Silver Dressings Improve Diabetic Wound Healing Without Reducing Bioburden

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Abstract: Introduction. Silver dressings are widely used in the treatment of chronic wounds to reduce bacterial bioburden. However, little is known about the mechanism of silver ions on the healing process. In this study, a mouse model of wound healing was used to examine the effect of silver dressings in normal and diabetic wounds. Methods. Two 5-mm full-thickness wounds were created on the dorsal skin of diabetic BKS.Cg- m+/+Leprdb/J mice (experimental group) and wild type C57BL/6 mice (control group), and treated with either a silver or gauze dressing. Measurement of wound areas by digital planimetry demonstrated faster healing in the silver-treated wounds of both diabetic and control mice. Results. Quantitative bacterial cultures showed a reduction of bioburden in silver-treated wounds in wild type mice. Unexpectedly, there was no decrease in bioburden in the silver-treated diabetic wounds compared to the control diabetic wounds, despite improved healing in the silver-treated diabetic wounds. Staphylococcus xylosus, a known biofilm producer, was the only bacteria identified in all the wounds. In vitro studies showed S. xylosus produced biofilms faster in higher glucose environments; this may explain the increased bioburden in the wounds in diabetic mice compared to wild type mice. Conclusion. The results demonstrate improved healing and reduced bioburden in normal wounds with silver dressings. In contrast, silver dressings improved healing in diabetic wounds despite no effect on bioburden, suggesting silver may have beneficial effects in addition to its antimicrobial properties.

Key words: biology of wound healing, silver dressings, diabetes, biofilm, bacteria

Impaired wound healing in patients with diabetes is a significant health problem. More than 80 million Americans have either diabetes or pre-diabetes. These patients have impairments in the mechanisms associated with wound healing. As a consequence, these nonhealing wounds lead to more than 70,000 lower limb amputations per year. Unfortunately, these numbers are expected to increase due to rising obesity rates. Impaired wound healing in diabetic patients is not completely understood. Normal wound healing involves angiogenesis, epithelialization, collagen deposi-
tion, and the formation of granulation tissue. Research has shown a significant decrease in all of these healing events in both diabetic humans and in diabetic animal models.2

Silver dressings are a popular wound treatment and have been utilized in advanced wound care for more than 40 years. Research has shown silver to be an effective antimicrobial agent against bacteria, viruses, yeast, and fungi.3-5 Silver ions can cause microbial cell death in 2 ways. First, silver ions can bind to the cell wall and disrupt cell wall integrity.6 In addition, silver ions can bind directly to DNA, which interferes with cell replication and transcription.7 Thus, the beneficial effect of silver dressings may be to reduce the bacterial load of the wound.

It has been suggested that silver may have other benefits to wound healing in addition to its antimicrobial activity. During the normal healing process a moderate amount of inflammation is necessary. Inflammatory cells, including neutrophils and macrophages, infiltrate the wound to destroy and phagocytose pathogens. In addition, they clean the wound by removing dying cells and debris. However, an excessive amount of inflammation can actually impede the healing process.8,9 Some studies have suggested that silver also aids in wound healing by reducing inflammation. For example, in a porcine model of contact dermatitis, pig ears treated with silver demonstrated improvements in overall healing. A reduction of inflammation in the silver-treated wounds was observed after 72 hours, as evidenced by increased apoptosis of inflammatory cells and decreased proinflammatory cytokines.10 In a separate porcine wound healing model, silver-treated wounds healed an average of 10 days faster, and this correlated with active fibroblasts, thicker granulation beds, and limited inflammation. The control wounds showed raised edges, edema, and full contracture, which are all signs of inflammation; however, the silver treated wounds showed no signs of contracture.11 Similarly, another porcine wound healing model showed reduced matrix metalloproteinases and erythema in full-thickness wounds treated with silver compared to controls. This was associated with increased apoptosis and increased numbers of fibroblasts, monocytes, and neutrophils in the silver-treated wounds.12 Finally, a murine and cavia model of allergic contact dermatitis showed silver-treated ears had a reduction in swelling, erythema, and decreased expression of tumor necrosis factor-alpha (TNF-α) and interleukin-20 (IL-20) compared to controls.13,14

Taken together, these findings suggest that silver ions may be able to impact the inflammatory response in skin. The ability to reduce the inflammatory response could potentially be a critical process in the ability of silver to facilitate wound healing. However, previous studies did not demonstrate that silver had anti-inflammatory properties independent of simply decreasing bioburden. More research is needed to delineate actual benefits of silver, if any, and the mechanisms by which it works. By fully understanding these mechanisms, practitioners could potentially maximize wound healing and decrease recovery times for patients across all medical disciplines.

Methods

Animals. All animal procedures were approved by the Missouri State University Animal Care and Use Committee prior to the start of the study. Healthy 8-week-old female adult mice were obtained from The Jackson Laboratory, Bar Harbor, ME. The diabetic mouse strain BKS.Cg-m+/+Leprdb/J (db/db) that shares many similarities with human adult onset diabetes was used as the experimental group. Wild type C57BLKS/J mice with the same genetic background as the db/db mice were used for controls. The animals were individually housed in the animal facility at Missouri State University, Springfield, MO, on a 12-hour light/dark cycle. The animals were allowed to acclimate for 7 days upon arrival and had access to food and water ad libitum.

Wound model. A mouse model of wound healing that closely mimics human wound healing was used.15 Prior to surgery, mice received a subcutaneous injection of buprenorphine (0.1ml/10g) and were anesthetized with 3% isoflurane gas anesthesia delivered through a precision vaporizer (Tec 3 Isoflurane Vapouser, VetQuip, Castle Hill, New South Wales, Australia). The animals were allowed to acclimate for 7 days upon arrival and had access to food and water ad libitum.
to remove hair. The skin was cleansed with 3 alternating scrubs (isopropanol, betadine, isopropanol) and draped. A punch biopsy tool was used to create 2 full-thickness wounds of 5 mm on the dorsal skin paramedian to the vertebral column just caudal to the most distal tip of the scapula. After the wound was created, a ring-shaped silicone splint was applied to the skin 2-3 mm beyond the perimeter of the wound. The silicone splint was affixed with tissue adhesive (VetBond, 3M Veterinary & Animal Care, St. Paul, MN) and 6 interrupted sutures. Wounds were dressed with either moistened gauze or moistened silver dressing (Choice Therapeutics, Wrentham, MA). The silver dressings consisted of a nylon fabric plated with silver ions. The gauze dressing consisted of a standard woven cotton fabric. Both dressings were sterilized and moistened with sterile water before use. A transparent film dressing (Tegaderm, 3M Health Care Skin & Wound Care, St. Paul, MN) was then used to cover both the moistened silver dressing and the moistened gauze dressing. The occlusive nature of the transparent film dressing ensured that all dressings stayed moist throughout the experiment. Dressings were checked twice daily to ensure they remained in place; all dressings were replaced after 7 days. During recovery, the mice were placed in individual cages under a warming lamp. Mice were observed daily for general health. Glucose levels of peripheral blood were measured using a blood glucose monitor and tests strips (Contour, Bayer Healthcare, Mishawaka, IN). Mice with blood glucose levels > 300 mg/dl were considered diabetic. Digital photographs were taken on days 2, 4, 6, and 8 to quantify wound healing. The moistened gauze and silver dressings were briefly removed for wound visualization and then immediately replaced. Both the control and silver-treated dressings were removed and replaced in the same way to control for the effect of brief dressing removal. Wound areas were measured by a digital planimetry program (SigmaScan Pro 5.0, SPSS Science, Chicago, IL), with calibrated digital photographs. To increase measurement accuracy, 10 different tracings were made with the digital planimetry program for each wound and then averaged.

Microbiology. Staphylococcus aureus (ATCC 6538), Staphylococcus epidermidis (ATCC 55984), and Serratia marcescens (ATCC 60) were purchased from the America Type Culture Collection, Manassas, VA. Surgical scissors were used to excise an area of skin that included the entire wound bed and 1 mm of tissue surrounding the wound. Staphylococcus xylosus was isolated from diabetic mouse (strain BKS.Cg-m+/+Leprdb/J) wounds cultured 5 days after wound creation. A microbial identification system (VITEK 2, Biomerieux, Durham, NC) was used to identify S. xylosus from homogenized tissue samples. The system used in this study measures 47 biochemical substrates including carbon source utilization, enzymatic activities, and resistant patterns.

**Corrected zone of inhibition test.** A corrected zone of inhibition (CZOI) test was used to determine the antibacterial properties of the silver dressing. This test is similar to the standard zone of inhibition test except zones are corrected to take into account the differences in shape of the hand-cut testing dressings. This test is a modification of the standard Kirby-Bauer test for antimicrobial sensitivity. Gram-positive bacterial isolates were grown in tryptic soy broth (Remel, Lenexa, KS) while the gram-negative bacteria was grown in nutrient broth (Remel, Lenexa, KS), both for 18-24 hours. Following overnight growth, 0.1 mL of gram-positive bacteria was streaked on tryptic soy agar (Remel, Lenexa, KS) and 0.1 mL of gram-negative bacteria was streaked on nutrient agar (Remel, Lenexa, KS) in 3 directions to form a confluent lawn. Silver dressings (n = 2) and gauze (n = 2) were cut into 0.5 in x 0.5 in squares and placed in the center of each lawn. The bacterial cultures were diluted serially in saline solution (Ricca Chemical Co, Arlington, TX) and 0.1 mL was plated on appropriate plates (gram-positive bacteria on tryptic soy agar and gram-negative bacteria on nutrient agar). Plates were incubated for 24 hours at 37°C (gram-positive) and 26°C (gram-negative). The CZOI was measured by measuring the zone of clearing across 1 direction and subtracting the width of the dressing. This measurement was repeated across the other direction and values were averaged. The CZOI reflects only the width of clearing around the dressing. The starting concentration of bacteria was determined by the following formula: N x DQ x 0.1, where N is

**Key Points**
- The diabetic mouse strain BKS.Cg-m+/+Leprdb/J (db/db) that shares many similarities with human adult onset diabetes was used as the experimental group.
- Wild type C57BLKS/J mice with the same genetic background as the db/db mice were used for controls.
- Wounds were dressed with either moistened gauze or moistened silver dressings.
of the dilution plate counted, V is the volume of the diluent used for tissue homogenization, and W is the weight of the sample in grams. The 10 factor accounts for the 0.1 mL volume of sample that was plated. Clinically significant quantitative culture values were operationally defined as > 10^5 cfu/g. 17,18

In vitro biofilm growth. Bacteria were grown in tryptic soy broth to a concentration of approximately 108 organisms per milliliter of broth. A modified tryptic soy broth was made by adding 17 g of cascin peptone, 5 g of sodium chloride, 3 g soy peptone, and 2.5 g dipotassium phosphate to 1000 mL of deionized water. Dextrose was added to achieve solutions with final glucose concentrations of 0 mg/dL, 125 mg/dL, 250 mg/dL, and 500 mg/dL. All solutions were sterilized by autoclaving at 121°C for 15 minutes. Sterile slides placed into a preweighed sterile tube containing 1 mL normal saline. This tube was then reweighed. The weight of the sample was obtained by subtracting the first weight from the final weight. The tissue was then homogenized using a pestle and tube homogenizer (Kontes Glass Inc, Vineland, NJ) for 30 seconds. Serial 10-fold dilutions of the homogenate were made in normal saline. Nutrient agar plates were inoculated with 0.1 mL of the homogenate as well as each serial dilution. Plates were placed in an incubator at 37°C for 24 hours, and colony counts were performed. The number of colony forming units (cfu) per gram of sample was calculated by the following equation: N x DQ x V x 10/W, where N is the number of colonies counted on a plate of a given dilution, DQ is the reciprocal of the dilution counted, and 0.1 is the volume of solution plated. Corrected zone of inhibition tests were conducted in duplicate. To confirm that the silver dressings killed the microorganism under the dressing and did not simply hinder bacteria growth due to pressure, a culture was taken from underneath each dressing, plated on both tryptic soy agar and nutrient agar and allowed to grow overnight at 37°C.

Quantitative culture technique. For quantitative cultures of wounds, surgical scissors were used to excise an area of skin that included the entire wound bed and 1 mm of tissue surrounding the wound. Quantitative cultures were obtained using a modification of a method developed by Buchanan. 16 Samples were
were placed in sterile Coplin staining jars containing the indicated broth. The bacterial solution was added to the jar to reach a final concentration of $10^7$ organisms/mL. Jars were placed on a shaker and incubated at 37°C. Slides were removed at the indicated times and stained for the presence of biofilm. Slides were coated with 10 mM cetylpyridinium chloride, allowed to air dry for 20-30 minutes, and then fixed by gently heating. After cooling, slides were stained with a 2:1 mixture of saturated aqueous Congo Red solution and 10% (v/v) Tween 80 for 15 minutes. Slides were carefully rinsed in distilled water, dipped into 10% (vol/vol) Ziehl carbol fuchsin, and carefully rinsed again in distilled water. Slides were then placed at 37°C and allowed to dry.

**Results**

Faster wound healing times were observed with silver dressings in wild type mice, which served as the control group. Figure 1 shows representative photos of wounds on the wild type mice during healing. Smaller wounds were observed in wild type mice treated with silver dressings ($n = 8$) compared to wounds with gauze dressings ($n = 8$) on day 4 ($t [18] = 20.45$, $P < 0.01$), day 6 ($t [18] = 24.15$, $P < 0.01$), and day 8 ($t [18] = 12.97$, $P < 0.01$), after initial wounding. The gauze dressing showed no ZOI across both bacteria (Figure 1C). The silver dressing showed a 2 mm ZOI against *S. marcescens* and a 4 mm ZOI against *S. aureus* (Figure 1C).

Silver dressings also improved healing in diabetic wounds. The experimental group (mean = 46.63g, SD = 1.98) had a statistically significant higher body weight compared to the control group (mean = 21.65, SD = 4.48) ($t = 26.39$, $P < 0.001$) (Figure 1A). In addition, the experimental group (mean = 425.52 mg/dl, SD = 109.81) had a statistically significant higher blood glucose level than the control group (mean = 108.35mg/dl, SD = 22.43) ($t = 16.63$, $P < 0.001$), which
confirmed the mice were diabetic (Figure 1B). The diabetic wounds (n = 8) were statistically smaller when treated with silver dressings compared to gauze dressings on day 12 (t [18] = 5.83, P < 0.01), day 15 (t[18] = 29.23, P < 0.01), and day 17 (t [18] = 18.04, P < 0.01) (Figure 2A and 2B). Diabetic wounds treated with silver were essentially closed by day 17 compared to diabetic wounds treated with gauze that remained open until day 22. Mann-Whitney U statistical tests confirmed the independent t-test results showing identical alpha values for both control group wounds (wild type mice) and experimental wounds (diabetic mice) (P < 0.01).

Quantitative cultures and bacteria sensitivity. All wounds were created and dressed using aseptic surgical procedures. Quantitative cultures of the wound biopsies on day 0 demonstrated that there were no detectable bacteria at baseline. To determine if the silver dressings improved healing by reducing the bacterial load of the wound, cultures were conducted at day 2 and day 5 post-wounding. In the control group, silver-treated wounds (n = 8) showed a reduction in the number of clinically significant samples (operationally defined as > 10^5 cfu/g) compared to gauze-treated wounds (n = 8) on day 2 and day 5 (Fisher’s exact, P = 0.05). However, there was little difference in the number of clinically significant values between silver- or gauze-treated wounds in the experimental group (Fisher’s exact, P > 0.05). Chi-square tests for independence were conducted on the logarithmic value of actual bacterial counts to examine differences between gauze- and silver-treated wounds for both control and experimental mice. Control group wounds treated with silver had significantly lower bacterial counts on day 2 (z = 3.03, P < 0.05) but not on day 5 (z = 1.50, P > 0.05) compared to gauze treated wounds. There was no difference in bacterial counts in diabetic wounds on day 2 (z = 0.46, P > 0.05) and 5 (z = 1.58, P > 0.05). Actual bacterial counts are shown in Figure 3. These results suggested that silver dressings are able to reduce bacterial growth in the control group wounds but not in the experimental wounds at day 2.

Wounds contain Staphylococcus xylosus. In order to determine why the silver dressings did not reduce the bacterial counts in the diabetic wounds, a culture and sensitivity assay on all the wounds was conducted to identify potential differences in the wound flora between the control and experimental groups. *Staphylococcus xylosus* was the only bacteria detected in each wound. Corrected zone of inhibition testing showed that *S. xylosus* was susceptible to the antimicrobial properties of silver in vitro. The silver dressing had an average CZOI of 5 mm while the gauze dressing had a zero CZOI against *S. xylosus* (Figure 4). This indicated that silver was able to kill *S. xylosus* at an initial bacterial concentration of 2.0 x 10^8 (cfu/ml). Silver was effective against *S. xylosus in vitro*, but this could be altered in vivo. Because the diabetic wounds have elevated...
wound glucose levels and *S. xylosus* thrives in high glucose environments, the authors wanted to determine if *S. xylosus* biofilms formed more readily in glucose-enriched environments compared to low glucose environments. Indeed, *S. xylosus* produced biofilms faster in higher glucose environments. Biofilms were not observed in 0 mg/dl glucose and until 10 hours of incubation. However, in higher levels of glucose, 250 mg/dl and 500 mg/dl, biofilm formation was observed after 3 hours of incubation (Figure 5). This may be a potential explanation of why silver was less effective against bacteria in high glucose diabetic wounds.

**Discussion**

This study shows that, in a mouse model, silver dressings improve healing times in both the control (wild type) group and experimental (diabetic) group when compared to gauze dressings. Silver-treated wounds in wild type mice showed a reduction in clinically significant quantitative culture values (> 10^5 cfu/g) on day 2 and day 5 compared to gauze-treated wounds. However, quantitative culture values showed a significant reduction only on day 2. Thus, the efficacy of the silver dressing was reduced over time, suggesting that silver dressings may need to be replaced after 48 hours to maintain antimicrobial efficacy. Surprisingly, silver dressings did not reduce the bioburden in diabetic wounds. There was statistically no difference in bacterial counts between silver- and gauze-treated wounds in diabetic mice. The lack of bioburden control in diabetic wounds might be due to resistant bacteria found only in the diabetic wounds. However, culture and sensitivity analysis showed that *Staphylococcus xylosus* was the only organism identified in wounds in both the control and experimental groups. The presence of *S. xylosus* was not unexpected because it is associated with mouse dermatitis. Nevertheless, it was surprising to identify only a single bacterial species, because most wounds contain multiple bacterial species. The paucity of bacterial species may be due, in part, to the fact that the mice are housed in specific pathogen-free facilities. Future studies using molecular techniques, such as real-time polymerase chain reaction may allow for more sensitive detection of other bacterial species.

Interestingly, *S. xylosus* is a biofilm producer. *A biofilm is a community of bacteria linked together by a layer of extracellular polysaccharides, which protects the bacteria from antibiotics and the host immune system. Normal host immune responses against bacteria include phagocytes (macrophages and neutrophils), antibodies, and complement proteins; these are all impaired when the bacteria have formed a protective biofilm.*

Increased biofilm production in diabetic wounds suggests the diabetic wound environment may be more conducive to bacterial growth compared to normal wounds. One potential difference could be glucose concentrations within the wound. Wound glucose concentrations are equal to blood glucose concentrations for the first 5 days after injury; they begin to decrease as the wound heals. This may be important because...
biofilm production has been shown to be dose dependent with higher levels of glucose producing thicker biofilms. Higher levels of glucose have been shown to induce biofilm formation mediated through the RBF gene in many staphylococci species. The RBF gene encodes a family of transcription regulators associated with biofilm formation. Increased glucose concentrations were associated with higher expression of the RBF gene and more biofilm production. Mutation in the RBF gene caused a reduction in biofilm production, indicating a critical role for the RBF gene in the formation of glucose-induced biofilms.

Silver’s microbicidal efficacy decreases when treating biofilms. An in vitro biofilm model showed that silver is only effective against mature P. aeruginosa biofilms at concentrations 10-100 times higher than the concentration required to kill planktonic bacteria. Based on these findings, silver concentrations in commercially available wound dressings may be too low to have antimicrobial activity against wound biofilms. This could be problematic because biofilm production is more prevalent in chronic wounds compared to acute wounds.

Conclusion

Patients with diabetes have impaired wound healing due to a delay in the onset of inflammation, and an increase in biofilm formation, which increases antimicrobial resistance and hinders the host immune response. Increased glucose levels may be responsible for the increased prevalence in diabetic wounds. Therefore, controlling glucose in patients with diabetes, either systemically, or at wound level, may be critical in the prevention of biofilm production rate. Significantly, this study demonstrates for the first time that silver dressings improve healing in diabetic wounds without decreasing bacterial bioburden in the wound. This supports the hypothesis that silver ions may have additional benefits for healing beyond antimicrobial activity. Further research will be needed to understand silver’s effect on the host immune and inflammatory responses involved in wound healing.

References

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