HISTOPATHOLOGY OF LESIONS IN SWINE EXPOSED TO A HEMOSTATIC BANDAGE COMPOSED OF SALMON THROMBIN AND FIBRINOGEN

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ABSTRACT

Hemostatic bandages composed of salmon thrombin and fibrinogen are very effective in stopping bleeding in a swine aortal injury model. However, because of concern that these foreign proteins may induce an immune response in the patients, we investigated the inflammatory response in pigs exposed to salmon thrombin/fibrinogen dressings. Two defined full-thickness skin lesions were surgically created on the backs of 25kg Yorkshire swine and one lesion was treated with a salmon protein- based dressing and the other with a control commercially-available pad. Animals were sacrificed at seven or twenty-one days and the lymphoid organs harvested for histopathological examination. The 21-day animals were given an additional boost of salmon thrombin/fibrinogen to simulate a second bandage application. Examination of the histology showed a cellular inflammatory response in treated and untreated animals that resolved by the 21-day stage. Lymph node and spleen samples showed germinal center formation in follicles, but the activity levels were higher in organs on the untreated side. Blood samples taken to assay for antibodies showed antibodies formed at low titers that recognized salmon fibrinogen and, following the booster shot, salmon thrombin.

1. INTRODUCTION

Bleeding from severe wounds is a major cause of preventable death from traumatic injuries on the battlefield (Champion et al. 2003). Control of hemorrhage is the initial step in field trauma care and having a widely deployable bandage to staunch blood loss will decrease loss of life. Hemostatic dressings based on coagulation proteins have been shown to be highly effective (Jackson et al. 1998: Pusateri et al. 2003). However, dressings based on human proteins have the disadvantages of high cost for the raw materials and the possibility of pathogen transmission. Even mammalian proteins carry the risk of disease transmission such as bovine spongy encephalitis (Aguzzi and Glatzel 2006). An alternative is a dressing composed of salmon fibrinogen and thrombin. These dressings are also effective in stopping bleeding in a swine aorta injury

model (Rothwell et al. 2005) and have been proposed as an alternative material for an active coagulative matrix. A possible drawback to this approach is that it is unknown if exposure to coagulation proteins isolated from highly divergent species such as teleost fish will provoke an immune response that could inhibit the normal host coagulation response.

The possibility of this type of response may not be unexpected because there is a history of adverse reactions to bovine proteins. Transfusion with bovine thrombin caused the production of antibodies against Factor V (Tarantino et al. 1997). This was ascribed to impurities in the preparations, however, even highly purified bovine thrombin has been reported to cause an anti-human Factor V antibody associated coagulopathy (Lawson et al. 2005). Even topical use of bovine thrombin has proven to cause allergic responses (Wai et al. 2003). These responses are not limited to coagulation proteins. In at least one report, sperm prepared for artificial insemination using bovine serum albumin induced an anaphylactic reaction (Orta et al. 2003). Ingestion of cow's milk has also been shown to elicit an immune response and to stimulate lymphocyte proliferation (Motrich et al. 2003).

The goal of this project was to determine if salmon thrombin and fibrinogen would cause the same type of response and to examine the cellular basis for that reaponse. We used histopathology to characterize the tissue response to salmon dressings in swine after excisional cutaneous surgery that created wounds with separated edges and found a lymphocyte response that included cellular proliferation and cytokine secretion. however, antibody production was limited and there were no signs of adverse immunological reactions to the dressings.

2. METHODS

2.1 Animal Care

All procedures were reviewed and approved by the Walter Reed Army Institute of Research Animal Care and Use Committee and performed according to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition. Animals were

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housed and all experiments occurred in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International. All swine were purchased through a USDA approved vendor and individually housed as previously described (Rothwell et al. 2002).

2.2 Surgical Procedures

Female Yorkshire swine (25-28 kg) were prepared for surgery and monitored during the procedure as described previously. A vascular access port catheter line was inserted into the vein using modified Seldinger technique (Knebel et al. 2006) and Fudge technique. A 16-18-gauge, 2.5-3" introducer needle was inserted into the jugular vein percutaneously, followed by a j-wire. An expander catheter was fed onto j-wire through the skin and into the jugular. The expander catheter was removed with j-wire remaining in the jugular vein and a central venous catheter was fed onto j-wire into jugular vein. The catheter was secured to subcutaneous tissues in a simple interrupted pattern with 3-0 PDS and the catheter is flushed with a citrate anticoagulant solution to verify placement into vein and to create a citrate lock. The catheter was then attached to the port, which was buried in a subcutaneous pocket on the shoulder.

Paired identical full thickness dermal wounds were surgically created on the right and left dorsal skin surface, paramedium to the spinal column in four pigs and monitored for 7 days. A second group of four pigs were subjected to a similar pair of skin lesions and monitored for 21 days. Animals in the 21-day group were injected with thrombin (60IU) and fibrinogen (200 µg) on day seven. The total number of wounds to evaluate in each time point was eight. The right dorsal lesion was bandaged with a dressing composed of lyophilized fibrinogen and thrombin applied to a full thickness dermal lesion approximately 2x2 cm. The left dorsal lesion was dressed with a commercially available, non-hemostatic bandage. At the end of the time point, the animals in each group were euthanized and the carcass presented for necropsy.

2.3 Tissue Preparation

At necropsy, each pig was placed in lateral recumbency and the skin defects measured, gross lesions noted and recorded, and gross photographs were captured. The tissue of the treated (right) and untreated (left) lesions, the pre-femoral lymph nodes, mesenteric lymph nodes and spleen were harvested for histopathology. The tissue samples were fixed in 10% neutral buffered formalin and then routinely trimmed, processed, and embedded in paraffin wax. A microtome was used to cut an approximately 4µm thick section which was mounted on a glass slide and stained with

hematoxylin and eosin (H&E). Histopathology assessment was performed using a light microscope. Evaluation parameters on the skin sections included comparison of the wound edge with the wound center of the right (treated) versus the left (untreated) side. Grading of the skin samples was performed for signs of superficial and deep inflammation, re-epithelialization, granulation tissue, fibrosis, crust formation and necrosis. The mesenteric lymph node, prefemoral lymph nodes and spleen from each animal was also evaluated.

3. RESULTS

3.1 Inflammation and Re-epithelialization of the Skin Lesion in the 7-day group

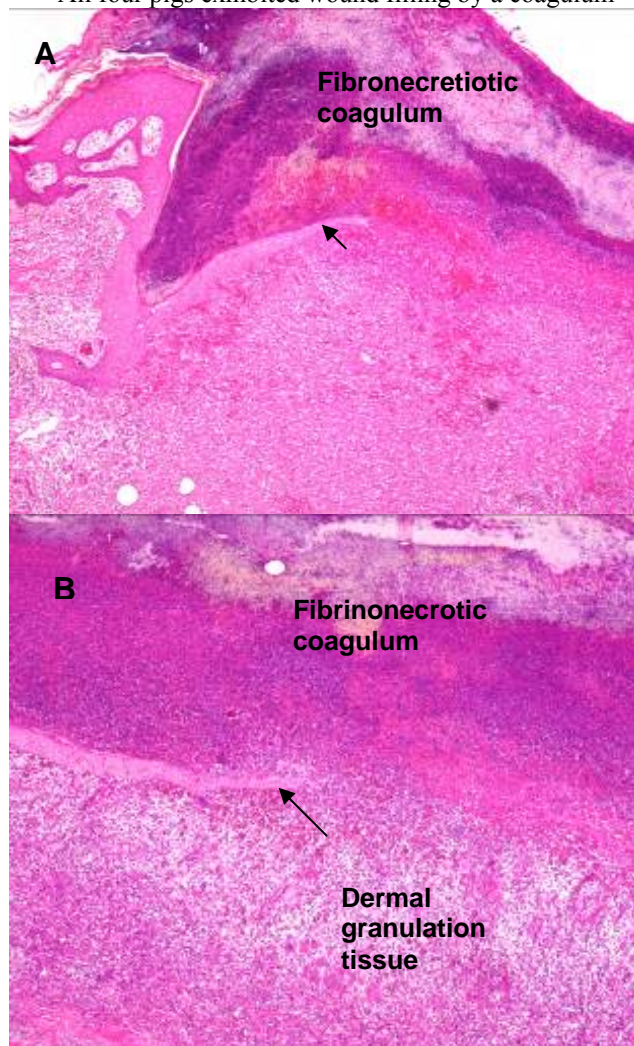All four pigs exhibited wound filling by a coagulum

Figure 1 Samples taken at 7 days from control (A) and salmon bandage-treated (B) injuries show fibronecrotic coagulum in both cases. Arrows show epithelial cell projections towards the wound center (H & E staining, 40x magnification).

composed of necrotic cellular debris, neutrophils, fibrin, hemorrhage, and edema (Figure 1). Granulation tissue and edema expanded the superficial dermis on both the right (treated) and left (untreated) sides subjacent to this fibrinonecrotic coagulum. Histologically, this granulation tissue was composed of many small caliber blood vessels lined by hypertrophied endothelium and oriented perpendicular to the skin surface. The edema widely separated dermal collagen and fibroblasts (Figure 2).
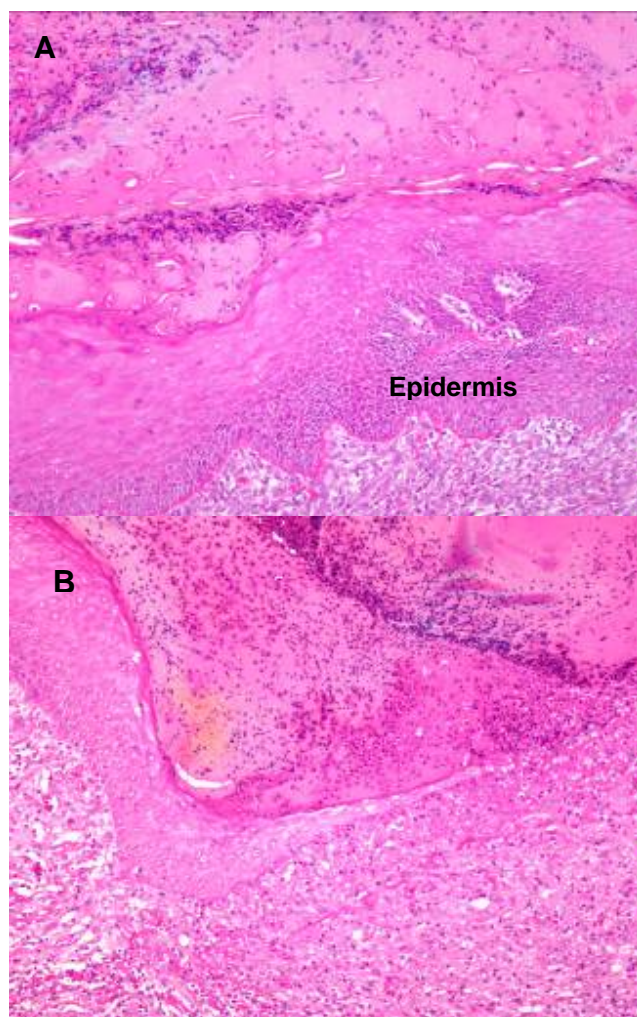

Figure 2 Samples taken at 7 days from control (A) and salmon bandage-treated (B) injuries show crust, epidermis and dermis at wound edge). (H & E staining, 100x magnification).

Evidence of fibroplasia, characterized by numerous plump, activated fibroblasts with deposition of abundant collagen, extended from the junction of the dermis deep to the panniculus adiposus. This fibroplasia was moderate in severity with a multifocal to diffuse distribution in all four pigs on both the right and left sides.

Superficial inflammation of all eight wounds was moderate to marked in severity and composed of primarily neutrophils, fewer macrophages and rare multinucleate inflammatory giant cells subjacent to the fibrinonecrotic scab. Deep inflammation was marked in one right (treated) lesion, and mild to moderate in the remaining right side lesions. Deep inflammation in the left (untreated) lesions was marked in two cases and mild to moderate in two cases.

An attempt at re-epithelialization on the wound edges was evident in all eight wounds at the 7-day time point. Typical findings at these margins included epidermal hyperplasia, acanthosis, spongiosis, deep rete ridges and dermal pegs, parakeratotic hyperkeratosis and projections of regenerative epithelial cells toward the wound center (Figure 3).
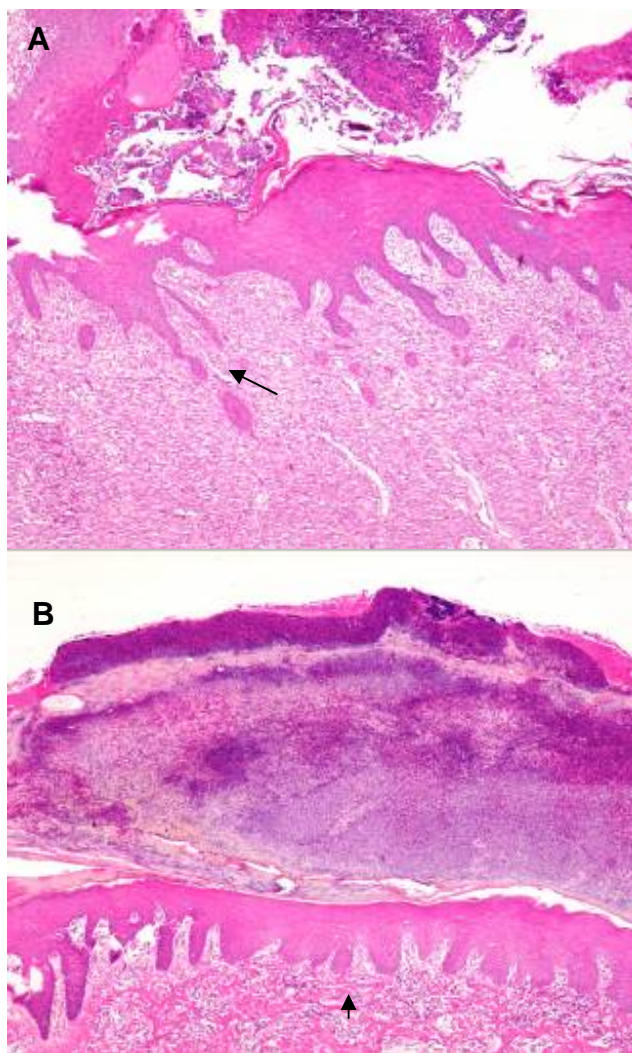

Figure 3 Samples taken at 7 days from control (A) and salmon bandage-treated (B) injuries show rete ridges of epithelial cells extending deep into the dermis (arrows). (H & E staining, 20x magnification).

To summarize the 7-day group, all wounds were filled with a fibrinonecrotic coagulum. Each wound exhibited superficial granulation tissue in the dermis on both the right (treated) and left (untreated) sides. There were numerous neutrophils, fewer macrophages and multifocal hemorrhage. Inflammation variably extended deep into the subcutis and was composed of lymphocytes, plasma cells and macrophages. Re-epithelialization at the margins and moderate fibroplasia was evident in all eight wounds.

3.2 Inflammation and Re-epithelialization of the Skin Lesion in the 21-day group

In the 21-day group, seven of eight wounds exhibited complete re-epithelialization that was covered by a surface clot similar in cellular composition to the 7-day wounds (Figure 4).
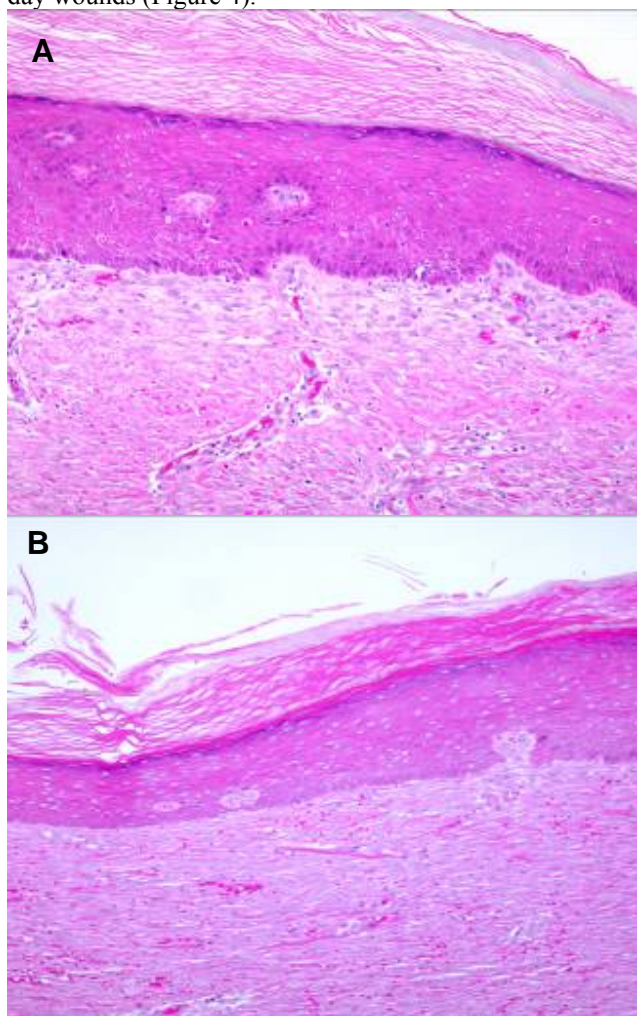

Figure 4 Samples taken at 21 days from control (A) and salmon bandage-treated (B) injuries show complete re-epithelialization. (H & E staining, 100x magnification).

In these seven cases, the superficial inflammation was minimal to mild. One left (untreated) case in the 21-day

group displayed incomplete re-epithelialization and the wound defect was filled by a fibrinonecrotic coagulum along with marked superficial inflammation. The wound edge in this case exhibited similar epithelial cell hyperplastic changes as the 7-day group.

All eight wounds exhibited mild amounts of dermal granulation tissue and deep inflammation that was composed of perivascular lymphocytes and macrophages. Fibroplasia and collagen deposition was brisk in comparison to the 7-day group (Figure 5). This change extended from the junction of the dermis to the subcutaneous fat in all eight wounds.
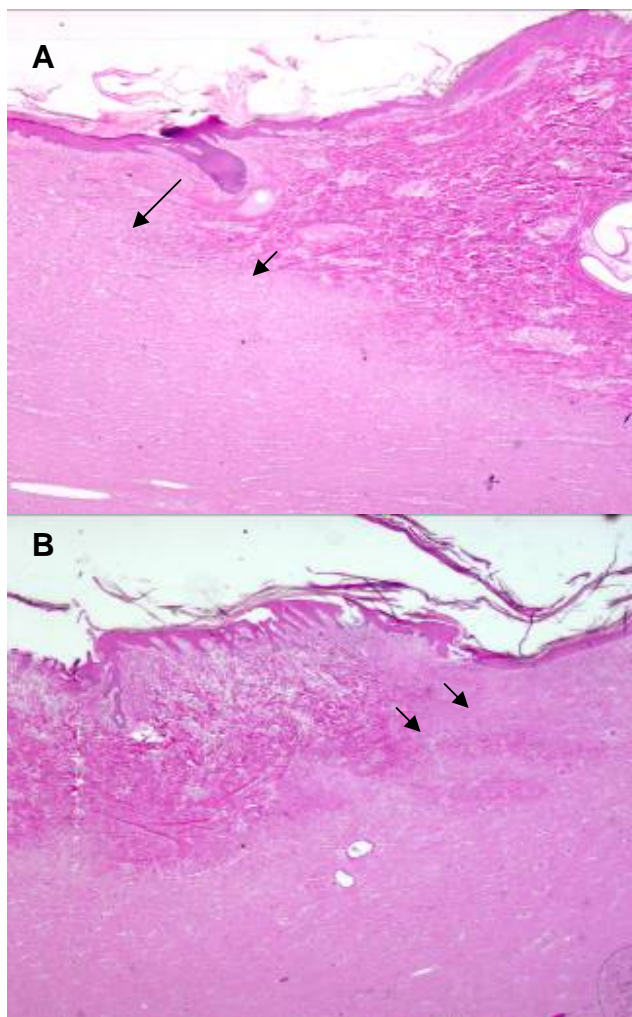

Figure 5 Samples taken at 21 days from control (A) and salmon bandage-treated (B) injuries show signs of fibroplasia (arrows). (H & E staining, 20x magnification).

Superficial inflammation was minimal to mild in the four right (treated) lesions and generally composed of few lymphocytes, plasma cells and macrophages. Superficial inflammation in the left (untreated) side varied from minimal in one case, mild in two cases and marked in one case (Figure 6). The minimal to mild cellular infiltrate was

similar to the right side wounds. However, the one marked case of inflammation in one left side wound was composed of numerous neutrophils with fewer dermal macrophages, lymphocytes, plasma cells and eosinophils. Neutrophils rarely formed intraepidermal pustules. Additionally, there was hemorrhage, fibrin and edema with necrosis in this wound.

Deep inflammation in the right (treated) side varied from minimal to mild in three cases and moderate in one case. This subacute inflammation was predominantly clustered around vessels. In the left (untreated) side, deep inflammation was minimal in two cases and moderate in two cases, subacute and primarily perivascular.

in the superficial dermis. The inflammation was primarily composed of mononuclear cells. Fibroplasia was abundant. One left side wound exhibited similar histopathology lesions as the 7-day group, including incomplete re-epithelialization and marked inflammation.

3. 3 Immune organ involvement

The lymph nodes and the spleen were examined for signs of activation. The mesenteric lymph nodes and spleen were found to be similar histologically among the four pigs in each group. The amount of white pulp (lymphoid tissue containing T and B lymphocytes) contained in the spleen increased slightly in the 21 day samples compared to the 7 day samples (Figure 7).
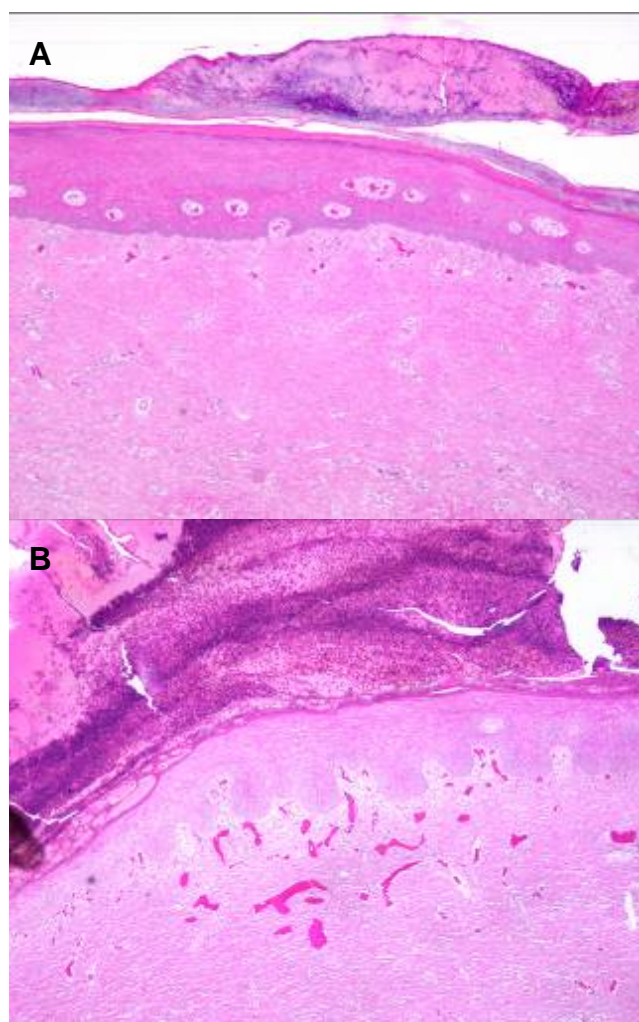

Figure 6 Samples taken at 21 days from control (A) and salmon bandage-treated (B) injuries show coagulum at the surface with some epidermal thickening. (H & E staining, 40x magnification).
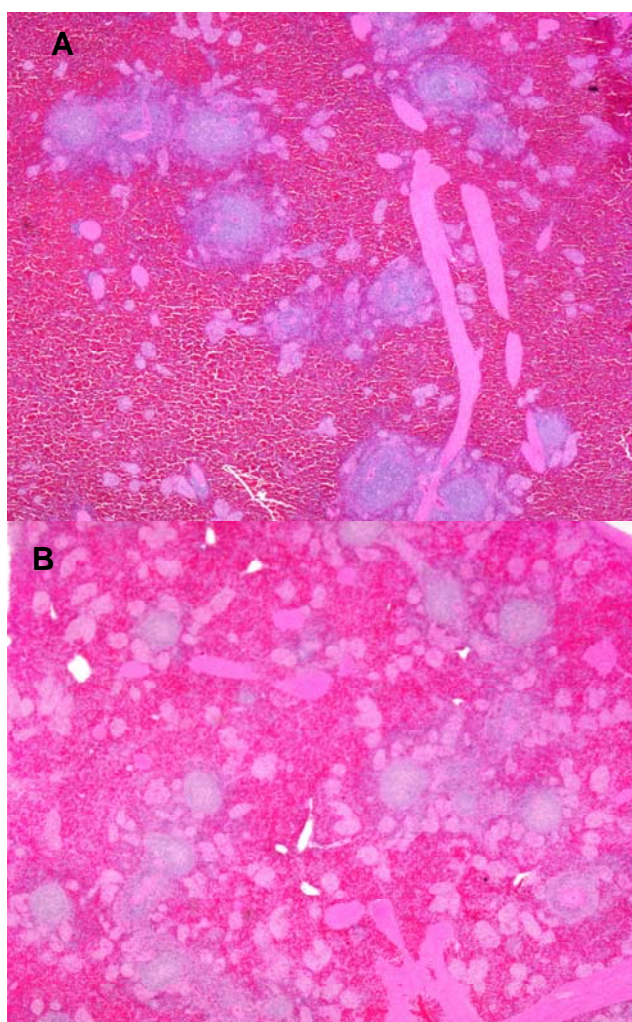

Figure 7. Samples taken at 7 days (A) and 21 days (B) after injury show coagulum at the surface with some epidermal thickening. (H & E staining, 100x magnification).

In summary, the 21-day group exhibited complete re-epithelialization in seven wounds, which was covered by a fibrinonecrotic scab. Granulation tissue was evident

Although mesenteric lymph nodes showed little difference between the 7-day and 21-day groups, the prefemoral lymph nodes did display differences. The node

from the control side generally exhibited fewer lymphoid follicles and decreased turnover of lymphoid cells than the prefemoral nodes from the treated side.
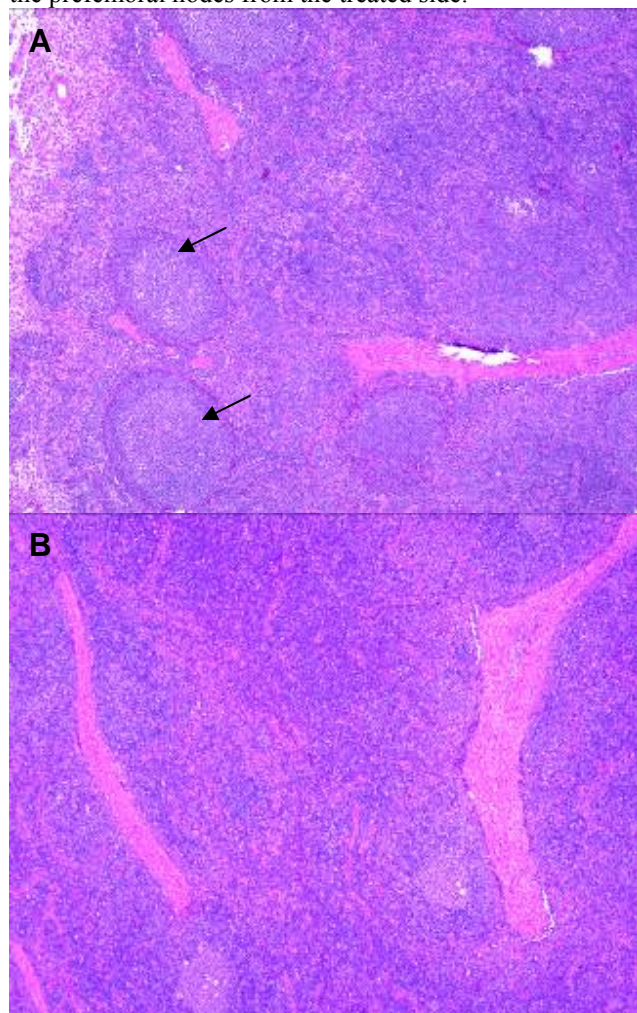

Figure 8. Samples taken at 7 days from salmon bandage-treated (A) and control (B) injuries. Arrows indicate follicles with germinal centers. (H & E staining, 100x magni-fication).

4. DISCUSSION

Because of the introduction of foreign proteins derived from the salmon blood into a wound site, we were concerned that the wound healing process may be impeded and that coagulopathy may be induced by initiation of an adverse immune response. To investigate these possibilities, we treated full thickness skin wounds with thrombin/fibrinogen dressings, control dressings, and compared the progress of wound healing and the state of activation of the lymph nodes and the spleen.

Skin wound repair is classically divided into two types, which are first intention and second intention. First intention wound healing occurs in skin when the edges are opposed, normally with sutures. Second intention wound healing occurs when there are unopposed edges. The cutaneous defect in second intention wound healing is typically larger and does not permit the use of sutures to close the wound (Kumar et al. 2005).
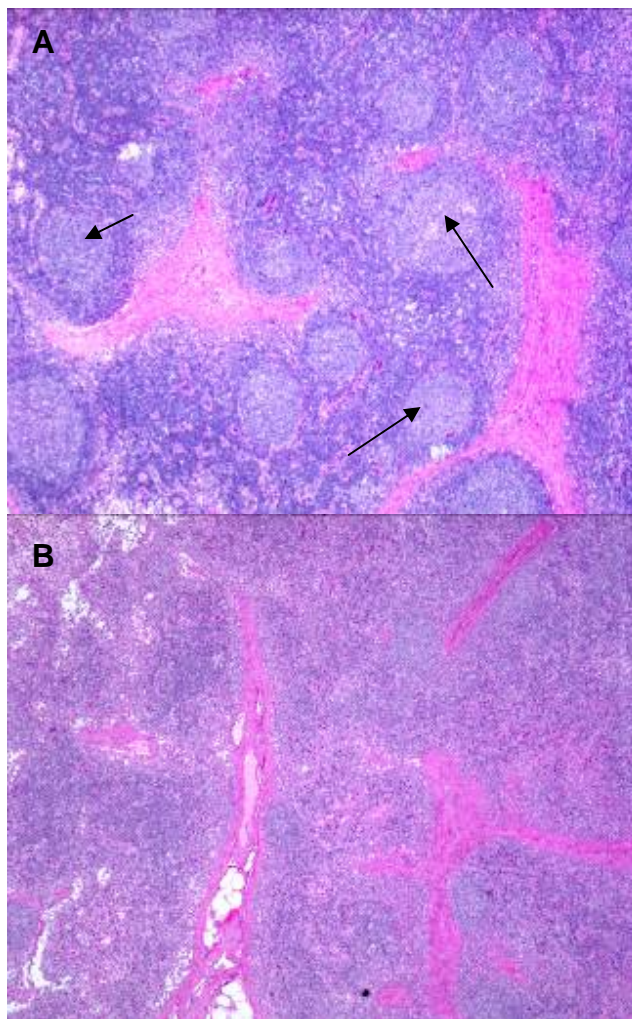

Figure 9. Samples taken at 21 days from salmon bandage-treated (A) and control (B) injuries. Arrows indicate follicles with germinal centers. (H & E staining, 100x magni-fication).

Cutaneous wound healing can be divided into three general phases: inflammation, proliferation and reorganization. These phases correlate to time-dependent steps in the wound healing process. After cutaneous injury, the clotting cascade is set in motion and a fibrin clot fills the wound defect. At the wound margins, neutrophil chemotaxis is initiated in response to inflammatory cytokines. Removal of necrotic cellular debris occurs. Neovascularization, consisting of small caliber blood vessels and fibroblasts, takes place via invasion of the dermis deep to the wound. As time progresses, the inflammatory cell infiltrates change from primarily neutrophils to macrophages (Bochsler and

Slauson 2002). The proliferation phase is characterized by a robust granulation tissue response with epithelial cell regeneration that attempts to close the gap created by the wound. Scar formation is the final step in reorganization where fibroblasts, collagen and myofibroblasts function in wound contraction (Kumar et al. 2005).

Excisional cutaneous wounds were surgically created in these eight pigs, bandaged with two different types of dressings, and monitored for two time points of seven or twenty-one days. The 7-day and 21-day time points generally followed the well-established models of cutaneous wound healing by second intention where there are separated edges and no surgical opposition. Cutaneous wounds healed by second intention follow a complex process in closing the defect. These types of wounds display a robust, localized inflammatory response, form abundant granulation tissue and have a thin epidermis overlying scar tissue. As expected, the 21-day group exhibited complete re-epithelialization in seven out of eight cases. Over time, these wounds would likely show signs of scarring with contracture if allowed to progress for additional weeks and months.

A notable histopathology difference in both the 7-day and 21-day time points was increased activation of prefemoral lymph nodes on the treated side in contrast to the nodes taken from the untreated side. This was not unexpected as it may reflect a greater degree of immune stimulation on the side exposed to the salmon protein bandages or it may be due to an increased coagulative response at the time of the initial wound, resulting from the fibrin/thrombin bandages. This observation warrants further investigation.

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