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Cytotoxic effects of chlorhexidine and nystatin on cultured hamster buccal epithelial cells

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Summary

Primary cultures of hamster buccal epithelial cell were used in this study to investigate the potential cytotoxicity of chlorhexidine and nystatin. Following exposure to 0–0.01% of chlorhexidine for 5–60 min, or following exposure to either nystatin suspension or solution ranging from 0–0.1% for 1 h, the viability of hamster buccal epithelial cells was measured by 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) hydrolysis and colony-forming efficiency. Chlorhexidine digluconate at concentrations greater than 0.005% was cytotoxic to the cells after a 1 h incubation. However, a 5 min contact with this drug did not cause significant effects when the concentration was less than 0.01%. A linear decrease in survival rate was observed when the cells were exposed to 100–200 μg/ml (0.01–0.02%) of nystatin solution. The inhibition of colony-forming efficiency by a nystatin suspension (ID_{50} = 634 μg/ml) was 8.3-fold lower than that of a nystatin solution (ID_{50} = 76 μg/ml). Results of this study suggested that buccal epithelial cells were relatively more resistant to toxic effects of chlorhexidine and nystatin than other cell types.

Introduction

Both chlorhexidine and nystatin are broad-spectrum antimicrobial agents that have been widely used against fungi infection. Chlorhexidine has been shown to be effective in the chronic treatment of candidiasis (Langslet et al., 1974; McGaw et al., 1985; Ferretti et al., 1987) and in inhibiting supragingival plaques (Loe et al., 1973; Grennstein et al., 1985). Nystatin is also considered for treating oral candidiasis (McCourtie et al., 1986) and systemic candidiasis (Mehta et al., 1987). Many in vivo animal studies and clinical investigations have provided a general picture of low toxicity of these two drugs (Davies et al., 1954; Seneca et al., 1955–1956). However, subsequent to a report of delayed wound healing after brief exposure to chlorhexidine (Mobacken et al., 1974), the in vitro cytotoxic effect of chlorhexidine has been investigated in several cell models, such as fibroblasts, Hela cells (Goldschmidt et al.,

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1977), and blood cells (Gabler et al., 1987). Most of the cytopathologic phenomena in cultured human fibroblasts and Hela cells occurred at the concentrations of chlorhexidine in excess of 0.005% and treatment times of up to 3 h. Effects of the drug on metabolic activity are observed within 30 s (Goldschmidt et al., 1977). The buccal epithelial cell, a cell subject to exposure to these drugs on intraoral cavity application, has not been examined for sensitivity to chlorhexidine or nystatin.

Based on the broad use of chlohexidine and nystatin in oral hygiene, an in vitro model of buccal epithelial cells has been used in this study to determine the cytotoxic effect of chlorhexidine and nystatin. The technique for isolation and culture of hamster buccal epithelial cell has been established by Tavakoli-Saberi and Audus (1989). The morphological and biochemical characteristics of this system were similar to those of freshly excised hamster buccal epithelium, presenting a suitable model for investigation of toxicity in vitro.

Materials and Methods

Materials

Chlorhexidine digluconate (20% aqueous solution), nystatin, and trypsin type III from pancreas, were obtained from Sigma Chemical Co. (St. Louis, MO). Heat-inactived fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT). Minimal essential medium (MEM) and Ham's nutrient mixture F12 were obtained from Hazleton Biologics, Inc. (Lenexa, KS). 3-(4,5-Dimethyl-2-thiazoyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was purchased from Eastman Kodak Co. (Rochester, NY). Neutral red, HCl, isopropanol, acetic acid, and ethanol were obtained from Fisher Co. (St. Louis, MO). All other chemicals and biochemicals were supplied by Sigma.

Media and drug solutions

Two kinds of cell culture media were used. Medium I was Eagle's MEM supplemented with 10% fetal bovine serum, 100 μg/ml penicillin G, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 300 μg/ml ascorbic acid, 2.2 g/l sodium bicarbonate, and 10 mM Hepes (pH 7.4). Medium II was Ham's F-12 supplemented with 10% fetal bovine serum, 15 mM Hepes, and 2 μg/ml fibronectin, plus antibiotics as in medium I.

All of the chlorhexidine digluconate and nystatin solutions and suspensions were made in medium II without serum. A 20% chlorhexidine digluconate solution was first prepared at 0.1% in distilled water, and then diluted in medium to the desired concentrations. Nystatin was dissolved in dimethyl sulfoxide (DMSO) before diluting in the medium. The final concentration of DMSO was less than 3% in the experiments and was not directly cytotoxic at the concentration used here. Nystatin suspensions were made by directly suspending nystatin in culture medium. Both nystatin solutions and suspensions were prepared immediately prior to cytotoxicity tests.

Isolation and culture of hamster buccal epithelial cells

Hamster cheek pouches epithelial cells were isolated and cultured as described by Tavakoli-Saberi and Audus (1989). In brief, cheek pouches were washed thoroughly with sterile swabs under tap water, and hairs overlaying the pouch were disinfected with 70% ethanol. Pouches were then everted, excised, and placed in cold Tricine buffer (pH 7.4). One longitudinal incision was made in each pouch following by repeated rinsing with Tricine buffer, The pouch were then cut into several pieces and immersed in Hank's PBS containing 0.25% trypsin at 4°C overnight.

Epithelial sheets were removed and shredded into about 1 mm^3 pieces, incubated with residual trypsin at 37°C for 30 min before being gently pipetted in medium II to disperse the cells. Cells were seeded in 96-well or 6-well plates coated with cross-linked rat-tail collagen and fibronectin and incubated in medium II at 95% humidity, 5% CO_2 at 37°C.

MTT cytotoxicity assay

The MTT assay measures the cleavage of the tetrazolium ring by various dehydrogenase enzymes in active mitochondria. Therefore, the re-

action only occurs in living cells (Mosmann, 1983). Hamster buccal epithelial cells were plated at $1-2 \times 10^5$ cells/cm^2 density in 96-well plates for 5 days before the MTT assay. The cell monolayers were washed with Hank's PBS before and after being exposed to 0.1 ml chlorhexidine or nystatin solutions or suspensions for 1 h. The cells were then cultured in 0.1 ml medium II containing 10 μl MTT stock solution (5 mg/ml in pH 7.4 PBS) at 37°C as the method developed by Mosmann (1983). After 4 h, 0.1 ml of acid-isopropanol (0.04 N HCl in isopropanol) was added to wells and incubated overnight at room temperature to dissolve the dark blue crystals produced by living cells. The color changes were determined from changes in absorbance at 540 nm using a microplate reader (Cambridge Series 700, Cambridge Technology Inc., Watertown, MA). Cell-free wells treated exactly as the cell-containing wells served as blank controls. For each experiment, a standard curve was constructed to describe the relationship between absorbance and viable cell number.

Colony-forming efficiency assay

Cells were plated at 1×10^5 cells/well in 6-well plates and incubated for 20 h before treatment. A 1 ml aliquot of either a chlorhexidine solution or nystatin suspension was added to each well for 1 h. Following the incubation each well was washed twice with Hank's PBS. The cells were then incubated in medium II containing epithelial growth factor (10 ng/ml) for 7 days before washing and fixing in 10% formalin. The cells were stained with 1% aqueous crystal violet. Cytotoxicity was determined by counting the colonies formed on drug-treated wells vs those formed on drug-free wells (Sundqvist et al., 1989).

Statistical analysis

Data are expressed as mean ± standard error of at least four replications. A one-way analysis of variance (ANOVA) followed by Scheffe's post-hoc test (ABSTAT, Anderson Bell software) were used to check the significance between control and drug-treated samples. Differences were considered statistically significant if $p < 0.05$.

Results and Discussion

The isolated hamster buccal cells grew to confluent monolayers in 4–5 days as observed under a phase-contrast microscope. The monolayers were morphologically epithelial-like (not shown). The histochemical and enzymatic characterization of these cells has been previously described by Tavakoli-Saberi and Audus (1989).

A linear decrease in absorbance was observed with chlorhexidine concentrations ranging from 0.005 to 0.01% as measured by the MTT assay (see Fig. 1). The dosage required to reduce cell survival by 50% (ID_{50}) was estimated by non-linear regression methods at 0.007% following a 1 h exposure to the drug. This was in agreement with the report by Goldschmidt et al. (1977) that the release of ^{51}Cr from human newborn fibroblasts could be detected at a concentration of chlorhexidine equal to or greater than 0.005% after a 3 h incubation. Similar results were also reported within the same study with human gingival fibroblasts and Hela cells. Thus, it is apparent that chlorhexidine concentrations greater than 0.005%

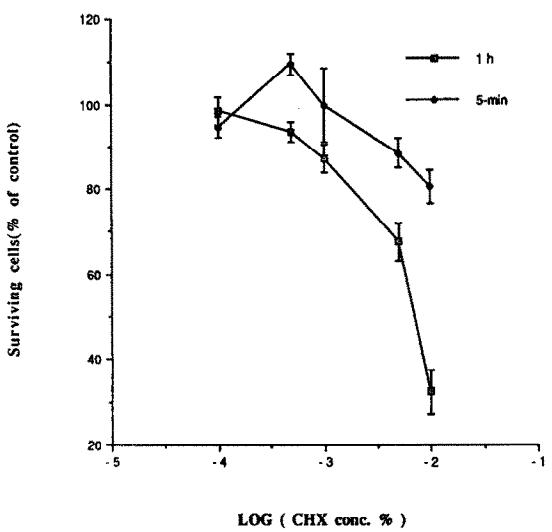

Fig. 1. Concentration-dependent effect of chlorhexidine digluconate on viability of hamster buccal epithelial cells after a 5 min or 1 h exposure to the drug measured by the 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2 *H*-tetrazolium bromide(MTT) hydrolysis assay ($n = 4$).

are toxic to a variety of cell types in vitro when exposure times are longer than 1 h.

In contrast, if human buccal epithelial cells were treated with chlorhexidine for only 5 min, a significant reduction in cell survival was not observed even with the concentrations as high as 0.01% (Fig. 1). Helgeland and co-workers (1971) found that concentrations of chlorhexidine from 0.05 mM (about 0.0045%) were toxic to human skin epithelial cells after a 5 min exposure. A possible explanation for the differences between our findings and those observed with skin epithelial cells is that the latter may be more sensitive than buccal epithelial cells. Audus et al. (1992) indicated that chlorhexidine reversibly altered the membrane-lipid packing order of the superficial hydrophobic regions of human buccal epithelial cells and suggested this as a potential mechanism of drug-induced toxicity. Only slight changes in the buccal epithelial cell membrane fluidity as reflected by changes in the fluorescence anisotropy after a 5 min exposure to concentration of chlorhexidine less than 0.01% were observed in the same study, consistent with the lack of effects on cell viability herein.

The relative colony-forming efficiency (as a percentage of that of control cells) vs chlorhexidine concentration is shown in Fig. 2. The growth and proliferation of the cells were inhibited by about 32% at 0.001% of chlorhexidine and by 95% at 0.005%. The ID_{50} was about 0.0016% which was slightly lower than the result from MTT assay. At this dosage, cells may apparently be prevented from proliferation without being killed immediately.

The effects of nystatin solutions on the viability of hamster buccal epithelial cells after a 1 h contact is shown in Fig. 3. Cell viability decreased almost linearly with nystatin solutions ranging from 50 to 200 μg/ml (0.005–0.02%). About 110 μg/ml (0.011%) of nystatin was required to reduce viability by 50%. A similar range of nystatin concentrations caused the significant decrease of fluorescence anisotropy, an indication of a decrease in membrane-lipid packing order (i.e., increased 'membrane fluidity') in human buccal epithelial cells (Audus et al., 1992), consistent with our MTT assay results. Mehta et al. (1987)

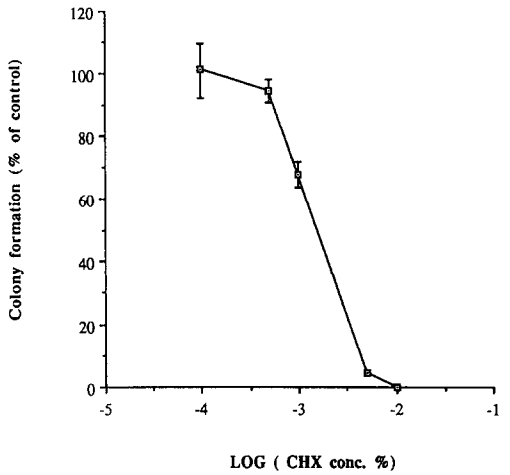

Fig. 2. Concentration-dependent effect of chlorhexidine digluconate on hamster buccal epithelial cell viability after 1 h exposure to the drug determined by colony-forming efficiency assay ($n = 4$).

investigated the toxicity of nystatin to human erythrocytes in vitro by measuring the release of hemoglobin from erythrocytes. They found a linear increase in lysis of human erythrocytes with nystatin concentrations from 60 to 120 μg/ml,

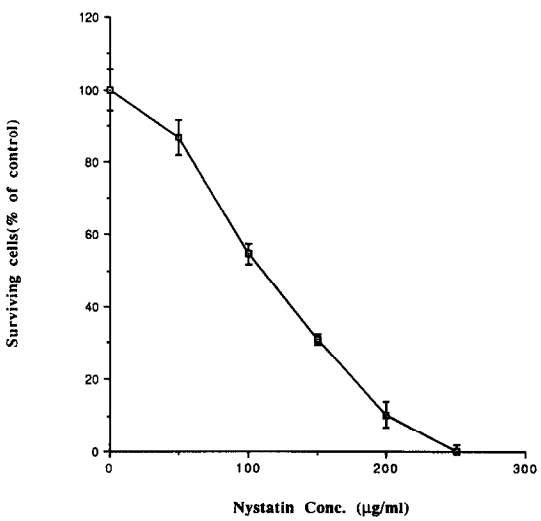

Fig. 3. Concentration-dependent effect of nystatin solutions on hamster buccal epithelial cell viability after 1 h exposure to the drug determined by the 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2*H*-tetrazoliumbromide (MTT) hydrolysis assay ($n = 4$).

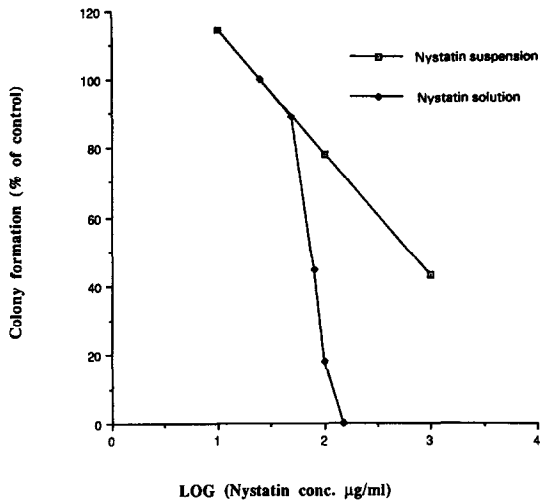

Fig. 4. The comparison of nystatin solutions with nystatin suspensions on the colony-forming efficiency of hamster buccal epithelial cells after 1 h exposure to the drug.

with a 100% lysis produced at 120 $\mu g/ml$. This is about one-half of the dose required for a 100% killing of the buccal epithelial cells. Here again, buccal epithelial cells seem to have a higher resistance to drug-induced loss of viability than other cell types. Since nystatin is more often prepared as a drug suspension rather than a solution, we compared cytotoxicities of nystatin suspensions and solutions using the colony-forming efficiency assay. Treatment with nystatin suspension and solution produced very different efficiencies as shown in Fig. 4. The ID_{50} of nystatin suspension was 634 $\mu g/ml$ (0.0634%), 8.3-fold greater than the ID_{50} of nystatin solution (about 0.0076%). Nystatin is quite insoluble in water, and thus is poorly absorbed from the gastrointestinal tract of either animals or man. Solubility differences may be one of the reasons that suspensions had little toxicity as described by Aguirregoicoa et al. (1989) and Seneca (1955–56) and in these studies.

In summary, chlorhexidine digluconate and nystatin reduce cultured hamster buccal epithelial cell viability in a concentration- and time-dependent manner. Shorter exposure times and lower concentrations were generally non-toxic to the cells. The cytotoxicity of nystatin suspension to hamster buccal epithelial cells was 8.3-fold lower than that of nystatin solution and 39.6-fold lower than that of chlorhexidine.

Acknowledgment

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