Comparison of the Effects of Antimicrobial Wound Dressings on Cell Viability, Proliferation, and Growth Factor Activity

Sarah Ramsay, Catherine Jelf, Duncan Aust, and Eric Roche

Healthpoint Research and Development, Fort Worth, TX

ABSTRACT

Successful management of wound bioburden is essential to proper wound healing, and silver dressings are well established for this medical need. Despite their popularity, silver dressings are not indicated for all wound healing situations. Certain silver dressings significantly delay reepithelialization in wound models (1), and silver nitrate has been found to be cytotoxic *in vitro* (1,2). Furthermore, heavy metals such as silver may interfere with the activity of commonly used protein therapeutics such as enzymatic debriders (3). A relatively new alternative to silver dressings is a polyvinyl alcohol (PVA) sponge complexed with the antimicrobial organic pigments methylene blue and gentian violet.

Three commercially available silver dressings and a pigment-complexed polyvinyl alcohol (PC-PVA) sponge were tested for their effects on cell viability and proliferation. In addition, because many wound care agents rely on growth factors for their healing activity, the effect of antimicrobial dressings on fibroblast growth factor (FGF) activity was evaluated. Extracts from the PC-PVA sponge were less cytotoxic than those from the silver dressings according to MTT assays. Similarly, extracts from the PC-PVA sponge exhibited less inhibition of cellular proliferation than two of the silver dressings as measured by BrdU assays. Furthermore, pre-incubating FGF with silver nitrate or extracts from a silver dressing substantially reduced the ability of the growth factor to promote cellular proliferation. FGF activity was not affected by incubation with gentian violet, methylene blue, or PC-PVA sponge extracts. These results suggest that the PC-PVA sponge offers an alternative to silver dressings that is less cytotoxic and less inhibitory toward cell proliferation. The PC-PVA sponge may also be more compatible with protein therapeutics and growth factors released by host cells to promote wound healing.

MATERIALS AND METHODS

MATERIALS

Dressings were obtained from Advanced Tissue (Little Rock Arkansas); human neonatal fibroblasts (HDFn) and supporting media from Cascade Biologics; BrdU (EMD Biosciences) and MTT (Chemicon) assays from VWR; and methylene blue, gentian violet, silver nitrate, and FGF from Sigma Aldrich.

CELLULAR VIABILITY AND PROLIFERATION ASSAYS (FIGURE 1)

Three 4 cm^2 samples were cut under aseptic conditions and each was incubated in 8 mls of Medium 106 plus LSGS for either 4, 8, or 24 hours at 37 °C, 5% CO_2. Media without dressing was used as a control and incubated in the same manner. HDFn were adhered to 96-well plates at a concentration of ~6500 cells/ well. Prior to treatment, cells were serum starved overnight in Media 106 with 0.1% BSA. For treatment, media was removed and replaced with 100 µl of dressing soaks and plates were incubated for 24 hours at 37ºC, 5% CO_2.

For the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay, MTT reagent was added during the final 3 hours of the assay and the assay was performed according to manufacturer's instructions. The final chromogenic readout reflects the conversion of insoluble formazan by live cells and is indicative of cellular viability. The mean values and standard deviations are from three independent experiments.

For the bromodeoxyuridine (BrdU) assay, BrdU was added during the final 24 hours of cell culture, and the assay was performed according to manufacturer's instructions. The final chromogenic readout at 450 nm reflects the incorporation of the analog into DNA by actively proliferating cells. The mean values are for two independent experiments with the range shown by error bars.

GROWTH FACTOR INTERACTION (FIGURE 4)

HDFn were adhered, starved, and treated as described above. FGF at a concentration of 1.3 µg/mL in serum free media was pretreated prior to assay of its activity in cell culture by mixing 1:1 with either dressing soaks or antimicrobial molecules and incubating for one hour at 37°C. Dressings were cut into 4 cm^2 squares and soaked in 8 mL of purified water for 24 hours at 37°C prior to growth factor incubation. 100 µg/ml solutions of gentian violet, methylene blue and silver nitrate were prepared in serum free media, resulting in a final FGF preincubation concentration of 50 µg/ml. The no treatment control contained FGF with serum free media only.

After one hour each growth factor solution was diluted 1:1000 in serum free media 106 to 50 ng/mL and 0.65 ng/mL for test articles and FGF, respectively. At this concentration test articles (GV, MB and SN) are not cytotoxic. Following 24 hour incubation cell proliferation was analyzed by BrdU assay as described above. Data are % relative to FGF alone, with means and standard deviations calculated based on 24 replicates from 3 independent experiments (8 replicates from each).

Figure 1: Assays for Cytotoxicity and Inhibition of Cell Proliferation

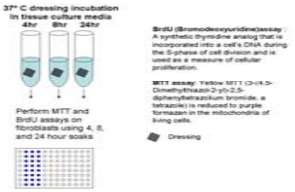
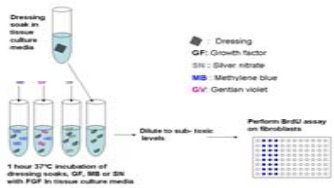
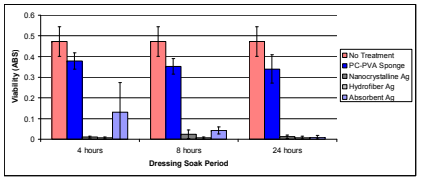


Figure 4: Assay for Growth Factor Inactivation



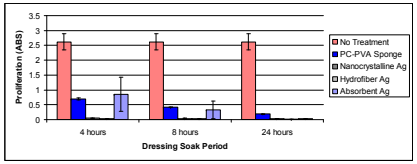
RESULTS

Figure 2: Reduction in Fibroblast Viability Caused by Dressing Soaks



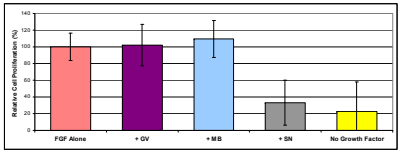
- Fibroblast viability was decreased by extracts from all tested dressings.
- Extracts from the three silver dressings had a much greater negative impact on cellular viability than extracts from the PC-PVA sponge.

Figure 3: Inhibition of Fibroblast Proliferation by Dressing Extracts



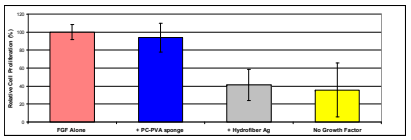
- Cellular proliferation was substantially decreased by all soaks.
- Extracts from nanocrystalline and hydrofiber silver dressings had a more deleterious effect on cellular proliferation than either the PC-PVA sponge or the absorbent silver dressing, especially at shorter soak times.

Figure 5: The Effect of Antimicrobial Molecules on FGF Activity



- Ionic silver in the form of silver nitrate inactivated FGF, causing a statistically significant reduction in activity to a level approaching that of a no growth factor control.
- Methylene blue and gentian violet, pigment components of the PVA sponge, did not reduce activity.
- After incubating silver with FGF, the mix was diluted 1000-fold and incubated for 24 hours in cell culture, yet no significant activity was recovered, suggesting inhibition is irreversible or high affinity.

Figure 6: Effects of Dressing Soaks on FGF Activity



- A soak from the hydrofiber dressing containing ionic silver produced a statistically significant reduction in FGF activity, leading to activity similar to a no growth factor control.
- Extract from the PC-PVA sponge did not significantly reduce FGF activity.

CONCLUSIONS

- The common silver dressings used in these studies contain silver in different forms and amounts. Nevertheless, extracts from a PC-PVA sponge were less deleterious to cell health and growth in our studies than extracts from silver dressings.
- The antimicrobial pigments in the PVA sponge do not appear to inhibit fibroblast growth factor activity. In contrast, ionic silver showed nearly complete inactivation of FGF.
- This inhibition of FGF by ionic silver appears to be either irreversible or high affinity inhibition.
- Cell survival, cell proliferation, and the activity of growth factors produced by host cells are all important for the process of wound healing. These dressing soak experiments indicate that compounds released from silver dressings have a more negative effect on these critical wound healing processes than those released from the PC-PVA sponge.
- In addition, exogenous growth factors are used clinically to promote wound healing. These results suggest that the PC-PVA sponge may be more compatible with growth factor therapy than silver dressings.

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2. Poon VK, Burd A. *In vitro* cytotoxicity of silver: implication for clinical wound care. *Burns*. 2004; 30: 140-7.
3. Falanga V. Wound bed preparation and the role of enzymes: a case for multiple actions of therapeutic agents. *Wounds*. 2002; 14(2): 47–57.

PC-PVA Sponge : (Pigment-Complexed Polyvinyl Alcohol Sponge)	Hydrofera® Blue foam dressing (Hydrofera, LLC), distributed by Healthpoint Ltd.
Hydrofiber Ag Dressing:	Aquacel® Ag hydrofiber dressing (Convatec)
Nanocrystalline Ag Dressing:	Acticoat™ Ag absorbent (Smith & Nephew),
Absorbent Ag Dressing:	Silvasorb® sheet (Acrymed)

Activity Against Wound Pathogens and Absorbency for Silver Dressings and a PVA Sponge Containing Organic Pigments

Stacey Solano-Kiedaisch, Catherine Jelf, Sarah Ramsay, Duncan Aust, and Eric Roche
Healthpoint Research and Development, Fort Worth, TX

ABSTRACT

Management of wound exudate and bioburden is fundamental to chronic wound care, and has led to increasing use of antimicrobial absorbent dressings. Silver is the principle antimicrobial used in dressings. An alternative is a dressing constructed of a polyvinyl alcohol (PVA) sponge complexed with the antimicrobial pigments gentian violet (GV) and methylene blue (MB). The capacity of this sponge to absorb fluid and thereby remove microbes from a wound was evaluated by soaking in phosphate buffered saline. The PVA sponge was roughly 1.5 to 4 times more absorptive per cm^2 than the tested silver dressings and also exhibited similar to superior absorbency compared to three highly absorptive dressings. The antimicrobial potential of the two pigments was compared to that of ionic silver by determining minimum inhibitory concentrations (MICs) against a range of wound pathogens. GV was generally the more active of the two pigments. GV was also more potent than silver nitrate (SN) against the assayed gram-positive bacteria with the greatest difference occurring against the most common wound pathogen, *Staphylococcus aureus*. The yeast *Candida albicans* was susceptible to GV in the same range as SN. SN was slightly more active than GV against most of the gram-negative bacteria tested. The antimicrobial effects of the PVA dressing and an ionic silver dressing were evaluated by fully absorbing bacterial suspensions, incubating for 24 hours at 37°C, extracting fluid, and performing quantitative microbiology. No viable *S. aureus*, *Pseudomonas aeruginosa*, or *Escherichia coli* were recovered from either dressing to the limit of detection. These studies suggest that a pigment-complexed PVA sponge offers a reasonable alternative to silver dressings by providing exceptional absorption coupled to broad spectrum antimicrobial activity.

MATERIALS

STRAINS
Microbes used in these studies were obtained from American Type Culture Collection (ATCC).

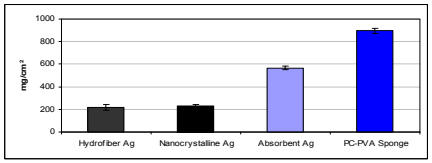
ABSORBANCE CAPACITY
A 4 cm^2 sample of each dressing was weighed and then placed into a weigh boat with 10 mL PBS (Figure 1) or PBS containing 10% fetal bovine serum (Figure 2). The weigh boats were placed in Petri dishes, covered with the dish lid, and incubated at 37°C for 24 hours. The dressings were then removed from the weigh boats, allowing excess fluid to drip off, and weighed. Values are the average of two independent experiments with the range shown by error bars.

MINIMUM INHIBITORY CONCENTRATIONS (MICS)
Broth microdilution MICs were performed in accordance with NCCLS Standards (1-2). Reported values and ranges are from 2-7 independent experiments, and control antimicrobials with quality control strains were within the acceptable range in all cases (3).

BACTERIAL SOAK AND RECOVERY
Bacterial colonies were suspended from agar plates and subsequently diluted to approximately $1x10^6$ cfu (colony forming units)/mL in PBS. A 700 µL volume of the suspension was applied to a 4 cm^2 sample of dressing in a sterile Petri plate. The dressings were incubated at 37°C for 24 hours. Fluid was extracted, and 10-fold serial dilutions were spotted onto dry agar media. Agar plates were incubated overnight at 37°C, and bacteria were counted at appropriate dilutions. Calculated cfu values are the average of 2-3 independent experiments, and 24-hour results were below the limit of detection (125 cfu) in all cases.

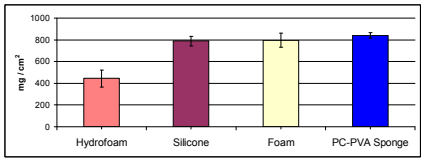
RESULTS

Figure 1: Superior Absorbency of PVA Sponge Compared to Common Silver Dressings



- Comparison of the 24-hour absorbency (weight fluid / area) of PBS.
- The least absorbent dressings were the hydrofiber Ag and nanocrystalline Ag, and the absorbent Ag and PVA Sponge were roughly 2.5X and 4X as absorbent.

Figure 2: Absorbency of PVA Sponge Compared to Other Highly Absorptive Dressings



- Comparison of the 24-hour absorbency (weight fluid / area) of PBS with 10% FBS.
- The absorbency of the PVA sponge was comparable to tested foam and silicone dressings, and nearly twice that of a hydrofoam dressing.

Table 1: MICs (µg/mL) for Pigments and Silver Nitrate Against Gram-Positive Species

		S. aureus ATCC 29213	*S. epidermidis* ATCC 12228	*E. faecalis* ATCC 29212	*S. pyogenes* ATCC 19615
Ionic Silver	Silver Nitrate	4-8	2-4	4-8	4
Organic Pigments	Gentian Violet	0.25-0.5	0.13-0.25	2-4	2-4
	Methylene Blue	16-32	8-16	4-32	1-8
Controls	Gentamicin	0.5-1	0.13	> 8	4-8
	Ciprofloxacin	0.5	0.25	1-2	0.25-0.5

- Genitan violet exhibited greater activity than methylene blue against tested microbes except for *Streptococcus pyogenes*, where activity was similar.
- Tested staphylococci were substantially more susceptible to GV than silver nitrate, and *Enterococcus faecalis* and *Streptococcus pyogenes* were marginally more susceptible.

Table 2: MICs (µg/mL) for Pigments and Silver Nitrate Against Gram-Negative Species

		E. coli ATCC 25922	*P. mirablis* ATCC 7002	*K. pneumoniae* ATCC 29019
Ionic Silver	Silver Nitrate	2-4	4-16	1-2
Organic Pigments	Gentian Violet	8-16	4-8	4
	Methylene Blue	> 128	> 128	> 128
Controls	Gentamicin	1	1-4	0.06-0.13
	Ciprofloxacin	0.016	0.06	0.016-0.03

- Similar activity for SN and GV was obtained against *Proteus mirabilis*.
- SN was slightly more potent than GV against *Klebsiella pneumoniae* and *Escherichia coli*.
- No activity was observed for methylene blue against gram-negative bacteria.

Table 3: MICs (µg/mL) for Pigments and Silver Nitrate Against Candida Species

		C. albicans ATCC 10231	*C. tropicalis* ATCC 750
Ionic Silver	Silver Nitrate	1-2	1
Organic Pigments	Gentian Violet	0.5	0.5-1
	Methylene Blue	64	128
Controls	Amphotericin B	0.03-1	0.5-1
	Fluconazole	0.5-1	0.25-0.5

- GV activity against *Candida albicans* was marginally greater than SN, but similar activity was observed against *Candida tropicalis*.
- Methylene blue exhibited modest activity against candida species.

Table 4: Recovery of Bacteria from Wound Dressings (cfu / mL)

	PC-PVA Sponge		Hydrofiber Ag	
	0 hr	24 hr	0 hr	24 hr
***S. aureus* ATCC 29213**	1.4×10^6	< 125	1.4×10^6	< 125
***E. coli* ATCC 25922**	6.8×10^5	< 125	6.8×10^5	< 125
***P. aeruginosa* ATCC 27853**	4.2×10^5	< 125	4.2×10^5	< 125

- Bacterial suspensions (~10^6 bacteria/mL) were absorbed into the pigment-complexed PVA sponge or a hydrofiber silver dressing.
- After incubation for 24 hours, no bacteria were recovered to the limit of detection for either dressing.

CONCLUSIONS

- The pigment-complexed PVA sponge exhibits greater absorbency compared to tested silver dressings, and similar or greater absorbency compared to other highly absorbent dressings.
- Of the two organic pigments complexed to the PVA sponge, gentian violet exhibited greater activity than methylene blue against all microbes except for *Streptococcus pyogenes*, where the two molecules were similar.
- MIC values for gentian violet and ionic silver in the form of silver nitrate against assayed gram-positive strains indicate a greater antimicrobial potency of GV against those species. In particular, GV exhibits a substantially more potent activity (~16 fold greater) against *Staphylococcus aureus* and *epidermidis* compared to SN.
- Antimicrobial activity of SN was generally greater than that of GV against gram-negative bacterial species, and within the same range as GV against the yeasts *C. albicans* and *C. tropicalis*.
- Both the pigment-containing PVA sponge and the hydrofiber Ag dressing effectively eliminated bacteria from fluid recovered 24 hours after absorbing bacterial suspensions into the dressings.
- These *in vitro* results suggest that the PVA sponge containing organic pigments can serve as an alternative to silver dressings by providing exceptional absorption combined with broad spectrum antimicrobial activity.

REFERENCES

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Absorbent Ag Dressing:	Silvasorb® sheet (Acrymed)
Hydrofoam Dressing:	Polymem® hydrofoam (Ferris MFG)
Silicone Dressing:	Mepilex® (Molnlycke)
Foam Dressing:	Allevyn™ (Smith & Nephew)