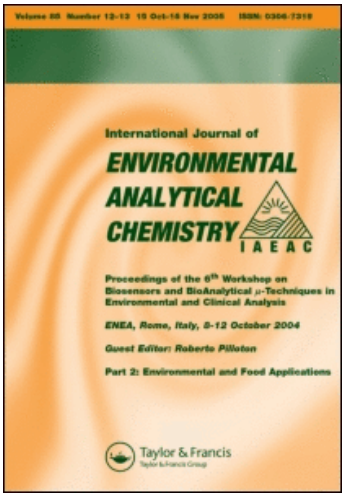

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Genotoxicity and Cytotoxicity of 4-Nonylphenol Ethoxylate on Lymphocytes as Assessed by the Comet Assay

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GENOTOXICITY AND CYTOTOXICITY OF 4-NONYLPHENOL ETHOXYLATE ON LYMPHOCYTES AS ASSESSED BY THE COMET ASSAY

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Surfactants, which are prevalent at industrial sites and in the environment generally, are potential risk factors in human carcinogenesis. The widespread industrial use of surfactants such as 4-alkylphenol ethoxylates and their prevalence in many cleaning products have provoked studies about surfactant concentrations in water and their toxicity levels. Up to now, these substances have mainly been tested on aquatic organisms. Though tests on human cell lines are rare.

The alkaline Comet assay was performed to evaluate the genotoxicity of 4-nonylphenol ethoxylate, a biodegradable product of 4-alkylphenol ethoxylate, in human lymphocytes. Concentrations tested ranged from 0.15 to 150 μg/mL.

Test concentrations of 10 to 15 μg/mL caused an increase level of DNA migration in human cells, but without inducing excessive toxicity (viability > 80%). Though induced levels of DNA migration starting at concentrations of 30 μg/mL may have been due to excessive levels of cytotoxicity (viability < 70%). Based on these data, 4-nonylphenol ethoxylate can induce DNA damage in human lymphocytes but at higher concentrations than are normally found in river or drinking water. However, considering the prevalence of surfactants, the measured genotoxicity of these substances is of concern. Further investigations on human target cells are necessary to evaluate the carcinogenic impact of surfactants and reconsider their environmental acceptance.

Keywords: Genotoxicity; Human lymphocytes; 4-nonylphenol ethoxylate; Comet assay

INTRODUCTION

In addition to tobacco smoke and alcohol consumption, many environmental and occupational xenobiotics have been identified as risk factors for tumor initiation [1]. Possible mechanisms include DNA damage leading to mutation.

Nevertheless, many other environmental substances still demand investigation of their genotoxic potential. One example is surfactants, a group of compounds

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found in household cleaning products and industrial settings (i.e., as a wetting agent to dissolve paper and as a paint emulsifier).

4-alkylphenol ethoxylates (APEOs), important representatives of this group, are flushed into waste waters and end up in sewage treatment plants, where they undergo stepwise biodegradation to become lipophilic and toxic compounds such as 4-nonylphenyl ethoxylate (NPEO) [2]. Being essential in aqueous solutions, these substances come into contact with the aquatic and terrestrial environment via waster water and by being potentially released through the spreading of wastewater treatment sludge for soil enrichment [3]. They can finally end up in drinking water, fish and other seafood.

The toxicity of APEO was determined in several aquatic species like salmon and common mussels [4]. A genotoxicity study on MCF-7 human breast tumor cells revealed genomic instability by telomeric associations and chromosomal aberrations caused by nonylphenol [5]. In a previous study on blood samples of 2 donors nonylphenol caused DNA damage using concentrations from 1 to 500 μM.

In addition to toxicity, the estrogenic and xenoestrogenic potential of surfactants has been demonstrated [6]. NPEOs showed xenoestrogenic behavior in aquatic organisms, induced proliferation in human breast cancer cell lines [5,7] and enhanced the excretion of estrogens in humans [8].

Because of their harmful character, APEOs and their metabolites have been partially replaced by other tensides. Nevertheless, due to their favorable emulsifying and surface-wetting ability, worldwide industrial production has persisted: In 1997, production was still at 1 18 000 tons in the European Union [9]. In Schleswig-Holstein, Germany, nonylphenol ethoxylate was found at concentrations between 0.002 and 0.001 μg/L in drinking water [2].

Information concerning APEOs and their metabolites is still incomplete and requires more research. Despite their widespread prevalence and human exposure, incomplete data were available regarding the biological behavior and impact of these substances on human cells. Human testing so far was performed on a human tumor cell line and lymphocytes of 2 blood samples only. Therefore, the aim of the present study was to assess the genotoxicity of 4-nonylphenyl ethoxylate measured in human cells of a larger patient collective using the alkaline Comet assay.

EXPERIMENTAL

Peripheral lymphocytes of 24 healthy donors were used for this study: 16 males, aged 20–60 (mean age 35) and 8 females, aged 21–61 (mean age 43.5). The patients had signed an informed consent statement. The Ethical Commission of the Medical Department, Ludwig-Maximilians-University Munich, approved the study.

A dose-response relationship in terms of genotoxicity was determined using concentrations of 4-nonylphenol ethoxylate ranging from 0.15 to 150 μg/mL. As a negative control, 40 μmol/mL DMSO, the solvent for 4-nonylphenol ethoxylate, was used.

Heparinized blood samples were obtained by peripheral venous puncture. Lymphocytes were separated on equal amounts of Lymphoprep (Nycomed; Oslo, Norway) using density gradient centrifugation (20 min, 2000 U/min, 20°C). After being washed with phosphate-buffered saline (PBS; Gibco BRL Lifetechnologies; Eggenstein, Germany) the cells were resuspended in fetal calf serum (FCS, Gibco) and dimethyl sulfoxide (DMSO; Merck; Darmstadt, Germany) and stored immediately

at −80°C. To preserve a physiological character of the samples, we did not use S9-mix for metabolic activation. After thawing, the lymphocytes were washed in PBS and resuspended in RPMl 1640 and 10% FCS (Gibco). Time interval between thawing and incubation of the lymphocytes was not more than 2 months. Storing lymphocytes as described above was shown not to influence genotoxicity testing using the Comet assay [10]. Before incubation, cell counts and viability testing were performed. Lymphocytes were incubated with 4-nonylphenol ethoxylate (Arkopal N-080, nonylphenolpolyglykolether with 8–11 ethoxyl (EO)-units; Clariant, Frankfurt, Germany) for 60 min. After this period cytotoxicity was re-examined using trypan blue staining.

The alkaline microgel electrophoresis assay (Comet assay) was used to measure DNA single strand breaks and alkaline labile sites on human lymphocytes *in vitro* [11,12].

Special comet slides, designed with a frosting of 5 mm along the long edges (76 mm × 26 mm; Langenbrinck; Emmendingen, Germany), were prepared with 85 μL of 0.5% normal melting agarose (Biozym; Hameln, Germany). The cell aliquots were suspended with 75 μL of 0.7% low melting agarose (Biozym) and applied to the prepared slides. Alkaline lysis with 10 mL DMSO, 1 mL Triton-X, and 89 mL alkaline lysis buffer (NaCl 0,9%, Na_2EDTA, Trizma base, *N*-Lauroylsarcosin sodium salt) was for 1 h. The slides were placed into a horizontal gel electrophoresis chamber (Renner; Dannstadt, Germany), positioned close to the anode and covered with alkaline buffer solution containing 10 mM NaOH and 200 mM Na_2EDTA at pH 13.2. After a 20-min DNA "unwinding" period, electrophoresis was started at 0.8 V/cm and 300 mA for 20 min. Following neutralization (Trizma base, pH 7.5; Merck), the slides were air dried and the DNA stained with ethidium bromide (Sigma). All slides were examined under a fluorescence microscope (magnification 40×; DMLB, Leica; Heerbrugg, Switzerland) with an adapted CCD camera (Cohu; San Diego, Calif., USA). Eighty cells were scored per sample.

Depending on the degree of strand-break induction, the DNA fragments demonstrate different types of migration within the electric field, creating so-called comets (Figs. 1 and 2), with single strand breaks and alkali labile sites in the tail of the comet. The comets were measured using an image analysis system (Komet 3.1; Kinetic Imaging; Liverpool, U.K.). To quantify extent of DNA migration, the *Olive Tail Moment* (OTM), which is the relative amount of DNA in the tail of the comet multiplied by the median migration distance [13], was used.

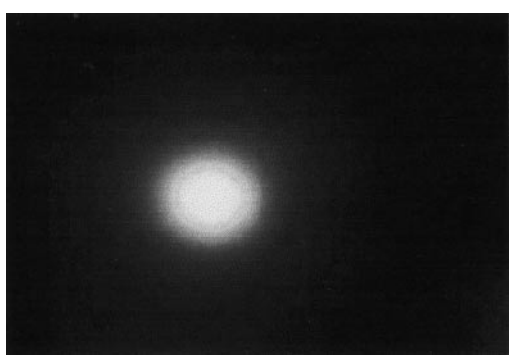

FIGURE 1 DNA of a cell with low damage after incubation with DMSO and fluorescent staining with ethidium bromide.

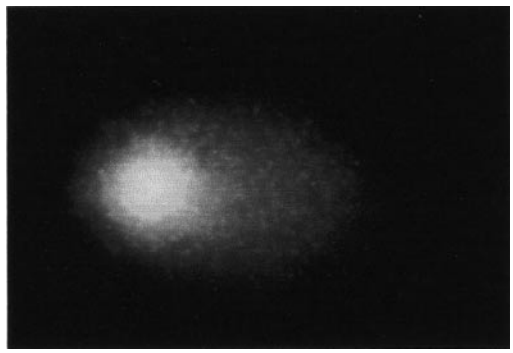

FIGURE 2 DNA of a cell with medium damage after incubation with 15 µg/mL 4-nonylphenol ethoxylate and fluorescent staining with ethidium bromide.

For statistical analysis, the SPSS^{TM} 11.0 program was used. To compare genotoxic impacts by different concentrations of the tested substance (4-nonylphenol ethoxylate *vs*. negative control), the Wilcoxon Test was applied.

RESULTS

Incubation with DMSO as negative control caused DNA migration in the alkaline microgel electrophoresis assay (Comet assay) defined as OTM: 2.34 (mean) ± 1.16 (standard deviation) and a mean viability of 89.29 ± 7.18% in peripheral lymphocytes.

Low DNA migration and high viability counts were achieved after incubation with 0.15, 1.5 and 5 µg/mL NPEO (OTM 3.35 ± 0.65; viability 87.2 ± 5.15%), (OTM 2.96 ± 1.04; viability: 89.2 ± 3.76%) and (OTM 3.09 ± 1.5; viability: 86.15 ± 7.7%) respectively. Concentrations of 10 and 15 µg/mL, however, resulted in enhanced strand break induction (OTM: 3.82 ± 1.62, 4.23 ± 1.15 and 4.39 ± 0.83) with relatively stable cytotoxicity levels (viability: 81.17 ± 3.82, 82.11 ± 6.05, 86 ± 5.35%).

Starting at a concentration of 30 µg/mL, viability was as low as 64.54 ± 17.49% with an OTM of 5.21 ± 2.75, respectively.

Higher DNA fragmentation was measured at concentrations of 45, 60 and 150 µg/mL (OTM: 7.25 ± 3.71, 8.70 ± 4.64 and 9.46 ± 1.58) though with high cytotoxic levels (viability: 48.15 ± 23.40, 44.0 ± 33.98 and 0 ± 0%).

Statistically significant genotoxicity was unveiled for concentrations of 10 µmol/mL ($p = 0.002$), 15 µmol/mL ($p = 0.011$), 30 µmol/mL ($p = 0.001$), 45 µmol/mL ($p = 0.001$), 60 µmol/mL ($p = 0.018$), 75 µmol/mL ($p = 0.043$) and 150 µmol/mL ($p = 0.018$) compared to the negative control.

Single results are listed in Table I. An overview of genotoxic and cytotoxic effects is given in Figs. 3 and 4.

DISCUSSION

The occurrence of malignant tumors in humans is associated with many exogenous risks, such as exposure to occupational and environmental chemicals. In particular, substances that are widely prevalent and used, e.g., metals, wood dust, polycyclic

TABLE I OTM values listed for each patient and concentration of 4-nonylphenol ethoxylate

Age	*Gender*	*Results (mean Olive Tailmoment)*									
48	w	2.11	2.44	3.08			3.87				
24	m	3	2.76	2.6			2.68				8
59	m	1.64	3.98	4.9			4.42				10.94
20	m	0.99	3.49	1.97			5.22				
21	w	2.53	4.08	2.27			6.24				
56	w	0.61					5	6.8	10.7	12.3	
55	w	1.2					2.4	3.8	6	11	
55	m	1.66			2.55	1.56	3.67				
33	m	2.36			2.91	4.22	4.56				
20	m	4.46			4.85	5.28		6.9	11.25		
49	m	5.64			6.91	7.38		11.45	14.65		
34	m	4.13			4.16	4.66		5.19	10.19		
46	m	1.38			1.77	2.25		3.11	4.91		
36	w	2.05			1.91	3.42		2.9	5.68		
22	m	1.37			1.3	2.07		2.04	2.43		
22	m	3.03			3.98	3.29		3.33	3.67		
60	m	1.2			2.38	2.36		1.9	2.37		
35	w	2.61			1.7	2.8		4.41	7.26		
46	m	1.36			2.37	5.57		7.04	4.54		
60	w	3.62			3.33	4.79		8.88	10.58		
26	m	2.34								6.14	9.1
58	m	2.12								5.34	8.97
20	m	1.55								17.8	10.31
46	m	1.76								3.75	9.75
36	w	2.31								5.85	13.6
		negative control	0.15	1.5	5	10	15	30	45	60	150
					concentration of 4-nonylphenol ethoxylate (μg/mL)						

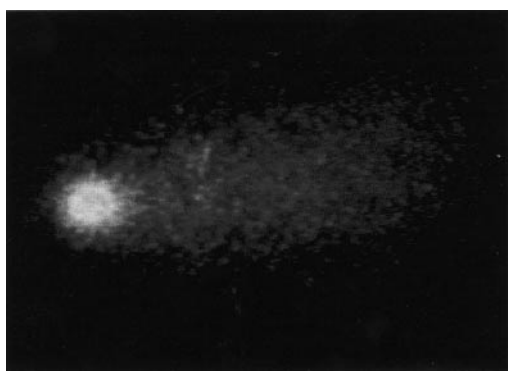

FIGURE 3 DNA of a cell with great damage after incubation with 45 μg/mL 4-nonylphenol ethoxylate and fluorescent staining with ethidium bromide.

aromatic hydrocarbons and nitroso compounds, have been found to be of carcinogenic risk to humans [1,14,15].

Alkylphenol-ethoxylates are common surfactants used in cleaning products and involved in many industrial processes requiring wetting agents and emulsifiers. APEOs and their biodegradable products have xenoestrogenic characteristics. Acting as estrogen agonists, NPEOs or 4-nonylphenol ethoxylate caused distinct changes and growth in germ cells of feathed minnows and extensive cell proliferation in the

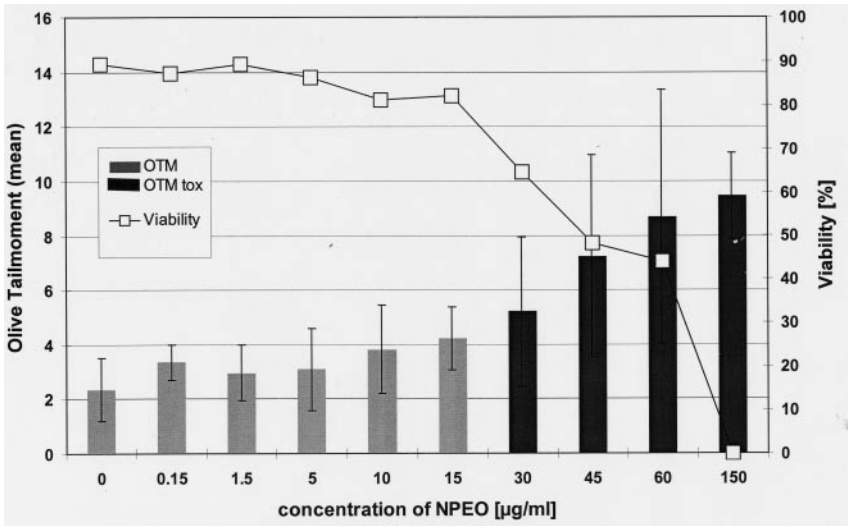

FIGURE 4 Genotoxicity and cytotoxicity of 4-nonylphenol ethoxylate. The light bars show the genotoxic impact of the substances with low cytotoxic effects (OTM), whereas dark bars demonstrate genotoxicity of 4-nonylphenol ethoxylate with accompanying increase of cytotoxicity (OTM tox).

mammary glands of noble rats [16]. In addition, some biodegradable products of estrogen-like APEOs, especially 4-nonylphenol ethoxylate, also demonstrate cytotoxic effects in aquatic organisms, which has led to increasing concern about the environmental hazards of these substances [2].

Under various test conditions, NPEO induced cytotoxicity in many aquatic organisms at concentrations ranging from 0.069 to 5.0 µg/mL [2,4]. Most detected concentrations of 4-nonylphenol ethoxylates in river water ranged from 0.00001 to > 0.1 µg/mL, lower than these cytotoxic levels [17].

One study showed significant genotoxic effects of nonylphenol (1, 10 and 50 µM) in human lymphocytes and sperm cells of a single donor tested [18]. Because of distinct differences of the chemical property compared to 4-nonylphenol ethoxylate, these results have low impact on the assessment of the risk potential of NPEOs. NPEO has shown its ability to alter cell cycle kinetics, to induce DNA damage and chromosomal aberrations tested on rodents [19]. Moreover, using the human breast cancer cells MCF-7, alterations of estrogen-responsive reporter genes were elucidated [20].

The present investigation on human lymphocytes showed a dose-dependent increase in DNA damage at concentrations starting at 5 µg/mL 4-nonylphenol ethoxylate, whereas lower concentrations (0.15–1.5 µmol/mL) showed no significant differences in genotoxicity compared to the negative control in our test system. Concentrations higher than 30 µg/mL with increasing genotoxic effects again were accompanied by excessive cytotoxicity (viability $< 70\%$). Viabilities below 70% are considered to be incompatible with reliable genotoxicity measurements using the Comet assay [21]. Concentration levels causing dose-dependent genotoxicity with viabilities higher than 70% in our investigation are comparable to the concentrations resulting in cytotoxicity in aquatic organisms (summarized in [2]).

The concentrations of 4-alkylphenol ethoxylates and their metabolites in river water are below acute cytotoxicity thresholds for aquatic organisms in most reported studies

and concentrations below 10 μg/mL had no significant genotoxic impact on human lymphocytes *in vitro*. Therefore concentration used in most industrial processes are below a genotoxic threshold. However, the estrogenic action and additional genotoxic potential measured in human lymphocytes might be of concern given the widespread prevalence of these chemicals in the environment and the bioaccumulation in the food chain. The application of additional types of testing to investigate a possible induction of genomic instability in human cell systems is warranted to analyze a risk profile for tumor initiation of 4-alkylphenol ethoxylate.

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