

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Genotoxic evaluation of occupationally and environmentally occurring biocides: the sterilizing agent ethylene oxide and the pyrethroid insecticide phenothrin

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The Examination takes place at the Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 1st April 2015, 11 am

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Introduction

Cancer is a leading cause of death worldwide, with 14.1 million new cases and 8.2 million deaths in 2012. Globally, 19% of all cancers are attributable to environmental and occupational chemical exposures, resulting in more than 1.5 million deaths each year. According to estimations, workplace carcinogens are accounted for approximately 2% to 8% of the global cancer mortality.

Meanwhile the amounts and kinds of chemicals in use are rapidly increasing, the knowledge on their hazardous properties is not in line with this development. There is still a large gap between the information we have and the information we need to control chemicals in order to protect human health. Investigation of the, among others, genotoxic features of certain substances is particularly important because induced DNA damage is closely associated with carcinogenesis so related information is necessary for the establishment of scientific basis for the assessment of cancer risk to humans.

The widespread production and use of biocidal substances underpin the importance of understanding the potential health risk of human exposure to these agents because some of them can attack cellular macromolecules, such as DNA. Other biocide compounds, such as pesticides, do not necessarily exert their effect through direct molecular damage; even though their genotoxic feature cannot be ruled out.

The knowledge of genotoxic effects of frequently used chemicals and molecular mechanisms responsible for DNA damage can aid in the design of efficient strategies that will prevent such damage from accumulating into mutations. A wide range of assays are presently used for the detection of direct genotoxic effects. One such method is the single cell gel electrophoresis assay, which is able to detect single strand breaks, double strand breaks and alkali labile sites.

Measurement of DNA damage by the comet assay

The alkaline version of the single cell gel electrophoresis assay (comet assay) is a cheap, sensitive, easy to perform and rapid technique to evaluate the DNA damage in individual cells. The fundamental principle of the test is to detect DNA damage by

monitoring movement of DNA fragments in an agarose gel. The negatively charged fragments of DNA are pulled toward the anode giving the nucleus its characteristic comet trace profile in which the nucleus represents the head of the comet and the migrated DNA represents the tail. The resulting fluorescently labelled comets can be either scored by visual examination or measured as morphological parameters calculated by image analysis software from the intensity profile. The most commonly used computed parameters include the comet tail length, the percentage of DNA in the tail and the tail moment. The latter can be defined as the product of the fraction of DNA in the comet tail and the tail length in μm .

The comet assay can be applied to a variety of studies including the investigation of genotoxicity, DNA repair, environmental and human biomonitoring as well as clinical studies. In addition to determining which chemicals can cause genetic damage, the assay can also provide useful information on the mechanism of damage, e.g. with use of specific endonucleases that can recognize various types of damaged bases. As single cells are visualized, it is also possible to detect intercellular differences in response to DNA damaging agents.

Ethylene oxide

Ethylene oxide (EO), a well-known sterilizing agent, was found genotoxic in various *in vitro* and *in vivo* test systems. Its DNA damaging effect is attributed to the direct alkylating properties of the molecule. EO is classified by the International Agency for Research on Cancer (IARC) as proven human carcinogen. Increased incidence of malignant neoplasm including, among others, lung cancer has been observed in workers exposed to ethylene oxide; however conflicting epidemiological evidence exists regarding its carcinogenic spectrum.

Characteristics, application, exposure

Ethylene oxide is the simplest cyclic ether and a very reactive alkylating agent due to its highly strained ring which can open easily. At room temperature, EO is a colorless gas with ether-like odor. It occurs naturally in the atmospheric air, being produced in small amounts by oxidation processes. EO also occurs in the exhaust gases of hydrocarbon

combustion, such as that of internal-combustion engines, as well as in tobacco smoke. A large quantity of EO is produced artificially in industrial processes. Its worldwide annual production was about 19 million tons in 2006.

Minor amounts (0.05%) of the annual production of ethylene oxide are used directly in the gaseous form for food disinfection and sterilization of heat- and/or moisture-sensitive medical equipment in hospitals. EO is an excellent sterilizing agent because of its effective bactericidal, sporicidal, and virucidal activity. Disadvantages of EO use are that it can leave toxic residues on sterilized items and it possesses several physical and toxicological hazards to personnel and patients, therefore the applications of this substance merit special attention.

Human exposure to EO occurs mainly through inhalation of occupationally polluted air by workers involved in ethylene oxide production or in the processing and use of this compound in occupational settings. Although, the majority of industrial operations in chemical plants are performed in closed systems nowadays, exposure can be still significant in sterilization plants and in health care sterilization facilities, particularly during unloading of the sterilized materials.

The 8-hour time-weighted average (8-h TWA) of industrial EO exposure levels typically ranged from undetectable level to 18 mg/m^3 , although in occasional work situations (loading of the gas, leaks, plant breakdown, etc.), the worst-case peak exposures were up to $17,300 \text{ mg/m}^3$. Airborne concentrations (8-h TWA) of EO measured in hospitals reached 124 mg/m^3 , but in exceptionally cases (improper operation of sterilizers, insufficient ventilation of sterilization or aeration area, inadequately adjusted instruments, etc.), significantly higher exposure levels (even up to several thousand mg/m^3) were also registered. Due to the inadequate operation of gas sterilizers in the pediatric ward of the County Hospital in the City of Eger, Hungary, the airborne concentration of EO exceeded 150 mg/m^3 during unloading of the sterilized materials as measured in 1992.

Based on experimental findings in animals and on epidemiological observations in humans, the U. S. Occupational Safety and Health Administration (OSHA) established a permissible exposure limit for occupational exposure to ethylene oxide of 1.8 mg/m^3 determined as an 8-hour time-weighted average concentration. Likewise in Hungary, the 8-hour maximum permissible exposure level of EO in the workplace air is 1.8 mg/m^3 . Being much more allowing, the Health and Safety Executive in the U. K. recommend 9.2 mg/m^3 airborne concentration of EO as a long-term workplace exposure limit (8-h TWA).

Health effects

Acute inhalation exposure to high levels of ethylene oxide can cause nausea, vomiting, respiratory tract irritation, bronchitis, pulmonary edema, emphysema and may lead to central nervous system depression and seizures. Dermal or ocular contact with solutions of EO may cause irritation of the eyes and skin, while long-term exposure to high levels of airborne EO can result in cataracts. Some evidence exists indicating that short- and long-term inhalation exposure can cause an increased frequency of miscarriages in female workers.

Genotoxicity, mutagenicity, carcinogenicity

As ethylene oxide is an extremely reactive, direct-acting alkylating agent, it attacks the nucleophilic groups of cellular macromolecules, including DNA. The mechanism of genotoxicity of EO is not fully characterized, but is thought to involve the formation of DNA adducts. The most abundant DNA adduct induced by ethylene oxide is N7-(2-hydroxyethyl)guanine (N7-HEG). Its mutagenic potential was investigated in a variety of *in vitro* and *in vivo* experiments which detected that EO can induce DNA strand breaks, HPRT mutations, micronucleus formation, chromosomal aberrations, and sister chromatid exchange in cultured cell model systems. EO was demonstrated to increase the frequency of DNA strand breaks, sister chromatid exchanges, chromosomal aberrations, HPRT, p53, H-ras and K-ras mutations, micronucleus formations, and changes in the expression of base excision DNA repair genes in experimental animals and humans *in vivo*.

The carcinogenic properties of EO were demonstrated by animal inhalation studies, where different types of neoplasms, including lung cancer, were developed; however, still limited evidence exists for the cancer causing ability of ethylene oxide in humans. According to human epidemiological findings, occupational exposure to ethylene oxide is principally associated with the development of lymphatic and hematopoietic tumors, but further studies identified additional sites of neoplasms, too, including breast and stomach cancer. Although the major portal of entry of EO exposure is the respiratory tract, there is still insufficient evidence that EO is able to induce tumors in the respiratory tract.

Nevertheless, significantly elevated mortality among EO-exposed hospital workers was observed in Hungary, which could be partly attributed to lung cancer.

It is widely accepted that ethylene oxide is a genotoxic agent, but its DNA damaging effect on human lung cells has not yet been extensively studied, despite the fact that these cells are the first barrier encountered by EO. Previous studies investigated the genotoxic property of ethylene-oxide in human fibroblasts and in breast epithelial cells *in vitro* with the use of single cell gel electrophoresis assay and found positive dose-response relationship. Nevertheless, no studies have been conducted in cells derived from the lung, the principal biological target of EO.

Phenothrin

Phenothrin, a synthetic pyrethroid pesticide, is widely-used to control agricultural and household insects, as well as to eliminate human louse infestation. It is classified by the United States Environmental Protection Agency (US EPA) as “not likely to be carcinogenic to humans”, although studies on the genotoxic effect of this insecticide are lacking.

Characteristics, application, exposure

Phenothrin, also known as sumithrin, is a colorless or pale yellow liquid possessing a slight odor. It belongs to the type I pyrethroid group of pesticides that does not contain cyano molecular group. Phenothrin was first synthesized in 1969, and has been used in various applications since 1977. Recent quantitative data on the production and use of phenothrin are not publicly available; its worldwide production level was estimated 70-80 tons per year in 1989 by the WHO. In all likelihood, its global use has greatly increased over the past decade as pyrethroid pesticides have emerged to substitute several organophosphate pesticide residential uses that are no longer available.

As an effective nerve stimulant, phenothrin influences the conduction of nerve impulses by forcing the sodium channels of insects to remain open and the consequent excessive sodium discharge eventually leads to paralysis.

Phenothrin is widely present in pesticide products that are used in commercial and industrial settings to control agricultural and household insects, as well as in infectious disease control of insect vectors. Furthermore, phenothrin has therapeutic applications; specifically it is used for eliminating human louse or scabies infestation, in which case it is formulated as a powder, shampoo, or lotion.

The general population may be exposed to phenothrin through multiple routes such as inhalation of household aerosol sprays, ingestion of food containing residual material, or dermal contact with pediculicides (medications used to treat lice and scabies infestations). Furthermore, occupational scenarios may also pose increased risk of exposure to phenothrin if no effective protective equipment is supplied to or used adequately by the workers who mix, load and apply the pesticide product.

Health effects

The sensitivity of human nerves to phenothrin is low, nevertheless studies reported general toxic effects of phenothrin observed in humans including symptoms like dizziness, salivation, headache, fatigue, diarrhea, and irritability to sound and touch.

Genotoxicity, mutagenicity, carcinogenicity

Although phenothrin was found to be non-mutagenic in *Escherichia coli* strains, its genotoxic potential could be evidenced in an *in vivo* animal study, in which phenothrin administered to rats intraperitoneally for 14 consecutive days caused oxidative DNA damage in the liver and kidney as measured by high performance liquid chromatography (HPLC). Further data provided by other mutagenicity and genotoxicity studies, especially on higher organisms, are lacking.

Its carcinogenic potential was investigated by *in vivo* animal studies, in two of which phenothrin increased the incidence of liver cancer; however, it did not achieve statistical significance. In an *in vitro* study, epithelial cells of the mammary gland were exposed to phenothrin and the results indicated an increased WNT10B proto-oncogene expression. On the basis of these limited findings, phenothrin has been classified by the United States Environmental Protection Agency (US EPA) as “not likely to be carcinogenic to humans”.

Aim and objectives

The purpose of our study was to characterize the genotoxic properties of the sterilizing agent ethylene oxide and the pyrethroid insecticide phenothrin in *in vitro* cellular models by using a modern, highly sensitive genotoxicity test.

To fulfill the aim, the following objectives were set:

- 1.1. To examine the susceptibility of lung epithelial cells to the alkylating insult of EO in an *in vitro* system with alkaline comet assay by comparing three cell populations: (1) cultured human lung epithelial cells which represent a useful *in vitro* model of the lung as the hypothesized target of EO exposure; (2) cultured human keratinocytes as non-established targets of EO; and (3) isolated human peripheral blood lymphocytes, a commonly used cell type in biomonitoring.
 - 1.2. To describe the susceptibility pattern of these three cell types towards the alkylating insult of EO by comparing it to the susceptibility of the same cell types towards the oxidative DNA damage induced by hydrogen peroxide (H₂O₂).
 - 1.3. To measure the *in vitro* active concentrations of EO with gas chromatography in order to determine the average exposure level during treatment that allows for modelling the *in vivo* internal dose.
 - 1.4. To reconsider the adequacy of the present (1.8 mg/m³) occupational exposure limit for ethylene oxide.
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- 2.1. To update our knowledge about the genotoxic properties of phenothrin as measured by the comet assay in *in vitro* cellular models of human peripheral blood lymphocytes and human hepatocytes.
 - 2.2. To make a preliminary evaluation on the appropriateness of the present regulations of phenothrin use.

Materials and methods

Cell cultures

Human peripheral blood samples were obtained by venipuncture into heparin-containing vacutainer tubes (BD Vacutainer Systems, Plymouth, UK) from 5 healthy volunteers (males, aged 25 to 30 years). Mononuclear white blood cells were separated from the erythrocytes by density gradient centrifugation using Histopaque-1077.

Human type II-like alveolar epithelial cells (A549) were kindly provided by the University of Birmingham, Institute of Occupational Health. The cells were originally derived from a lung adenocarcinoma and are extensively used as an *in vitro* model system to study human respiratory epithelial cell biology.

HaCaT cells, spontaneously immortalized human keratinocytes were a gift from the Department of Dermatology, Medical and Health Science Center, University of Debrecen. This cell line is a widely accepted cellular equivalent of human keratinocytes.

The human hepatoblastoma-derived cell line (HepG2) was purchased from ATCC (Manassas, VA, USA). It provides a frequently used *in vitro* model in human toxicological studies on liver cells.

In vitro treatment

The day before the experiment, the adherent cells (A549, HaCaT and HepG2) were seeded for treatment into 6 wells (2×10^5 cells/well) of a 12-well plate and allowed to attach overnight and grow to 80–90% confluence. Isolated human peripheral blood lymphocytes were partitioned at a cell density of 2×10^5 cells/ml medium into 6 wells of a 12-well-plate on the day of the experiment.

Treatment concentrations of EO (0–500 μ M) and H_2O_2 (0–10 μ M) showed no evidence of cytotoxicity. Aliquots of EO and H_2O_2 solution at different concentrations were added to the cell cultures (human peripheral blood lymphocytes, A549 and HaCaT) for 1 h at 37°C.

Treatment doses of phenothrin that showed no sign of considerable cytotoxicity were 0, 20, 50, 100, 500, and 1000 μ M. Aliquots of different concentrations of the

phenothrin solution and the methanol solvent control were added to the cell cultures (human peripheral blood lymphocytes and HepG2) and incubated for 1 h at 37°C.

Detection of ethylene oxide concentration by gas chromatography

Gas chromatography (GC) was used to monitor the active concentration of EO during treatment. EO concentration in aqueous solution was determined using a HP 5890 gas chromatograph with flame ionization detector equipped with HP 7673 autosampler (Hewlett-Packard, Wilmington, USA) and a split injector. The GC was equipped with a HP-PLOT U, bonded divinylbenzene/ethylene glycol dimethacrylate capillary column with 30 m column length, 0.32 mm diameter and 10 µm film thickness (Agilent Technologies, Palo Alto, CA, USA). The stationary phase separated polar molecules and allowed the detection of EO in aqueous solution with high efficiency and thermal stability.

EO concentration measurements were carried out under the same conditions as the genotoxic investigations. Following incubation for 0, 15, 30, 45, or 60 min at 37°C, 1 ml medium was pipetted into plastic Eppendorf tube, cooled to 0°C and centrifuged. The supernatant medium was transferred into 1.5 ml glass screw-cap vials sealed with Teflon-lined septa for GC analysis. The time-weighted average exposure level of EO for the whole incubation period was assessed by integrating the data from the investigated incubation time points.

Cytotoxicity test

Before and after treatment, aliquots of cells were subjected to cytotoxicity assay. Calcein AM and 7-AAD fluorescent dyes were used to colabel the cells. Calcein AM is a non-polar compound that passively crosses the plasma membrane of living cells, where it is cleaved by intracellular esterases to reveal a very polar derivative of fluorescein (calcein) that remains trapped in the cytoplasm. 7-AAD is a DNA intercalating dye, which is able to permeate membranes of dead and dying cells, but cannot penetrate plasma membranes of live healthy cells.

Genotoxicity test

The alkaline version of the single cell gel electrophoresis assay (comet assay) was performed immediately after chemical treatment following the procedure described by *Singh et al.* with slight modifications. Degreased frosted slides were preliminarily covered with 1% normal melting point agarose (NMA). After solidification, the gel was scraped off the slide. The slides were then coated with three layers: 1% NMA covered with 0.75% low melting point agarose (LMA) containing the cells ($\sim 2 \times 10^4$ per slide) and topped with 0.75% LMA layer. After solidification, the embedded cells were lysed at 4°C for at least 1 h, shielded from light. After lysis, the DNA was allowed to unwind for 20 min in the alkaline electrophoresis buffer and subjected to electrophoresis in the same buffer for 20 min at 0.8 V/cm and 300 mA in a horizontal electrophoresis chamber. Finally, the slides were rinsed gently three times with neutralization buffer to remove excess alkali and detergent. After drying, each slide was stained with ethidium bromide and stored in a humidified container at 4°C until analysis.

For the detection of oxidative DNA damage, Fpg, a lesion specific restriction endonuclease that can recognize oxidized purines and pyrimidines, was applied. After lysis, two additional steps were incorporated in the comet assay: slides were washed three times in 1X FLARE buffer over a 30 minute period at room temperature, and then incubated for 45 min at 37°C with Fpg diluted in enzyme reaction buffer. Slides treated with buffer alone were applied as negative control. The slides were then processed as described earlier.

Image and data analysis

The fluorescence signal was detected at 400x magnification using a Zeiss Axioplan epifluorescent microscope equipped with a 50 W mercury lamp and a CCD camera connected to an image analysis system.

To determine cytotoxicity, FITC filter for Calcein AM and TRITC filter for 7-AAD was applied to excite the colabeled cells. Survival rate was determined by visual examination of 10 randomly selected non-overlapping fields per slide. Each field contained 10 to 30 images. Cell viability is expressed as the mean of the proportions of living cells

from repeated experiments. The proportions of living cells observed in technical replicates were subjected to statistical analysis.

For the assessment of genotoxicity, samples were excited by using FITC filter. Comet Imager v.2.2.1. software was used to analyze 2 x 50 randomly captured comets from duplicate slides and compute the DNA damage parameters during automatic measurement process. Percentage of DNA in the tail (tail DNA %, TD), tail length (μm , TL) and tail moment (a combined descriptor considering both tail length and the fraction of DNA migrated in the tail) were measured to quantify DNA damage. The results are presented as mean of the median values of DNA damage parameters from repeated experiments. The medians of technical replicates were subjected to statistical analysis.

Experiments with EO and H_2O_2 were independently carried out three times on A549 and HaCaT cells and five times on human peripheral blood lymphocytes from a healthy volunteer. Investigations with phenothrin were independently performed three times on HepG2 cell line and five times on human peripheral blood lymphocytes from five different donors.

Simple linear regression (Pearson test) was used to determine the association between DNA damage levels and concentrations of the examined toxicants. Means of cell viability (cytotoxicity) and medians of DNA damage (genotoxicity) induced by various concentrations of the chemical agents in repeated experiments were statistically compared to that of untreated cells using two-sample, one-tailed Student's *t*-test. Statistically significant difference was accepted at 5% significance level.

Results

Ethylene oxide

Internal dose of ethylene oxide

The concentration of EO in the cell culture medium decreased gradually during the 1 hour incubation period in a time-dependent manner as detected by gas chromatography. A rapid decrease of EO concentration was observed in the beginning of the incubation period, but the rate of decline slowed down considerably as time progressed. The proportion of the initial amount of the genotoxic agent remaining in the cell culture fluid at the end of the exposure decreased with increasing initial doses, that is, higher initial doses of EO demonstrated more extensive drop. In fact, 18% (20 μ M), 35.8% (50 μ M), 44.5% (100 μ M) and 52.5% (500 μ M) of EO evaporated from the medium into the headspace over 1 hour. The average acting concentrations (16.4 μ M, 32.1 μ M, 55.5 μ M, 237.5 μ M) for the total incubation period were considerably lower than the initial concentrations.

Ethylene oxide-induced cytotoxicity

The viability of cells treated with EO or used as controls was found to be over 88% in all cases. 1-h exposure to ethylene oxide proved to induce limited cell death in a concentration dependent manner.

Alkylating DNA damage induced by ethylene oxide

Exposure of the three cell types to the alkylating agent EO in the 0-237.5 μ M concentration range showed dose-dependent increase of DNA damage measured by tail DNA and tail length.

Lung epithelial cells have nearly linear dose-response relationship with the applied concentrations. EO induced a considerable increase (more than 8-fold) of tail DNA values in the 0-237.5 μ M concentration range which was statistically significant already at

the lowest used concentration (16.4 μM). Lung epithelial cells also had the highest absolute values of tail DNA in the 16.4-55.5 μM concentration range among the used cell types. The tail length indicated statistically significant increase (8-fold increase over the background) from 55.5 μM concentration. Treatment with EO induced the longest comet tails in lung epithelial cells at each concentration when compared to the other two cell types.

In lymphocytes and keratinocytes, statistically significant increase of TD values was observed at 32.1 μM and 55.5 μM concentrations, respectively. TL values showed statistically significant increases from 16.4 μM in lymphocytes and from 237.5 μM in keratinocytes. Compared to the other cell types, keratinocytes had the lowest DNA damage levels in the upper concentration range (32.1-237.5 μM).

Linear regression analyses revealed a statistically significant positive correlation of DNA damage with increasing EO concentrations in all three cell types with the steepest slope in lung epithelial cells (TL, $\beta = 0.029$) and lymphocytes (TD, $\beta = 0.211$).

Hydrogen peroxide-induced cytotoxicity

The viability of cells treated with H_2O_2 or used as controls was found to be over 87% in all cases. 1-h exposure to hydrogen peroxide induced only limited dose-dependent cell death in the applied concentration range.

Oxidative DNA damage induced by hydrogen peroxide

To characterize the susceptibility of the three cell types to oxidative DNA damage in contrast to alkylating effect, cells were exposed with H_2O_2 in a concentration range of 0 to 10 μM .

H_2O_2 induced dose-dependent increase of DNA damage in all the three cell types. Lung epithelial cells had the lowest level of DNA damage over the whole concentration range. The increase of DNA damage values was negligible in lung cells and in keratinocytes at low concentrations (1, 2 μM). Statistically significant increase of tail DNA and tail length values were noted at 2 μM in lymphocytes, but only at higher concentrations (5, 10 μM) in the other two cell types. A statistically significant linear correlation between DNA damage and H_2O_2 concentrations was found in each cell type with the lowest increment in lung epithelial cells (TD, $\beta = 1.441$; TL, $\beta = 0.293$).

Although without statistical significance, H₂O₂ treatment increased the level of oxidative DNA damage recognized by Fpg in lymphocytes and keratinocytes, indicating the presence of oxidized pyrimidine and purine bases. The extent of Fpg-dependent DNA damage in lymphocytes and keratinocytes increased with increasing concentrations of H₂O₂. In lung epithelial cells, the Fpg-dependent oxidative DNA insult was minimal.

Phenothrin

Phenothrin-induced cytotoxicity

1-h phenothrin exposure induced slight cytotoxicity in human peripheral blood lymphocytes and human hepatocytes in a concentration-dependent manner. All samples had relatively high, over 77%, viability after treatment.

DNA damage induced by phenothrin

Phenothrin exposure induced dose-dependent increase of DNA damage in both cell types measured as tail DNA and tail length. The finding clearly indicates the genotoxic potential of this pyrethroid pesticide.

Continuous rise of DNA damage values of both cell types was observed in the whole concentration range. Human peripheral blood lymphocytes had considerably higher absolute values of both parameters than hepatocytes in the upper concentration range (50-1000 μ M). The lowest concentration of phenothrin that produced a statistically significant increase in DNA damage was 50 μ M and 20 μ M in human peripheral blood lymphocytes, while in hepatocytes it was 50 μ M and 100 μ M as assessed by tail DNA and tail length, respectively.

There was statistically significant positive correlation between DNA damage and phenothrin concentration in human peripheral blood lymphocytes as well as in hepatocytes. The slope of the linear regression line for both indicators was found to be steeper in human peripheral blood lymphocytes (TD, $\beta = 0.066$; TL, $\beta = 0.005$).

Oxidative DNA damage induced by phenothrin

The nature of phenothrin-induced DNA damage was further investigated with the modified comet assay, using the lesion specific restriction endonuclease Fpg. Treatment with phenothrin apparently increased the level of oxidized DNA bases in both cell types, although Fpg cleavage could not induce statistically significant increase in the DNA damage levels. The extent of Fpg-detected DNA damage showed clear dose dependence.

Significant linear correlation was observed between the oxidative DNA damage and phenothrin concentrations in human peripheral blood as well as in liver cells. The slope of the linear regression line was found to be higher in lymphocytes ($\beta = 0.001$) in the applied concentration range. Results indicate less oxidative DNA insult in liver cells than in lymphocytes.

Discussion

The increasing production and use of chemicals, including biocidal substances, underpin the importance of understanding the potential health risk of human exposure to these agents. Initial genotoxic events play a crucial role in the development of malignancies; therefore, detailed information on the genotoxic properties of certain biocides is essential for the development of effective preventive measures or treatments against cancers.

Contribution to the knowledge about the genotoxic properties of two widely used biocidal compounds that can be encountered not only in certain occupational settings but also in the general environment, deriving both from natural sources and from human activities, serves our aim. Ethylene oxide was used in this work as a “model agent” to study the role of alkylating effects in the development of lung cancer. The genotoxic potential of the pyrethroid insecticide phenothrin was also investigated to allow for evaluating the genotoxic risk associated with phenothrin use.

Ethylene oxide genotoxicity

Ethylene oxide is an important gaseous alkylating biocide compound, particularly used in low-temperature chemical sterilization processes for a variety of heat sensitive materials. The lung epithelium is the first barrier that encounters inhalant toxins, which are associated with the development of respiratory cancer. Various *in vivo* animal experiments have indicated that inhalative exposure to EO may eventually lead to lung cancer, but its ability to cause lung cancer in humans has not been unequivocally demonstrated yet. Although its genotoxic ability is well established, no previous studies have examined the EO-induced DNA damage in cells derived from the lung, as the primary biological target of local effect. An important additional reason for investigating the EO-induced genotoxic effect in lung epithelial cells was that the sensitivity of lung tissue to alkylative DNA-damaging agents is still not fully elucidated.

Our results revealed pronounced DNA damage in lung epithelial cells induced by EO over the whole concentration range used. The smallest dose that resulted in a significant genotoxic effect was as low as 16.4 μM . Lymphocytes showed slightly less susceptibility to

the alkylating effect of this compound. Compared with the other two cell types, keratinocytes proved to be relatively insensitive to EO-mediated DNA damage, which achieved significance only at high concentrations. Our findings support previous observations of increased level of DNA strand breaks induced by the alkylating agent sulfur mustard in lung epithelial cells and white blood cells. In an earlier study, the specific DNA damaging ability of EO in various human cell lines was investigated with the comet assay, where the susceptibility pattern to EO in lymphocytes and keratinocytes was remarkably similar to those observed in our experiments.

In contrast to the high susceptibility towards the alkylating effects of EO, lung epithelial cells were considerably insensitive against the DNA damaging effect of the oxidative agent H₂O₂, which is reflected in the relatively low level of DNA damage induced in them compared with the other two cell types. The lowest dose of H₂O₂ that resulted in a statistically significant increase of DNA strand breaks in lung cells was 5 µM, while in lymphocytes it could already be observed at a lower dose (2 µM).

The detection of Fpg-dependent DNA sites revealed considerable H₂O₂-induced oxidative damage in lymphocytes and keratinocytes, while there was basically no oxidative damage found in lung epithelial cells over the applied concentration range. The possible explanation for the increased resistance of this cell type against oxidative insult might be that pulmonary cells are constantly under oxidative stress, and therefore they are equipped with a wide range of antioxidant defense mechanisms. Our results are in agreement with the general concept about the resistance of lung epithelial cells to H₂O₂.

A remarkable finding of our study is that the treatment concentrations of EO substantially changed in the cell culture medium during incubation, as measured by gas chromatography. This observation must be taken into consideration when modelling the internal dose. The average exposure levels of EO were significantly lower than the initial concentrations, which can be explained by the highly volatile property of EO.

The relatively low average EO concentrations that directly affected the cells in our experiments are representative to the serum levels of occupationally exposed individuals, which largely depends on the actual airborne concentration of EO. *Brugnone et al.* monitored occupational exposure to EO by measuring the concentrations in the ambient air and blood and found that the EO concentration in the blood was, on average, 3.3 times higher than its concentration in the air. On the basis of this finding, the lowest internal dose (16.4 µM) that caused significant genotoxic effect in our *in vitro* study is equivalent to 233

mg/m³ (20 ppm) EO concentration in the workplace air, a level that has been detected several times in occupational settings. Taking into consideration the uncertainty factors applied for carcinogens based on a nonlinear low-dose extrapolation and for inter-individual variability, our findings support the appropriateness of the 1.8 mg/m³ level of the present occupational exposure limit for EO in the USA and Hungary, but draw attention to the unduly high (9.2 mg/m³) long term exposure level of this agent in the United Kingdom.

In conclusion, the lung epithelial cells demonstrated increased sensitivity to the alkylating effect of EO, but considerable resistance to the oxidative DNA damage induced by H₂O₂. These observations support the assumption that lung epithelial cells can be susceptible targets of an EO-mediated alkylating insult and EO may contribute to the development of lung cancer.

Phenothrin genotoxicity

The synthetic pyrethroid insecticide phenothrin is commonly used to kill household insects and mosquitoes, although its direct DNA-damaging effect is not fully characterized. Only one published study is available that assesses its genotoxic risk by measuring oxidized DNA bases in rat liver and kidney with the use of HPLC. The mutagenicity of commonly used insecticides containing phenothrin in 0.2% (~ 6 mM) concentration was previously investigated with Ames spot forward mutation assay and yielded negative results. Based on animal studies, phenothrin exposure has been related to the development of liver cancer in mice and rats, although the increase of cancer incidence was statistically not significant between the control and exposed groups. Another investigation linked phenothrin with breast cancer. Other studies have not supported the cancer-causing ability of this insecticide.

According to our results, acute phenothrin exposure can lead to a significant, concentration-dependent increase of DNA damage in the absence of marked cytotoxicity in peripheral blood lymphocytes and hepatocytes under the applied conditions. The lowest concentrations of phenothrin that resulted in a statistically significant DNA damage were 20 μM and 50 μM in lymphocytes and in hepatocytes, respectively. Hepatocytes proved to be less sensitive towards the genotoxic effect of phenothrin than lymphocytes what may be explained by the rich detoxification mechanisms of HepG2 cells. The oxidative genotoxic

effect of phenothrin could be evidenced in both cell types, although it did not reach statistical significance. Nonetheless, the results point out the potential of phenothrin to induce genotoxic damage. Such information is first provided by our study and may infer important consequences, since significant effects were observed at relatively low (micromolar) doses.

The general population is exposed to phenothrin primarily from incidental ingestion of remaining residues after residential pest control or from use of commercial pediculicides, but significant exposure may occur in certain occupational settings, too. Measurements of urinary metabolites provide useful biomarkers of exposure; however, there is insufficient information at this time to allow for correlation of the amount of metabolites measured in the urine to the body burden or to the level of environmental exposure to phenothrin. It would be speculative to estimate the amount of urinary metabolites produced by the doses applied in our experimental system in the absence of applicable toxicokinetic models of phenothrin metabolism in humans. Data exist only for some other pyrethroid pesticides, the residues of which have been measured in the urine and plasma of industrial workers. Concentrations of fenvalerate and cypermethrin were reported to reach 0.044 μM in urine and 1.08 μM in plasma, respectively. However, the toxicokinetic properties of these pesticides may differ from that of phenothrin.

In conclusion, our findings provide one of the first evidences that the pyrethroid insecticide phenothrin has detectable genotoxic potential. The effective doses used in our study are in the low micromolar range, close to, although higher than the internal dose of phenothrin as it can be estimated by extrapolation from the limited data on endogenous concentrations of other synthetic pyrethroids. Nevertheless, due to the stochastic nature of genotoxic effect that has no threshold of safety, the DNA-damaging potential cannot be ruled out at lower concentrations, too. The limited data available about the toxic effects of phenothrin and the positive results reported here with the agent used on primary and secondary cell cultures call for further studies to characterize the genotoxic properties of this pesticide.

Conclusions and recommendations

- Alkaline comet assay proved to be a useful and sensitive method to quantify DNA damage at the single cell level, allowing for the *in vitro* identification of susceptible cell types towards distinct DNA damaging mechanisms.
- Ethylene oxide and hydrogen peroxide induces dose-dependent DNA damage in human lung epithelial cells, peripheral blood lymphocytes, and keratinocytes without considerable cytotoxic effect in the 0-237.5 μM and 0-10 μM concentration range, respectively.
- Lung epithelial cells demonstrate increased sensitivity to the alkylating effect of EO, but increased resistance to the oxidative DNA damage induced by H_2O_2 .
- Lung epithelial cells can be susceptible targets of an EO-mediated alkylating insult therefore EO exposure may contribute to the development of lung cancer.
- Our findings support the appropriateness of the 1.8 mg/m^3 level of the present occupational exposure limit for EO in the USA and Hungary, but draw attention to the unduly high (9.2 mg/m^3) long term exposure level of this agent in the United Kingdom.
- Phenothrin induces dose-dependent DNA damage in the absence of marked cytotoxicity in human peripheral blood lymphocytes and hepatocytes in the 0-1000 μM concentration range.
- Phenothrin can cause detectable oxidative DNA damage in the examined cell types.
- It is advisable to reconsider the health hazards of phenothrin in regard to its DNA damaging potential.
- In order to minimize the risk of exposure and related health effects, cautious use of phenothrin containing products, especially the avoidance of direct human applications, can be recommended.



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List of publications related to the dissertation

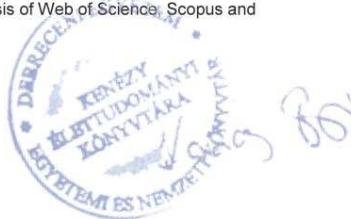
1. **Nagy, K.,** Rác, G., Matsumoto, T., Ádány, R., Ádám, B.: Evaluation of the genotoxicity of the pyrethroid insecticide phenothrin.
Mutat. Res. Genet. Toxicol. Environ. Mutagen. 770, 1-5, 2014.
DOI: <http://dx.doi.org/10.1016/j.mrgentox.2014.05.001>
IF:2.22 (2012)
2. **Nagy, K.,** Ádány, R., Szűcs, S., Ádám, B.: Susceptibility of lung epithelial cells to alkylating genotoxic insult.
Environ. Mol. Mutagen. 54 (8), 682-689, 2013.
DOI: <http://dx.doi.org/10.1002/em.21800>
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Presentations at conferences

Nagy K, Ádám B, Ádány R: oral: Tüdő és vastagbél hámsejtek érzékenysége etilén-oxid genotoxikus hatásával szemben. IV. Conference of the Hungarian Association of Public Health Schools (NKE), 2-4 Sept, 2010, Szombathely, Hungary

Nagy K, Ádám B, Ádány R: poster: Increased role of the alkylating effect of ethylene oxide in the genotoxic damage of lung epithelial cells. V. Conference of the Hungarian Association of Public Health Schools (NKE), 31 Aug – 2 Sept, 2011, Szeged, Hungary

Nagy K, Ádám B, Szűcs S, Ádány R: poster: Toxicokinetic monitoring of human cell cultures exposed to ethylene oxide. VI. Conference of the Hungarian Association of Public Health Schools (NKE), 5-7 Sept, 2012, Budapest, Hungary

Nagy K, Rác G, Takashi M, Ádány R, Ádám B: oral: A szintetikus peszticid fenotrin genotoxikológiai jellemzése üstökös elektroforézis segítségével. VII. Conference of the Hungarian Association of Public Health Schools (NKE), 4-6 Sept, 2013, Kaposvár, Hungary

Nagy K, Ádány R, Szűcs S, Rác G, Takashi M, Ádám B: oral: Munkahelyi és egyéb környezeti expozíciókban azonosított biocid vegyületek genotoxikológiai jellemzése: a sterilizáló szerként használt etilén-oxid és a szintetikus piretroid fenotrin. VIII. Conference of the Hungarian Association of Public Health Schools (NKE), 27-29 Aug, 2014, Nyíregyháza, Hungary

Nagy K, Ádány R, Szűcs S, Ádám B: poster: Susceptibility of lung epithelial cells to alkylating genotoxic insult. 50th Congress of the European Societies of Toxicology (EUROTOX), 7-10 Sept, 2014, Edinburgh, Scotland