

# Cytotoxicity to Cultured Human Keratinocytes of Topical Antimicrobial Agents<sup>1</sup>

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Cultured skin grafts administered clinically for closure of burn wounds may be contacted by topically applied antimicrobial agents. A study was performed to assess whether commonly used topical antimicrobial agents are toxic to cultured human keratinocytes (HK) *in vitro*. Serum-free MCDB 153 culture medium containing Neosporin G.U. irrigant (Neomycin, 40 µg/ml—polymyxin B sulfate, 200 units/ml) and a standard tissue culture antimicrobial agent of penicillin (10,000 units/ml)—streptomycin (10,000 µg/ml)—amphotericin B (25 µg/ml) had no effect on the keratinocyte growth rates when compared to standard MCDB 153 medium without antibiotics. Medium containing Sulfamylon (mafenide acetate, 0.85%), Polysporin (polymyxin B sulfate, 1 × 10<sup>4</sup> units/ml—bacitracin, 500 units/ml), gentamicin sulfate (0.1%), modified Dakins solution (25%), and acetic acid (0.25%) all showed statistically significant ( $P < 0.01$ ) decreases in keratinocyte growth rates. This data suggests that commonly applied antimicrobials may not be appropriate for cultured grafts in the concentrations that are used clinically. © 1990 Academic Press, Inc.

## INTRODUCTION

Coverage of the excised wound is a major thrust in burn care today. Early excision of full-thickness injuries, with adequate coverage, has led to increased survival of extensively burned patients [1-3]. Early excision imposes a significant requirement for coverage of excised areas, especially if donor sites for autologous skin grafts are limited. Conventional treatment for the temporary closure of these wounds has been dominated in recent years by cadaveric allografts. A variety of biologic and synthetic dressings have also been developed and tested clinically [4, 5]. More recently a number of research centers, including our own, have had success with the development of skin substitutes [6-9].

Skin substitutes have taken many forms, including cultured epidermal sheets [6, 10], collagen-GAG matrix

with Silastic membrane [11, 12], allogenic dermis with cultured keratinocytes [7, 13], and collagen gels with cultured keratinocytes [8] to name a few. Our model employs a collagen-GAG matrix containing autologous fibroblasts and autologous keratinocytes attached *in vitro* to an external surface [9, 14]. The keratinocytes are restricted to the surface by a nonporous collagen-GAG film applied to one side of the collagen-GAG sponge. This model has proven to be successful in our athymic mouse model [15] and in limited clinical studies [16].

With the increasing number of burn centers employing skin substitutes, the clinical administration and management of these relatively fragile grafts needs to be addressed. It is reported that these grafts are more prone to destruction by infection than are split thickness skin grafts [4, 17]. Appropriate topical antimicrobial agents and postoperative dressings for cultured skin substitutes have not been rigorously tested. The present study evaluates the cytotoxic effects of widely used antimicrobial agents on cultured human keratinocytes *in vitro*.

## MATERIALS AND METHODS

**Tissue culture conditions.** Human keratinocyte (HK) cultures were initiated from surgical discard skin or were obtained from the UCSD Regional Tissue Bank with informed consent of the donor's family. HK were grown in serum-free nutrient medium MCDB 153 containing 0.2 mM/liter calcium, increased amounts of selected amino acids [18], 10 ng/ml epidermal growth factor (GIBCO Laboratories, Grand Island, NY), 5 µg/ml insulin, 0.5 µg/ml hydrocortisone, 0.1 mM/liter ethanolamine, 0.1 mM/liter phosphoethanolamine, 0.5% (vol/vol) bovine pituitary extract and penicillin (10,000 units/ml)—streptomycin (10,000 µg/ml)—amphotericin B (25 µg/ml) (PSF) (GIBCO Laboratories) as an antibiotic-antimycotic agent [19-21]. Keratinocytes were incubated at 37°C, 5.0% CO<sub>2</sub>, and saturated humidity.

**Experimental conditions and assay of HK growth.** HK were inoculated into 60-mm petri dishes (Lux 5220, Miles Scientific, Naperville, IL) from subconfluent primary

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cultures at a density of  $5 \times 10^2/\text{cm}^2$ . The medium was changed to a non-PSF medium on Day 3 postinoculation. On Day 4, medium was changed again and antimicrobial test agents were titrated in at decreasing half-log concentrations, beginning with a concentration of test agent most closely approximating the clinical dose. At this time, baseline cell counts (Coulter Electronics, Inc., Hialeah, FL), osmolarity (Advanced Digimatic Osmometer, Model 3DII, Advanced Instruments, Inc, Needham Heights, MA), and pH (Corning pH Meter 145, Corning, Littleton, CO) were measured for each agent at its highest concentration. Controls included medium with no antimicrobial agent, standard cell culture antimicrobial agent (PSF), highest measured osmolarity (added NaCl), and medium-high osmolarity (added NaCl; Fig. 1). HK were then harvested on Day 8 and counts were obtained using a Coulter counter. Data points of all assays are expressed as means  $\pm$  standard error of the mean (SEM) of triplicate dishes, from duplicate assays using separate strains of HK.

**Antimicrobial agents.** Serial dilutions of agents with half-log concentrations were prepared. Table 1 lists the individual agents, together with their highest concentration tested and the clinical dose commonly administered.

## RESULTS

The results of the experimental data are compiled in Table 2. This table lists percentage cell growth, or proliferation of human keratinocytes as compared to standard MCDB 153 without PSF. Standard error of the mean is included for each value. Osmolarity and pH are included for each agent at their highest tested concentration ( $X$ ). Pairwise comparison (Student's  $t$  test) of each agent to the control was performed. Statistically significant ( $P < 0.05$ ) reduction in growth of cultured keratinocytes is noted (\*).

*Sulfamylon (mafenide acetate, Winthrop-Breton Laboratories, New York, NY).* The data show Sulfamylon to

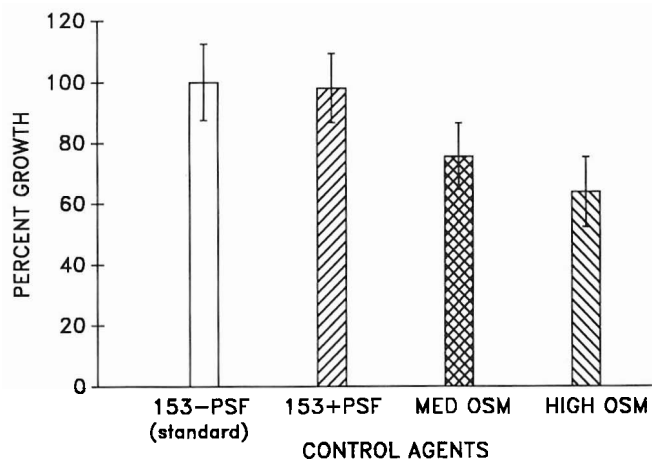


FIG. 1. Effect of medium osmolarity, high osmolarity, and standard tissue culture antimicrobial agent on keratinocyte growth.

TABLE 1

Agent	Highest test dose	Clinical dose
Sulfamylon (mafenide acetate)	0.85%	8.5%
Polysporin		
Polymyxin B sulfate	$1 \times 10^4$ units/ml	$1 \times 10^4$ units/ml
Bacitracin	500 units/ml	500 units/ml
Neosporin G.U. irrigant		
Neomycin sulfate	40 $\mu\text{g}/\text{ml}$	40 $\mu\text{g}/\text{ml}$
Polymyxin B sulfate	200 units/ml	200 units/ml
Gentamicin sulfate	1.0 mg/ml (0.1%)	1.0 mg/ml
Acetic acid	0.25%	0.25%
Modified Dakins solution	25%	25-50%

decrease cell growth across a broad range of concentrations (Fig. 2). The beginning concentration was 0.85%, which is already  $10\times$  less than the clinical dose, and was chosen in order to keep osmolarity closer to the physiologic range. At  $X^{-2}$  (or  $1000\times$  less concentrated than clinical doses) Sulfamylon continued to reduce cell growth to a statistically significant level. The highest concentration medium of mafenide acetate had a pH of 7.36, which should be nontoxic. The corresponding medium's osmolarity was 398, which is less than the high osmolarity control of 401. However, cellular growth ( $6.5 \pm 0.89$ ) was significantly ( $P < 0.05$ ) less than for this control ( $64 \pm 6.5$ ). Therefore, reduction in HK growth can be attributed to Sulfamylon.

*Polysporin (Burroughs Wellcome Co., Research Triangle Park, NC).* Polysporin consists of two antibiotics: bacitracin (500 units/ml) and polymyxin B sulfate (10,000 units/ml) in a white petrolatum base. For this study, a combination of these two antibiotics in clinically comparable doses was employed—bacitracin (Pharma-Tek, Inc., Huntington, NY) and polymyxin B sulfate (Roerig, New York, NY). The data on this combination of bacitracin and polymyxin B sulfate showed marked inhibition of proliferation at the clinical doses (Fig. 3). At  $X^{-1}$  (10-fold dilution), the relative growth was only 11%. This effect was almost reversed by  $X^{-1.5}$ , with an increase to 83% cell growth. Because this sample contained two drugs, each was tested separately in the standard assay protocol. Bacitracin alone showed decreased cell proliferation at the higher concentrations, but returned to nonsignificant levels by  $X^{-1}$ . By contrast, Polymyxin B sulfate had low cell growth until  $X^{-1.5}$ , at which concentration growth returned to control levels. Osmolarity and pH were normal. Overall, the combination of bacitracin and polymyxin B sulfate, as found in Polysporin, decreased keratinocyte proliferation at the higher concentrations. This reversed, but only at the lower concentrations, with polymyxin B sulfate causing the greater reduction of HK growth.

*Neosporin G.U. irrigant (Burroughs Wellcome Co., Research Triangle Park, NC).* The data on G.U. irrigant shows it to have little, if any, negative effect on the proliferation of keratinocytes in this assay (Fig. 4). Polymyxin

**TABLE 2**  
**Effects of Antimicrobial Agents on Growth of Cultured Keratinocytes**

Agent	mOs	pH	% Cell growth relative to standard concentration				
			X	X <sup>-0.5</sup>	X <sup>-1</sup>	X <sup>-1.5</sup>	X <sup>-2</sup>
MCDB 153-PSF	343	7.50	100 ± 7.5				
MCDB 153 + PSF	343	7.52	107 ± 7.4				
153 + high mOs	401	7.50	64 ± 12				
153 + med mOs	371	7.50	76 ± 11				
Polysporin	347	7.42	9.7 ± 0.43*	6.6 ± 0.24*	11 ± 0.77*	83 ± 13	68 ± 9.8*
Bacitracin	365	7.44	15 ± 3.5*	65 ± 8.6*	85 ± 12	105 ± 13	85 ± 12
Polymyxin B sulfate	360	7.54	8.9 ± 0.44*	5.9 ± 0.56*	43 ± 6.2*	103 ± 14	105 ± 14
Neosporin G.U.	339	7.50	106 ± 12	87 ± 12	98 ± 12	79 ± 10	80 ± 12
Gentamicin	333	7.32	6.4 ± 0.62*	50 ± 1.9*	95 ± 1.7	108 ± 2.0	106 ± 2.8
Sulfamylon	398	7.36	6.5 ± 0.89*	11 ± 2.0*	27 ± 4.9*	50 ± 6.6*	67 ± 9.5*
Modified Dakins	445	7.87	11 ± 0.29*	17 ± 1.7*	11 ± 0.61*	69 ± 1.0*	94 ± 0.66
Acetic acid	385	5.20	6.0 ± 0.22*	59 ± 2.4*	101 ± 1.9	102 ± 1.6	104 ± 2.2

\* P < 0.05.

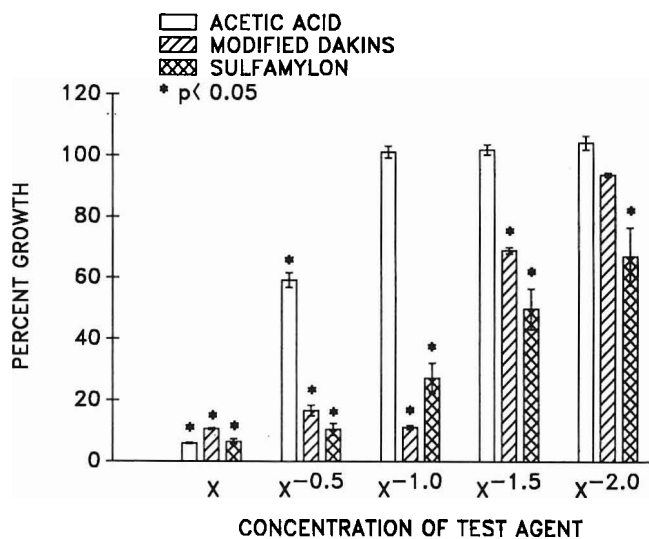
B sulfate had no negative effect, which correlates well with the data on polymyxin B at similar concentrations in polysporin. A level of 200 units/ml would be comparable to between X<sup>-1.5</sup> and X<sup>-2</sup> on the polymyxin B curve (Fig. 3), which yielded normal cell numbers. Neomycin sulfate, as a component of G.U. irrigant, also had no negative effect on cell proliferation, as no inhibition was noted in the combination. Osmolarity and pH were normal.

*Gentamicin sulfate* (Elkins-Sinn, Inc. Cherry Hill, NJ). The standard dose of gentamicin as a topical agent is 1.0 mg/ml (0.1%), which was the highest concentration (X) tested. Cell proliferation was inhibited at this concentration, but by X<sup>-1</sup> dilution, total cell numbers had returned to control values (Fig. 4). Osmolarity was slightly

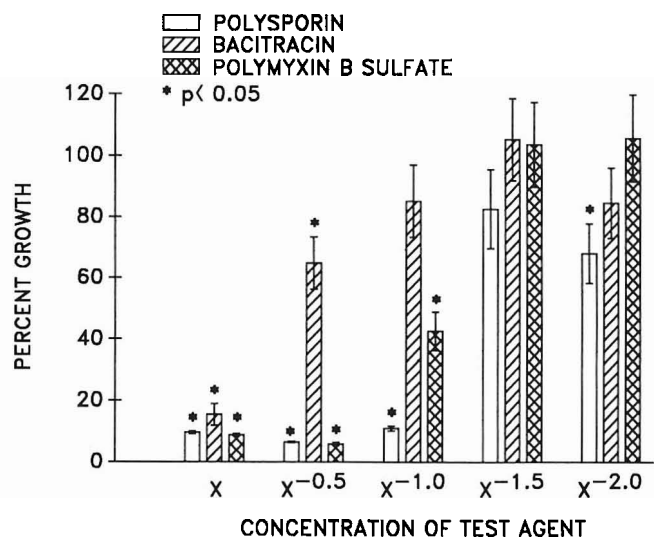
low, but should not have had an adverse effect. The pH was slightly acidic (7.32), but other agents in this pH range or lower (acetic acid at X<sup>-1</sup>) did not show an adverse effect on proliferation.

*Acetic acid.* Acetic acid used clinically for irrigation is supplied as a 0.25% solution. An acidic pH (5.20) was found at the highest strength tested (0.25%). This acidic medium definitely reduces cell proliferation (Fig. 2). At X<sup>-1</sup> dilution (or a concentration decrease of 10-fold) the pH had risen to 7.18 and cell proliferation is comparable to that of controls.

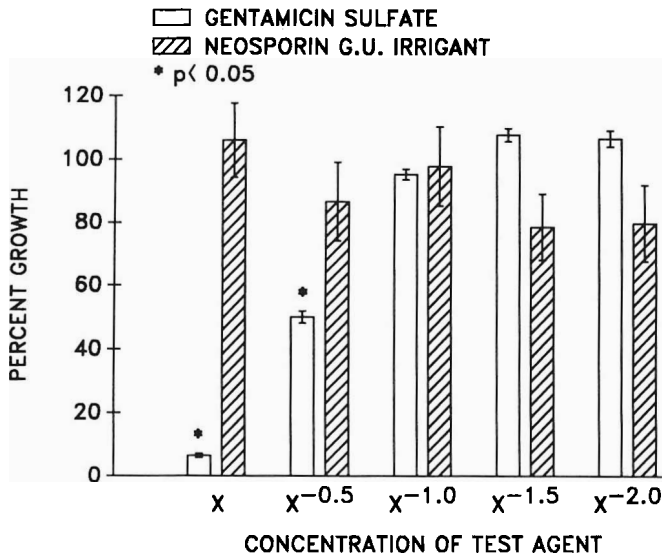
*Modified Dakins solution.* This compound is made from household bleach, baking soda, and water. The standard solution is 12 ml bleach (Clorox Bleach, 5.25%



**FIG. 2.** Effect of concentration of antimicrobial agents on cultured keratinocyte growth. "X" equals highest concentration of tested agent. Exponent equals dilution factor.



**FIG. 3.** Effect of concentration of antimicrobial agents on cultured keratinocyte growth. "X" equals highest concentration of tested agent. Exponent equals dilution factor.



**FIG. 4.** Effect of concentration of antimicrobial agents on cultured keratinocyte growth. "X" equals highest concentration of tested agent. Exponent equals dilution factor.

sodium hypochlorite, The Clorox Co., Oakland, CA), 120 ml water, and 10 ml baking soda. This concentration of solution is referred to as full strength modified Dakins solution. The highest value tested was 25%, or  $\frac{1}{4}$  strength, due to its high osmolarity and pH. Our data show that this solution reduces keratinocyte proliferation until it reaches the  $X^{-2}$  dilution (0.25% strength solution or 0.00125% sodium hypochlorite; Fig. 2). There are also considerable problems with the 25% solution of high osmolarity (445 mOs) and basic pH of 7.87. At  $X^{-1}$  (10-fold dilution), the osmolarity is down to a more acceptable 387 and the pH is 7.66. The agent is still completely inhibitory to proliferation of keratinocytes at this dilution.

## DISCUSSION

The data in this paper show that many of the commonly used topical antimicrobial agents have profound cytotoxic effects on human keratinocyte growth *in vitro*. For this study, emphasis was placed on evaluating these agents under the clinical conditions to which the cultured skin substitutes will be subjected. The role of antimicrobial agents in burn care has been one of controlling the inevitable microbial colonization of both eschar and open, debrided wounds. The design of an effective antimicrobial must include broad spectrum of activity, the ability to penetrate the eschar without having significant systemic absorption, low local histotoxicity, low systemic toxicity, and minimal selection of resistant organisms. Other practical matters include ability to obviate pain, low cost, number of dressing changes, and availability [22]. In treating cultured grafts, cytotoxicity is crucial, as these grafts are more fragile initially than split thickness skin

grafts. Lineweaver showed that human fibroblast toxicity exceeded bacterial toxicity with some antimicrobial agents [23]. Therefore, the agents which have been used clinically to control and treat bacterial colonization may not be the most appropriate choices to keep cultured skin substitutes both viable and free from infection. Each agent must act within the wound healing environment in such a way as to minimize the histotoxicity while maintaining its antimicrobial effectiveness.

The effects which are seen as reduced growth rates of cultured keratinocytes are not necessarily due to the direct toxic nature of each agent. Successful tissue culturing of keratinocytes requires careful attention to many biologic parameters [24]. Of these, osmolarity and pH are two of the more important. In this study, controls for osmolarity have been included by adding NaCl in an amount to correlate with the highest measured osmolarity of the agents. A control using the standard MCDB 153 with penicillin-streptomycin-amphotericin B (PSF) was also included (Fig. 1).

Sulfamylon (mafenide acetate) is applied as an 8.5% cream or mixed with saline to form a slurry. It has bacteriostatic properties against gram-negative and gram-positive organisms, especially *Pseudomonas aeruginosa* and some anaerobes. Mafenide acetate as a slurry, or in aqueous form as we tested it, could potentially be used as an antimicrobial on cultured grafts. The data, though, show substantial reduction of keratinocyte proliferation across a broad dilutional range, thus making this agent not desirable in the initial treatment of these grafts.

Polysporin is supplied as an ointment and is used widely for minor cuts, burns, and abrasions. In burn care it is often used as a topical antimicrobial on superficial-partial thickness burns and on burns to the face. Bacitracin is antibacterial *in vitro* against many gram-positive and a few gram-negative organisms. Polymyxin B sulfate is effective against all gram-negative bacilli except *Proteus* species.

Neosporin G.U. irrigant is an antibiotic combination commonly used in urinary bladder irrigation. The two ingredients are neomycin sulfate and polymyxin B sulfate. The clinical doses are achieved once the supplied concentrate is diluted in 1 liter of sterile saline. Polymyxin B sulfate has been discussed previously, but is found in lower concentration in G.U. irrigant than in polysporin. Neomycin sulfate, an aminoglycoside, is effective on gram-positive cocci, gram-negative bacilli, and gram-negative cocci. Systemic complications of these two agents include nephrotoxicity, neurotoxicity, neuromuscular blockade, and hypersensitivity reactions. In the intact bladder, there is minimal systemic absorption. When applied to open wounds, burns, and granulating surfaces there is risk of considerable systemic absorption.

Gentamicin sulfate is another aminoglycoside with actions and adverse effects similar to neomycin sulfate. It is used as a parenteral agent in most cases, but also as a topical agent, especially in its cream form. It is an excel-

lent agent for gram-negative bacilli and has moderate activity against some gram-positive cocci.

The agents polysporin, neosporin G.U. irrigant, and gentamicin have not often been used in large burns as topical antimicrobial agents. These drugs could be absorbed in large quantities, with systemic side effects being a great risk. However, they could be used for short durations in treating the cultured grafts early postplacement, when they are especially susceptible to infection.

From the data, Neosporin G.U. irrigant does not effect keratinocyte proliferation and would be a good choice. Gentamicin sulfate, at lower concentrations, would also be a potentially useful agent. Again, these agents should be used cautiously, for a limited time, to avoid their potential systemic toxicities.

Acetic acid has been shown to have no gross or microscopic effect on reepithelialization of donor sites when compared to saline [25]. Dilute acetic acid has been used clinically on cultured epithelial autografts by Gallico *et al.* [26]. They state that topical antibiotics were toxic to the autografts in the first week postplacement, but that after 1 week dilute acetic acid or silver nitrate (AgNO<sub>3</sub>) allowed epithelialization to progress. Clinicians often use this at  $\frac{1}{4}$  strength, which is effective against a variety of gram-positive and gram-negative organisms. In this study, acetic acid only reduced cell proliferation at the higher concentrations. It would be an acceptable agent at  $\frac{1}{10}$  strength to treat these grafts, and there is no apparent need to wait 1 week as previously reported [26]. Whether acetic acid continues to act as an effective antimicrobial at this concentration needs further study.

Modified Dakins solution (active ingredient, sodium hypochlorite) is an effective antimicrobial topically and has been used for sterilization and disinfection for many years. This agent has also been used to effectively decontaminate human skin prior to harvesting basal cells for the use of cultured epidermal grafts [27]. Cotter *et al.* also showed that 0.5% sodium hypochlorite was toxic to basal cells if treated for 14 days [27]. This concentration of sodium hypochlorite corresponds to full strength modified Dakins solution. From our data, this agent is quite toxic to keratinocytes and is not an appropriate agent for these grafts.

Silver sulfadiazine and silver nitrate are agents which are used as topical antimicrobials in burn care. Silver sulfadiazine as a 1% cream (Silvadene) is the mainstay in topicals used today. Dr. Charles Fox, who first synthesized silver sulfadiazine [28], supplied material for use in powdered form. It is currently not used as a topical agent on newly placed cultured grafts, but testing this material was attempted. Silver nitrate (0.5%) is used as an aqueous solution by some centers for the treatment of burns. Gallico *et al.* [26] state that silver nitrate was toxic to cultured epithelial grafts in the first week, but thereafter did not inhibit epithelialization of the wound. Absorption of silver through the wound is inhibited by its precipitation with chloride and proteinate salts [22]. We found that this also

happened *in vitro* with the chloride and proteins in tissue culture medium. Silver nitrate was readily soluble in water, but silver sulfadiazine powder was not soluble in base, acid, or other solvents which could facilitate its use in this experiment. Further study of these agents will require another assay format.

Contamination of burn wounds by one or more microbial organisms can cause failure of skin grafts. Cultured skin substitutes described to date have greater susceptibility to destruction by infection than split-thickness skin grafts [4, 17]. The data in this study show that, at very low concentrations, many of these antimicrobial agents tested are nontoxic to the keratinocytes. But at these dilutions, the individual agents may be below the MIC value required to be effective and may indeed lead to an environment capable of producing resistance to the respective agents. Resolution of this problem requires identification of drugs which at defined concentrations allow acceptance and persistence of cultured skin grafts, while arresting or reversing the progression of microbial infections already present. This study takes an initial step toward enhancing the survival of cultured skin substitutes in combination with topical agents used clinically to control wound infection.

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