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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List of abbreviations

7-AAD: 7-aminoactinomycin D

8-oxodG: 8-oxodeoxyguanine

A549: adenocarcinomic human alveolar basal epithelial cell line

AP site apurinic or apyrimidinic site

CA chromosome aberration

Calcein AM: acetomethoxy derivate of calcein

CAREX: International Information System on Occupational Exposure to Carcinogens

CCD: charge-coupled device

CDCA: chrysanthemumdicarboxylic acid

DALYs: disability-adjusted life years

DNA: deoxyribonucleic acid

DSBs double strand breaks

EO: ethylene oxide

FITC: fluorescein isothiocyanate

Fpg: formamidopyrimidine DNA glycosylase

GC: gas chromatography

H_2O_2: hydrogen peroxide

HaCaT: human keratinocyte cell line

HepG2: human hepatocarcinoma cell line

HPLC: high performance liquid chromatography

HPRT: hypoxanthine phosphoribosyltransferase

IARC: International Agency for Research on Cancer

LM: low melting point agarose

MCF-7: human breast cancer cell line (Michigan Cancer Foundation-7)

MN micronucleus

N7-HEG: N7-(2-hydroxyethyl)guanine

NIOSH: National Institute for Occupational Safety and Health

NMA: normal melting point agarose

O6-meG O6-methylguanine

OSHA: Occupational Safety and Health Administration

PBS: phosphate buffered saline

PCBs: polychlorinated biphenyls

ROS reactive oxygen species

SCE sister-chromatid exchange

SEM: standard error of the mean

SSBs single strand breaks

SV-40: simian vacuolating virus 40

TD: tail DNA %

TL: tail length

TRITC: tetramethylrhodamine isothiocyanate

TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling

TWA: time-weighted average

US EPA: United States Environmental Protection Agency

UV ultra violet light

WHO: World Health Organization

Introduction

Cancer is a leading cause of death worldwide, with 14.1 million new cases and 8.2 million deaths in 2012 [1]. Globally, 19% of all cancers are attributable to environmental and occupational exposures, resulting in more than 1.5 million deaths each year [2]. According to the latest publications, workplace carcinogens are accounted for approximately 2% to 8% of the global cancer mortality [3, 4]. In 2010, there were globally an estimated 118,097 deaths and nearly 2.7 million disability-adjusted life years (DALYs) from neoplasms due to exposure to occupational carcinogens [5]. Since 1971, more than 900 agents, mixtures, and exposure situations have been evaluated by the WHO's International Agency for Research on Cancer (IARC), of which more than 450 have been identified as carcinogenic, probably carcinogenic, or possibly carcinogenic to humans based on findings from experimental studies in animals and epidemiological studies in humans. Currently, 113 agents are classified by the IARC as proven carcinogenic to humans, including a number of substances found in the environment and work settings such as benzene, cadmium, ethylene oxide, formaldehyde, nickel compounds, polychlorinated biphenyls (PCBs), tobacco smoke, vinyl chloride, benzo[a]pyrene, etc. Reports on certain identified environmental factors that can increase the risk of human cancer are regularly published as IARC Monographs [6].

Nowadays, use of chemicals forms a vital part of our life as it provides basic conditions of existence and determines life standard. Most of the people encounter them every day, either at work or elsewhere. The rapid global industrialization exacerbated the chemical risks in workplaces and so it also increased work-related health and safety problems. Exposures to hazardous chemicals in occupational settings usually tend to be higher and more durable than those in the ambient environment. Therefore, workers may be at much higher risk of chemical-related diseases than the general population. Nevertheless, occurrence of harmful substances is unavoidable in other areas of life, too. Chemical products for domestic use, including insecticides, herbicides and home cleaning products, as well as presence of toxic contaminants in food or drinking water pose permanent health risk to the general public. To prevent the possible acute and chronic chemical-induced effects on humans and to control the safe handling and use of substances, chemical safety measures gain more and more importance [7].

Despite the increasing amounts and kinds of chemicals in use, the knowledge on their hazardous properties is not in line with the industrial 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 [8]. Research efforts to expand the knowledge of these substances are particularly important to provide sufficient scientific base for risk assessment; therefore, among others, the genotoxic features of chemicals need to be investigated.

Contribution to the knowledge about the genotoxic properties of two widely used biocide 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 the above 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.

Experimental background

The genetic information is stored in deoxyribonucleic acid (DNA) molecules, which are under constant attack as a consequence of normal cellular metabolism, as well as exposure to genotoxic agents. Unrepaired DNA damage can eventuate in mutations that alter the genetic information encoded within DNA. Mutation denotes any changes in the genetic material of an individual cell or organism, ranging from single nucleotide changes to the gain or loss of entire chromosomes, that can be passed on to future cells or organisms. Mutations can lead to missing or malformed protein product of a certain gene, or can lead to cancer if they occur in specific areas of DNA that control cell growth, death, differentiation and repair. Cells have consequently evolved complex mechanisms to protect their genetic material against mutations. Defects in the cellular response to DNA damage can result in genomic instability, a hallmark of cancer cells. Cells respond to genotoxic damage by invoking DNA repair pathways and initiating DNA damage signalling cascades, or inducing programmed cell death (i.e. apoptosis) [9].

The term "genotoxicity" is broadly used for all lesions in the genetic material or in the genetic processes, such as DNA repair, which are not necessarily associated with mutagenicity. Contrarily, the term "mutagenicity" refers only to the induction of permanent

transmissible changes in the amount or structure of the genetic material of a cell or organism. Thus, genotoxicity covers a broader spectrum of endpoints than mutagenicity [10].

DNA damage

DNA can be damaged in a number of ways. As it is inherently unstable molecule, spontaneous damage due to replication errors, deamination, depurination and oxidation is aggravated by the additional effects of radiation and environmental chemicals. Hundreds of different DNA damage products have been documented; however, these lesions can be categorized into a few major groups without attempting to be comprehensive.

Simple adducts

DNA adducts are a form of DNA damage caused by covalent attachment of a chemical moiety to DNA. Some of these DNA adducts have been proposed as useful biomarkers of exposure to environmental toxins as well as of carcinogenic risk [11, 12].

Oxidation

DNA bases can be oxidized by a variety of mechanisms. Reactive oxygen species (ROS), including singlet oxygen ($\cdot O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\cdot OH$), are the primary instigators of oxidative DNA damage [13]. ROS are generated from cellular metabolism, including oxidative respiration and lipid peroxidation, or can be induced by indirect effects of chemicals, as well as ionizing and ultra violet light (UV) irradiation [14, 15]. Reactive oxygen species are normally kept in balance by antioxidant enzymes (superoxide dismutase, catalase, peroxidases) or radical scavengers (glutathione, melatonin, vitamins A, C and E) [16]. Chemicals may induce ROS artificially by enzymatic conversion to secondary reactive products and/or free radicals, or by disturbing antioxidant defence and enzyme functions [17]. Oxidative DNA damage includes a variety of lesions, including abasic sites, base adducts, base modifications, sugar lesions, base-protein cross-links, single strand breaks (SSBs), and double strand breaks (DSBs) [18]. The most powerful ROS responsible for the direct damage to DNA are the hydroxyl radical and singlet oxygen that induce, among others, the formation of the most common adduct, 8-oxodeoxyguanine (8-oxodG), that has been used as a biomarker for oxidative damage [19]. The oxidized guanine bases no longer

have three hydrogens available for binding with cytosine therefore binds preferably to adenine and result in guanine to thymine transition mutations if not repaired [20]. The mutagenic potential of DNA oxidation is clearly evidenced by the mutagenicity of ionizing radiation which produces DNA oxidation [21].

Alkylation

Alkylation is the transfer of an alkyl group from one molecule to another, leading to various types of adducts on the heterocyclic bases or backbone. Methylation is the simplest type of alkylative modification. The N 7 position of guanine is the most vulnerable site on DNA, N7-methylguanine is thereby the most abundant alkylation product; however, it is relatively innocuous and is removed mostly through spontaneous depurination. Methylation of the O 6 position of guanine induces O6-methylguanine (O6-meG) adduct, the mutagenic potential of which is relatively higher because it mispairs with thymine during DNA replication, which gives rise to a transition mutation of G:C to A:T [22]. The phosphodiester DNA backbone is also sensitive to alkylation damage, which can lead to the cleavage of the backbone [23].

Alkylating agents are prevalent in the environment and are widely used in chemical industry, as well as for chemotherapeutic and sterilizing purposes [24-26].

Hydrolysis

Hydrolysis of DNA is also a type of primary DNA damage, formed both endogenously and induced by different types of exogenous agent. (·OH) radicals remove hydrogen from the deoxyribose-phosphate backbone causing DNA cleavage between the deoxyribose sugar and nucleobases, which creates apurinic or apyrimidinic site (AP site). AP sites unrepaired can result in mutation during semi-conservative replication as a random nucleotide base will be inserted into the strand synthetised opposite them [18, 27].

DNA cross-links

Formation of DNA cross-links has been shown to be enhanced by various chemicals that produce bulky, large-size adducts, as well as by exposure to UV irradiation. UV light principally induces cyclobutane pyrimidine dimers that are dimeric photoproducts between adjacent pyrimidine bases on the same DNA strand [28]. The presence of large DNA adducts

or DNA cross-links can hinder the progression of DNA polymerases during replication and also interfere with chromosome segregation during mitosis [27].

DNA strand breaks

DNA strand break (single and double) may be created by ionizing radiation, industrial chemicals, reactive oxygen species, excessive base excision repair, replication of single strand DNA breaks, collapsed replication forks, inhibition of DNA polymerase and topoisomerase. In addition, most of the DNA alterations mentioned above can potentially be transformed to single or double strand breaks, as the DNA repair machinery incises damaged DNA in order to remove and replace it with an undamaged DNA sequence [10]. Unrepaired DNA double strand breaks (DSBs) may result in structural chromosome abnormalities, whole or partial chromosome loss, and genetic recombination, but can also lead to the breakdown of DNA replication, causing apoptosis to prevent a possible mutation being passed on during replication [29].

Mismatches of DNA

Errors during DNA replication are the endogenous source of mismatched bases. It occurs when wrong DNA base is stitched into place in a newly forming DNA strand, or a DNA base is skipped over or mistakenly inserted. Genotoxic agents are also able to cause errors in DNA replication by inhibiting enzymes involved in the replication process [29].

DNA repair pathways

Cells have multiple strategies for responding to DNA damage; including initiation of transient cell cycle arrest, utilizing specific DNA repair pathways or undergoing apoptosis. If the exposure to DNA damage persists, it may fix alterations incorporated into the genome as mutations. To ensure stable maintenance and inheritance of the genetic material, several DNA repair pathways are employed to repair DNA lesions, depending on the type of the damage:

- direct DNA damage reversal
- homologous recombination

- non-homologous end-joining
- DNA mismatch repair
- nucleotide excision repair
- base excision repair

Most DNA lesions are repaired efficiently, with half-life varying from 4 min for base damage to 90 min for DSBs [30]. Alkylating agent induced methyl-DNA lesions can be repaired by direct reversal, applying DNA-alkyltransferases that are capable to remove the alkyl group in a one-step reaction. Products of alkylation damage are also repaired by the nucleotide or base excision repair pathway [23]. DSBs are repaired exclusively by either homologous recombination or non-homologous end joining pathways [31]. DNA mismatch repair primarily restores single base mismatches and single base loops, or insertion and deletion loops [29]. Nucleotide excision repair is the principal way by which human cells remove bulky adducts or UV-induced cyclobutane pyrimidine dimers from DNA, but it repairs essentially all DNA lesions. Recognition of the damage leads to removal of a short single-stranded DNA segment that contains the lesion. The undamaged single-stranded DNA remains and DNA polymerase uses it as a template to synthesize a short complementary sequence. Final ligation to complete nucleotide excision repair and form a double stranded DNA is carried out by DNA ligase. The base excision repair pathway is involved in repairing simple DNA changes such as single strand breaks, and simple DNA adducts arising from oxidative and alkylating damage, as well as mispaired or inappropriate bases. The process is initiated by the action of specific DNA repair enzymes, the DNA glycosylases, which recognize and remove specific damaged or inappropriate bases, forming AP sites. An AP endonuclease is then employed to cleave the DNA backbone which causes a single strand break. This gap in the DNA is then filled by DNA polymerase and ligated by DNA ligase [29].

A number of chemicals have been shown to alter DNA repair functions, by modifying the structure or capacity of repair enzymes, or by changing their expression on a gene level, indirectly contributing to the accumulation of DNA damages. Failure in the DNA repair mechanisms can lead to mutagenesis and ultimately carcinogenesis [32].

Chemical carcinogenesis

Chemicals may induce cancer by genotoxic or non-genotoxic mechanisms. Chemical carcinogenesis is a complex, multi-stage process leading to malignant cell transformation which includes initiation, promotion and progression [33]. The initial events typically involve genotoxic damage to cellular DNA, leading to a mutation, which can give the cell a selective growth advantage and/or an inability to regulate growth. Alterations of genes that control the cell cycle or cell differentiation can be associated with neoplastic development. A constant activation of proto-oncogenes or inactivation of tumor-suppressor genes as a result of mutation can result in a clonal cell population with a proliferative or survival advantage that can be expanded in the tumor promotion stage. Promotion does not involve a direct genotoxic event. It is defined as the clonal expansion of initiated cells, induced by a promoting agent, resulting in a preneoplastic lesion. Progression is marked by a permanent selective growth of preneoplastic cells into neoplastic cells. The alterations that bring about progression can arise from continued exposure to the carcinogen, additional spontaneous or induced mutations, or genomic instabilities [34]. The agents that effect the transition from the promotion stage to the progression stage are termed progressor agents while agents that effect the transition of normal cells to the progressive stage are termed complete carcinogenic agents [10].

Genotoxicity and mutagenicity assays

Investigation of genotoxicity is particularly important because it is closely associated with carcinogenesis and it is necessary for the establishment of scientific basis for the assessment of cancer risk to humans from exposure to chemicals. It is undertaken for two main reasons:

- to detect chemicals that might cause genetic damage,
- to detect chemicals that might be carcinogenic (based on the assumption that mutagenesis is a key event in the process of carcinogenesis).

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 this damage from accumulating into mutations.

A wide range of assays are used currently for the detection of genotoxic and mutagenic effects. The sensitivity and specificity of these tests, with respect to test species and genetic endpoint, are highly variable. There are three categories of genotoxicity assays: those that measure change at the chromosomal structure level, those that measure change at the level of genes, and those that measure alterations at the level of the DNA molecule. These can be combined either as a test battery or in a tiered system, however, a battery of tests can also be ordered in a tiered system [35].

Basic tests typically used in the first or screening phase of investigation are microbial gene mutagenicity assays and tests for primary DNA damage in mammalian cells, as these are the most rapid and least expensive laboratory methods. The most commonly used microbial gene mutagenicity assay is the *Salmonella* (Ames) short-term mutagenicity test which uses various histidine-dependent *S. typhimurium* strains as indicator organisms for mutagenic events [36]. Ideal method for detecting DNA damage at a single cell level is the single cell gel electrophoresis assay (comet assay) which is able to detect single strand breaks, double strand breaks and alkali labile sites [37]. Other methods include: the detection of DNA adducts or the detection of unscheduled DNA synthesis that occurs in response to DNA damaging exposures [38, 39], the alkaline-elution assay [40] which measures the rate in which single strands of broken DNA pass through a filter, the alkaline unwinding assay [41] which measures the rate at which double-stranded DNA unwinds in alkali dependent on the number of strand breaks, zonal centrifugation [42] which measures the average molecular weight of DNA fragments, sedimentation of nucleoids [43] which measures the distance nucleoids sediment in a sucrose gradient dependant on the amount of DNA supercoiling, and the DNA precipitation assay [44] which measures the percent of undamaged DNA precipitated after centrifugation. Although these methods have been shown to provide a sensitive measure of the overall DNA damage to cells, there are several limitations when compared to the comet assay. For example, typically large number of cells is required, radiolabeling of DNA does not permit analysis of DNA damage in noncycling cells, and information on the response of individual cells is not possible.

The second or confirmation tier is where positive results are confirmed using mammalian systems. The most often applied tests are mammalian gene mutation assays and *in vitro* cytogenetics assays, such as chromosome aberration (CA) assay, sister-chromatid exchange (SCE) assay, and the micronucleus (MN) assay. The CA assay assesses chromosome damage in metaphase cells using light microscopy. It is sensitive to agents which break the DNA strand directly. On the other hand the assay is time-consuming,

technically demanding, and therefore expensive [45]. SCE can sensitively detect chemical mutagens that interfere with the DNA structure by alkylating bases, or by intercalating between the double helix of DNA [46]. The MN assay is capable for the detection of chemicals which induce formation of small membrane bound DNA fragments, i.e. micronuclei, in the cytoplasm of interphase cells. Micronuclei originate mainly from chromosome breaks or whole chromosomes that fail to engage with the mitotic spindle (due to damage to the mitotic mechanism) when the cell divides. The MN assay does not require metaphase spread for analysis, and is therefore simpler. In addition, the simplicity of scoring and its wide applicability in different cell types make it a useful tool to assess cytogenetic abnormality [47].

In the third or final phase of investigation one or more *in vivo* tests are utilized in order to evaluate possible mechanism of genotoxicity and provide a test model in which additional relevant factors (absorption, distribution, metabolism, excretion) that may influence the genotoxic activity of a compound are explored [35].

Measurement of DNA damage by the comet assay

The single cell gel electrophoresis assay or comet assay is a cheap, sensitive, easy to perform and rapid technique to evaluate the DNA damage in individual cells [48]. Its development began with the work of Östling and Johanson in which they embedded irradiated cells in agarose and observed that nucleoids with damaged DNA were stretched toward the anode, while undamaged nucleoids had round figure [49]. The cell had the appearance of a comet in which the nucleus represents the head of the comet and the migrated DNA represents the tail (Fig. 1).

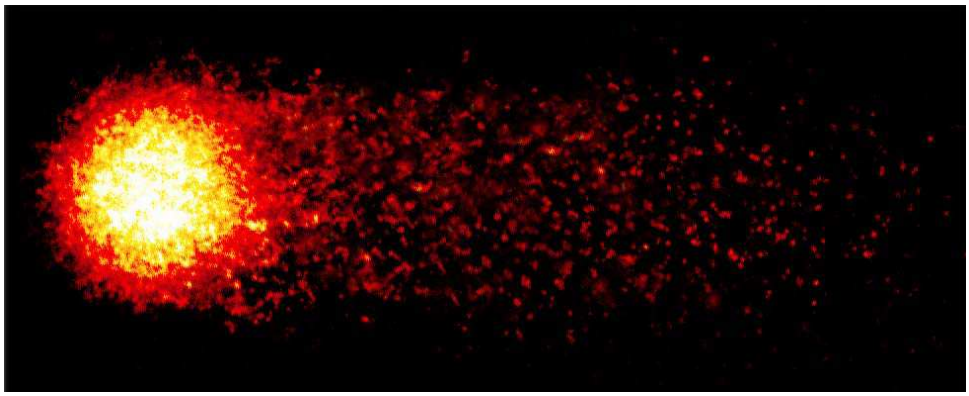

Figure 1. *Image of a damaged cell nucleus in the comet assay.*

They also observed that the amount of DNA migration toward the anode increased in irradiated cells in a dose-dependent manner. In 1988, Singh et al. modified the assay by introducing an alkaline condition during the electrophoresis at pH > 13 [37]. The latter version is commonly referred to as the alkaline comet assay and permits the detection and quantification of not only DNA single and double strand breaks but also alkaline-labile sites induced by a series of physical and/or chemical agents [50]. Alkaline-labile sites are a consequence of DNA depurination and modification of the sugar moiety, which result in chemical instability and breakage of the phosphodiester backbone during treatment with alkali [13]. Due to the larger spectrum of detectable DNA lesions, the alkaline comet assay is more commonly used. By utilizing the comet assay it is possible to detect low levels of DNA damage in individual cells therefore only a small number of cells are needed in each experiment and almost any eukaryotic cell types can be used. As single cells are visualized, it is possible to detect intercellular differences in response to DNA damaging agents [51]. In addition, the observation of DNA damage is less subjective than that of other short-term genotoxicity assays; comet assay is faster and easier to perform, it has a higher statistical power and the possibility of automation [52].

The fundamental principle of the test is to detect DNA damage by monitoring movement of DNA fragments in an agarose gel. The basic steps include acquisition of a single cell suspension, preparation of microscope slides, cell lysis, enzyme treatment (optional), alkali unwinding, electrophoresis, neutralization and DNA staining for visualization of the “comet”. Cells can easily be obtained from cell cultures, from whole blood, or from tissues by enzymatic digestion and suspended in either PBS or their respective media. Once a single cell suspension is prepared, the cells are embedded in low melting agarose and mounted on a microscope slide. After the agarose has cooled and solidified, the slides are subjected to a prechilled lysis solution containing detergent and a high salt concentration which compromise the cellular and nuclear membranes and extract the nuclear proteins. When cell lysis is complete, the slides are placed in an alkaline solution of pH>13. The purpose of this step is to allow the DNA double helix to relax and unwind, permitting the detection of single strand breaks and alkaline-labile sites. After alkali unwinding, the cells are subjected to electrophoresis in the alkaline solution. The negatively charged fragments of DNA are pulled toward the anode giving the nucleus its characteristic comet trace profile. Finally, the agarose gel is gently neutralized to remove alkali and detergents to avoid interference with the DNA-specific fluorescent staining. The resulting 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 percentage of DNA in the tail (the fraction of nuclear DNA that has migrated during electrophoresis from the nucleus to the tail), the comet tail length (the maximum distance that the damaged DNA migrates from the leading edge of the head) and the tail moment. Percentage of DNA in the tail and comet tail length are the measures that seem most linearly related to dose and the easiest to understand, as well as give clear indication of what the comets actually look like. Tail moment can be defined as the product of the fraction of DNA in the comet tail and the tail length in μm, and is therefore a valuable measure to use as it takes into consideration the density of strand breaks in one measure [48, 53].

The comet assay can be applied to a variety of studies including the investigation of genotoxicity and DNA repair as well as clinical studies [54-57]. 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 [58]. Alkylating agents generate alkylated DNA bases that may be sites of DNA excision repair. Incomplete excision repair sites are a source of DNA strand breaks that are detected in the comet assay. In order to detect specific DNA damage, Collins et al. [59] developed a modified version of the comet assay by introducing an enzymatic DNA digestion step, for example formamidopyrimidine DNA glycosylase (Fpg) treatment, for the detection of oxidized purines and pyrimidines. The main substrate of Fpg is 8-oxodG, which is probably the most abundant biomarker of oxidative DNA damage (Fig. 2) [19]. Comet assay can also be effectively used in environmental as well as in human biomonitoring studies, since it can be easily performed on human blood samples from individuals suspected to sustain exposure to DNA damaging agents [60].

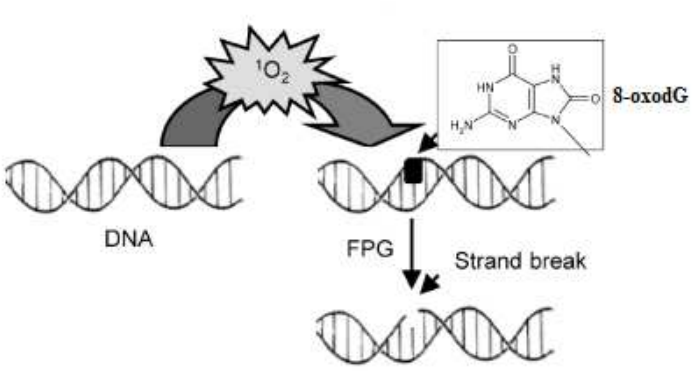

Figure 2. *Schematic representation of DNA oxidation by $^1O_2$ leading to the formation 8-oxodeoxyguanine which is recognized by Fpg enzyme that cleaves DNA in the site of the lesion.*

Modified from Berra C.M. et al.[61].

The primary disadvantage of comet assay is its low specificity that may be associated with cytotoxicity. Based on experimental results, the maximum concentration of test substance should allow for more than 75% viability in order to avoid false positive responses due to cytotoxicity [62]. Other limitations of the assay are that it cannot measure the fidelity of repair of DNA strand breaks and the necessity for single cell suspension [48].

Ethylene oxide

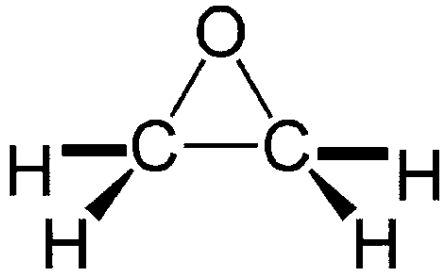

Figure 3. *Chemical structure of ethylene oxide.*

Physical, chemical properties

Ethylene oxide (EO), also known as ethene oxide or oxirane, has the molecular formula of H_2COCH_2. It is the simplest cyclic ether and a very reactive alkylating agent due to its highly strained ring which can open easily (Fig. 3). EO is a colorless gas at room temperature and atmospheric pressure, but it condenses at low temperatures into liquid. Its boiling point is 10.7°C. The liquid has a characteristic ether-like odor. It is miscible in all proportions with water, alcohol, ether, and most organic solvents. Its vapours are flammable and explosive. EO is a very versatile compound, storing considerable energy in the ring structure. Its reactions proceed mainly via ring opening and are highly exothermic. Under appropriate conditions, EO is known to undergo a variety of reactions, such as isomerization, polymerization, hydrolysis, combustion, and decomposition which produce a considerable energy.

Occurrence

Ethylene oxide occurs naturally in the atmospheric air, being produced in small amounts by oxidation processes; in addition, it is formed endogenously as a metabolite in certain plants and microorganisms. It also occurs in the exhaust gases of hydrocarbon combustion, such as that of internal-combustion engines, as well as in tobacco smoke. It can form spontaneously from manure and sewage sludge, too. A large quantity of EO is produced artificially in industrial processes.

Production and application

Ethylene oxide was first discovered by Wurtz [63] in 1859 by liquid phase oxidation using potassium hydroxide to eliminate hydrochloric acid from ethylene chlorohydrin. Nowadays, EO is exclusively produced by direct oxidation of ethylene in the presence of a silver catalyst [64]. Its worldwide annual production was about 19 million tons in 2006 [65].

The major industrial application of EO is as a raw material in the production of several industrial chemicals and intermediates, including ethylene glycols (used in the production of antifreeze, polyester and polyethylene terephthalate, liquid coolants and solvents), polyethylene glycols (used in perfumes, cosmetics, pharmaceuticals, lubricants, paint thinners and plasticizers), ethylene glycol ethers (used as a key component of brake fluids, detergents, solvents, lacquers and paints), ethanol amines (used in the manufacture of soap and detergents), and ethoxylates in the manufacture of detergents, surfactants, emulsifiers and dispersants (Fig. 4) [65].

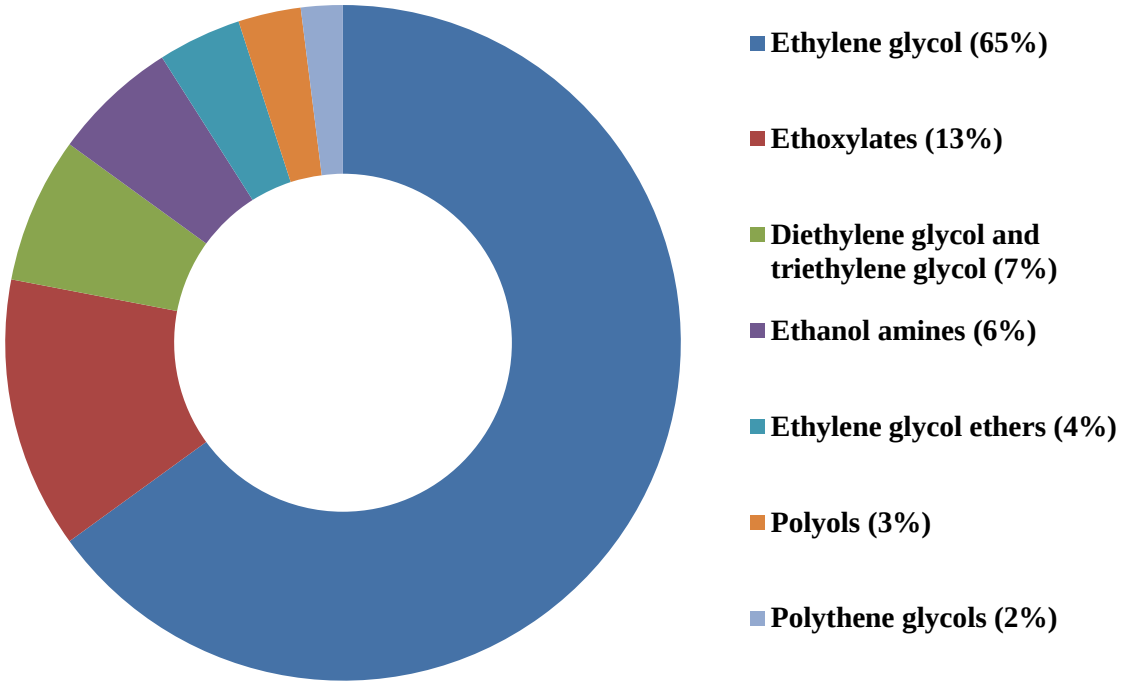

Figure 4. *World industrial use of ethylene oxide (2006).*

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 [25, 64]. EO is an excellent sterilizing agent because of its effective bactericidal, sporicidal, and virucidal activity.

The EO sterilization is a low temperature chemical sterilization method which takes longer time than steam sterilization, typically 18-24 hours for a complete cycle. Temperatures reached during sterilization are usually in the 50-60°C range. EO gas must be introduced into the partially evacuated workspace of the sterilizer at the concentration of 750-1200 mg/l and must have direct contact with microorganism on the items to be sterilized. Due to the highly flammable and explosive nature of EO in air, it must be used in an explosion-proof sterilizing

chamber in a controlled, well-ventilated environment. Items sterilized by this process must be packaged with wraps and be aerated. The aeration may require a long time (16-18 hours in aeration chamber) to make sterilized items safe for handling and patient use. There are gas sterilizers available that use a mixture of EO with formaldehyde [66, 67].

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 [68].

Exposure data

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 [25, 64].

According to the estimation of the National Institute for Occupational Safety and Health (NIOSH), in 1990 about 250,000 workers in the USA were annually exposed to EO, 75,000 of whom were sterilizer operators or hospital workers [69]. In 15 member states of the European Union (1990-93), 47,000 workers, including 22,000 medical workers, were exposed to ethylene oxide estimated by the CAREX exposure information system [70].

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 mg/m^3 [64, 71]. Airborne concentrations (8-h TWA) of EO measured in hospitals reached 124 mg/m^3, but in exceptional 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 [72].

Based on experimental findings in animals and on epidemiological observations in humans that proved EO presents a health hazard to workers, 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 [73]. Likewise in Hungary, the 8-hour maximum permissible exposure level of EO in the workplace air is 1.8 mg/m^3 determined by the Hungarian Joint Decree between the Ministry of Health and the Department of Social and Family Affairs of the Occupational Chemical Safety (No. 25/2000). 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) [74].

Ethylene oxide is not only an important exogenous toxicant, but it is also generated in small quantity by the cytochrome P450 2E1 conversion of ethylene, which is formed *in vivo* during normal physiologic processes, including methionine oxidation, lipid peroxidation, and the metabolizing activity of intestinal bacteria [75-78]. Cigarette smoke also contains EO [64]. Practically everyone is, therefore, exposed to a certain extent to EO during their lifetime.

Metabolism

After inhalation, ethylene oxide passes through the pulmonary alveoli and enters the circulation. 20-25% of inhaled EO that reaches the alveolar space is exhaled, while 75-80% is taken up by the body. It is very soluble in blood and is rapidly distributed to various body tissues following its absorption. According to a toxicokinetic study by Brugnone et al. [79], the average blood concentration of ethylene oxide is approximately 3.3 times higher than its environmental air concentration, reflecting the high solubility of ethylene oxide in blood.

There are two possible pathways (enzymatic and non-enzymatic) responsible for ethylene oxide metabolism in humans (Fig. 5). EO can be converted either to ethylene glycol through hydrolysis by reaction with water and chloride or to thioethers by conjugation with glutathione. Ethylene glycol is the major metabolic product of ethylene oxide hydrolysis that can be excreted in the urine or further metabolised by alcohol dehydrogenase to glycoaldehyde, which is transformed to glycolic acid and glyoxal and then to glyoxylic acid. The toxic glyoxylic acid that suppresses oxidative cell metabolism is eventually detoxicated via several metabolic pathways. Enzymatic deactivation occurs by glutathione conjugation yielding S-2-hydroxyethyl-glutathione, S-2-hydroxyethyl-cysteine and S-2-hydroxyethyl-mercapturic acid. Glutathione conjugation prevents ethylene oxide from covalently binding to cellular proteins and nucleic acids. About two-thirds of the population possess the ability to

enzymatically conjugate EO with glutathione, while the remaining one-third is out of this ability that may increase an individual's susceptibility to the toxic effects of EO [64, 80].

After metabolized, EO metabolites are excreted primarily through the urine. The half-life of absorbed ethylene oxide in humans has been estimated to be less than 1 hour [64].

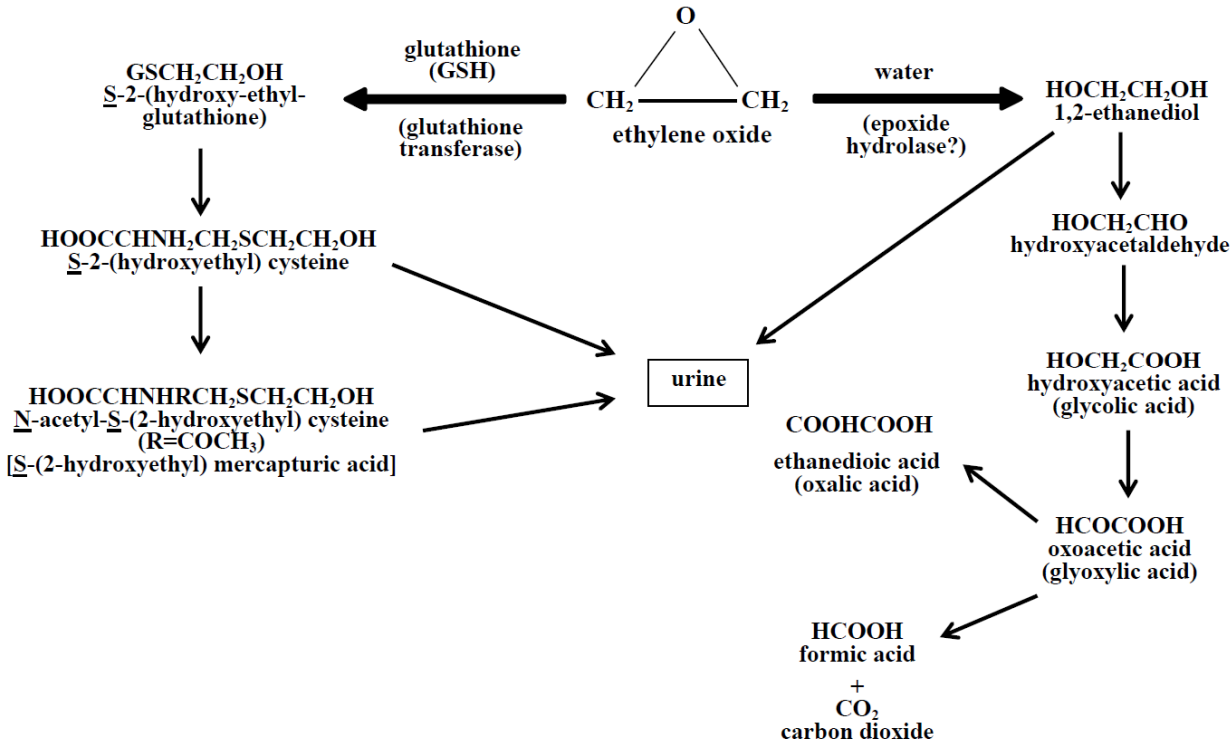

Figure 5. *Metabolic pathway of ethylene oxide.*

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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 [81, 82].

Genotoxicity, mutagenicity, carcinogenicity

As ethylene oxide is an extremely reactive, direct-acting alkylating agent, it attacks the nucleophilic groups of cellular macromolecules, including DNA [83]. 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) [84]. Its mutagenic potential was investigated in a variety of *in vitro* and *in vivo* experiments which detected that EO can induce DNA strand breaks [85], HPRT mutations [86], micronucleus formation [87], chromosomal aberrations [87, 88], and sister chromatid exchange [89] in cultured cell model systems. EO was demonstrated to increase the frequency of sister chromatid exchange [90], p53, H-ras and K-ras mutations [91, 92], micronucleus formation [90], HPRT mutation [93], and changes in the expression of base excision DNA repair genes [94] in experimental animals *in vivo*, as well as DNA strand breaks [95], chromosomal aberrations [96], micronucleus formation [97], sister chromatid exchange [98], and N-ras and p53 gene expression alterations [99] in humans. The carcinogenic properties of EO were demonstrated by animal inhalation studies, where different types of neoplasms [100, 101], including lung cancer were developed [102], however, still limited evidence exists for the cancer causing ability of ethylene oxide in humans.

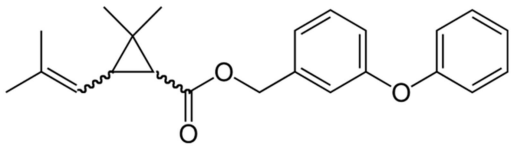
According to human epidemiological findings, occupational exposure to ethylene oxide is principally associated with the development of lymphatic and hematopoietic tumors [103-105], but further studies identified additional sites of neoplasms, too, including breast [104, 106, 107] and stomach cancer [108]. Although the major portal of entry of EO 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 [109].

Based on the limited evidence of carcinogenicity from human epidemiological studies, and sufficient evidence of carcinogenicity from studies in experimental animals, EO has been classified by the IARC as “carcinogenic to humans” (group 1) in 1994 [64].

It is widely accepted that ethylene oxide is a genotoxic agent [64], 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 [85] and in breast epithelial cells *in vitro* with the use of single cell gel electrophoresis assay [110], 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



Physical, chemical properties

Figure 6. *Chemical structure of phenothrin.*

Phenothrin, also known as sumithrin, has the molecular formula of $C_{23}H_{26}O_3$, and the molecular weight of 350.46 g/mol (Fig. 6). It is a colorless or pale yellow liquid possessing a slight odor. It belongs to the pyrethroid group of pesticides that have stereoisomers due to the asymmetric carbon structure. Phenothrin has four different types of stereoisomers, such as 1R *trans* (1), 1R *cis* (2), 1S *trans* (3), and 1S *cis* (4) isomers (Fig. 7).

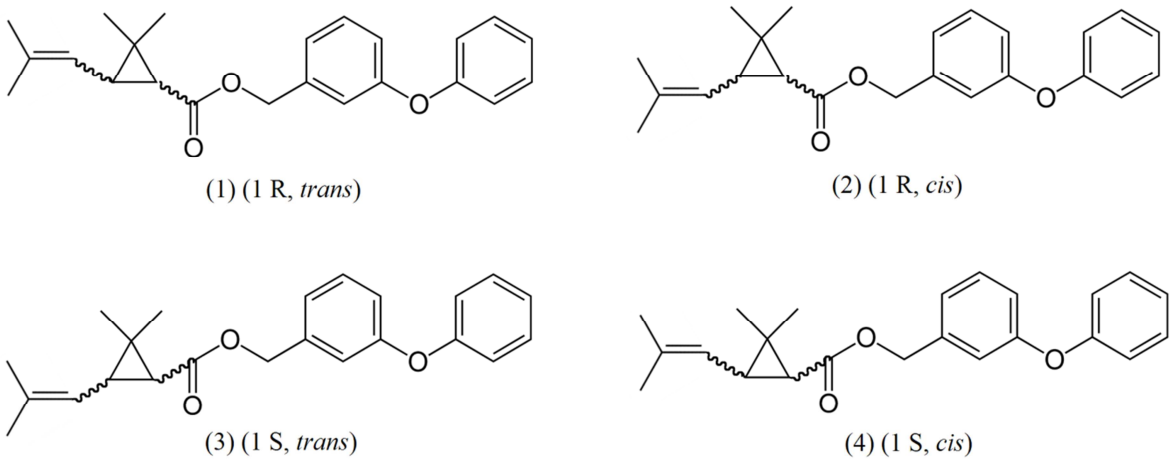


Figure 7. *Chemical structure of the four stereoisomers of d-phenothrin.*

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The commercially used d-phenothrin is a mixture of isomers 1R-*trans* and 1R-*cis* in 4:1 ratio. It is poorly soluble in water (2 mg/litre at 25°C), but can be dissolved in organic solvents such as acetone, xylene, hexane or methanol. It is stable in the air but unstable if exposed to light and alkaline conditions.

Historically, pyrethroids are synthetic analogues of pyrethrins, insecticidal substances obtained from the flowers of a species of chrysanthemum (*Chrysanthemum cinerariaefolium*). The majority of pyrethroids were created by modifying the chrysanthemic acid moiety of the pyrethrin I and esterifying the alcohols. Synthetic pyrethroids have been developed in order to improve the specificity and activity of pyrethrins, while maintaining the high knockdown and low terrestrial vertebrate toxicity. Phenothrin is an ester of chrysanthemic acid (2,2-dimethyl-3-(2,2-dimethylvinyl)-cyclopropanecarboxylic acid) and 3-phenoxybenzyl alcohol.

Pyrethroids are classified according to their structure and toxicology, including those lacking the cyano group on the phenoxybenzyl moiety (type I) and those with a cyano group on the phenoxybenzyl moiety (type II). Phenothrin belongs to the type I pyrethroid group which does not contain cyano molecular group. Other type I pyrethroids are allethrin, tetramethrin and permethrin. Type II pyrethroids include deltamethrin, cyphenothrin, cypermethrin and fenvalerate. The first manufactured pyrethroid was allethrin that was used worldwide from the 1950's. From 1950 to 1970, resmethrin, tetramethrin and phenothrin were developed by applying further various alcohol substituents. These first generation pyrethroids are still widely used, but they are photolabile, therefore, their half-life is only hours if exposed to light. In outdoor condition, the half-life of phenothrin is less than 1 day. Radiolabeled phenothrin was observed to be degraded in the soil after 1 to 2 days. However, when it was used under flood conditions, degradation was slower (2 weeks to 2 months).

Type II pyrethroids were developed to improve photo stability mainly by modifying chrysanthemic acid of the pyrethrin molecule. Further modification by dihalovinyl analogue was used to increase stability even more. Permethrin was developed based on the dihalovinyl analogue modification. In addition, structural modifications with halogenated vinylcyclopropylcarboxylates were applied to ensure widespread application in agriculture. The products created using halogenated vinylcyclopropylcarboxylates are cypermethrins, cyfluthrin and cycloarthrin. The half-life of type II pyrethroids can be as long as one hundred days in the soil after used in agricultural application [111, 112].

Production and application

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 [112]. 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 it 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 [113].

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. It can be applied in pesticide mixtures as a synergist or alone as direct insecticide for both indoor and outdoor pest control. For example, to control mosquitoes and prevent transmission of arboviruses after Hurricane Isabel, the Virginia Department of Health sprayed residents with phenothrin in 2003 [114]. 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 [112, 115].

Exposure data

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). According to deterministic exposure assessments, several residential scenarios would result in exposures of concern, especially the incidental ingestion of residues by toddlers. Application of pediculicides is considered to be a significant source of residential phenothrin exposure, too, due to direct dermal contact. 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. Since the environmental persistence of phenothrin ranges from 1 to 2 days, the exposure from residues in food or drinking water is expected to be very low [112, 115]. At present, reliable quantitative data on exposure are not available to allow for the characterization of the dose-response relationship.

Metabolism

Phenothrin is metabolized rapidly by hydrolytic cleavage of the ester bond, followed by oxidation and glucuronidation yielding to the common urinary metabolites *cis*- and *trans*-chrysanthemumdicarboxylic acid (*cis*- and *trans*-CDCA). The *trans* isomers are metabolized more rapidly than *cis* isomers, and excreted mainly in the urine, while metabolites of the *cis* isomers are excreted mainly in the feces. The half-life of the urinary excretion varies from 4 to 12 hours after exposure. *Trans*-CDCA is used as a biomarker for internal dose assessment of certain pyrethroid insecticides [116].

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 [117]. Dermal exposure may lead to local paresthesia around the exposed skin surface [118].

Genotoxicity, mutagenicity, carcinogenicity

Although phenothrin was found to be non-mutagenic in *Escherichia coli* strains [119], 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) [120]. Further data provided by other mutagenicity or 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 [121, 122]. 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 [123]. 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” [115].

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 by means of the alkaline comet assay in 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 (H_2O_2).

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/m^3) occupational exposure limit for ethylene oxide.

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

Chemicals

Ethylene-oxide and chemicals used for the alkaline comet assay were purchased from Sigma-Aldrich Chemie GmbH (Heidelberg, Germany). D-*trans*-Phenothrin was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The endonuclease formamidopyrimidine DNA-glycosylase (Fpg, FLARE™ Module) was acquired from Trevigen (Gaithersburg, MD, USA). Acetomethoxy derivate of calcein (Calcein AM) and 7-aminoactinomycin D (7-AAD) fluorescent dyes were purchased from Biotium (Hayward, CA, USA). The cell culture medium and the supplements were provided by Gibco (Paisley, UK).

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. The buffy-coat was aspirated and resuspended in RPMI 1640 medium containing 10% fetal calf serum.

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.

A549 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin, while HaCaT and HepG2 cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum,

100 U/ml penicillin and 100 µg/ml streptomycin. The cell lines were grown as monolayer in T_{25} and T_{75} flasks (TPP, Trasadingen, Switzerland) at a temperature of 37°C in a humidified incubator with 5% CO_2 atmosphere and were passaged twice a week.

In vitro treatment

The day before the experiment, the adherent cells (A549, HaCaT and HepG2) were seeded for treatment into 6 wells ($2x10^5$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 $2x10^5$ cells/ml medium into 6 wells of a 12-well-plate on the day of the experiment.

Treatment concentrations of EO (0-500 µM) and H_2O_2 (0-10 µM) that showed no evidence of cytotoxicity were previously determined by Trypan blue exclusion assay. Stock solution and dilution series (100 µM, 10 µM, 1 µM)of the two agents were freshly prepared before each experiment. Due to the highly volatile nature of EO (boiling point: 10.7°C), gas-tight pre-cooled syringe (Hamilton, Reno, NV, USA) was used to weigh it into the cell culture medium at 0°C in glass screw-cap vial sealed with Teflon-lined septa. Aliquots of EO solution at different concentrations were added to the cell cultures (human peripheral blood lymphocytes, A549 and HaCaT) for 1 h at 37°C. During incubation, the plate and the plastic tubes were hermetically sealed to limit EO evaporation from the sample. Treatment with different concentration of H_2O_2 was conducted in the same way with the exception that the dilution and weighing procedure were carried out at room temperature.

Treatment doses of phenothrin (0-1000 µM) that showed no sign of considerable cytotoxicity were previously determined by Trypan blue exclusion assay. Stock solution and dilution series (100 µM, 10 µM, 1 µM) were made in methanol. 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. The methanol content in the cell culture medium was 10% (v/v) for each treatment, the concentration that was found in previous experiment to be non-genotoxic and non-cytotoxic.

Following incubation, adherent cells were washed and scraped from the wells to avoid trypsin-induced DNA damage. All the cell cultures were centrifuged and resuspended in serum-free medium at a cell density of 2000 cells/µl. Cell viability was assessed before and after the treatment by combined fluorescent staining with Calcein-AM and 7-AAD and was over 80% in all cases. Incubation was stopped on ice to avoid DNA repair.

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 chromatography integration was achieved using a Packard Bell Packmate computer equipped with HP ChemStation chromatography software (Rev. A.0603). Nitrogen was used as the carrier gas at 2 ml/min and it produced a column head pressure of 52 psi at 120°C. The injection volume was 1 µl (using 5 µl autosampler syringe), at a split ratio of 1:10. The injector temperature was 180°C and the detector temperature was 200°C. 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 and cooled to 0°C. The cellular components were removed by centrifugation at 10.000 rpm for 1 min and by application of wool inlet liner to prevent column occlusion. The supernatant medium was transferred into 1.5 ml glass screw-cap vials sealed with Teflon-lined septa for GC analysis. The used column allowed for direct injection of samples containing large amount of water, therefore extraction procedure was unnecessary. The amount of EO in the sample presented by the area under the elution curve was calculated using a calibration curve that was provided by measurements on a series of known aqueous EO dilutions in the range of 10 to 2000 µM. 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.

To characterize the possible confounding effect of endogenous EO produced by cellular metabolic mechanisms, the intracellular levels of EO were determined. Cells were lysed by using MagNA Lyser Green Beads (Roche Diagnostics GmbH, Mannheim, Germany) to liberate EO generated physiologically by the cells. The homogenate was then ultracentrifuged to separate the supernatant that was then directly injected into the gas

chromatograph. Sample preparation was conducted at 0°C to keep EO in solution. The endogenous level of EO was below the detection limit in each cell type.

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.

Both fluorescent dyes were dissolved in PBS to a final concentration of 2 µM each. 200 µl of this working solution was added to the cell pellets ($1x10^5$ cells) then incubated for 30 minutes at 4°C, shielded from light. The labeled cells were washed and resuspended in ice cold PBS buffer. 40 µl of cell suspension was plated on microscope slide for immediate microscopic examination.

Cell survival was determined to exclude on-going cell death at the end of the chemical treatment when genotoxicity test was performed so that the confounding effect of apoptotic DNA damage could be excluded.

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. [37] 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 (~ 2 x 10^4 per slide) and topped with 0.75% cell-free LMA layer. After solidification, the embedded cells were lysed (2,5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris base, pH 10, 1% sodium N-lauroyl sarcosinate and 1% Triton X-100 added fresh) 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 (300 mM NaOH, 1 mM Na_2EDTA, pH 13) and subjected to electrophoresis in the same buffer for 20 min at 0.8 V/cm

and 300 mA in a horizontal electrophoresis chamber (Bio-Rad, Richmond, CA, USA). Finally, the slides were rinsed gently three times with neutralization buffer (0.4 M Tris base-HCl, pH 7.5) to remove excess alkali and detergent. After drying, each slide was stained with ethidium bromide (20 µg/ml) 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 [19]. After lysis, two additional steps were incorporated in the comet assay: slides were washed three times in 1X FLARE buffer (1 mM HEPES-KOH, pH 7.4, 100 mM KCl) over a 30 minute period at room temperature, and then incubated for 45 min at 37°C with Fpg diluted in enzyme reaction buffer (1X FLARE buffer plus 1X BSA). Concentrations of the enzyme were prepared according to the protocol provided by the manufacturer. 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 (Carl Zeiss GmbH, Germany) equipped with a 50 W mercury lamp and a CCD camera (IMAC-CCD, Computer Systeme, Germany) connected to an image analysis system.

To determine cytotoxicity, FITC filter (absorbance wavelength: 467-498 nm) for Calcein AM and TRITC filter (absorbance wavelength: 532-554 nm) 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 (Fig. 8). 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.

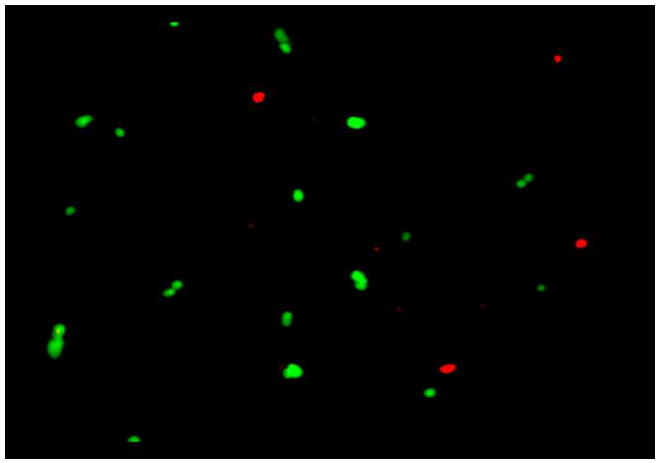


Figure 8. *Microscopic image of combined fluorescence stained human peripheral blood lymphocytes.*

For the assessment of genotoxicity, samples were excited by using FITC filter. Comet Imager v.2.2.1. Software (MetaSystems GmbH, Germany) was used to analyze 2 x 50 randomly captured comets from duplicate slides and compute the DNA damage parameters in an automatic measurement process. The software displayed the intensity curve of the whole image (yellow line) and of the intensity of the head located around the highest intensity density (red line) after background correction. The difference between the two intensity curves (blue line) provided the intensity of the tail (Fig. 9). DNA damage parameters were automatically calculated from the intensity signals. 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.

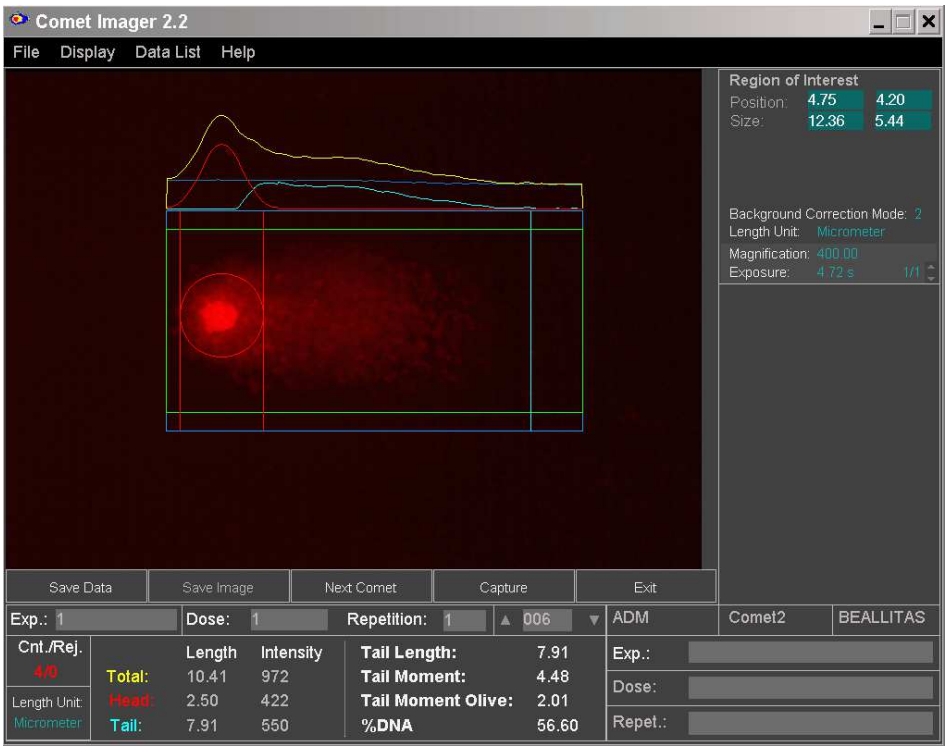


Figure 9. *Measurement of the DNA damage parameters of a lymphocyte nucleus using Comet Imager Software 2.2.1.*

From each sample, the medians of the DNA damage parameter values were used as central values since data had not normal distribution and medians are less sensitive to outliers due to skewed data distribution than means [124]. 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.

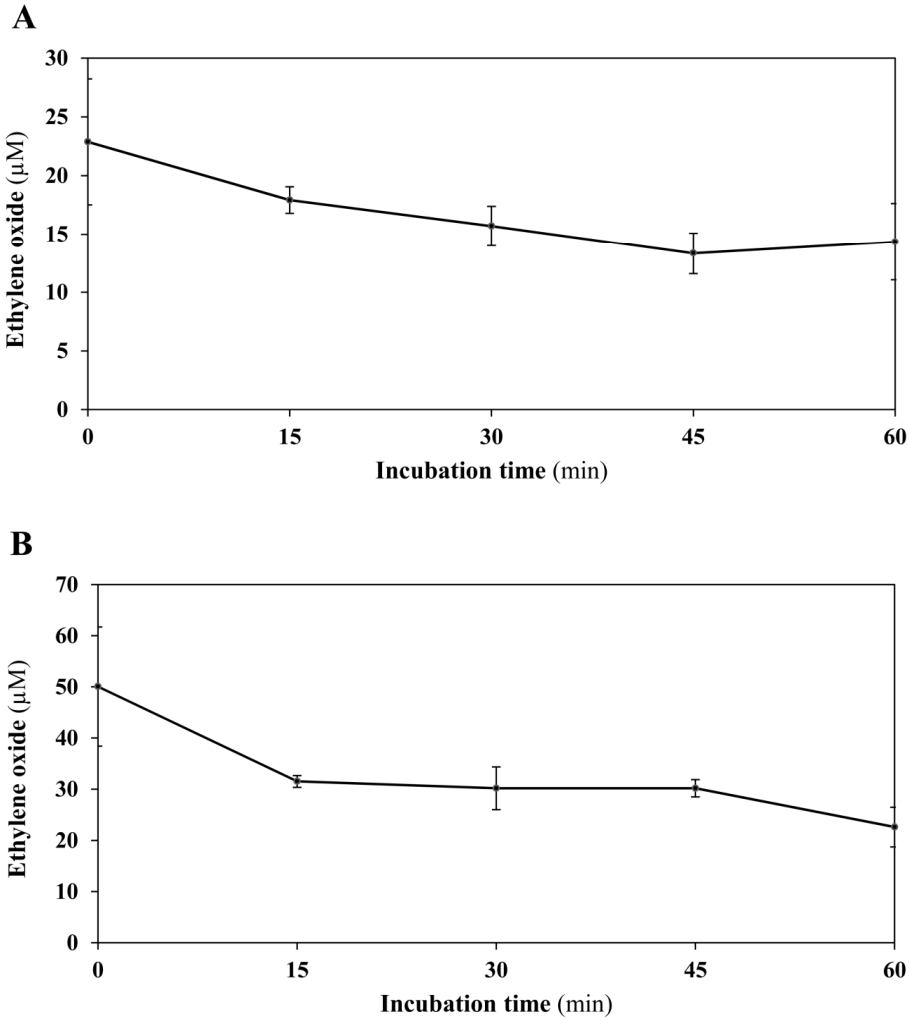
Simple linear regression (Pearson test) was used to determine the association between DNA damage levels and concentrations of the examined toxicants. Means of cell viability (cytotoxicity) and medians of DNA damage (genotoxicity) induced by various doses of the chemical agents in repeated experiments 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 (Fig. 10).



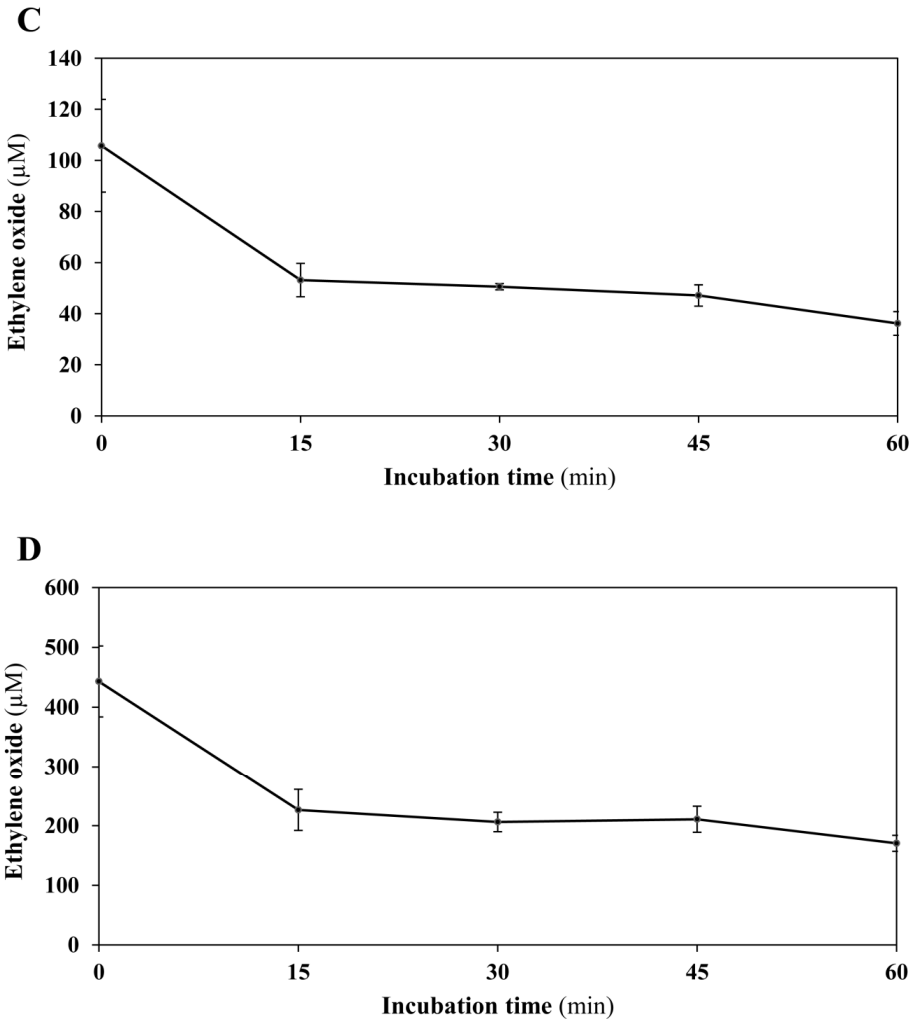


Figure 10. *Time-dependent concentration changes of ethylene oxide in the cell culture medium detected by gas chromatography. Initial treatment concentrations of EO were 20 µM (A), 50 µM (B), 100 µM (C) and 500 µM (D). Samples from the cell culture medium incubated in sealed 12-well plates for 0, 15, 30, 45, 60 min at 37°C were centrifuged, then subjected into gas chromatograph with flame ionization detector. Data are means ± SEM (n=3).*

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 µM), 35.8% (50 µM), 44.5% (100 µM) and 52.5% (500 µM) of EO evaporated from the medium into the headspace over 1 hour.

The overall active concentration that models internal dose was estimated by calculating the area under the concentration-time curve (time-weighted average). The average acting concentrations for the incubation period were considerably lower than the initial concentrations as presented in Table I.

Table I. *Acting concentration of ethylene oxide during incubation for 1 hour at 37°C.*

Initial concentration (µM)	Acting concentration (µM)*
20	16.4
50	32.1
100	55.5
500	237.5

*Time weighted average over 1 h

Ethylene oxide-induced cytotoxicity

The viability of cells treated with EO or used as controls was assessed by combined fluorescent staining method and found to be over 88% in all cases. All three cell types showed high initial viability (>94%). 1-h exposure to ethylene oxide proved to induce limited cell death in a concentration dependent manner (Fig. 11). Statistically significant decrease of the cell viability could only be observed in lymphocytes and lung epithelial cells at the concentration of 237.5 µM. Keratinocytes showed slightly less viability than the other two cell types, however without statistically significant differences. The results of the cytoxicity assay suggest that at the end of treatment there was no considerable cell death detectable.

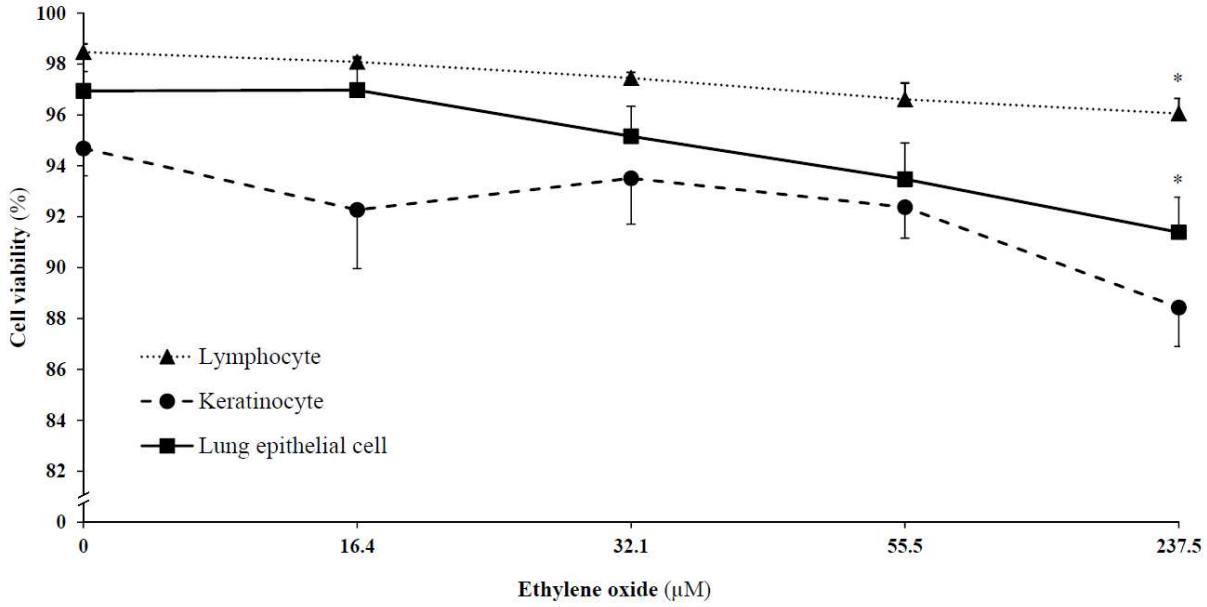


Figure 11. *Effect of ethylene oxide treatment on cell viability. The data points represent the means ± SEM of repeated experiments. Statistically significant difference (*P<0.05, **P<0.01, ***P<0.001) from corresponding untreated control was determined by Student's t-test.*

Alkylating DNA damage induced by ethylene oxide

Exposure of the three cell types to the alkylating agent EO in the 0-237.5 µM concentration range showed dose-dependent increase of DNA damage measured by tail DNA and tail length as shown in Figure 12.

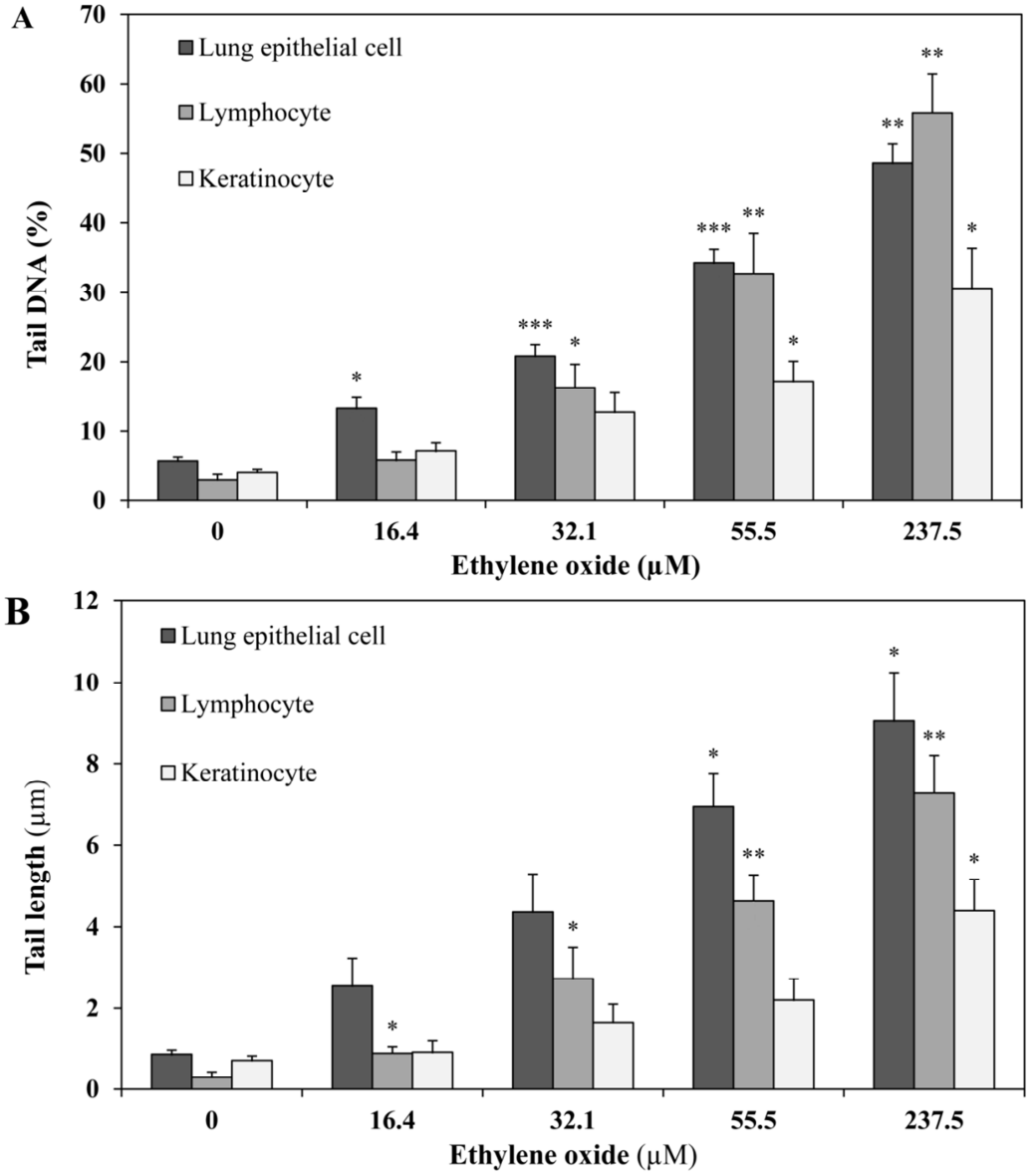


Figure 12. *DNA damage induced by 1 h exposure to sub-cytotoxic concentrations of ethylene oxide in lung epithelial cells, lymphocytes and keratinocytes, measured as tail DNA (A) and tail length (B) in comet assay. Data are averages of median values of repeated experiments (+ SEM indicated by error bars). Statistically significant increase (*P<0.05, **P<0.01, ***P<0.001) was determined by comparing the values of DNA damage induced by various doses of ethylene oxide to the background level of untreated cells by Student's t-test.*

All cell types show nearly linear dose-response relationship with the applied doses. EO induced a considerable increase (more than 8-fold) of tail DNA values in lung epithelial cells in the 0-237.5 µM 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 dose. 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 (Table II). The slope of the linear regression line of both DNA damage parameters was found to be higher in lung epithelial cells (0.158 and 0.029) than in keratinocytes (0.102 and 0.014) in the applied concentration range. Compared with lung cells, lymphocytes had a steeper slope of the TD parameter (0.211), but less increment when considering the TL parameter (0.026).

Table II. *Simple linear regression analysis of the DNA damage (tail DNA %, tail length) induced by 1 h exposure to sub-cytotoxic concentrations of ethylene oxide in the examined cell types.*

Variable	*r**	*β***	*p*-value
Lung epithelial cell			
tail DNA %	0.887	0.158	1.03E-05
tail length (µm)	0.797	0.029	3.74E-04
Lymphocyte			
tail DNA %	0.895	0.211	9.35E-08
tail length (µm)	0.855	0.026	1.56E-06
Keratinocyte			
tail DNA %	0.861	0.102	3.63E-05
tail length (µm)	0.869	0.014	2.58E-05

**r*, Pearson's correlation coefficients
***β*, regression coefficient

Hydrogen peroxide-induced cytotoxicity

The viability of cells treated with H_2O_2 or used as controls was assessed by combined fluorescent staining method. It was found to be high in untreated cells (>95%). 1-h exposure to hydrogen peroxide induced limited cell death (over 87% viability in all treated cases) in a concentration dependent manner (Fig. 13). Statistically significant decrease of the cell viability could be observed in lymphocytes and keratinocytes in the 2 to 10 µM concentration range at the end of the treatment. The same for lung epithelial cells could be noticed from 5 µM.

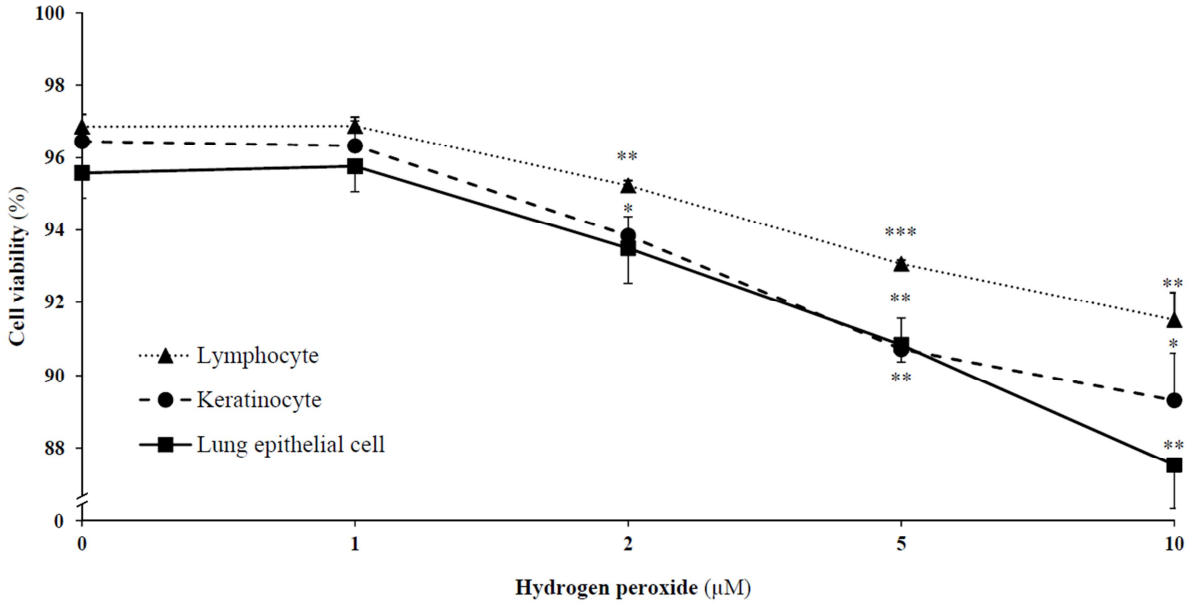
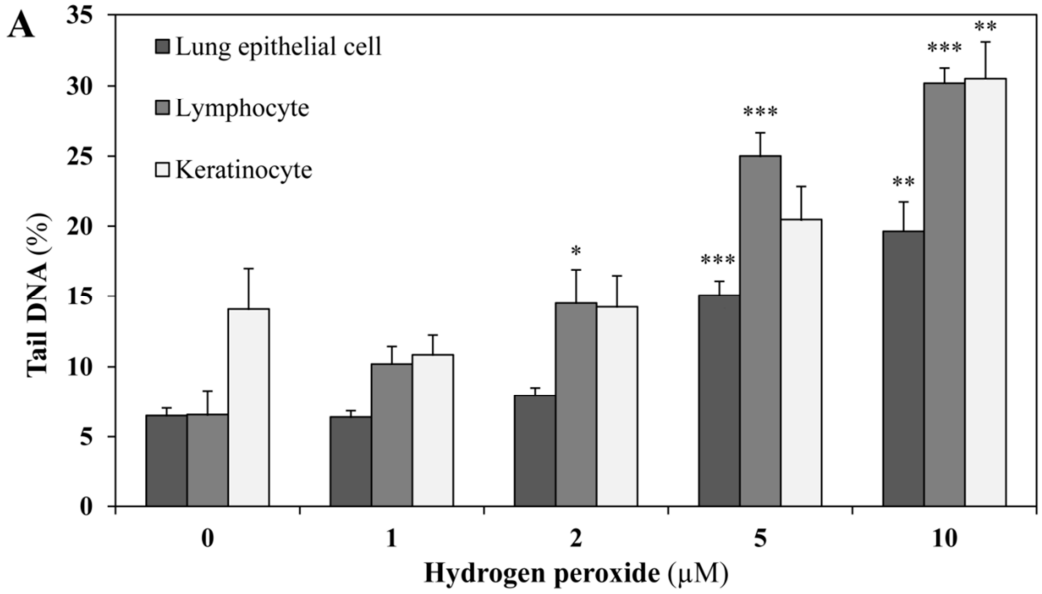


Figure 13. *Effect of hydrogen peroxide treatment on cell viability. The data points represent the means ± SEM of repeated experiments. Statistically significant difference (*P<0.05, **P<0.01, ***P<0.001) from corresponding untreated control was determined by Student's t-test.*

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. The oxidative properties of EO were also investigated, but there was no EO-induced Fpg-dependent oxidative DNA damage observed in the examined cells in the applied concentration range.

Figure 14 shows dose-dependent increase of DNA damage induced by H_2O_2. 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 doses in the other two cell types. The lowest concentration of H_2O_2 that resulted in a statistically significant increase of tail DNA in lung epithelial cells and tail length in keratinocytes was 5 µM. Only treatment with a relatively high dose of 10 µM H_2O_2 caused a significant increase in tail DNA content in keratinocytes and in tail length in lung epithelial cells.



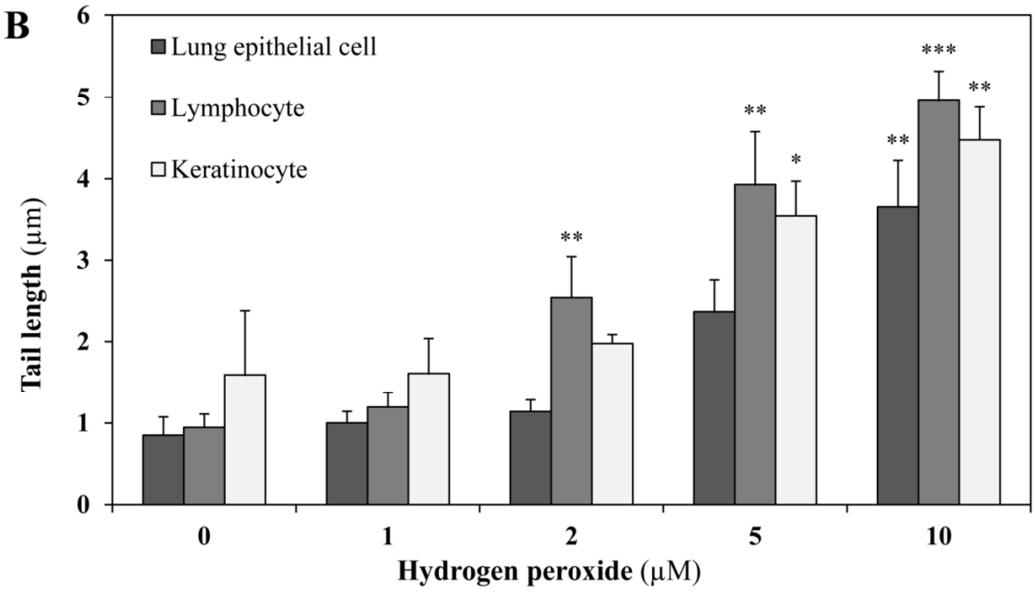


Figure 14. *DNA damage induced by 1 h exposure to sub-cytotoxic concentrations of hydrogen peroxide in lung epithelial cells, lymphocytes and keratinocytes, measured as tail DNA (A) and tail length (B) in comet assay. Data are averages of median values of repeated experiments (+ SEM indicated by error bars). Statistically significant increase (*P<0.05, **P<0.01, ***P<0.001) was determined by comparing the values of DNA damage induced by various doses of hydrogen peroxide to the background level of untreated cells by Student's t-test.*

A statistically significant linear correlation between DNA damage and H_2O_2 concentrations was also found in each cell type examined (Table III). Lung cells had the least steep slope of both TD and TL parameters (1.441 and 0.293, respectively). Conversely, steadily rising DNA damage levels were characteristic for lymphocytes with the highest slope of TD and TL values (2.375 and 0.409, respectively).

Table III. *Simple linear regression analysis of the DNA damage (tail DNA %, tail length) induced by 1 h exposure to sub-cytotoxic concentrations of hydrogen peroxide in the examined cell types.*

Variable	*r**	*β***	*p*-value
Lung epithelial cell			
tail DNA %	0.934	1.441	1.56E-09
tail length (µm)	0.895	0.293	9.78E-08
Lymphocyte			
tail DNA %	0.909	2.375	2.77E-08
tail length (µm)	0.895	0.409	9.31E-08
Keratinocyte			
tail DNA %	0.865	1.876	8.22E-07
tail length (µm)	0.829	0.314	6.18E-06

**r*, Pearson's correlation coefficients
***β*, regression coefficient

Fpg-dependent oxidative DNA damage induced by H_2O_2 in the examined cell types can be expressed as the difference of tail moment values detected with or without Fpg digestion in the comet assay (Fig. 15). Although no statistically significant differences in the DNA damage were observed between controls and Fpg-treated cells measured by tail moment, H_2O_2 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 increased with increasing doses of H_2O_2. A similar tendency was characteristic for keratinocytes. In lung epithelial cells, the oxidative DNA insult was minimal. There was practically no extra DNA damage recognized by Fpg in the applied concentration range.

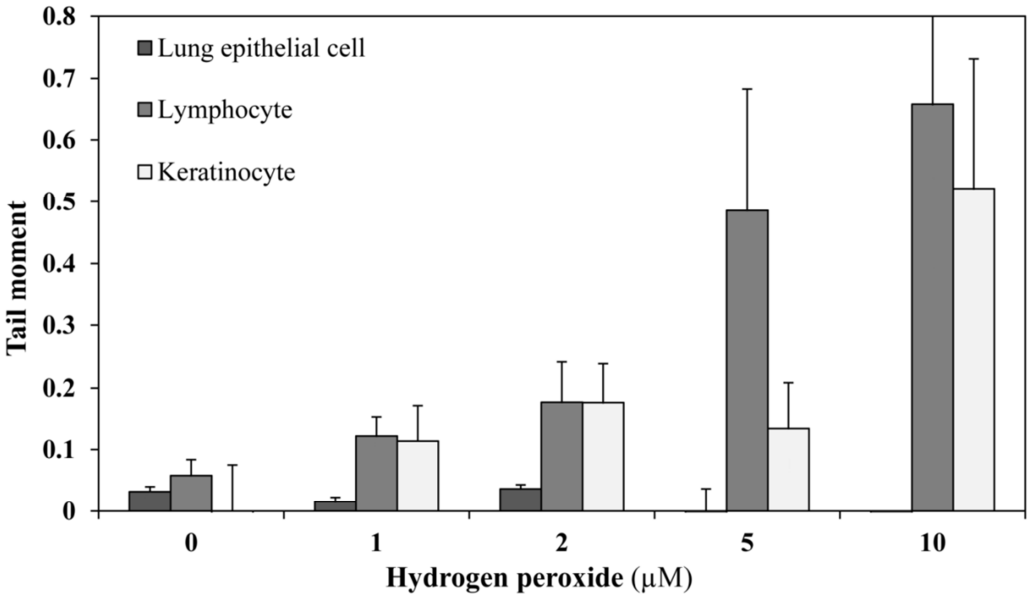


Figure 15. *Oxidative DNA damage induced by 1 h exposure to sub-cytotoxic concentrations of hydrogen peroxide in lung epithelial cells, lymphocytes and keratinocytes. Data are means of differences of corresponding median values detected with and without Fpg modification of the comet assay in repeated experiments (+ SEM indicated by error bars).*

Phenothrin

Phenothrin-induced cytotoxicity

Both human peripheral blood lymphocytes and human hepatocytes showed high initial viability (>90%) in all experiments. 1-h phenothrin induced limited cell death in human peripheral blood lymphocytes and human hepatocytes in a concentration-dependent manner (Fig. 16). Although their dose-response curves follow similar pattern, statistically significant decrease of the cell viability could only be observed in lymphocytes from the concentration of 50 µM. All samples had relatively high, over 77%, viability remaining after treatment.

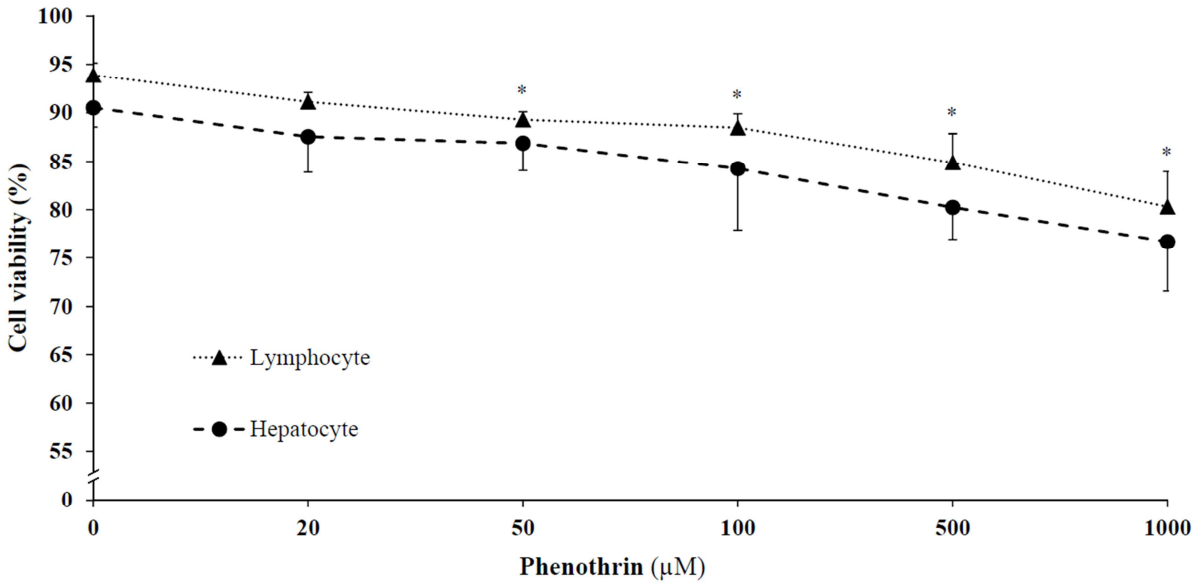


Figure 16. *Effect of phenothrin treatment on cell viability. The data points represent the means ± SEM of repeated experiments. Statistically significant difference (*P<0.05, **P<0.01, ***P<0.001) from corresponding untreated control was determined by Student's t-test.*

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 (Fig. 17).

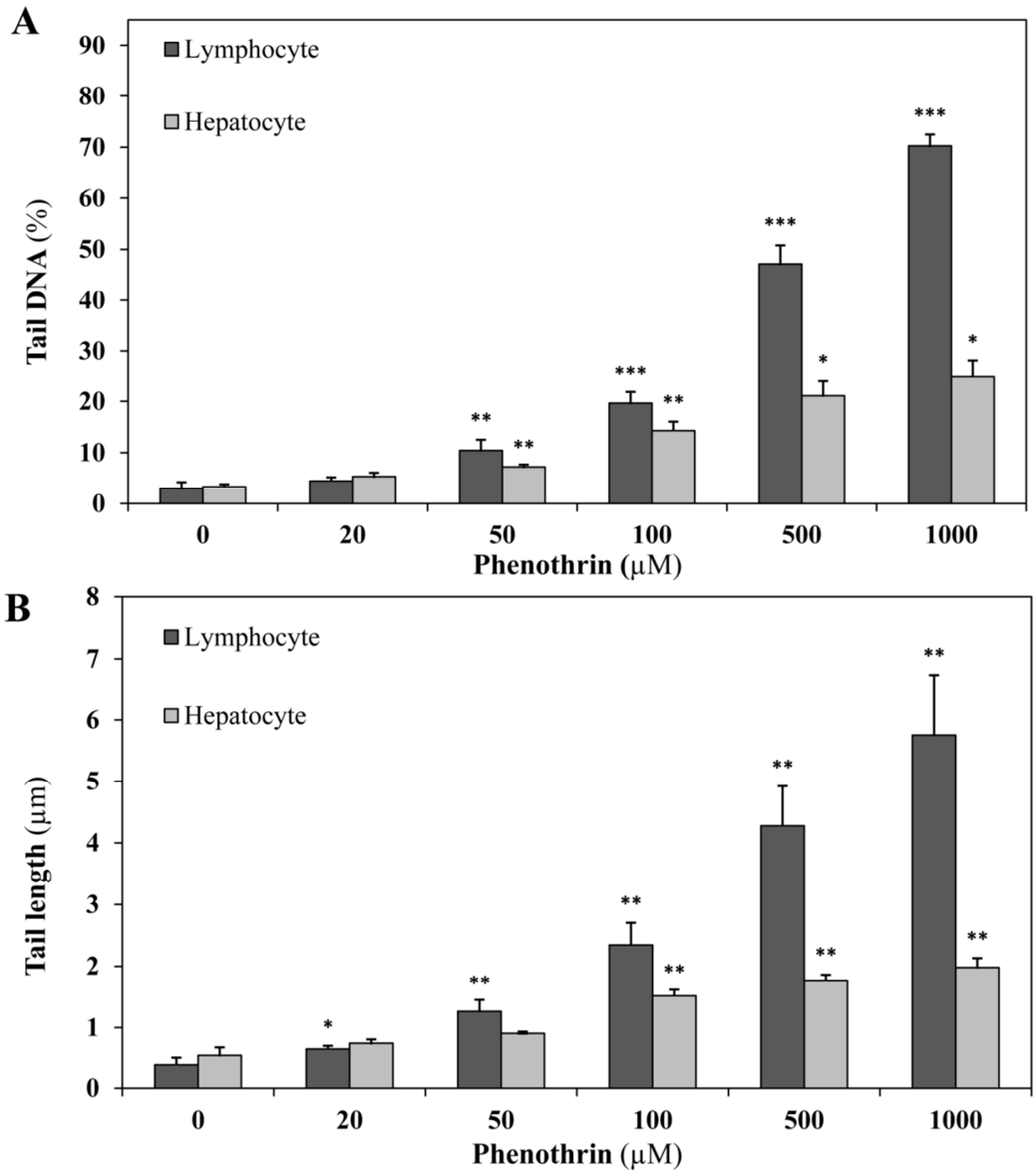


Figure 17. *DNA damage induced by 1-h exposure to sub-cytotoxic concentrations of phenothrin in human peripheral blood lymphocytes and cultured hepatocytes measured as tail DNA (A) and tail length (B) in comet assay. Data are means of median values of repeated experiments (+ SEM indicated by error bars). Statistically significant increase (*p < 0.05, **p < 0.01, ***p < 0.001) was determined by comparing the values of DNA damage induced by various doses of phenothrin to the background level of untreated cells by Student's t-test.*

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 µM). The lowest concentration of phenothrin that produced a statistically significant increase in DNA damage was 50 µM and 20 µM in human peripheral blood lymphocytes, while in hepatocytes it was 50 µM and 100 µM 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 (Table IV). The slope of the linear regression line for both indicators was found to be steeper in human peripheral blood lymphocytes.

Table IV. *Simple linear regression analysis of the DNA damage (tail DNA %, tail length) induced by 1 h exposure to sub-cytotoxic concentrations of phenothrin in the examined cell types.*

Variable	*r**	*β***	*p*-value
Lymphocyte			
tail DNA %	0.982	0.066	4.31E-04
tail length (µm)	0.957	0.005	2.62E-03
Hepatocyte			
tail DNA %	0.912	0.020	0.011
tail length (µm)	0.848	0.001	0.032

**r*, Pearson's correlation coefficients
***β*, regression coefficient

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. Fpg-dependent oxidative DNA damage in the examined cell types was expressed as the difference of tail moment values detected with or without Fpg digestion in the comet assay (Fig. 18). 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.

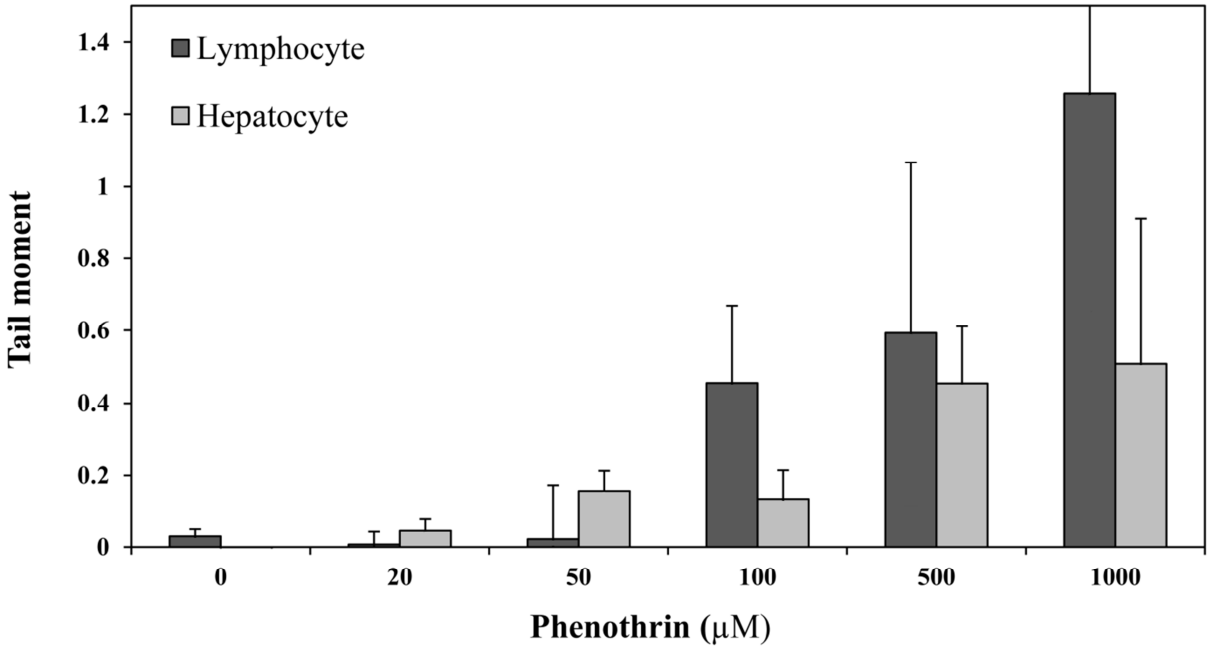


Figure 18. *Oxidative DNA damage induced by 1-h exposure to sub-cytotoxic concentrations of phenothrin in human peripheral blood lymphocytes and cultured hepatocytes measured as tail moment in comet assay. The bars represent the additional damage detected by restriction endonuclease Fpg. Data are means of differences of corresponding median values detected with and without Fpg modification of the comet assay (+ SEM indicated by error bars).*

Significant linear correlation was observed between the oxidative DNA damage and phenothrin concentrations in human peripheral blood as well as in liver cells (Table V). The slope of the linear regression line was found to be higher in human peripheral blood

lymphocytes than in hepatocytes in the applied concentration range. Results indicate less oxidative DNA insult in liver cells than in lymphocytes.

Table V. *Simple linear regression analysis of the DNA damage (tail moment) induced by 1 h exposure to sub-cytotoxic concentrations of phenothrin in the examined cell types.*

Variable	*r**	*β***	*p*-value
Lymphocyte tail moment	0.959	0.0011	2.46E-03
Hepatocyte tail moment	0.924	0.0005	8.33E-03

**r*, Pearson's correlation coefficients
***β*, regression coefficient

Discussion

A large number of chemicals occurring in various environments of our life, including the workspace, can react with the DNA molecule and result in structural and functional changes in several genes that could contribute to the development of malignancies [125]. Initial genotoxic events play a crucial role in the process of chemical carcinogenesis [126]; therefore, detailed knowledge on the genotoxic properties of environmental and occupational agents is essential for the development of effective preventive measures or treatments against cancers.

The widespread production and use of biocidal substances underpin the importance of understanding the potential health risk of human exposure to these agents. According to their mechanism of action, several types of biocides can be distinguished. Some of them can attack cellular macromolecules, such as DNA, what explains the antimicrobial efficacy of many sterilants and disinfectants. Other biocide compounds, such as pesticides, do not necessarily exert their effect through direct molecular damage [127]; even though their genotoxic feature cannot be ruled out.

One of the purposes of our studies was to investigate the susceptibility of lung cells to biocide-induced alkylating DNA insult. We used the alkaline comet assay to describe the dose-response characteristics of EO-mediated DNA damage in three human cell populations, in lung epithelial cells, in peripheral blood lymphocytes and in keratinocytes. The susceptibility pattern of these cell types towards the alkylating insult of EO was compared with their susceptibility pattern towards the oxidative DNA damage induced by H_2O_2. The pyrethroid insecticide phenothrin that has been classified as a non-genotoxic agent so far was the other target of our studies. Its DNA damaging potency was measured by the comet assay in cellular models of human peripheral blood lymphocytes and hepatocytes in order to review the genotoxic hazard of phenothrin use.

Ethylene oxide induced alkylating DNA damage

Ethylene oxide is an important alkylating biocide compound with a worldwide demand of 19 million tons in 2006. It is mainly used in low-temperature chemical sterilization processes for a variety of heat sensitive materials such as agricultural and medical devices.

EO, that was found genotoxic in various *in vitro* and *in vivo* test systems, sterilizes products in gaseous form by means of an alkylation reaction that destroys organisms' ability to reproduce [64].

Alkylation is one of the most common types of DNA damage that can lead to mutations and cancer [128]. The lung epithelium is the first barrier that encounters inhalant toxins, such as polycyclic aromatic hydrocarbons (e.g. benzo[*a*]pyrene) which are associated with the development of respiratory cancer [129]. Various *in vivo* animal experiments have indicated that inhalative exposure to EO may eventually lead to lung cancer [64], but only very limited data on humans are available in the literature suggesting that lung cancer cases might be associated with exposure to EO [130]. The ability of EO 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 as measured by tail DNA, although their tail length values increased significantly from the lowest applied dose, too. EO has been reported to cause significant increase of DNA damage in peripheral blood mononuclear cells measured by the comet assay, but only at high concentrations in excess of 450 µM [131]. 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 a previous observation of increased level of DNA strand breaks induced by the alkylating agent sulfur mustard in lung epithelial cells measured by the TUNEL assay [132]. The genotoxicity induced by alkylating insult was investigated in white blood cells by Ludlum et al. [133], where exposure to sulfur mustard generated DNA adducts similarly to the EO effect. 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 [110].

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 H_2O_2, 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 H_2O_2 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). Low concentrations of H_2O_2 (1-2 µM) induced minimal increase of DNA damage in lung epithelial cells and keratinocytes, but the response to higher doses (5-10 µM) differed in the two cell types, where keratinocytes proved to be more sensitive against H_2O_2 insult than lung epithelial cells.

The detection of Fpg-dependent DNA sites revealed considerable H_2O_2-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 intra- and extracellular antioxidant defense mechanisms, e.g. they contain high levels of glutathione that plays an important role in the antioxidant defense [134, 135]. Our results are in agreement with the general concept about the resistance of lung epithelial cells to H_2O_2 [136]. An *in vitro* study used the comet assay to investigate the oxidative DNA damage induced by 0.2 to 1 mM H_2O_2 in SV-40 transformed lung epithelial cells and, consistent with our findings, reported resistance towards the oxidative insult [137]. Oxidative DNA damage in rat type II pulmonary epithelial cells was detected by gas chromatography in a study that applied considerably higher, millimolar, H_2O_2 concentration [138]. It can be assumed that the antioxidant defense system was able to protect the A549 cells against oxidative insult in the low concentration range of H_2O_2 that was used in our investigations.

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.

A confounding factor in assessing genotoxic risk associated with EO exposure may be its endogenous level; however, that was found below the detection limit in each cell type. According to previous investigations, endogenously formed EO induced minimal level of DNA lesions (~ 1 to 10 N7-HEG adducts /10^7 nucleotides) in lymphocytes [139, 140]; consequently, endogenous EO concentrations are unlikely to pose a considerable genotoxic burden in our *in vitro* model system.

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 depend on the actual airborne concentration of EO [141]. Recent literature data indicate that occupational settings still remain a major source of ethylene oxide exposure, especially in health care and the related industries where workers may be directly exposed to EO. Its concentrations measured in work airspace were up to several thousand mg/m^3 occasionally; however, assessment of correlation between exposure dose and *in vivo* dose requires prudent consideration [142]. 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 [79]. 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/m^3 (20 ppm) EO concentration in the workplace air, a level that has been detected several times in occupational settings [64]. 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/m^3 level of the present occupational exposure limit for EO in the USA [73], but draw attention to the unduly high (9.2 mg/m^3) long term exposure level of this agent in the United Kingdom [74].

In conclusion, the developed *in vitro* system was able to detect the DNA damaging effect of EO and H_2O_2, exerting alkylating and oxidative DNA damage, respectively. The lung epithelial cells demonstrated increased sensitivity to the alkylating effect of EO, but considerable resistance to the oxidative DNA damage induced by H_2O_2. These observations support the assumption that lung epithelial cells can be susceptible targets of an EO-mediated alkylating insult. While the findings do not provide conclusive evidence for a causal link due to the limitations of using an *in vitro* model, they support the concept that 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 study found that phenothrin administered intraperitoneally for 14 consecutive days caused statistically significant, dose-dependent increase of oxidative DNA damage in both organs [120]. 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 [119]. 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 [121, 122]. Another investigation linked phenothrin with breast cancer. Phenothrin has been shown to mimic estrogenic activity in MCF-7 human breast carcinoma cells by increasing the level of WNT10B proto-oncogene expression [123]. Other studies have not supported the cancer-causing ability of this insecticide [112, 143].

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 [144]. 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 [115]. 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. Results of a single study in humans following inhalation exposure to pyrethroid sprays containing phenothrin as a component report that the mean concentration of pyrethroid metabolite *trans*-CDCA in urine was 1.1 µg/l urine; notwithstanding, the proportion of phenothrin in the pyrethrum mixture was unknown [116]. 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 [145, 146]. 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

The identification of carcinogenic chemicals acting via genotoxic mechanisms still remains a major challenge. 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.

Our confirmation of the appropriateness of the existing occupational exposure limit for ethylene oxide provides supportive evidence for the level of threshold as well as proves the adequacy of the comet assay to be used in the establishment and verification of occupational limits and strategies for biomonitoring of chemical exposures in the workplace so as to reduce the genotoxic risk of individuals as far as reasonably practicable.

In the light of our findings on phenothrin, it is advisable to reconsider the health hazards of this pesticide, especially in regard to its DNA damaging potential. First and foremost, 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 recommend.

Summary

More than 1 million people die annually worldwide from malignant diseases caused by chemical carcinogens. According to the multistage model of carcinogenesis the initial events of cancer development typically involve damage to cellular DNA that can be triggered by certain genotoxic agents.

We conducted *in vitro* experiments using the single cell gel electrophoresis assay to investigate the genotoxic properties of two biocides, ethylene oxide and phenothrin, 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. One of our aims was to investigate the role of ethylene oxide-induced DNA damage in the development of lung cancer by characterizing the susceptibility of lung epithelial cells, peripheral blood lymphocytes and keratinocytes towards the ethylene oxide-mediated alkylating and the hydrogen peroxide-mediated oxidative DNA insult and by comparing the susceptibility pattern of these cell types towards the distinct DNA damaging mechanism. Another aim of our studies was to investigate the genotoxic potential of the pyrethroid insecticide phenothrin in cellular models of human peripheral blood lymphocytes and human hepatocytes in order to reconsider the genotoxic risk associated with phenothrin use.

Ethylene oxide induced statistically significant increase in DNA damage at a low concentration (16.4 µM) in lung epithelial cells and in lymphocytes. In keratinocytes, significant genotoxic effect was detected only at a higher dose (55.5 µM). Contrarily, increased resistance of lung epithelial cells was observed against hydrogen peroxide-mediated oxidative insult. These results suggest an increased sensitivity of lung epithelial cells towards the alkylating effect of ethylene oxide, supporting the possible role of ethylene oxide and other alkylating genotoxic agents in the induction of lung cancer.

Phenothrin induced statistically significant DNA damage from concentrations 20 µM and 50 µM in human peripheral blood lymphocytes and hepatocytes, respectively, as well as oxidative DNA damage could be detected in both examined cell types. The findings provide evidence for the genotoxic properties of phenothrin and point out the importance of considering the use of phenothrin with caution.

The genotoxic evaluation of the studied chemicals is inevitable for the appropriate hazard identification and risk assessment of their use, as well as for the design and implementation of effective occupational and environmental preventive measures against the development of chemical-induced cancers.

Magyar nyelvű összefoglaló

A világon évente egy milliónál is többen halnak meg rákkeltő kémiai anyagok okozta rosszindulatú megbetegedésekben. A karcinogenezis többlépcsős modellje szerint a rosszindulatú daganatok kialakulásának kezdeti lépéséért elsősorban DNS mutációk a felelősek, melyek gyakorisága megnövekszik genotoxikus expozíciók hatására.

Kutatásaink során két, egyes munkahelyeken és olykor a mindennapi környezetünkben is előforduló biocid vegyület, az etilén-oxid és a fenotrin genotoxikus képességét tanulmányoztuk laboratóriumi körülmények között üstökös elektroforézis segítségével. Egyik célunk az volt, hogy megvizsgáljuk az etilén-oxid alkiláló hatásának a tüdőrák kialakulásában betöltött szerepét tüdő laphámsejteknek, perifériás limfoctáknak és keratinocitáknak az etilén-oxid alkiláló és a hidrogén-peroxid oxidatív típusú DNS károsító hatásaival szembeni érzékenységi mintázatának összehasonlítása révén. Kutatásaink másik célja a szintetikus peszticid fenotrin használatához kapcsolódó genotoxikus kockázat újraértékelése volt a vegyület DNS károsító hatásának humán perifériás limfocitákon valamint hepatocita sejtvonalon történő vizsgálata révén.

Már alacsony dózisú etilén-oxid expozíció hatására (16,4 µM) megfigyelhető volt a DNS károsodást jelző üstökös paraméterek értékeinek statisztikailag szignifikáns növekedése tüdő laphámsejtek és limfociták esetében, míg keratinocitákban szignifikáns genotoxikus hatás csak magasabb dózisnál (55,5 µM) alakult ki. Ezzel ellentétben a tüdő laphámsejtek fokozott rezisztenciát mutattak a hidrogén peroxid oxidatív típusú DNS károsító hatásával szemben. Az eredmények a tüdő laphámsejtek etilén-oxid alkiláló hatásával szembeni fokozott érzékenységét jelzik, utalva az etilén-oxidnak és egyéb alkiláló genotoxikus ágenseknek a tüdőrák kialakulásában betöltött lehetséges szerepére.

A fenotrin szignifikáns genotoxikus hatást fejtett ki perifériás limfociták esetében 20 µM, míg májsejtek esetében 50 µM koncentrációtól, illetőleg mindkét sejttípusban kimutatható mértékű oxidatív típusú DNS károsodás volt megfigyelhető. Megfigyeléseink alátámasztják a fenotrin DNS károsító képességét és felhívják a figyelmet a vegyület körültekintő használatának fontosságára.

A vizsgált ágensek genotoxikus hatásának jellemzése elengedhetetlen feltétele a használatukhoz kapcsolódó veszély azonosításának, a kockázat becslésének és az ezek ismeretén alapuló hatékony rákmegelőző foglalkozás- és környezet-egészségügyi intézkedések megtervezésének és végrehajtásának.

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Publications

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List of publications related to the dissertation

1. **Nagy, K.**, Rácz, 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
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2. **Nagy, K.**, Ádány, R., Szűcs, S., Ádám, B.: Susceptibility of lung epithelial cells to alkylating genotoxic insult.
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Appendix