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USE OF RAINBOW TROUT PRIMARY EPIDERMAL CELL CULTURES AS AN ALTERNATIVE TO IMMORTALIZED CELL LINES IN TOXICITY ASSESSMENT: A STUDY WITH NONOXYNOL

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Abstract—Increased concern has centered around environmental contaminants that exert estrogenic effects. A class of nonionic surfactants, the nonylphenol ethoxylates (such as the compound nonoxynol), are an example of one such group of contaminants. We studied the effects of nonoxynol (α-(4-nonylphenyl)-ω-hydroxypoly-(oxy-1-ethanediyl)) on an epithelial primary culture. The culture system that was employed is a development of an in vitro technique for the culture of rainbow trout (*Onchorynchus mykiss*) epithelium, one that allows for the culture of both epithelial cells and goblet cells. The LC50 after 24 h was found to be 16.6 μmol/L. The LC50 after 48 h was 12.9 μmol/L. Nonoxynol was found to exhibit an acute lethal dose of 75 μmol/L. A decrease in the number of goblet cells present in the system was observed as exposure to nonoxynol increased. Nonoxynol was found to kill epithelial cells mainly by necrosis. A comparative study of the effects of nonoxynol on the epithelium papulosum cyprini cell line was also carried out. The LC50 of nonoxynol on this cell type was 4.1 μmol/L, with an acute lethal dose of 10 μmol/L.

Keywords—Primary epidermal cell cultures Epithelium papulosum cyprini Nonoxynol Goblet cells Necrosis

INTRODUCTION

Nonoxynol—α-(4-nonylphenyl)-ω-hydroxypoly-(oxy-1-ethanediyl)—is a nonionic surfactant mixture that is prepared by reacting nonylphenol with ethylene oxide [1]. Recent discoveries have led to concerns regarding the environmental safety of a major class of nonionic surfactants, of which nonylphenol is a member, the alkylphenol polyethoxylates [2]. These are used in a variety of products, including institutional cleaning agents, textiles, agricultural chemicals, plastics, paper products, household cleaning agents, and personal care products [3]. Over one billion pounds of nonylphenol polyethoxylates are produced annually. Because of legislative measures and the implementation of technologies for the protection and rehabilitation of the environment, the actual levels of contaminants have decreased in European countries. Thus, the emphasis in the development of ecotoxicology assessment methods has moved from the measurement of acute, lethal effects of single chemicals to the early detection and evaluation of chronic sublethal stresses of chemical mixtures [4,5]. For a given class of chemicals, the relationship between toxicity and physical and chemical characteristics could allow the potential impact of members of that class to be predicted. Such quantitative structureactivity relationships could lead to rapid economical hazard evaluations to indicate those chemicals that will require in-depth study. In order to establish these relationships, rapid and inexpensive toxicity tests are required to screen many compounds [6]. Isolated cells that maintain the essential traits of the in vivo state during culture in vitro provide an excellent experimental approach to use in establishing diagnostic markers, such as early indicators of exposure and early indicators of effect. These isolated cells can be directly exposed to toxicants so that the toxicokinetic phase is easy to model; they allow for the establishment of the dose and time dependencies of the cellular response to single toxicants and toxicant mixtures without confusing systematic influences, and they are well suited for analysis of mechanisms, be it mechanisms of disease or mechanisms of tolerance [7]. Although the lack of systematic influences may be a problem, in vitro systems do provide a useful aid to hazard definition in addition to in vivo studies.

In a previous study [8], an in vitro technique was developed for the culture of rainbow trout (*Onchorynchus mykiss*) epithelia. In the broad perspective of environmental toxicology and biohazard assessment, epithelial cells are highly significant biological indicators, since all the substances that are received or exerted by the body must transverse an epithelium [9]. Epithelial cells form the barrier between an individual and its environment and are a primary target for pollution-related carcinogenesis [10,11]. Because the characteristics of established cell lines deviate substantially from those of their normal counterparts, fish-derived primary cells in culture may represent an alternative for toxicity studies. If differentiated properties are lost, it is difficult to relate the cultured cells to functional cells in the tissue from which they are derived [12]. Primary cells can be made to express a high number of differentiated characteristics [8].

One of the breakdown products of nonoxynol is 4-nonylphenol. 4-Nonylphenol has been found to be weakly estrogenic in fish [13,14]. Exposure of wildlife to environmentally persistent estrogenic chemicals can result in deleterious reproductive consequences [15]. A major effect of the exposure of male and female teleosts to estrogen is the stimulation of the synthesis and secretion of vitellogenin in the liver [16]. Exposure of the male rainbow trout to 30 μg/L 4-nonylphenol for 3 weeks significantly induced hepatic vitellogen synthesis and inhibited testicular growth. The threshold concentration

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for this induction was estimated to be 10 μg/L [15]. It was demonstrated that detergents can produce toxic effects on keratinocytes at concentrations as low as 3 μg/L [17].

This study reports the use of primary tissue cultures as an ecotoxicology assessment tool using the pollutant nonoxynol.

MATERIALS AND METHODS

Primary culture

Healthy rainbow trout were obtained from a commercial fish farm in Roscrea, Ireland. Epithelial cell outgrowths were obtained from explants using a modification of the technique developed previously [8], as summarized below. A section of skin from the dorsal side was taken, cleaned of muscle tissue, and chopped into 2 mm^2 pieces. Individual tissue fragments were placed in 24 cm^2 Nunc flasks (Biosciences, Dublin, Ireland) containing 2 ml of initiating medium containing serum, as reported in [8]. The cultures were incubated at 19°C in an atmosphere containing 5% CO_2 in a refrigerated incubator (Leec, Nottingham, UK). No medium changes were given, as this arrested cell growth.

Established cell line

The epithelium papulosum cyprini (EPC) cells originated from carp epidermal herpes-virus–induced hyperplastic lesions [18] and have epithelial-like morphology. They were maintained as a monolayer in BHK-21 medium (Gibco Life Technologies, Paisely, Scotland) supplemented with 10% fetal calf serum, 0.025 mol/L Hepes buffer solution, 5 ml L-glutamine (200 mM), and 5 ml penicillin/streptomycin (5000 IU/ml; all from Gibco Life Technologies). The cultures were incubated at 30°C.

Nonoxynol solution preparation

Nonoxynol (Igepal C210) was dissolved in a known volume of dimethylsulfoxide. Sequential dilutions were made prior to the addition to the cultures in volumes of 50 μl per flask—the control contained 50 μl of dimethylsulfoxide.

Cytotoxicity endpoints

Cell growth reduction. The reduction of cell growth in cultures induced by nonoxynol was investigated. Four days after attachment, the perimeter of the cultures was outlined on the bottom of the flask with a permanent marker. Cells that had grown out of the explant formed a whitish area that could be observed with the naked eye. The cultures were then treated for 24 and 48 h with the following sequential concentrations of nonoxynol: 0, 1.25, 2.5, 10, 12.5, 25, 50, and 75 μmol/L (three cultures per dose point). The experiment was repeated twice. The cultures were fixed after exposure in 10% buffered formalin and were counterstained in hematoxylin. The outgrowth area of the culture was estimated using a 1 mm^2 grid by counting the number of squares covered by the explant outgrowth. The area before treatment was also measured. The results are given as a percentage of the control survival rate. The mean values and standard errors were calculated for survival. This method takes into consideration the fact that primary cultures from the same animal do not grow uniformly under the same conditions. The LC50 data was calculated using a probit analysis computer program (LC50 program, U.S. Environmental Protection Agency, Washington, DC).

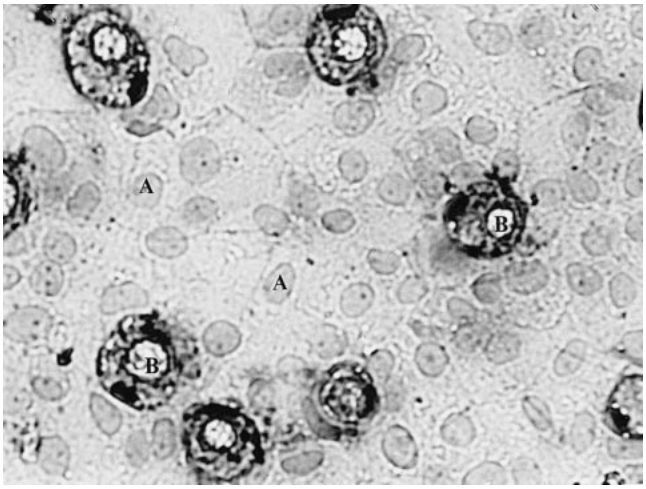

Fig. 1. Normal rainbow trout epithelial skin cell culture showing (**A**) normal epithelial cells and (**B**) goblet cells.

Morphology

The types of cells present in each explant were also counted using a Leica image analyzer (Leica Microsystems Holdings, Ernst-Leitz-Strasse, Germany). Five fields, each 0.55 mm^2, were analyzed from each of the explants described above, two from the inner region, two from the outer region, and one from the midregion. The types of cells identified were classified as normal epithelial (Fig. 1), goblet (Fig. 1), necrotic (Fig. 2), and apoptotic cells. Goblet cells were recognizable in their epithelium based on their basophilic cytoplasm, their basally located nuclei, and the accumulation of mucus secretory granules that filled and distended their apex to give the cells their characteristic goblet shape [19]. Cells were classified as ''undergoing necrosis'' if they exhibited the following features: swelling of the cytoplasm and organelles, with only slight changes in the nucleus, or organelle dissolution and rupture of the plasma membranes, resulting in leakage of the cellular contents into the extracellular space [20]. Cells were classified as ''undergoing apoptosis'' if they showed evidence of two or more of the following: cell volume shrinkage and picnotic nucleus (chromatin condensation), blebbing of the cytoplasm, nuclear fragmentation, and development into apoptopic bodies [21,22].

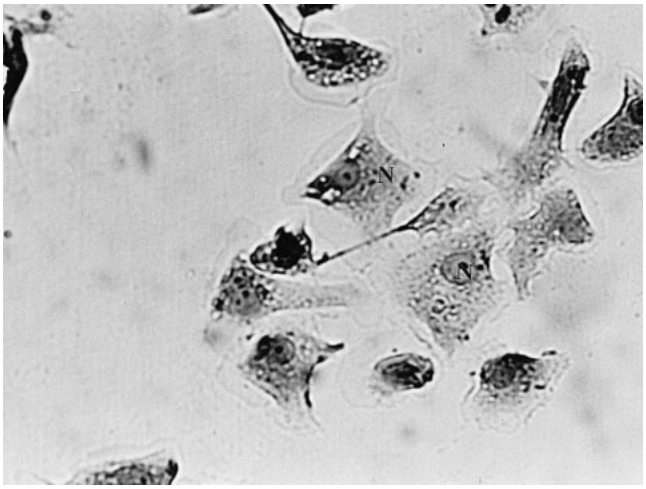

Fig. 2. Rainbow trout epithelial cell culture following exposure to 50 μM nonoxynol showing necrotic cells (N).

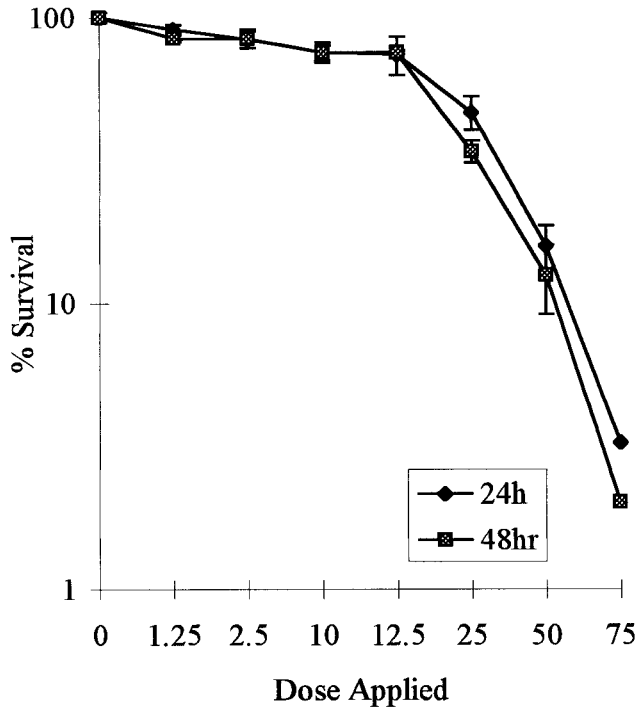

Fig. 3. Percent (%) survival of rainbow trout epithelia with nonoxynol explanatory legend: shows the percentage survival of rainbow trout primary epithelial tissue cultures following treatment with nonoxynol. The results are expressed as mean ± SEM for $n = 6$. The LC50 value for 24 h of exposure is 16.6 μmol/L; the LC50 value for 48 h of exposure is 12.9 μmol/L (probit analysis).

Clonogenic assay

Reduction in colony-forming ability was assessed using the clonogenic assay established by Puck and Marcus [23]. Appropriate numbers of cells were seeded into 25 cm^2 flasks with 5 ml of the appropriate medium, such that about 100 viable colonies could be expected to form after about 7 d. After 4 h, different concentrations of nonoxynol were added in volumes of 50 μl per flask (1%, w/v). Colonies were stained with 15% carbol Fushchin after 20 d of incubation. The toxicity of nonoxynol was assessed by determining the colony-forming efficiency of these cells during exposure and by relating the percentage of colonies to untreated control figures.

The survival fraction was calculated using the following formula.

$$\%SF = \frac{\text{no. of colonies counted}}{\text{number of cells plated}} \cdot \frac{100}{\text{Control plating efficiency}}$$

RESULTS

Primary culture growth data

Rainbow trout primary epithelial cell cultures were found to exhibit a decreased sensitivity to nonoxynol compared with the EPC cell line. Nonoxynol had a LC50 of 16.6 μmol/L after 24 h. Figure 3 shows that a statistically insignificant ($p < 0.05$) increase in cell death was observed over the next 24 h; the LC50 for 48 h of exposure was 12.9 μmol/L. Nonoxynol tested on the primary cell system was found to have an acute lethal dose of 75 μmol/L. The threshold level of response, the level of exposure at which a significant decrease in survival begins to occur, is 12.5 μmol/L for both 24 and 48 h of exposure. The LC50 values are based on the overall area of the culture, inclusive of both epithelial cells and goblet cells.

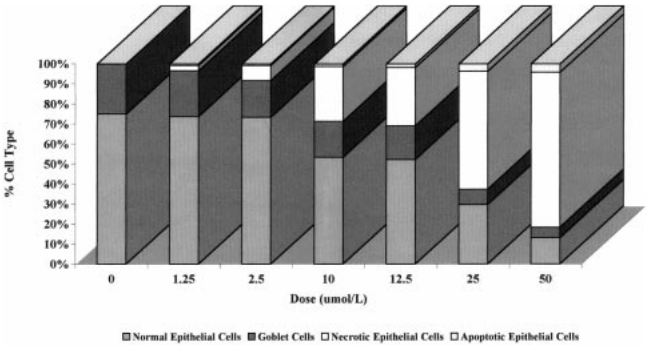

Fig. 4. Type of cells per culture following treatment with nonoxynol—24 h. Each value represents the mean for five (0.55-mm) grids counted in $n = 6$ replicate cultures.

Primary culture cell death type

The average number of cells per field in the control cultures was 31.25 ± 1.20. The number of cells per field decreased as the chemical treatment dose increased. The number of cells per field in the cultures treated with 50 μmol/L nonoxynol was 16.6 ± 1.71. Figures 4 and 5 show that an increase in epithelial necrotic cells was observed with increasing nonoxynol exposure in primary cell cultures. Epithelial cells located on the outside edge of the culture underwent necrosis, primarily at lower doses. A significant increase in those cells undergoing necrosis was observed at 10 μmol/L after 24 h (0 to 6.9 ± 2.5 cells/field) and 48 h (0.1 to 4.7 ± 1.5 cells/field) exposure to the pollutant. Only a slight increase in apoptotic cell death was observed with increasing nonoxynol exposure (0 to 1.7 ± 0.4 cells/field after 48 h exposure to 50 μl nonoxynol).

Relative effect on mucus cells versus epithelial cells

Decreases in the number of goblet cells per field from 7.7 ± 1.2 to 1.0 ± 0.8 (24 h) and from 8.4 ± 1.7 to 0. 8 ± 0.5 (48 h) were also observed as the nonoxynol dose was increased (Figs. 4 and 5). This decrease was observed uniformly across the explant. The threshold level of this decrease was found to be 12.5 μmol/L for 24 h of exposure to nonoxynol. The threshold level of this decrease after 48 h of exposure was slightly lower (10 μmol/L). The goblet cells release their secretory contents onto the culture surface. Emptying of the goblet mucous cells results in their loss and in a consequent decrease in

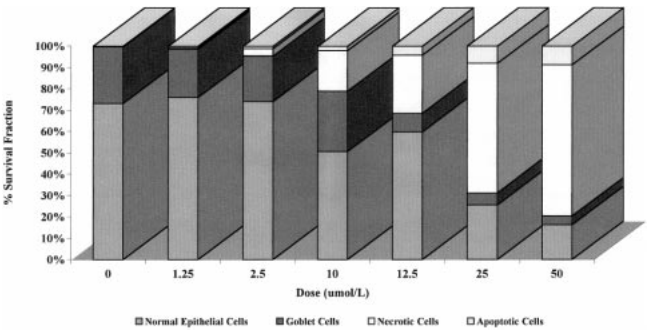

Fig. 5. Type of cells per culture following treatment with nonoxynol—48 h. Each value represents the mean for five (0.55-mm) grids counted in $n = 6$ replicate cultures.

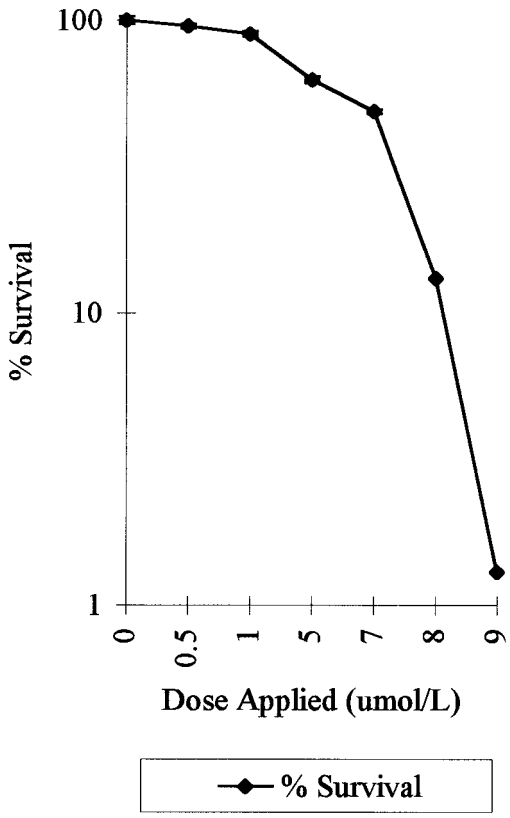

Fig. 6. Percent (%) survival of the epithelium papulosum cyprini (EPC) cell line following treatment with nonoxynol. These results are expressed as mean ± SEM for $n = 9$. The LC50 by probit analysis is 4.1 μmol/L.

their density. The percentage of epithelial cells per field in the cultures was found to increase by 19.9% for 24 h of exposure and by 22.4% for 48 h of exposure as the dose of pollutant increased.

Cell line plating efficiencies

Figure 6 shows the response of the EPC cell line to nonoxynol. The LC50 of nonoxynol acting on the cell line was 4.1 μmol/L. The acute lethal dose of nonoxynol on the cell line was 10 μmol/L. The mean plating efficiency of the control cells was 21.41 ± 0.71%.

DISCUSSION

Rainbow trout primary epithelial cell cultures were found to exhibit a decreased sensitivity to nonoxynol when compared with the EPC cell line studied. A similar relationship was observed when primary rainbow trout skin cultures were treated with cadmium and nickel [24,25]. Although the cell line is more sensitive than the primary cultures to the lethal effects of nonoxynol, primary cultures displayed differential responses of the various cell types present in the culture and were a more representative model of in vivo response. Both epithelial and goblet cells were present in the system [8]. As exposure levels to nonoxynol increased, the quantity of goblet cells decreased. Goblet cells produce mucus, and mucus production is a well-established defense mechanism of epithelial surfaces against pathogens and pollutants [26]. The goblet cells release their secretory contents onto the culture surface, thus forming a thick slimy protective coating in an attempt to prevent deleterious effects associated with the detergent medium [27]. It was observed that the goblet cells were decreasing in number because they were exocytosing their contents. In 1988, Roy observed that an anionic detergent induced similar significant changes in the number and size of goblet mucous cells in the opercular epidermis [27]. This study adds further evidence that mucus production occurs in the presence of an environmental pollutant, thus resulting in a decrease in the quantity of goblet cells present in the skin culture. Decreases in goblet number and density in rainbow trout primary skin culture could be important markers of the toxicity of aquatic pollutants.

An increase in necrotic epithelial cells was observed with increased dose exposure. The number of apoptotic epithelial cells remained extremely low and constant with dose exposure. These results suggest that nonoxynol kills rainbow trout skin epithelial cells through the process of necrosis. In a previous study, a loss of protein mioties was observed from the club cells and epithelial cells of the opercular epidermis as a result of exposure to an anionic detergent [28]. Rapid decreases in protein, ribonucleic acid, and DNA levels occur during necrosis [20]. Other organic and inorganic chemicals have been shown to induce similar responses in fish [29,30]. Exposure to waterborne Hg has been demonstrated to induce cellular necrosis and increased mucus secretion by gill cells [31,32].

The results of this study demonstrate that primary cell cultures are a more appropriate model for toxicity risk assessment of specific in vivo situations than are continuous cell lines, for the reasons stated above. If cultured under the appropriate conditions, the cells retain their full in vivo characteristics for up to 14 d, thus allowing sufficient time for investigation of the immediate in vitro effects of toxicants. The cells in the outgrowth area had a high rate of mitotic activity and were actively dividing [8]. The toxicity curves showed similar slopes to those obtained from cell lines—but with different critical concentrations. Other work carried out by our group (with this system) has shown that changes in glycogen deposits, stress proteins, and oncogene expression can also be studied using this system [5,24,25,33]. The use of short-term cytotoxicity assays for the initial screening of chemicals not only aids in establishing priorities for the selection of chemicals that should be tested further in vivo but also accelerates the time during which potential toxicants can be evaluated [34]. The primary cell culture system used in this study is an extremely useful tool for such assays. The primary cell culture system could also be utilized for different areas of study. These include multiple assays/investigations on homologous cultures derived from in vivo exposure, which may be a major step in solving the problem of comparing in vitro effects with that in vivo effects. The cultures can also be infected with viruses, and may therefore provide excellent opportunities for research into fish diseases.

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