# Cytotoxicity Testing of Topical Antimicrobial Agents on Human Keratinocytes and Fibroblasts for Cultured Skin Grafts

Steven T. Boyce, PhD, Glenn D. Warden, MD, and Ian Alan Holder, PhD
*Cincinnati, Ohio*

**Cultured epidermal skin has become an adjunctive therapy for treatment of major burn injuries, but its effectiveness is greatly limited because of destruction by microbial contamination. To evaluate candidate antimicrobial agents for use with cultured skin, a combined cytotoxicity-antimicrobial assay system was developed for determination of toxicity to cultured human keratinocytes and fibroblasts and for determination of susceptibility or resistance of common burn wound organisms. Candidate agents including chlorhexidine gluconate, polymyxin B, mupirocin, sparfloxacin, or nitrofurazone were tested separately for inhibition of growth of human cells and for inhibitory activity to microorganisms with the wet disk assay. The data showed that (1) chlorhexidine gluconate (0.05%) was uniformly toxic to both cultured human cells and microorganisms; (2) nitrofurazone (0.02%) had dose-dependent toxicity to human cells and limited effectiveness against gram-negative microorganisms; (3) sparfloxacin (30 μg/ml) had low toxicity to human cells and retained antimicrobial activity against both gram-positive and gram-negative bacteria; (4) polymyxin B (400 U/ml) was not toxic to human cells and had intermediate effectiveness on gram-negative bacteria; and (5) mupirocin (48 μg/ml) had no toxicity to skin cells and had uniform effectiveness against *Staphylococcus aureus* including methicillin-resistant *Staphylococcus aureus*. Selection of topical antimicrobial drugs by these assays may improve effectiveness of cultured skin for burns and may be used to control other surgical wound infections. (J BURN CARE REHABIL 1995;16:97-103)**

Recent advances in the grafting of burns and giant congenital nevi include cultured epithelium[1-3] alone or in combination with biopolymer implants[4-6] or allodermis.[7,8] However, incomplete epidermal barrier and lack of vascular and immune components at the time of grafting make all models[9-11] of cultured skin more subject to destruction by burn organisms than native skin grafts. These biologic deficiencies also make cultured epidermal keratinocytes more subject to cytotoxicity of topical antimicrobial agents. Most parenteral antimicrobial drugs are effective and have low toxicity, but topical use may induce resistant organisms that complicate treatment of sepsis. Therefore requirements for topical antimicrobial agents for cultured skin are low toxicity to cultured human skin cells, high activity against common burn wound organisms, and no overlap of activity with parenteral drugs.

Assays for agents that meet these requirements have tested cytotoxicity to cultured human fibroblasts[12] or keratinocytes,[13] antimicrobial activity on microorganisms,[14-16] or spectra of toxicity to combinations of fibroblasts and burn organisms.[17-20] This article describes a combined cytotoxicity-antimicrobial assay system in which candidate agents may be tested for toxicity to cultured human epidermal keratinocytes and fibroblasts and for antimicrobial activity to common burn wound organisms. Results of this study also identify candidate agents that qualify for evaluation in vivo with cultured skin.

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*Reprint requests: Steven Boyce, PhD, Shriners Burns Institute, Research Department, 3229 Burnet Ave., Cincinnati, OH 45229-3095.*
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**Table 1.** Antimicrobial agents tested

| Agent | Highest test concentration | pH* | mOsm* |
|---|---|---|---|
| Antiseptic | | | |
| Chlorhexidine gluconate | 0.5% (wt/vol) | 7.36 | 331 |
| Antibiotics | | | |
| Polymyxin B | 4000 U/ml | 7.45 | 328 |
| Mupirocin | 160 μg/ml | 7.45 | 335 |
| Sparfloxacin | 100 μg/ml | 7.66 | 334 |
| Nitrofurazone | 0.2% (wt/vol) | 7.45 | 744 |

*pH and osmolarity of highest test concentrations in culture medium for epidermal keratinocytes.

## MATERIAL AND METHODS

**Experimental Design.** The experimental algorithm by which candidate antimicrobial agents are qualified as having no or low toxicity to cultured human skin cells, and antimicrobial activity to common burn wound organisms has been described elsewhere.[17] After determination of the highest concentration of a candidate antimicrobial agent that was not cytotoxic to cultured keratinocytes and fibroblasts, an equal or lower concentration was tested for antimicrobial activity on representative samples of microorganisms isolated from burn wounds.

**Antimicrobial Agents.** Table 1 lists antimicrobial agents tested, the highest test concentrations, and their pH and osmolarity values in cell culture medium. All agents tested except sparfloxacin are used as active antimicrobial compounds in formulations available for clinical use: chlorhexidine in Hibiclens antimicrobial skin cleanser (Stuart Pharmaceuticals, Wilmington, Del.); polymyxin B in Neosporin GU irrigant (Burroughs-Wellcome & Company, Research Triangle Park, N.C.); mupirocin in Bactroban ointment (SmithKline Beecham, Philadelphia, Pa.); and nitrofurazone in Furacin soluble dressing (Roberts Pharmaceuticals, Eatontown, N.J.). Sparfloxacin, an experimental quinolone antimicrobial, was supplied by Parke-Davis Company, Ann Arbor, Michigan. All antimicrobial agents in pure powder form were diluted to the experimental concentrations with sterile saline solution for susceptibility testing against microorganisms or in aqueous culture medium (see following) for cytotoxicity testing. To increase the solubility of nitrofurazone the initial amount of dry powder was dissolved in 1 ml propylene glycol before it was diluted in saline solution or culture medium to the experimental concentrations. For human cell assays each agent was diluted serially at half-log concentrations to 100 times the highest test dose into respective growth media for keratinocytes or fibroblasts. For antimicrobial assays the clinically relevant concentrations were tested on clinical isolates of burn organisms (see following).

**Human Cell Assay.** Human epidermal keratinocytes and dermal fibroblasts were isolated from surgical discard tissue obtained with approval of the University of Cincinnati Institutional Review Board. Epidermal keratinocytes were grown as described previously[21,22] in serum-free MCDB (molecular, cellular, and developmental biology) 153 medium with 0.2 mmol/L calcium and increased amino acids[23]; they were supplemented with 0.5% bovine pituitary extract, 1 ng/ml epidermal growth factor, 5 μg/ml insulin, and 0.5 μg/ml hydrocortisone. Dermal fibroblasts were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 1 ng/ml epidermal growth factor, 5 μg/ml insulin, and 0.5 μg/ml hydrocortisone. Keratinocytes or fibroblasts were inoculated into 60 mm petri dishes at $2 \times 10^4$ cells/dish ($1 \times 10^3$ cells/cm$^2$) into the respective culture media described previously. These media also contained a combination of penicillin (10,000 U/ml), streptomycin (10,000 μg/ml), and amphotericin B (25 μg/ml). Cells were incubated 4 days at 37° C in 5% $CO_2$ with saturated humidity. Cell counts were performed on a Coulter model $Z_{BI}$ particle counter (Coulter Electronics, Inc., Hialeah, Fla.). On day 4 media were changed to remove penicillin-streptomycin-amphotericin B, baseline cell counts were performed, and test compounds were titrated into the cell cultures. Cells were incubated 4 additional days, harvested, and counted.[13] Each experimental condition was performed in triplicate, and each experiment was repeated once ($n = 6$).

**Wet Disk Assay.** Representative strains of gram-positive *(Staphylococcus aureus)* and gram-negative

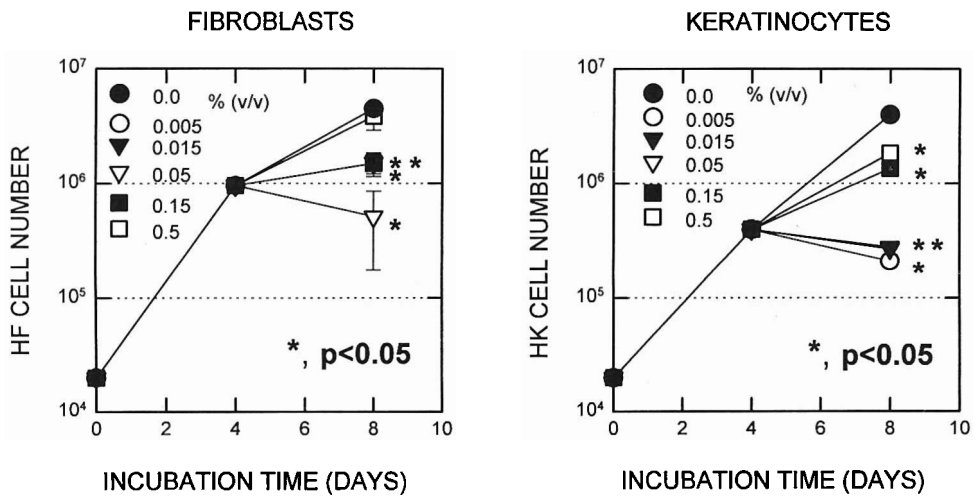


**Figure 1.** Dose response of cultured human keratinocytes and fibroblasts to chlorhexidine gluconate. Test conditions were initiated on culture day 4. All concentrations are cytotoxic to both cell types. Apparent reduction of toxicity at concentrations of 0.15% and 0.5% is artifactual counting of chemical precipitate in culture medium.

*(Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterobacter cloacae, Escherichia coli)* bacteria were isolated from wounds of patients with burns at the Shriners Burns Institute, Cincinnati Unit. Petri dishes (150 × 15 mm) containing Mueller-Hinton agar (Becton-Dickinson Microbiology Systems, Cockeysville, Md.) were inoculated by uniformly swabbing the plate surface with a suspension of each organism diluted to 0.5 McFarland density units. Sterile 6 mm filter paper disks were placed on the microbial lawns and received 25 μl of the highest test dose of antimicrobial solution. Dishes were incubated at 37° C overnight, and zone of clearing was measured in diameter.[15]

**Data Collection, Analysis, and Interpretation.** Data collected from human cell assays were tested for significance ($p < 0.05$; analysis of variance) compared with cultures that received no drug. Antimicrobial agents tested in wet disk assays were scored as effective, if the zone of clearing was 2 mm or greater in diameter. This zone diameter indicated whether the test agent demonstrated antimicrobial activity. By evaluation of combined results from both assays, dosages of test agents that had no significant toxicity to cultured cells and retained antimicrobial activity in wet disk assays could be considered for topical use with cultured skin.

## RESULTS

**Human Cell Assays.** Table 1 lists the pH and osmolarity of the highest concentrations of agents tested on human skin cells. Ranges of pH were 7.36 for chlorhexidine to 7.66 for sparfloxacin. Osmolarity ranged from 328 for polymyxin B to 335 for mupirocin except for nitrofurazone, which was very hypertonic at 744 mOsm. Figure 1 shows that chlorhexidine gluconate at concentrations as low as 0.005% (wt/vol) inhibits growth of both fibroblasts and keratinocytes. Higher concentrations of chlorhexidine gluconate (i.e., 0.15% to 0.5%) generated counts that were increased by artifact. Those concentrations formed particulate precipitate that was counted by the Coulter counter but contained no cultured cells. Titration of polymyxin B (Figure 2) over two log dilutions cause no inhibition of growth of fibroblasts, but did cause significant growth inhibition of keratinocytes at the highest test dose (4000 U/ml). Figure 3 shows that mupirocin (160 μg/ml) was completely nontoxic to both fibroblasts and keratinocytes at the concentrations tested. Similarly sparfloxacin was nontoxic to cell growth at all concentrations up to 100 μg/ml as shown in Figure 4. Nitrofurazone (Figure 5) showed concentration-dependent toxicity to both fibroblasts and keratinocytes and had very limited solubility at concentrations higher than those clinically relevant (0.02% /wt). Although these results do not distinguish whether detected cytotoxicity results from nitrofurazone or the propylene glycol vehicle, toxicity of the formulated agent at clinically relevant concentrations disqualifies this compound for use with cultured skin grafts.

**Wet Disk Assays.** Table 2 summarizes results of

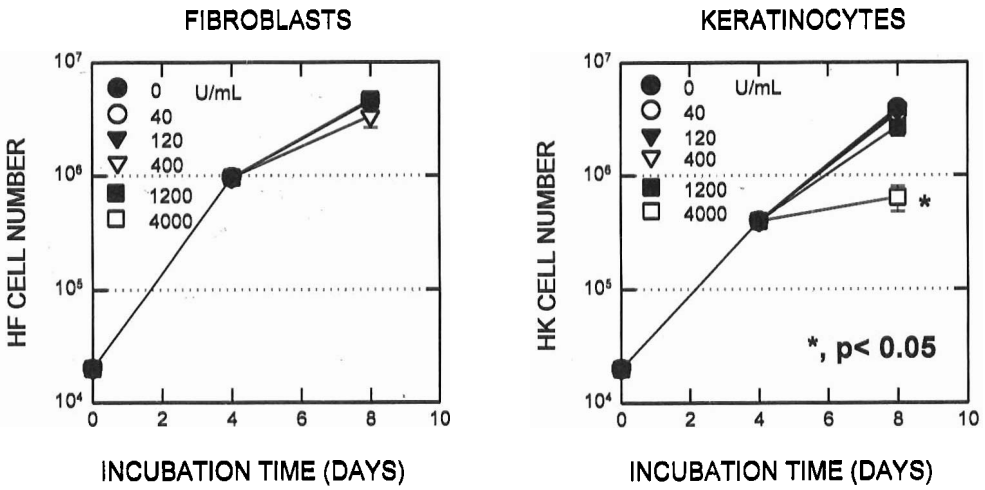


**Figure 2.** Dose response of cultured human keratinocytes and fibroblasts to polymyxin B. Test conditions were initiated on culture day 4. Cytotoxicity to keratinocytes is detected at 4000 U/ml.

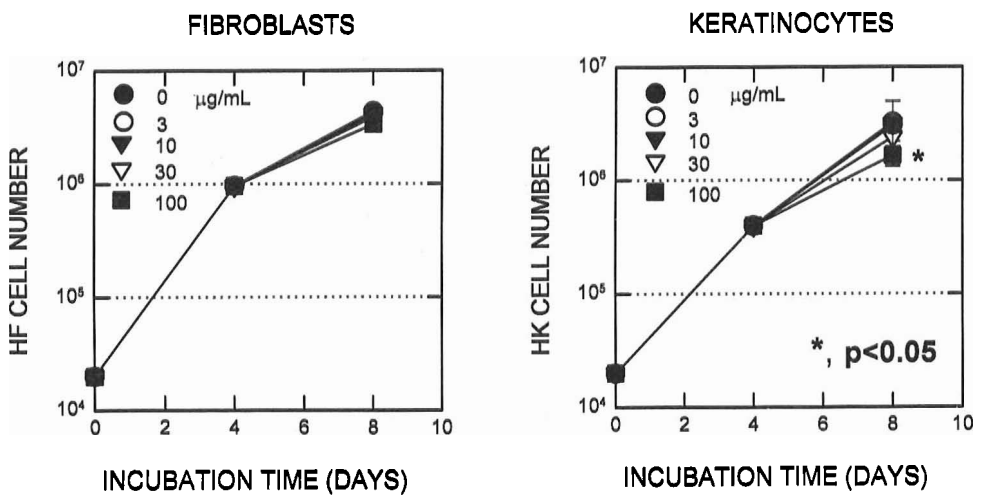


**Figure 3.** Dose response of cultured human keratinocytes and fibroblasts to sparfloxacin. Test conditions were initiated on culture day 4. Low but significant amounts of growth inhibition to keratinocytes occurs at 100 µg/ml. All other concentrations tested are nontoxic to both cell types.

wet disk assays of clinical isolates of microorganisms from burn wounds. Chlorhexidine (0.05% wt/vol) is uniformly effective (14/14) against all *Staphylococcus aureus* strains tested and against eight of 13 gram-negative organisms. Polymyxin B (400 U/ml) is moderately effective (12/22) against gram-negative organisms and is known not to be active against gram-positive organisms. Sparfloxacin (30 µg/ml) is uniformly effective against 12 *Staphylococcus aureus* and against 22 gram-negative bacteria tested. Mupirocin (48 µg/ml) is uniformly effective against all eight *Staphylococcus aureus* strains and was not tested against gram-negative organisms. Nitrofurazone (0.02% wt/vol) is moderately effective (four of eight) against *Staphylococcus aureus* and was not tested against gram-negative organisms.

## DISCUSSION

Although various models of cultured skin have been demonstrated to close wounds permanently,[1,4,7] all are more subject to destruction by common burn

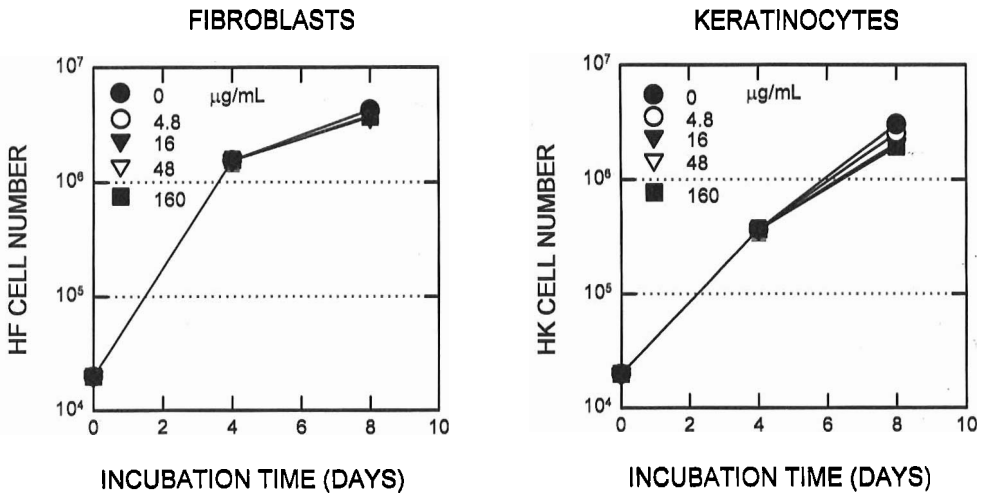


**Figure 4.** Dose response of cultured human keratinocytes and fibroblasts to mupirocin. Test conditions were initiated on culture day 4. No cytotoxicity is detected at concentrations tested.

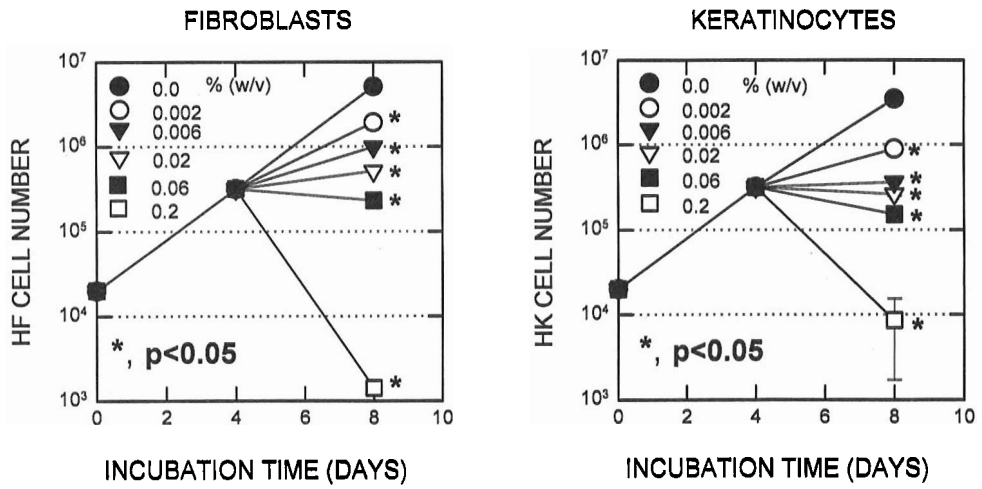


**Figure 5.** Dose response of cultured human keratinocytes and fibroblasts to nitrofurazone in propylene glycol. Test conditions were initiated on culture day 4. Nitrofurazone formulated in propylene glycol generates concentration-dependent cytotoxicity to both cell types.

organisms than are conventional skin grafts. This general limitation of cultured skin results from incomplete epidermal barrier at grafting, absence of vascular plexus and immune cells, and consequent protraction of engraftment by several days. These biologic deficiencies of cultured skin account for its vulnerability to microbial destruction and impose additional surgical and postsurgical requirements for protection of the cultured cells, until stable vasculature develops and epidermal tissue becomes keratinized. However, it has been shown[12,13,19] that topical antimicrobial agents (i.e., mafenide acetate, silver sulfadiazine, clorhexidine gluconate [Hibiclens]) that are used successfully with native skin grafts are toxic to cultured keratinocytes and are associated with failure of cultured grafts. Therefore quantitative assays are needed to select antimicrobial agents in appropriate dosages that have no or low toxicity to transplanted cells and that retain a broad spectrum of antimicrobial activity. Results presented here demonstrate that a combined assay system can determine concentration ranges of candidate drugs that have low toxicity to human cells and retain antimicrobial activity against burn wound microorganisms.

**Table 2.** Wet disk assay

| Agent and dose | Organisms (effective/no. tested) | | Totals |
|---|---|---|---|
| | Gram-positive* | Gram-negative* | |
| No drug | 0/12 | 0/4 | 0/12 |
| Chlorhexidine 0.05% (wt/vol) | 14/14 | 8/13 | 22/27 |
| Polymyxin B 400 U/ml | ND | 12/22 | 12/22 |
| Sparfloxacin 30 μg/ml | 12/12 | 22/22 | 34/34 |
| Mupirocin 48 μg/ml | 8/8 | ND | 8/8 |
| Nitrofurazone 0.02% (wt/vol) | 4/8 | ND | 4/8 |

*ND,* Not determined.
*Randomly selected clinical isolates from patients with burns at Shriners Burns Institute, Cincinnati Unit. Gram-positive organisms included only *Staphylococcus aureus.* Gram-negative organisms included *Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterobacter cloacae,* and *Escherichia coli.*

This combined assay system can both qualify and disqualify either individual agents or combinations of agents for topical use with cultured skin cells for wound treatment. Although chlorhexidine is uniformly effective against a wide variety of burn organisms, it is disqualified from consideration, because it is also highly cytotoxic to cultured keratinocytes and fibroblasts (Figure 1, Table 2). Conversely polymyxin B, sparfloxacin, and mupirocin are qualified as antibiotics that have low toxicity to skin cells within certain concentration ranges and retention of antimicrobial activity against burn organisms in those ranges (Figures 2, 3, 4; Table 2). In related studies combined cytotoxicity-antimicrobial assays also identified that addition of an antimycotic agent, nystatin, to antibiotic formulations does not increase cytotoxicity to transplanted cells nor reduce the antimicrobial activities of selected antibiotics.[17] In addition, amphotericin B in low concentrations (i.e., 1 μg/ml) is not cytotoxic to cultured cells[24] and can be used to manage fungal contamination in wounds. Therefore these assays identify stringent requirements for selection of antimicrobial agents that may avoid cytotoxicity to cultured skin. However, it is expected that as cultured skin substitutes are developed with more biologic homology to native skin (i.e., improved keratinization and barrier function), stringency for clinical management of cultured and native skin grafts may also become more homologous.

Other factors not addressed in this study that are important to clinical management of microbial contamination of cultured skin include but are not limited to (1) nonoverlapping antimicrobial activity with parenteral treatment of burn sepsis; (2) evaluation of combinations of agents to cover both gram-positive and gram-negative organisms; (3) delivery of adequate levels of antimicrobial formulations to the site of the microbial contamination; and (4) microbial monitoring of wound sites chosen for patient grafting with cultured skin cells to determine the types and sensitivities of organisms to noncytotoxic formulations of topical antimicrobials. Delivery of novel formulations to the site of contamination is a complex question that requires dedicated studies to address. To understand and regulate drug delivery future studies should address wound bed preparation and pretreatment, vehicles and protocols for administration of novel formulations, penetration and half-life of compounds in the wound, and effective dose at the graft-wound interface. Finally, monitoring of patient wounds to determine type and sensitivity of microbial contamination is a routine clinical procedure with which the cytotoxicity-antimicrobial assays described here can be easily coordinated. Any novel formulations of topical antimicrobials[25-27] qualified by the assays described here may be added to the sensitivity testing of organisms for identification of whether the formulations are suitable for use with cultured skin in particular patient cases.

In conclusion this study demonstrates a direct and inexpensive assay system by which topical antimicrobial agents may be selected for use with cultured skin grafts for burn wound treatment. Data from this assay system show that three antibiotics (polymyxin B, mupirocin, sparfloxacin) within specific concentration ranges are nontoxic to cultured human cells and are effective against a broad spectrum of burn organisms and that chlorhexidine gluconate and nitrofurazone are cytotoxic to cultured skin cells at clinically relevant concentrations. The data suggest that polymyxin B, mupirocin, and sparfloxacin alone or in combination may have prospective use as topical antimicrobials on cultured skin for burns. Application of these findings may improve engraftment and

survival of cultured skin for burns and may assist in the identification of qualified formulations of antimicrobial agents for management of other surgical infections.

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