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Genotoxic effects of the insect repellent n, n-diethyl-meta-toluamide (DEET) and detection of retinoblastoma gene expression in human lymphocytes: A pilot study

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Abstract

N, N-diethyl-meta-toluamide (DEET), has been used as the active ingredient in the majority of the commercial insect repellents for decades. Its extensive usage is related to the total protection that provides against a broad spectrum of biting insects. In the last few years, residues of DEET have been detected all over the world in various aquatic environments including drinking water. Consequently, DEET has become an emerging contaminant and the evaluation of its potential effects on human genetic material -presents major scientific interest.

The genotoxic and cytotoxic activity of DEET at different concentrations (i.e. 50, 100, 200 and 300 μg/ml) was evaluated by employing the Cytokinesis Block MicroNucleus (CBMN) assay in cultured human lymphocytes. DNA was extracted from all samples, and DNA methylation was performed. The expression of retinoblastoma (RB) gene was assessed by real-time RT-PCR.

The obtained results showed low gene expression irrespective of DEET concentration, therefore, further studies are required to draw definite conclusions. It is worth noting that there are no previous studies correlating DEET and its influence on RB gene expression. Our findings on the genotoxic and cytotoxic effects of DEET in cultured human lymphocytes suggest the potential human health risks of DEET. It is proposed that DEET should be handled with care, in order to minimize its environmental and human risk.

Key words: Genotoxicity, N,N-diethyl-meta-toluamide (DEET), retinoblastoma, micronuclei, lymphocytes

introduction

DEET (or N, N-diethyl-m-toluamide, also known as N, N-Diethyl 3-methylbenzamide) is the most effective and widely used insect repellent, with over 200 million users worldwide. DEET was one of the first synthetic insect repellents originally developed by the United States Department of Agriculture and first used by the U.S. military in 1946, registered for use by the general public in 1957, and has since sold on the market for almost a half a century. The products are available in 5% to 100% DEET concentrations and come in a variety of forms: aerosol, pump spray, lotion, creams, liquids, sticks, roll-ons, wipes, and bracelets for the hand. The US Centers for Disease Control and Prevention (CDC) estimates that 30% of Americans use DEET-based insect repellents to prevent mosquito bites, as well as other insect bites, such as ticks, which are responsible for transmitting Lyme disease (Borreliosis).[1,2] It is estimated that 23% -29% of children in the US are exposed to DEET. It is estimated that about 30% of the American population and about 25% of the UK use insect repellents that contain DEET at least once a year.[3] The benefits obtained by the use of insect repellents are certainly significant, however, their extensive usage[2] can cause potential effects on the human health.

In the present study, the expression of the RB1 gene as well as the Cytokinesis Block MicroNucleus (CBMN) assay in cultured human lymphocytes were used to study the potential mutagenic, genotoxic and cytotoxic effects of DEET.

Retinoblastoma is a malignancy that occurs because both copies of the RB1 gene that normally suppresses retinoblastoma are lost in the developing retinal cell in embryos, babies and young children. Retinoblastoma, as mentioned, can have bilateral or unilateral localization, and in 5% of children with hereditary retinoblastoma (H1), it is associated with a midline brain tumor.[4,5] Without timely and effective treatment, retinoblastoma can spread through the optic nerve to the brain, or through the blood to the bone marrow leading to death.[6]

The genotoxic, cytotoxic and mutagenic activities of certain agro-pharmaceuticals have been studied both with in vitro and in vivo systems, using indicators of genetic damage such as micronuclei (MN), single cell gel electrophoresis (SCGE), chromosomal aberrations (CA) and sister chromatid exchanges (SCE).[7-17] The CBMN assay in human lymphocytes, developed by Fenech and Morley (1985), uses cytochalasin-B, an inhibitor of actin polymerization, to prevent cytokinesis without blocking nuclear division.[18-20] As a result, binucleated (BN) cells are produced, which are scored for the presence of MN.[19,21,22]

Material and Methods

DNA isolation and methylation

DNA was extracted from all samples, using the appropriate kit from Invitrogen (Pure Link Genomic DNA) and the extraction was performed according to manufacturer's protocol. DNA methylation of samples was realized using Qiagen's Kit Epitect Bisulfite Kit. All DNA samples were stored at -20°C.

PCR

Real-time PCR

The expression RB gene, was assessed by real-time RT-PCR using specific primers synthesized by TIB-MOLBIOL (Berlin, Germany). All primers were used at a concentration of 20 pmol/μl in each reaction. Quantitative real-time PCR was performed in a final reaction volume of 20 μl in a LightCycler 480 white 96-multiwell plate (Roche Diagnostics, Mannhein, Germany). All samples were run in duplicate, and no-template controls were included in all runs to exclude possible DNA contamination. The RT-PCR mixture for RB gene contained Kappa SYBR Fast 2x, 0.5 μl for each primer, up to 20 μl total volume of reaction H2O and 2 μl DNA.

The sequences of the primers are:
RBS CAATTGGTGAATGATCATTCGGGAC
RBA TTTGGGACTCTCCTGGGAGATGT

CBMN assay in cultured human lymphocytes

Blood samples were obtained from two healthy non-smokers, male donors who had received no medication and had no health problems one month before blood sampling. Donors were aged between 20 and 28 years. Whole blood (0.5 ml) was added to 6.5ml Ham's F-10 medium (Invitrogen), 1.5 ml fetal calf serum (Invitrogen), and 0.3ml phytohemagglutinin (Invitrogen) to stimulate cell division. The selected concentrations of DEET were added 41h post culture initiation. DEET final concentrations were 50, 100, 200 and 300 μg/ml of culture medium. Three hours after the addition of the chemicals 6μg/ml cytochalasin-B (Sigma) were added (44h post culture initiation). Cells collected by centrifugation at 72 h post culture initiation, fixed with freshly made methanol/acetic acid (Riedel-de Haën/Merck) mixture (3:1v/v) after mild hypotonic treatment, were stained with Giemsa (Fluka).[9,14] Experiments were performed in duplicate. At least 1000 BN cells with preserved cytoplasm were scored per slide, for each donor and for each case, in order to calculate the frequency of MN. Standard criteria[22] were used for scoring MN. The Cytokinesis Block Proliferation Index (CBPI), given by the equation CBPI = [M1 + 2M2+ 3(M3+ M4)]/N, where M1, M2, M3 and M4 correspond to the numbers of cells with one, two, three, and four nuclei and N is the total number of cells,[23] was calculated by counting at least 2000 cells, for each donor and for each case, in order to determine possible cytotoxic effects.

Ethics statement and CBMN assay in human lymphocytes

The study was approved by the Ethical Committee of the University of Patras. After informed consent, two healthy, nonsmoking male individuals (<30 years) were used as blood donors to establish whole blood lymphocyte cultures. According to the donors' declaration, they were not exposed to radiation, drug treatment or any viral infection in the recent past.

Results

Expression of the retinoblastoma (Rb) gene

All samples were shown to have an expression corresponding at less than 50 copies of RB, regardless of the concentration of the insect repellent DEET indicating a low expression of the gene (Tables 1, 2). G6PDH was used as a housekeeping gene and was expressed in all samples.

Table 1. Presentation of the results of the expression of the Rb gene using the real time PCR technique.

SAMPLE	NO OF CYCLES
A1	33,82
A2	35,12
A3	37,85
A4	38,24

Table 2. Presentation of the results of the expression of the Rb gene using the real time PCR technique.

SAMPLE	NAME	CYCLES
A1	1	31,24
A2	1'	34,68
A3	2	37,68
A4	2'	-
A5	3	40,82
A6	3'	-
A7	4	34,55
A8	4'	35,81
A9	5	-
A10	5'	40,63

Genotoxic and cytotoxic effects on human lymphocyte cultures

DEET induced a statistically significant increase in the MN frequency compared to the control at the highest tested concentrations of 200 and 300 μg/ml (Figure 1). The cytotoxic effect of the DEET was evaluated by the determination of CBPI index. Regarding the cytotoxic index, statistically significant differences on CBPI were detected between control cultures and all the tested concentrations of DEET (Figure 2).

Statistical analysis

Statistical analysis of MN data was made by the G-test for independence on 2x2 tables. This test is based on the general assumption of the x2 analysis but offers theoretical and computational advantages.[24] The G-test was evaluated using the data analysis statistical software Minitab.

Discussion

Environmental concerns about DEET usually focus on its ability to accumulate in surface waters and sediments and the potential risks to aquatic organisms. It is generally recognized that a large percentage of DEET sprayed on the skin is flushed from the shower, bath, or swimming pool. DEET-sprayed garments are then washed, so the residue may be transferred to wastewater treatment plants or, in some cases, released directly into surface water or soil through sewage.[25]

The insect repellent DEET as described above is a widespread biocidal insect repellent and enters the aquatic environment mainly through urban wastewater where it has an increased residence time. It has also been detected in seawater, rivers, treated effluents as well as drinking water treated with conventional water treatment methods. Thus, its behavior in the environment, but also its effects on humans are of particular interest.[26,27]

Most of the evidence for the toxic effects of DEET in humans comes from cases of ingestion of the chemical. Swallowing can lead to hypotension, convulsions / seizures and coma within just 1 hour. Deaths have

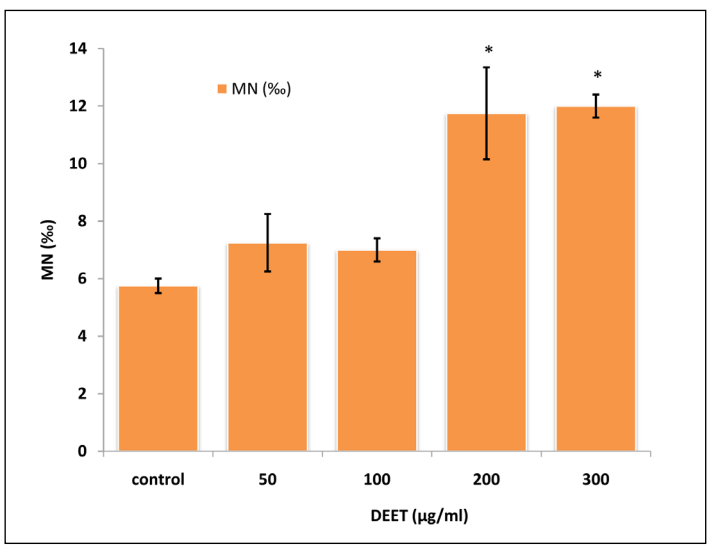

Figure 1. Frequencies (‰) of micronuclei (MN) values in cultured human lymphocytes which have been treated with DEET (50, 100, 200 and 300 μg/ml). *Significant difference in relation to control at $p<0.01$.

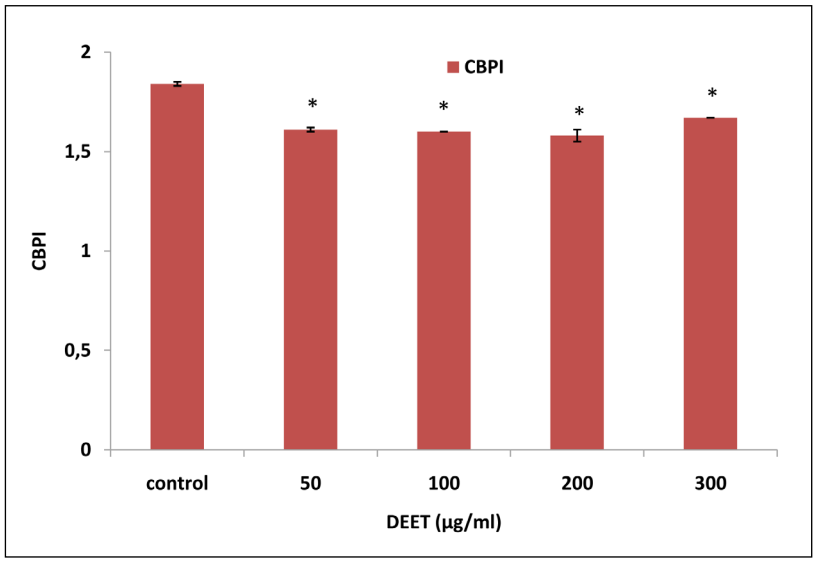

Figure 2. Cytokinesis block proliferation (CBPI) index values in cultured human lymphocytes which have been treated with DEET (50, 100, 200 and 300 µg/ml). *Significant difference in relation to control at p<0.001.

been associated with serum concentrations of 1 mmol / L. The mechanism leading to convulsions is unknown. They can occur as soon as 1 hour, as well as 48 hours after ingestion. Although convulsions can theoretically occur more frequently in people using DEET with concomitant use of drugs that reduce the rate of convulsions (e.g., bupropion, antipsychotics, systemic steroids, and anti-malarial agents), there are no confirmed interactions. Psychosis was described in an adult who had applied a product containing 70% DEET on the skin. Also, contact dermatitis after skin application has been described, as well as generalized itching and generalized angioedema. Conjunctival lesions may occur after eye exposure.[1]

The retinoblastoma-derived cell is most likely a precursor photoreceptor cell that develops cone and has both lost the alleles of the RB1 tumor suppression gene and remains in the inner nuclear layer of the retinal retina.[28-30] The RB1 gene on chromosome 13q14 encodes the pRb protein, a tumor suppressor protein that helps control cell proliferation. Thus, loss of this protein leads to uncontrolled cell proliferation and oncogenesis.[6] The incidence is higher in developing countries and in some Central and South American countries retinoblastoma is one of the most common solid malignancies in children.[31] The reason for this higher impact is unclear. Low socioeconomic status and the presence of human papillomavirus sequence in tumor tissue (retinoblastoma) have been implicated.[32] Africa and Asia have a 70% mortality rate compared to developed countries where this percentage is below 5%.[33]

The cell that is sensitive to cancer is present in the retina of young children, before birth, until about 7 years of age. Rarely, retinoblastoma is first diagnosed in the elderly, who probably previously had a detectable small tumor (retina) that existed in childhood and later became active.[34,35] In the most economically developed countries, the average age of onset of bilateral localized tumor is 12 months and 24 months for bilateral localization.[36]

In the present study a low expression of the Rb rgene, was shown probably inducing a low expression of the corresponding protein, which prevents

uncontrolled cellular cell proliferation. Decreased pRb function can cause cell cycle deregulation and thus lead to malignancy. Genetic inactivation of the pRB gene through mechanisms like methylation is one of the main reasons for the development of retinal tumor. It would be interesting in the future to study the effect of DEET on other proteins with which pRb interacts with transcription suppression (hBRM, BRG1, HDAC1 and SUV39H1) and neoplasm development.

Few studies have examined the genotoxicity of DEET in *in vitro* assays using human cells and no studies were located regarding genotoxic effects in humans exposed to DEET.[27] Exposure of primary human nasal mucosal cells from the inferior and the middle turbinate to concentrations of DEET ranging from 0.5 to 1.0 mM for 60 minutes induced significant DNA damage, as quantified by the comet assay. The results provide some evidence for the potential carcinogenicity of these agents to human nasal mucosal cells.[37] In the recent study by Legeay et al. (2016), the authors suggested pro-angiogenic properties of DEET in human health. The findings demonstrated that DEET specifically stimulates endothelial cells that promote angiogenesis which increases tumor growth.[38]

There are limited data in previous reports in mammalian *in vitro* and *in vivo* approaches with regard to the genotoxic effects of DEET. In Sprague-Dawley rats was applied a single dermal dose of 400 mg DEET/kg in 70% ethanol and the urine was collected and analyzed for the biomarker of DNA damage 8-hydroxy-2'-deoxyguanosine. The results showed a significant increase ($p<0.05$) in the levels of the biomarker in urine over a 72-hour period after dosing.[39] On the contrary, other studies with mammalian cells such as primary rat hepatocytes and Chinese hamster ovary cells, did not induce unscheduled DNA synthesis and chromosomal aberrations, respectively.[40]

The statistically significant genotoxic induction at DEET concentrations of 200 and 300 μg/ml as well as the increased MN frequencies at 50 and 100 μg/ml (Figure 1), corroborate previous reports with regard to the genotoxic action of DEET. In addition, the statistically significant cytotoxic induction at all the tested concentrations of the DEET reveals the ability of DEET to enhance cytotoxic effects (Figure 2). It is worth noting that our present results are the only available findings of the genotoxic and cytotoxic effects of DEET on cultured human lymphocytes.

Considering the limited reports existing in the literature regarding the possible genotoxic and cytotoxic effects of DEET in humans, DEET should be handled with care, in order to minimize its environmental and human risk. From that point of view, its potential impact to the environment, the organisms and human health must be further investigated and confirmed.

Conclusions

The present study provides for the first time in the literature significant results and conclusions regarding the effect of DEET on the human gene of retinoblastoma.

In general, a low expression of the retinoblastoma gene (Rb) gene regardless of the concentration of the insect repellent DEET and the methylation status of the samples and an increased cell proliferation and possible oncogenesis were observed

More specifically, the retinoblastoma gene is expressed in a very small percentage and therefore, the corresponding protein is poorly produced leading to an uncontrolled cell proliferation and thus to the formation of retinoblastoma tumor. Inactivation of pRb function by binding of viral oncoprotein is also present in other neoplasms, and thus the action of DEET on this oncogene may adversely affect other organs.

In addition, the present study provides data that

can cover the existing gaps in the literature regarding the genotoxic and cytotoxic activities of the DEET on human cells. Based on the results, human health risks posed by short and long-time exposures to DEET can be proposed.

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Conflict of Interest

The authors declare that they have no competing interest.

Author Contributions

ED, MA, DV and SS designed the study; ED, FK and DM performed the experiments, FK, MA, DM and DV analyzed the data; SS, MA, DV, DM and PD wrote the manuscript. All authors reviewed the manuscript.

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