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Surfactant 4-nonylphenyl-polyethylene glycol stimulates reactive oxygen species generation and apoptosis in human neuroblastoma cells

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ABSTRACT

The formulation of most pesticides is proprietary and individual components are therefore not generally known. In a preliminary study, we identified six compounds that are often present in pesticides, of which 4-nonylphenyl-polyethylene glycol (NP-40) was found to be the most toxic. In this study, we investigated the toxicity of NP-40 and underlying mechanism in neuronal SK-N-SH cells. Exposure to NP 40 at concentrations higher than 60 μmol/L for 24 hr decreased cell viability. The cytotoxicity of NP-40 was time- and concentration-dependent. Nuclear fragmentation and chromatin condensation were apparent starting at 50 μmol/L NP-40, and increased at higher concentrations. The expression of apoptotic factors including p53 and B-cell lymphoma (Bcl)-2-associated X protein was upregulated, while that of the anti-apoptotic marker Bcl-2 was downregulated at 80 μmol/L NP-40. Cytochrome c release was observed from 80 to 100 μmol/L by confocal microscopy. Caspase-9 and -3/7 activities increased according to concentration, and fluorescence-activated cell sorting analysis showed that apoptosis was induced at 50 μmol/L and was increased at 80 μmol/L. Our findings indicate that NP-40 stimulates the mitochondrial-mediated apoptosis pathway and reactive oxygen species production in a concentration-dependent manner, and suggest that antioxidant administration may be an effective treatment for patients with acute NP-40 poisoning.

Introduction

The ingestion of pesticides is a common method of suicide in numerous Asian countries (Bradberry et al., 2004; Mann and Bidwell, 1999; Moon and Chun, 2010). Patients who have ingested pesticides are candidates for aggressive therapy, which can improve their chances of survival. It is therefore important to be aware of the toxicity of all chemical components of pesticides.

Surfactants act as detergents, wetting agents, dispersants, and emulsifiers and are therefore widely used chemicals. For example, surfactants are frequently included in herbicide formulations as emulsifiers. Therefore, when pesticides are ingested intentionally or accidently, the potential for surfactant toxicity should be considered by the treating physician (Mann and Bidwell, 1999).

The harmful effects of surfactants have been characterized *in vitro* and include the inhibition of microbial dehydrogenase

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and algae nitrogenase activities (Margesin and Schinner, 1998) as well as cellular injury caused by mitochondrial dysfunction (Hsu and Youle, 1997). In contrast, *in vivo* evidence is limited owing to ethical considerations as well as technical limitations in terms of quantifying surfactant levels in body fluids such as plasma or urine. An additional challenge to the study of surfactant toxicity in humans is the variety of surfactant chemical structures. In addition, pesticide formulations are not disclosed by manufacturers, leaving physicians to treat patients without important information about constituent compounds. We have been able to identify six surfactants that are commonly included in pesticide formulations in Korea. Four of the six surfactants were ionic including sodium dodecylbenzenesulfonate (LAS), dioctyl sulfosuccinate sodium salt (DOSS), poly(acrylic acid) sodium salt (PAASS), lignosulfonic acid sodium salt (LSASS) while two were nonionic including decaethylene glycol monododecyl ether ($C_{12}E_{10}$) and 4-nonylphenyl-polyethylene glycol (NP-40).

Our preliminary study showed that 4-nonylphenyl-polyethylene glycol (NP-40) was the most toxic of these compounds. The present study investigated the mechanisms of cellular toxicity of NP-40, which can aid in the development of therapeutic strategies in cases of acute pesticide poisoning.

1. Experimental

1.1. Materials

NP-40, 3-(4, 5-dimethylthiaxol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), dimethylsulfoxide (DMSO), Trypan Blue, and Hoechst 33258 were obtained from Sigma-Aldrich (St. Louis, MO, USA); 2,7-dichlorofluorescein diacetate (H2DCFDA) was purchased from Invitrogen (Carlsbad, CA, USA); and primary antibodies against p53, B cell lymphoma (Bcl)-2, Bcl-2-associated X protein (Bax), β-actin, and cytochrome c were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Abcam (Cambridge, MA, USA). Horseradish peroxidase (HRP)-conjugated anti-rabbit and -mouse IgG were obtained from Santa Cruz Biotechnology; and Alexa Fluor 488 goat anti-mouse IgG was purchased from Invitrogen.

1.2. Methods

1.2.1. Cell lines

In a preliminary experiment, we investigated six surfactants commonly used as pesticide additives in Korea for their cytotoxicity against cells of different origin, including lung epithelial, hepatocellular, liver fibroblast-like, and neuronal cells. NP-40 was cytotoxic to these cell lines at very low concentrations; however, SK-N-SH human neuroblastoma cells showed the greatest sensitivity to the effects of NP-40. This result is consistent with the fact that unconsciousness and apnea are frequently manifested by patients with surfactant poisoning. Based on these findings, we used SK-N-SH in our experiments.

1.2.2. Cell culture and treatment

SK-N-SH cells were purchased from the Korean Cell Line Bank (Seoul, South Korea) and maintained in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37°C under saturating humidity in an atmosphere of 5% CO_2 and 95% air. An NP-40 stock solution was prepared in water and diluted in cell culture medium as required.

1.2.3. Cell viability and cytotoxicity assays

To investigate the effect of NP-40 on cell viability, cells were seeded at a density of 2×10^4 cells/well overnight until they reached 70%–80% confluence. Following treatment, 20 μL of 5 mg/mL MTT solution was added to each well, followed by incubation for 4 hr. The culture medium was then aspirated and 100 μL of DMSO was added to each well to dissolve the formazan crystals that had formed. The absorbance of the resultant solution was measured at 590 nm using a Victor X3 multilabel plate reader (Perkin Elmer, Waltham, MA, USA) (Zerin et al., 2015).

Cytotoxicity was determined by measuring lactate dehydrogenase (LDH) activity. Briefly, 50 μL of a cell suspension containing 2×10^4 cells were seeded overnight and then treated with 50 μL of the test chemical for indicated times. Three background control groups consisted of cells incubated in 100 μL of medium only as well as low- and high-concentration controls with 50 μL each of the cell suspension and medium. The high-concentration control sample was also treated with 5 μL lysis solution and incubated for 15 min. An equal volume of freshly prepared reaction mixture (Roche, Pleasanton, CA, USA) was added to each well according to the manufacturer's protocol, followed by incubation for 30 min in the dark. The absorbance was measured at 490 nm using a Victor X3 multilabel plate reader after adding 50 μL of the stop solution. The background value was subtracted from all readings.

$$\text{Cytotoxicity} = \frac{\text{sample value–low control}}{\text{high control–low control}} \times 100\%$$

was calculated as a percentage with the following equation:

1.2.4. Intracellular reactive oxygen species generation assay

To measure reactive oxygen species (ROS) generation, 2×10^4 cells were seeded in a black cell culture plate overnight, then treated with 10 μmol/L H2DCFDA in 1× Hank's balanced salt solution (HBSS) containing 0.1 mg/mL glucose for 30 min at 37°C. The cells were then washed with 1× HBSS and treated with NP-40. We used hydrogen peroxide (H_2O_2) and N-acetyl-L-cysteine (NAC) as ROS inducer and inhibitor, respectively. ROS generation was measured at the indicated times using the Victor X3 multilabel plate reader at excitation and emission wavelengths of 485 and 530 nm, respectively.

1.2.5. Chromatin condensation detection assay

To detect condensed chromatin, 1×10^5 cells were seeded overnight in a 24-well plate containing 10-mm glass coverslips until they reached 70%–80% confluence, followed by treatment with desired concentrations of NP-40 for the specified periods. Cells were then fixed with 4% paraformaldehyde for 15 min, washed with phosphate-buffered saline (PBS), then stained with 1 μL of Hoechst 33258 (5 mg/mL) in 1 mL basal medium for 10 min in the dark. The cells were then mounted in fluorescence mounting medium (Dako, Glostrup, Denmark) and observed