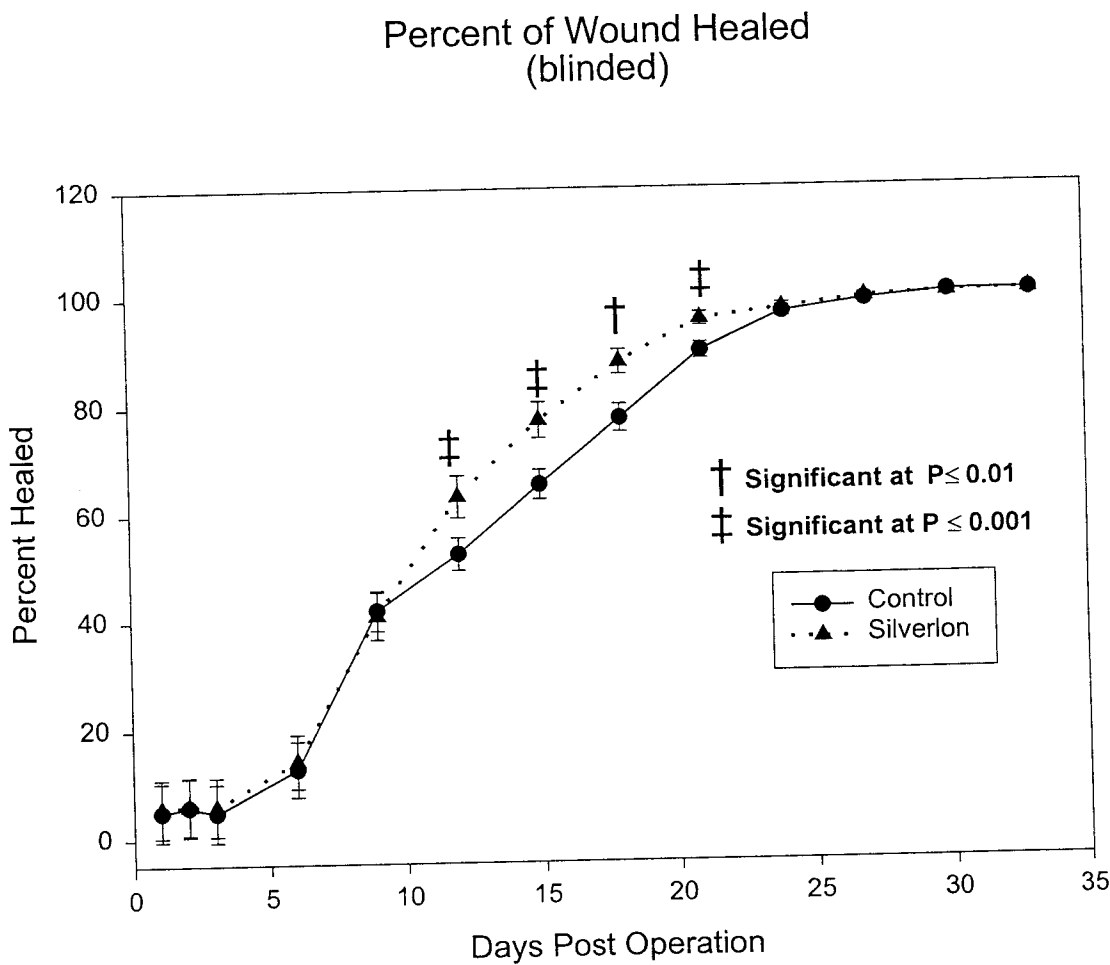


Figure 10