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The differential cytotoxicity of antiseptic agents

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The cytotoxicity of the antiseptic agents noxythiolin and chlorhexidine has been evaluated in-vitro using a range of tissue culture cell lines of differing degrees of neoplasticity. Noxythiolin appeared more cytotoxic than did chlorhexidine when tested against established neoplastic cell lines. By contrast, noxythiolin was not cytotoxic to normal control (non-neoplastic) cells. For chlorhexidine, the cytotoxic activity against control cells was similar to that observed for neoplastic cell lines. The results confirm an earlier observation of limited antitumour activity of noxythiolin solutions and, on the basis of differential cytotoxicity, confirm that noxythiolin is free from adverse effects against normal tissues and is safe for use as an antimicrobial agent applied to peritoneal surfaces and the healing wound.

The efficacy of topical antiseptic agents must be considered in relation to their potentially detrimental effects on wound healing. Various commonly used antiseptic agents, including eusol, povidone-iodine, hydrogen peroxide, chloramine and chlorhexidine gluconate, have been studied with respect to their possible adverse effect on granulation tissue using an in-vivo model (Brennan & Leaper 1985). All agents caused some adverse effects but with the hypochlorite antiseptics eusol and chloramine, blood flow in the capillary circulation of granulation tissue was inhibited and wound healing significantly delayed. The results highlight the fine balance of the relationship between bacterial killing and adverse tissue toxicity, a principle recognized by Fleming (1919).

Noxythiolin, *N*-methyl-*N'*-hydroxymethylthiourea, (Noxyflex S, Geistlich Sons Limited, Chester) is a commonly used topical antiseptic agent having a wide spectrum of antimicrobial activity. Additionally, noxythiolin possesses anti-endotoxic activity of potential clinical value (Wright & McAllister 1976) and has marked anti-adhesive properties both with regard to the adherence of bacteria and fungi to epithelial cell surfaces (Gorman et al 1986) and in the prevention of peritoneal adhesions (Browne & Stoller 1970; Pickard 1972). Clinical applications for noxythiolin solutions include peritoneal (Browne & Stoller 1970; Antos 1980) and bladder (Sutherland 1967; Harper 1981) irrigation and treatment of the infected wound.

Clinical experience with noxythiolin solutions suggests that there is little or no adverse effect toward normal tissues. However noxythiolin solutions have been demonstrated to possess some degree of cytocidal activity against tumour cells both in-vitro (Jamieson 1972) and in-vivo (Desai & Jamieson 1973). In-vitro cytocidal activity against tumour cells derived from colorectal carcinoma has also been confirmed (Umpleby & Williamson 1984). The apparent disparity of data for noxythiolin cytotoxicity may be interpreted as evidence for the possibility of potential impairment of wound healing when noxythiolin is used as a conventional antibacterial agent for application to normal tissues. The differential cytotoxicity of noxythiolin, in comparison with the antibacterial agent chlorhexidine, has therefore been re-evaluated using a range of tissue culture cell lines of differing degrees of neoplasticity to investigate further the potential adverse effects of these agents on the healing wound.

Methods

In-vitro cytotoxicity testing was performed using monolayers of tissue culture cell lines of differing degrees of neoplasticity. The epithelial-like cell line HEp2 (American Type Culture Collection CCL 23) (passage number 376) derived from a human laryngeal carcinoma and the MRC-5 line of human embryonic lung fibroblasts (ATCC CCL 171) (passage number 26) were used as controls. Cell monolayers were grown at 35 °C in borosilicate tubes containing Earle's minimum essential medium (MEM) incorporating 10% foetal bovine serum (FBS). Medium for MRC-5 cells was additionally supplemented with 1% non-essential amino acids (NEAA). Antibiotics were not added to any tissue culture medium.

A second strain of human embryonic lung fibroblasts, Flow 2002 (Flow Laboratories Limited, Hertfordshire, UK), was used (passage number 17) representing non-neoplastic cells having a finite life in-vitro. In addition, two reference strains of human skin fibroblasts, CRL 1510 (ATCC CRL 1510) (passage number 7) and Malme-3 (ATCC Human Tumor Bank 102) (passage number 12) (Fogh & Trempe 1975) were also included, these being specially selected as normal, non-neoplastic control cell lines. Growth medium for Flow 2002 and CRL 1510 cells was as for MRC-5 cells whilst for the Malme-3 cells the growth medium was McCoy's medium 5A supplemented with 10% FBS. For cytotoxicity testing, growth medium used for all cell lines was replaced with Earle's MEM plus 5% FBS and 1% NEAA 24 h before use.

Monolayers of approximately 1 cm^2 surface area and confluence of 80–100% were used throughout and all cytotoxicity testing was performed at 35 °C. Test solutions of noxythiolin (Noxyflex S) 1% w/v, noxythiolin

1% w/v plus amethocaine hydrochloride 0·004% w/v (Noxyflex) and chlorhexidine gluconate 0·05% w/v were freshly prepared in double glass-distilled water. Saline (0·85%) was used as negative control and the appropriate maintenance medium as blank for each cell line. Monolayers were aseptically drained of maintenance medium and 1 mL aliquots of test or control solutions were added with brief agitation and contact times of 1, 5, 10, 20, 30 and 60 min. Tubes were held in angled racks to ensure contact of cells with tissue culture media and test solutions at all times. All tests were performed in triplicate. Following exposure to test and control solutions, cell monolayers were drained and washed twice with the appropriate pre-warmed maintenance medium. Finally, 1 mL of maintenance medium was added and the monolayers incubated at 35 °C for a further 18 h.

Monolayers were observed for evidence of cytotoxicity by direct light and phase contrast microscopy at 1 and 18 h after exposure to test solutions. Subsequently, the supravital dye, trypan blue, was added to the tissue culture medium, without trypsinization, at a final concentration of 0·5% (w/v). After a further 30 min incubation the number of non-viable cells, showing uptake of trypan blue, was estimated as a percentage of the residual adherent cell population.

Results

Both the negative control (saline) and blank (complete growth medium) solutions showed no evidence of cytotoxicity at up to 60 min following challenge when compared with untreated cell monolayers. Toxic granulation and marked vacuolation of HEp2 and MRC-5 cells was observed following exposure to 1% w/v noxythiolin solution for periods in excess of 10 min. At exposure times greater than 20 min, toxic granulation and vacuolation was increased and approximately 40% of cells became detached from the monolayer. Trypan blue uptake was observed in 70% of the remaining adherent cell population. Similarly, trypan blue uptake was uniformly observed in detached cells indicating loss of viability. By contrast, there was a reduction in the observed cytotoxicity against Flow 2002 cells with only minimal toxic granulation and vacuolation after 60 min exposure to noxythiolin, no detachment of the monolayer and only 40% of cells showed uptake of trypan blue. The normal, non-neoplastic, control cells CRL 1510 and Malme-3 exhibited no evidence of cytotoxic damage after 60 min exposure to noxythiolin and <20% trypan blue uptake. Noxythiolin plus amethocaine gave results identical to those described for noxythiolin alone.

Chlorhexidine 0·05% (w/v) exhibited marginally less cytotoxic activity against HEp2 and MRC-5 cells following exposure for 60 min than that observed with noxythiolin. Brief exposure (<20 min) did not result in toxic granulation although by 60 min, chlorhexidine had produced a marked detrimental effect though marginally less than that with noxythiolin. The rate of cell detachment was less with chlorhexidine although 50% of the remaining adherent cells showed evidence of trypan blue uptake. In contrast to the results obtained for noxythiolin, chlorhexidine did not show any significant reduction in cytotoxic activity against the normal non-neoplastic cells, CRL 1510 and Malme-3, with toxic granulation and vacuolation being observed after 20 min exposure, detachment of approximately 50% of cells following 60 min exposure and trypan blue uptake observed in 40 and 30% of the residual adherent cells, respectively (Table 1). Noxythiolin solutions did not exhibit any cytotoxic activity against the CRL 1510 and Malme-3 cell lines when compared with the results obtained for neoplastic cells; toxic granulation and vacuolation of cells was not observed following 60 min exposure to 1% w/v noxythiolin solutions, detachment of cells from the monolayer did not occur and the proportion of adherent cells showing uptake of trypan blue was not significantly increased above that seen with blank and control solutions. The intermediate lines of non-neoplastic cells having a high passage number, MRC-5 and Flow 2002, exhibited reduced cytotoxic damage when compared with HEp2 although for the MRC-5 line these differences were minimal.

Table 1. The differential cytotoxicity of noxythiolin (N) and chlorhexidine (C) tested against cell lines of differing neoplasticity (60 min exposure).

	Toxic						Trypan blue uptake	
	Vacuolation		Granulation		Detachment			
	N	C	N	C	N	C	N	C
Hep2	††	††	†††	††	†††	††	70%	50%
MRC-5	††	††	††	††	††	†	70%	50%
Flow 2002	†	†	††	†	*	††	40%	40%
CRL 1510	*	†	*	††	*	††	<20%	40%
Malme-3	*	†	*	††	*	††	<20%	30%

* <25%, † 25–50%, †† 50–75%, ††† >75% of cells affected.

Discussion

The results of this study confirm the earlier observations of limited cytocidal activity of noxythiolin and chlorhexidine solutions (Jamieson 1972; Desai & Jamieson 1973; Umpleby & Williamson 1984). The activity of these agents is such that they may be suitable for use in the prevention of anastomotic recurrence in colorectal carcinoma due to re-implantation of viable exfoliated tumour cells (Umpleby & Williamson 1984). Application may be local, by application to the resection margins before anastomosis, or in the case of noxythiolin by intraperitoneal instillation of up to 1 L of 1% w/v solution before closure of the abdominal cavity. The cytotoxic activity of these agents, however, must be considered also with regard to their potential adverse effects on non-neoplastic tissues and, as widely used agents in the management of the infected wound, on the normal mechanisms of wound repair.

The HEp2 cell line derived from a laryngeal carcinoma is an established permanent line having clearly malignant characteristics (Norryd & Fjelde 1963). Exposure to both noxythiolin and chlorhexidine solutions at concentrations equivalent to those employed prophylactically and therapeutically in wound management clearly results in rapid and marked cytocidal effects. Similar cytotoxic activity was observed against the MRC-5 cell line (Jacobs et al 1970). Although non-neoplastic, this line which was used at passage number 26 shows increasing aneuploidy and polyploidy with repeated population doublings. The similar fibroblast cell line, Flow 2002, used at passage number 17 showed a decrease in cytotoxic damage following exposure to noxythiolin whilst with the normal reference strains of non-neoplastic human skin fibroblasts, CRL 1510 and Malme-3, used at low passage numbers, no cytotoxic activity was observed for noxythiolin. By contrast, chlorhexidine appeared cytotoxic to all cell lines used in this study.

Using the rabbit ear chamber as a model for in-vivo cytotoxicity testing of a wide range of antiseptic agents, Brennan & Leaper (1985) observed inhibition of capillary blood flow in granulation tissue and inhibition of subsequent wound repair with hypochlorite-based antiseptics. By contrast, the effects of chlorhexidine were less marked in this model; noxythiolin was not included in this report. Clearly, in-vivo testing for cytotoxicity of locally applied antiseptic preparations is desirable. However, in-vitro cytotoxicity testing using readily available tissue culture cell lines has become widely established although the results may not be applicable to the in-use situation.

The earlier observations of antitumour activity of noxythiolin when tested in-vitro particularly in the absence of control studies using non-neoplastic cell lines, are theoretically in direct conflict to the use of this compound as a wide spectrum antimicrobial preparation for wound management. The minimum active concentration of noxythiolin showing antitumour activity is in the range 0·0025% (HeLa) to 1·5% (Ehrlich ascites) depending on the malignant cell line used. However, the results obtained clearly show the differential nature of the cytotoxicity of noxythiolin, in contrast to that of chlorhexidine, and provide strong evidence for the lack of potential deleterious effects of noxythiolin on normal tissues. Clearly, cytotoxicity testing using tissue culture cell lines is limited in scope when compared with an in-vivo model (Brennan & Leaper 1985). The results obtained from in-vitro studies may not invariably be appropriate for extrapolation to the in-vivo situation due to profound differences in the susceptibility of cell lines of differing degree of neoplasticity. However, the results obtained in this study, whilst confirming limited anti-neoplastic activity, clearly show the absence of demonstrable cytotoxic activity of noxythiolin toward reference strains of normal, non-neoplastic cell lines at a concentration of 1% (w/v). The data support the continued use of solutions of noxythiolin as an antimicrobial agent without evidence of detrimental effect against normal tissue cells.

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