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METABOLISM AND *IN VITRO* ASSESSMENT OF THE MUTAGENIC ACTIVITY OF URINARY EXTRACTS FROM RATS AFTER INHALATION EXPOSURE TO 1-METHYLNAPHTHALENE

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Abstract

Objectives: 1-Methylnaphthalene (1-MN) is composed of 2 benzene rings and belongs to polycyclic aromatic hydrocarbons. The metabolism of 1-MN in laboratory animals and bacteria leads to the formation of 1-naphthoic acid (1-NA). **Material and Methods:** In this study the distribution of 1-NA in lung, liver, spleen, kidney and urinary excretion of 1-NA in rats after single and repeated inhalation exposure to 1-MN vapors were investigated. The activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and cytochrome were measured of the rats. Genotoxic effects were evaluated with the *in vitro* micronucleus test on V79 hamster fibroblasts. **Results:** The concentrations of 1-NA in the tissues of rats after single and repeated exposure to 1-MN were dependent on the exposure dose. High levels of 1-NA were found in kidneys of animals after the single and repeated exposure to 1-MN. With an increase of 1-MN dose, an increase in the activity of cytochrome P450 (CYP1A1 and CYP1A2) was observed in the liver of rats. Compared to control animals, significantly higher ALT activity was noted in serum of rats exposed to 1-MN. The micronuclei frequency in V79 cells exposed to 1-MN (in the range of analyzable concentrations; i.e., 5–25 μg/ml) did not differ significantly from the vehicle control, whereas urine extracts from rats exposed to 1-MN induced a significant increase in the frequency of micronuclei compared to urine extracts from the group of control animals. **Conclusions:** Metabolism of 1-MN in rats after the inhalation exposure leading to 1-NA was mainly observed during the first day after the end of exposure. It is likely that 1-MN metabolites present in rat urine can induce the increased micronuclei frequency as was shown in V79 cells. Int J Occup Med Environ Health. 2022;35(6):731–46

Key words:
rats, inhalation, micronucleus test, toxicokinetics, 1-methylonaphthalene, 1-naphthoic acid

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INTRODUCTION

1-Methylnaphthalene (1-MN) belongs to a group of chemicals called polycyclic aromatic hydrocarbons (PAHs) with 2 benzene rings. It is a chemical that pollutes drinking water, soil, air, seas and bottom sediments (sludge) [1–7]. The environmental contamination by 1-MN is mainly associated with the petroleum industry producing various organic solvents, gasoline and heating oils [1,8,9].

1-Methylnaphthalene can be found in different environmental conditions at potentially hazardous concentrations. The content of 1-MN in soil samples from Antarctica (oil contaminated) was in the range of 299–2820 ng/g dry weight of soil [1]. Measurements of indoor and outdoor atmosphere in the North Central Part of India showed that the average concentration of 1-MN was 205 ng/m^3 in kitchen, 140 ng/m^3 in living room and 337 ng/m^3 in outdoors at urban site, whereas at roadside site it was 163 ng/m^3, 184 ng/m^3, and 143 ng/m^3 in kitchen, living room and outdoors respectively [5]. The air levels of occupational and environmental exposure to 1-MN were in the range of 24–35 000 ng/m^3 in the 3 selected areas of the airport [4]. In Poland, the maximum allowable concentration (MAC) value calculated for 1-MN in time-weighted average (TWA) is 30 mg/m^3 [10].

After releasing into the environment, 1-MN may undergo biological transformation. Anaerobic bacteria (2 bacterial cultures) during their cultivation metabolized 1-MN mainly to 1-naphthoic acid (1-NA) [11]. Pseudomonas putida CSV86 (soil bacterium), metabolizes 1-MN via 2 different catabolic pathways where the final metabolites are 3-methylcatechol and 1-NA [12,13]. Cunninghamella elegans in 1-MN transformed mainly the methyl group (–CH_3) to form 1-hydroxymethylnaphthalene. Other identified metabolites of 1-MN included: 1-NA, 5-hydroxy-1-naphthoic acid and phenolic derivatives of 1-MN [14]. Similarly, cyanobacteria metabolized 1-MN by introducing a hydroxyl group (–OH) to the methyl group and formed 1-hydroxymethylnaphthalene. Two-ring hydroxyl metabolites have not been detected [15]. The guinea pig metabolized 1-MN mainly to 1-NA. In addition, 2 metabolites such as methylnaphthol and methylnaphthalene-dihydrodiol were detected in smaller amounts [16]. In urine samples of smokers and non-smokers, 5 metabolites of 1-MN and methyl isomers of naphtols were determined andmethyl naphtols were suggested to be used as a potential biomarker of non-occupational exposure to 1-MN [17].

There was an increased frequency of sister chromatid exchanges (SCEs) in human lymphocytes exposed *in vitro* to 1-MN, that was observed at each concentration of 1-MN used [18]. The frequency of SCE observed did not exceed twice that of the control group. The authors suggested that 1-MN cannot be classified as a potential genotoxic substance.

Murata et al. [19] reported that in the mice fed a diet containing 0.075% or 0.15% of 1-MN a positive effect was observed in some mutagenicity endpoints. Although 1-MN was positive in some mutagenicity endpoints (the SCE test), however negative effects were observed in the Ames test [20,21]. The 1-MN and 1-NA were not classified by International Agency for Research on Cancer (IARC) and US Environmental Protection Agency (EPA), whereas American Conference of Governmental Industrial Hygienists (ACGIH) classifies 1-MN into the carcinogenicity category TLV-A4 [22,23].

The aim of this study was to analyze the concentration of 1-NA in the lung, liver, spleen, kidney and urine of rats once and repeatedly exposed to vapors of 1-MN. A liver is the main organ that metabolizes xenobiotics. In the rat liver, CYP1A1 and CYPA2 are responsible for bioactivation of polycyclic aromatic hydrocarbons. Therefore, it was decided to study the CYPs (in liver) and liver toxicity markers (ALT and AST in the plasma) [24].

The activity of ALT and AST was determined in the serum of rats. The activity of cytochrome P_{450}1A in the liver of rats was determined for 2 isoforms, CYP1A1 and CYP1A2. The genotoxic effect in the *in vitro* micro-

nucleus test of the rat urine extracts was investigated on V79 hamster fibroblast cells.

MATERIAL AND METHODS

Chemicals

1-Methylnaphthalene (CAS #90-12-0, purity ≥98%) was supplied by Riedel-de Hanën (Seelze, Germany). 1-Naphthoic acid (CAS #86-55-5, purity >97%) and N,O-Bis(trimethylsilyl)trifluoroaceramide (CAS #25561-30-2, purity ≥98%) were purchased from Fluka (Buchs, Switzerland). 4-Methylphtalic acid (CAS #4316-23-8, purity ≥99%) was supplied by Aldrich (Steinheim, Germany); ethyl alcohol, concentration 95%, was from Polmos (Warszawa, Poland).

For *in vitro* studies a stock solution of 1-MN was prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Saint Louis, USA) at the concentration of 1 g/ml. Cyclophosphamide (CP) (CAS #50-18-0, Sigma-Aldrich #L5178V, Saint Louis, USA) was dissolved in DMSO at the concentration of 1 g/ml at 37°C and stored in dark up to 1 week in aliquots in –20°C. Mitomycin C (MMC) (CAS #50-07-7, Sigma-Aldrich #M4287, Saint Louis, USA) was dissolved in deionized water at the concentration of 500 μg/ml and stored in aliquots in –20°C. Cytochalasin B (CB) (Sigma-Aldrich #C6762, Saint Louis, USA) was prepared in DMSO at the concentration of 2 mg/ml and then diluted with 0.9% NaCl to the concentration 270 μg/ml and stored in aliquots in –20°C. Stock solution of propidium iodide (PI) (4 mg/ml), was dissolved in DMSO and stored in aliquotes in –20°C. It was diluted in Dulbecco's Phosphate Buffered Saline (PBS) (Gibco, Grand Island, USA) to the concentration of 50 μg/ml before using and such solution was stored at 4°C up to 1 month.

Glucose 6-phosphate (G-6-P) and β-nicotinamide adenine dinucleotide phosphate sodium salt (β-NADP-Na) were purchased from Sigma-Aldrich, whereas potassium chloride (KCl) was obtained from POCh S.A. (Gliwice, Poland). Stock solutions of G-6-P, β-NADP-Na and KCl were prepared in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich #D5671, Saint Louis, USA) at the concentration of 0.6 mmol, 0.03 mmol and 150 mmol, respectively. Rat Liver S9 fraction in KCl (prepared from animals induced with Aroclor 1254) was obtained from MP Biomedicals (Solon, USA).

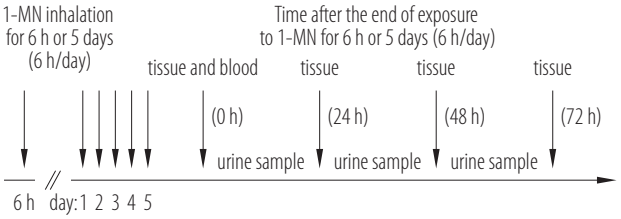
Animal groups and collection of biological material

Male Wistar rats IMP: WIST (2–3 months old) were exposed to 1-MN vapors at the nominal concentration of 0 mg/m^3, 50 mg/m^3, or 200 mg/m^3 in the nose-only inhalation system for 6 h (the single exposure) or 5 days (6 h/day, the repeated exposure). Eighty-four male Wistar rats were used in the experiment. The animals were given standard laboratory food and water *ad libitum*, except for the time when they were exposed to 1-MN vapors. After a 1-week acclimation, animals were divided into 21 treatment groups (4 rats each) and were subjected to single or repeated exposure to 1-MN. The animals were divided into 2 groups:

– the control groups (16 rats) – they were placed in the nose-only inhalation system, but no 1-MN was introduced into the inhalation chamber in the dosing air,
– the exposed group (68 rats) – they were exposed to 1-MN in the nose-only inhalation system.

In animals that were decapitated immediately after the termination of single or repeated inhalation exposure to 1-MN vapours, blood, liver, kidneys, spleen and lungs were collected after 24, 48 or 72 h (Figure 1). The samples were stored in glass vessels at –20°C until the analysis. In other animals, urine samples were collected during 3 days after termination of single or repeated inhalation exposure to 1-MN. During repeated exposure to 1-MN, the urine samples were collected between the consecutive 6-h exposures. For collection of urine samples, the rats were placed singly in metabolic cages (Tecniplast, Buguggiate VA, Italy). The collected urine samples were stored in glass vessels at –20°C until the analysis.

For assessment of the mutagenic activity, rat urines were collected during 24 h after termination of the repeated



Time of examination after exposure given in brackets.

Figure 1. Schematic diagram of the experimental protocol, in the study conducted at the Nofer Institute of Occupational Medicine, Łódź, Poland, in 2014

inhalation (5 days) to 1-MN (50 mg/m^3 or 200 mg/m^3). A urine sample collected from every rat was divided into:

- 8 ml of urine sample were extracted with 20 ml of diethyl ether for 10 min. The ether layer of 10 ml was collected and after its evaporation, the residue was used for *in vitro* study. For *in vitro* experiments, evaporated urine extracts (obtained from 4 ml of the ether extracts) were dissolved directly in cell culture medium in volume of 4 ml per sample and used for cell treatment in volume of 2 ml per well (2 replicates),
- 2 ml urine sample were hydrolyzed for selected ion mode (SIM) monitoring as was described in the "Analysis for 1-NA in biological material" part.

The Local Ethics Committee for Experiments on Animals approved the study protocol (opinion No. 22/ŁB 544/2011).

Inhalation and exposure monitoring

The exposure concentrations of 1-MN were chosen based on the Polish MAC for 1-MN in the air determined for TWE that was 30 mg/m^3 [10]. Two values of inhalation exposure to 1-MN were selected:

- slightly higher than the MAC (50 mg/m^3),
- 4 times higher (200 mg/m^3).

Inhalation exposure of rats was performed using the TSE Systems Head Nose Only Exposure Units (TSE Systems, Bad Homburg, Germany). Animals were exposed to 1-MN vapors in dynamic airflow of at least 15 air changes/h. During the exposure, the rats were placed in glass restrainer tubes. Temperature, humidity and airflow in the head nose-only exposure unit were monitored during each exposure period (TSE Daco Software, Bad Homburg, Germany). Vapours were generated by a metering unit equipped with a syringe pump and ultrasonic nebulizer. The desired concentrations of vapors were obtained by diluting them with the air.

1-Methylnaphthalene vapour samples (0.5 l) were absorbed on 2 ml liquid sorbent (ethyl alcohol). Concentrations of the vapors in the exposure chamber were measured every 30 min by gas chromatography (Hewlett-Packard 6890, Wilmington, USA) with a flame ionization detector (FID) using capillary column (HP-5, 50 m × 0.32 mm × 1.05 mm). The operating conditions were: carrier gas – helium, constant flow mode, column flow 1.4 ml/min; make-up gas (helium) 30 ml/min; air 300 ml/min; oven 110°C; inlet split 230°C, detector 260°C.

Analysis for 1-NA in biological material

Metabolite (1-NA) of 1-MN was measured in urine, lung, liver spleen and kidney samples by means of gas chromatography (Agilent Technologies 6890N, Santa Clara, USA) with mass selective detector (MSD 5973 Network), using 4-methylphtalic acid (Aldrich #34,830-9, Steinheim, Germany) as an internal standard and 1-NA (Fluka #70389, Buchs, Switzerland) as a standard.

Tissues (0.2–1.5 g) or urine sample (2 ml) were hydrolyzed (2 ml of 11 mol NaOH, 2 h at 95°C). After cooling, 5 ml of 6 N H_2SO_4 with 0.5 g NaCl was added and then extracted (10 ml diethyl ether, 10 min). The ether layer of 5 ml was collected after evaporation of diethyl ether, the residue was silylated for 30 min (70°C) with 0.5 ml N,O-Bis(trimethylsilyl)trifluoroaceramide [25].

Samples were separated, using a HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm); the programmed temperature: initial oven, 80°C/4 min; rate A: 5°C/min to 100°C, held 1 min; rate B: 15°C/min to 240°C, held 15 min.

Split injection with a split ratio of 20:1 and helium at the constant flow of 0.5 ml/min was used as carrier gas; inlet split 250°C, MS transfer line 250°C, MS source 230°C and MS quad. 150°C. Experimental samples were analyzed in a SIM monitoring: m/z 127, 155, 185, 229 and 244 for 1-naphtolic acid and m/z 147 for 4-methylphtalic acid. The limit of detection of 1-NA was 0.01 μg/g wet tissue and 0.01 μg/ml urine analysis.

Biochemical analysis for enzyme activities in biological material

The ALT and AST activity in serum of rats were determined using commercial kits produced by BioMar (Gliwice, Poland). Normal and pathological serum were used for quality control. Cytochrome P_{450} 1A (CYP1A) subfamily in mammals consists of 2 isoforms, CYP1A1 and CYP1A2. Activity of the enzymes in liver of rats was measured using the kinetic method described by Dey et al. [26] and Zamaratskaia et al. [27] with ethoxyresorufin (CAS #5725-91-7, Sigma-Aldrich #46121-5MG-F, Saint Louis, USA) and methoxyresorufin (CAS #5725-89-3, Sigma-Aldrich #69125-5MG, Saint Louis, USA) as reagents. The fluorescence of both enzymatic reactions was measured at 535 nm and 590 nm as excitation/emission wave lengths, protein concentration was analyzed with Bradford reagent. Bovine serum albumin was used as standard.

Urine extracts sample

For *in vitro* experiments, evaporated urine extracts (obtained from 4 ml of the ether extracts) were dissolved directly in cell culture medium in volume of 4 ml/sample and used for cell treatment in volume of 2 ml/well (2 replicates).

Cell system

V79 cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC #86041102, University of Wales, UK) and were maintained in DMEM supplemented with 10% heat-inactivated Fetal Bovine Serum (Gibco#10270-106), 2 mmol L-glutamine, 25 mmol HEPES (Sigma-Aldrich #H0887, Saint Louis, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich #P0781, Saint Louis, USA) at 37°C in an atmosphere of 5% CO_2. The doubling time of the cells were determined as 12 h.

Cytokinesis-block micronucleus (CBMN) assay in V79 cells

The CBMN assay, performed according to the then current guideline OECD TG 487 [28], was used to assess the induction of micronuclei by (part A) 1-MN (3 h; with and without metabolic activation) and (part B) urine extracts of rat exposed to 1-MN (24 h; without metabolic activation). On the day prior to the treatment, V79 cells were plated onto 6-well plates (2 wells per sample) at a density of 25×10^4 cells/well/2 ml or 6×10^4 cells/well/2 ml for the short (3 h) or long (24 h) treatment, respectively.

The formulation of metabolic activation (S9 mix), used in the part A of the study, was as follows: G-6-P (0.6 mmol), β-NADP-Na (0.03 mmol), KCl (150 mmol) and S9 fraction in a 1:1:1:2 ratio. The final concentration of S9 fraction in the exposure culture was 2%.

The test samples in the part A (the assessment of mutagenic potential of 1-MN in V79 cells after 3-hour treatment in the presence of S9 mix) were as follows: culture medium only (control of cell system – Cs), culture medium with vehicle, i.e., DMSO (the concentration of the vehicle was the same as in all test samples, i.e., 0.25%), the range of 1-MN concentrations (5–160 μg/ml) and a positive control (CP, 7.5 μg/ml).

The test samples in the part B (the assessment of mutagenic potential of urine extracts from 1-MN treated rats in V79 cells after 24-hour treatment in the absence of S9 mix) were as follows: culture medium only (Cs), a positive control (MMC, 0.05 μg/ml), extracts of urine collected from unexposed rats, extracts of urine from the 1-MN

treated rats. Four extracts per dose (0 mg/m^3, 50 mg/m^3 or 200 mg/m^3) and the duration of the exposure (a single or quintuple treatment) was assessed.

After the 3-hour and 24-hour treatments, the supernatant above the cells (treatment medium) was discarded, the cells were washed with fresh medium and cultured for another 24 h in fresh medium with CB (6.75 μg/ml) to induce binucleation of dividing cells. After that, the cells were trypsinized according to a standard protocol (0.25% of trypsin/EDTA; 3 min), centrifuged, treated with cold hypotonic solution (0.075 M KCl), fixed 3 times with methanol:acetic acid (1:9 v/v) and stored at +4°C at least overnight. Microscope slides prepared from the cell pellets were air-dried, coded and stored at +4°C until analysis. The slides were stained with PI for 5 min and analyzed under the OLYMPUS IX70-S8F2 (Olympus Optical Co. LTD, Tokyo, Japan) fluorescent microscope using 400× magnification. For each individual rat the MN frequency was assessed in 1000 binucleated lymphocytes per culture; 2 cultures per extract/sample. Micronuclei were scored according to criteria determined by Fenech et al. [29] To ensure that the treatments were conducted at accepted levels of cytotoxicity, replication index (RI) and the cytokinesis-block proliferation index (CBPI) and cytostasis were quantified based on results from 500 cells/replicate culture (1000 cells/treatment) according to formulas given in OECD TG 487 [28]. A test sample was considered to be positive if a statistically significant increase in the frequency of binucleate cells with micronuclei at ≥1 concentrations was observed.

Statistical analysis

Statistical analysis was performed with the use of the analysis of variance (ANOVA) test and t-test extended with all pairwise comparisons (Sigma-Stat 4.0 for Windows, Systat Software, San Jose, USA). $P < 0.05$ was considered significant.

RESULTS

General observations

All rats survived the inhalation exposure to 1-MN. Table 1 shows the target and actual concentrations of 1-MN in the inhalation chambers as well as the mean values of body weight and time of collecting biological material after the completion of the single or multiple inhalation exposure to 1-MN vapors.

Kinetics

The target exposure to 1-MN vapor concentrations were 50 mg/m^3 and 200 mg/m^3. The actual concentrations in the chamber during single and repeated inhalation exposures (M±SD) were respectively 50.3±10.6, 53.7±4.1, 225.4±17.8, 194.5±10.8 and 198.1±9.1. The relative temperature in the chamber was kept at 20–24°C, and the humidity was 39–43%.

Table 2 shows the weights of internal organs and the relative weights of the lungs, liver, spleen and kidneys of the animals after inhalation exposure to 1-MN vapors. Significant reductions in absolute and relative spleen weights were noted in rats exposed to 1-MN at 50 mg/m^3 and 200 mg/m^3 for 5 days compared to the control group. A significant increase in the absolute weight of the liver were noted in the groups of males exposed to 1-MN at a concentration of 50 mg/m^3 and 200 mg/m^3 for 6 h compared to the control group. A significant increase in the absolute weight of the kidney was noted in the group of rats exposed to 1-MN at a concentration of 200 mg/m^3 for 6 h compared to the control group. There were no significant differences in the absolute and relative lung weights of the exposed animals compared to the control animals.

In the tissues and urine of control animals, 1-NA was not found. The concentrations of 1-NA in the tissues of rats after the single and repeated exposure to 1-MN at the concentrations of 50 mg/m^3 and 200 mg/m^3 are presented in Table 3. The concentration of 1-NA in the tissues of rats depended on the concentration of 1-MN and

Table 1. Air concentration of 1-methylnaphthalene (1-MN) in inhalation chambers, body weight of rats and biological material collected after termination of single or repeated exposure and during repeated exposure to 1-MN, in the study conducted at the Nofer Institute of Occupational Medicine, Łódź, Poland, in 2014

Material collecting time	Body weight [g] (M±SD)					
	0 mg/m^3 (control)	50.3±10.6 mg/m^3 (6 h)	53.7±4.1 mg/m^3 (5 days)	0 mg/m^3 (control)	225.4±17.8 mg/m^3 (6 h)	194.5±10.8 mg/m^3 (5 days)
0 h – tissue		302±18			296±5	
24 h – tissue and 0–24 h – urine		321±14			308±10	
48 h – tissue		298±12			330±16	
72 h – tissue, 24–48 h and 48–72 h – urine		292±16			297±14	
0 h – tissue and 0 h – blood	247±11		249±15	240±15		255±18
6 days and 0–24 h – urine, 24 h – tissue	251±20		241±3	286±19		259±15
48 h – tissue			256±17			250±12[a]
72 h – tissue, 24–48 h and 48–72 h – urine			255±10			264±21[a]
6 days – urine						277±20[a]

Tissue – lungs, liver, spleen and kidney.
Data for 4 rats.
[a] Animals exposed to 1-MN vapors at concentration of 198.1±9.1 mg/m^3.

the duration of exposure. High levels of 1-NA were observed in kidneys after the single and repeated exposure immediately after the end of exposure to 1-MN vapors at both concentrations applied.

After repeated exposure to 1-MN vapors at a concentration of 50 mg/m^3 and 200 mg/m^3, the determined concentrations of 1-NA were significantly higher in the spleen and kidneys of rats compared to the single exposure to 1-MN. The concentrations of 1-NA in the lungs after repeated exposure to 1-MN at the dose of 50 mg/m^3 and in the liver at the dose of 200 mg/m^3 were significantly higher compared to the concentrations of 1-NA in lungs and liver of animals after single exposure to the same 1-MN exposure.

After 24 h from the end of single and multiple exposure to 1-MN vapors at a concentration of 50 mg/m^3 and 200 mg/m^3, a high concentration of 1-NA was found in the lungs, and in the kidneys after the repeated exposure to 1-MN at the concentration of 200 mg/m^3. Low concentrations of 1-NA were detected in the liver after the single and multiple exposure to 1-MN at the concentration of 200 mg/m^3 and the multiple exposure to 1-MN at the concentration of 50 mg/m^3. Similarly, low concentrations of 1-NA were determined in the spleen after the repeated exposure to 1-MN at a concentration of 200 mg/m^3.

After 24 h from the end of exposure to 1-MN at the concentration of 50 mg/m^3 in the kidneys and spleen of rats, the concentrations of 1-NA were below the quantification limit of the analytical method used. No 1-NA was detected 72 h after the end of exposure in all analyzed tissues of animals exposed to 1-MN.

Elimination

Table 4 shows the urinary excretion of 1-NA in rats after the single and repeated exposure to 1-MN vapors. The concentration of 1-NA in the urine of treated rats de-

Table 2. Absolute and relative weight of lung, liver, spleen and kidney of rats after exposure to 1-methylnaphthalene (1-MN), in the study conducted at the Nofer Institute of Occupational Medicine, Łódź, Poland, in 2014

1-MN concentration / material collecting time	Organ weight (M±SD)															
	lung				liver				spleen				kidney			
	control		studied		control		studied		control		studied		control		studied	
	absolute [g]	relative [g/100 g b.w.]	absolute [g]	relative [g/100 g b.w.]	absolute [g]	relative [g/100 g b.w.]	absolute [g]	relative [g/100 g b.w.]	absolute [g]	relative [g/100 g b.w.]	absolute [g]	relative [g/100 g b.w.]	absolute [g]	relative [g/100 g b.w.]	absolute [g]	relative [g/100 g b.w.]
50 mg/m^3																
6 h	1.36 ±0.11	0.55±0.04			9.38±0.64	3.81±0.41			0.80±0.05	0.33±0.03			1.88±0.08	0.76±0.05		
0 h			1.34±0.21	0.44±0.05			11.74±0.87*	3.89±0.39			0.61±0.01	0.23±0.02*			2.13±0.21	0.71±0.06
24 h			1.54±0.19	0.48±0.06			13.43±0.99*	4.20±0.46			0.69±0.09	0.21±0.02*			2.25±0.26	0.70±0.09
5 days																
0 h			1.45±0.29	0.58±0.12			8.35±1.28	3.34±0.34			0.51±0.04*	0.20±0.01*			1.69±0.19	0.68±0.05
24 h			1.26±0.05	0.52±0.03			8.75±0.83	3.63±0.32			0.55±0.07*	0.23±0.03			1.84±0.07	0.76±0.02
200 mg/m^3																
6 h	1.52±0.29	0.64±0.16			9.38±0.94	3.92±0.45			0.67±0.04	0.28±0.03			1.82±0.16	0.76±0.10		
0 h			1.22±0.22	0.42±0.07			11.46±0.73*	3.93±0.25			0.74±0.06	0.25±0.02			2.14±0.15*	0.73±0.05
24 h			1.35±0.18	0.44±0.06			12.80±1.10*	4.17±0.36			0.70±0.21	0.23±0.07			2.16±0.18*	0.70±0.06
5 days																
0 h			1.33±0.14	0.54±0.03			8.76±0.46	3.44±0.22			0.54±0.11	0.21±0.03*			1.79±0.12	0.70±0.01
24 h			1.57±0.40	0.59±0.13			9.90±0.12	3.83±0.18			0.47±0.10*	0.18±0.04*			1.87±0.22	0.72±0.09

Data for 4 rats.

* $p < 0.05$ – significantly different from the control group.

Table 3. Tissue distribution of 1-naphtholic acid (1-NA) in rats (N = 4/group) after single (6 h) and repeated (5 days, 6 h/day) exposure to 1-MN vapor in the study conducted at the Nofer Institute of Occupational Medicine, Łódź, Poland, in 2014

Material collecting time/ 1-MN concentration/ exposure time	1-NA in tissues [μg/g w.t.] (M±SD)			
	lung	liver	spleen	kidney
After the end of exposure				
50 mg/m^3				
6 h	0.195±0.057	0.240±0.075	0.064±0.031	1.420±0.452
5 days	0.350±0.054**	0.277±0.054	0.277±0.025**	3.039±0.331**
200 mg/m^3				
6 h	0.570±0.349	0.813±0.134	0.143±0.053	3.880±1.394
5 days	0.528±0.212	1.173±0.152*	0.610±0.102**	8.206±1933*
After 24 h of exposure				
50 mg/m^3				
6 h	0.075±0.021	n.d.	n.d.	n.d.
5 days	0.135±0.075	0.045±0.010	n.d.	n.d.
200 mg/m^3				
6 h	0.110±0.041	0.033±0.017	n.d.	0.135±0.189
5 days	0.393±0.267	0.060±0.047	0.063±0.017	0.144±0.138

n.d. – no data.
* $p < 0.05$ vs. 6 h; ** $p < 0.001$ vs. 6 h.

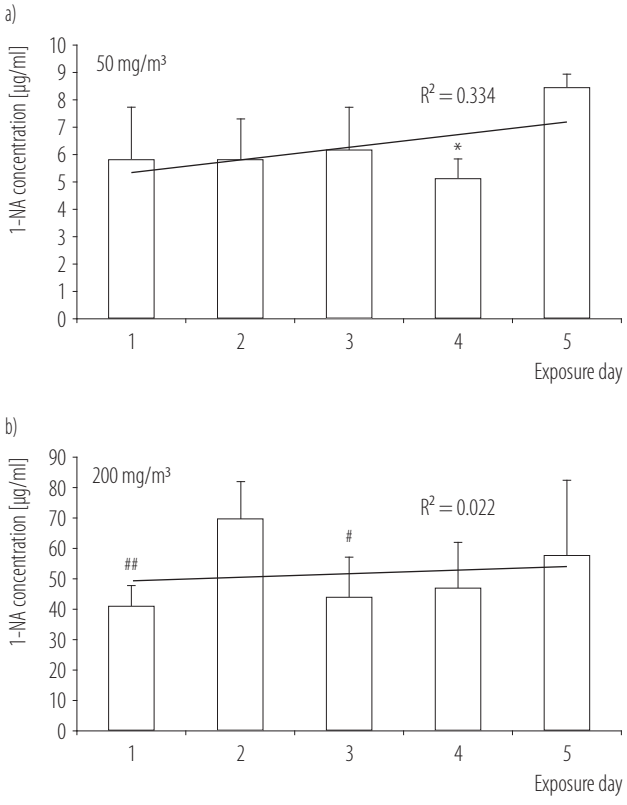
Table 4. Excretion of 1-naphtholic acid (1-NA) with rat urine (N = 4/group) after the single (6 h) and repeated (5 days, 6 h/day) inhalation exposure to 1-methylnaphthalene (1-MN) vapor in the study conducted at the Nofer Institute of Occupational Medicine, Łódź, Poland, in 2014

1-MN concentration/ exposure time	1-NA in urine [μg/ml] (M±SD)		
	0–24 h	24–48 h	48–72 h
50 mg/m^3			
6 h	5.77±1.94	0.18±0.12	0.10±0.04
5 days	8.44±0.49*	0.33±0.15	0.06±0.07
200 mg/m^3			
6 h	40.90±6.90	0.90±0.37	0.21±0.07
5 days	57.90±24.70	1.87±1.55	0.48±0.20*

Urine collection time: 0–24 h – first day, 24–48 h – second day, 48–72 h – third day.
* $p < 0.05$ vs. 6 h.

pended on the concentration and the duration of exposure to 1-MN vapors. In collected urine samples (3 days; 24 h/day), it was found that urinary 1-NA excretion was mainly in the first day (approx. 95%) after the end of exposure to 1-MN.

Figure 2 shows the urinary concentration of 1-NA in rats during multiple inhalation exposures to 1-MN vapors. During the 5 days of observation, a similar concentration of 1-NA in the rat urine was found, which was dependent on the exposure to 1-MN vapors. The coefficient of correlation (R^2) between the concentrations of 1-NA and the day of the measurement at the concentration of 50 mg/m^3 was 0.3348 and was many times higher than R^2 after the exposure to 200 mg/m^3 which was 0.0223.



* Significantly different from day 5 of exposure at $p < 0.05$.
\# Significantly different from day 2 of exposure at $p < 0.05$.
\## Significantly different from day 2 of exposure at $p < 0.001$.

Figure 2. Concentration of 1-naphthoic acid (1-NA) (M±SD) in rat (N = 4/group) urine collected during repeated inhalation exposure (5 days, 6 h/day) to 1-MN vapor at the target concentration of a) 50 mg/m³ and b) 200 mg/m³, in the study conducted at the Nofer Institute of Occupational Medicine, Łódź, Poland, in 2014

Biochemical analysis

Table 5 shows the results of biochemical parameters determined in serum and liver of control rats and rats repeatedly exposed to 1-MN vapors after the end of 5 days of exposure. Along with an increase in exposure to 1-MN vapors, a significant increase in the activity of cytochrome P_{450} (isoforms: CYP1A1 and CYP1A2) was found in the liver of rats exposed to 1-MN compared to the activity of cytochromes in the liver of control rats. Compared to the control group, a significantly higher activity of ALT was noted in serum of rats exposed to 1-MN at the dose of 200 mg/m³. There were no significant differences in the activity of AST in serum of rats from the control group compared with the group exposed to 1-MN vapors.

Micronucleus test

The *in vitro* assessment of micronucleus frequency in V79 cells exposed to 1-MN showed no difference compared with the vehicle control. Positive control induced an approx. Nine-fold increase in micronuclei frequency compared to the vehicle control (Table 6).

Table 7 shows the results of the effect of rat urine extracts in V79 cells on the frequency of micronuclei. A significant increase in binulceated cells with micronuclei after exposure to the urine extracts from animals after single exposure to 1-MN at the concentration of 50 mg/m³ and to urine extracts from animals after single and repeated exposure to 1-MN at the concentration of 200 mg/m³ was shown. Significantly higher number of cells with micronuclei were found in V79 cells treated with the urine extract of animals repeatedly exposed to 1-MN at a concentration of 200 mg/m³ compared to the urine extract of animals exposed to 1-MN at a concentration of 50 mg/m³.

DISCUSSION

In animals exposed to 1-MN vapors, no macroscopic changes in tissues were found. Similarly to guinea pigs and some bacteria, 1-NA was the metabolite of 1-MN detected in rats after inhalation exposure [11–14,16]. There was a rapid elimination of 1-NA from the examined tissues and rat urine after single or multiple exposure to 1-MN. Approximately 95% of the metabolite of 1-MN was excreted within the first day after the end of exposure. Such a rapid elimination of the metabolite is associated with the observed early and rapid elimination of 1-MN from blood, tissues and urine of rats after single and repeated inhalation exposure to 1-MN vapors at concentrations of 50 mg/m³ and 200 mg/m³ [30]. 1-Methylnaphthalene

Table 5. Biochemical parameters in rats exposed to 1-methylnaphthalene (1-MN) for 5 days in the study conducted at the Nofer Institute of Occupational Medicine, Łódź, Poland, in 2014

1-MN concentration	CYP1A1 [pmol ethoxyresorufin /min/mg protein] (M±SD)[a]	CYP1A2 [pmol methoxyresorufin /min/mg protein] (M±SD)[a]	Alanine aminotransferase (ALT) [U/l] (M±SD)[a]	Aspartate aminotransferase (AST) [U/l] (M±SD)[a]
0 mg/m^3 (control)	185.8±14.8	1524±139	150.5±15.0	65.4±15.5
50 mg/m^3	213.9±47.0	2347±130**	155.3±60.5	57.6±12.3
200 mg/m^3	246.4±20.3*	2615±221**	210.6±10.8**	71.6±15.2

ALT and AST activity in serum of rats.
CYP1A1 and CYP1A2 activity in liver of rats.
* $p < 0.05$ vs. control; ** $p < 0.001$ vs. control.
[a] For 4 rats.

Table 6. Effect of 1-methylnaphthalene (1-MN) exposure on the micronucleus frequency in binucleated V79 cells, the cytokinesis-block proliferation index and cytostasis following the 3-hr treatment in the presence of metabolic activation (S9 mix), in the study conducted at the Nofer Institute of Occupational Medicine, Łódź, Poland, in 2014

Sample	Binucleated V79 cells [n/2000 cells]		Cytokinesis-block proliferation index	Cytostasis [%]
	with micronuclei	micronuclei		
Vehicle control (DMSO) 0.25%	34	34	2.0	0
Positive control (CP) 7.5 µg/ml	203	314	2.0	0
1-MN concentration				
160 µg/ml	n.a.	n.a.	1.0	100
80 µg/ml	n.a.	n.a.	1.0	98
50 µg/ml	n.a.	n.a.	1.1	88
25 µg/ml	25	25	1.9	1
10 µg/ml	31	31	2.0	0
5 µg/ml	20	20	2.0	0

CP – cyclophosphamide; DMSO – dimethyl sulfoxide.
n.a. – not analyzed.

and its metabolite did not accumulate in the analyzed tissues of laboratory animals.

Świercz and Wąsowicz [30] reported a high 1-MN concentration in fat tissue of rat after single and repeated exposure to 1-MN vapor at target concentration of 50 mg/m^3 and 200 mg/m^3. Authors suggested that, 1-MN was rapidly eliminated from the blood and tissues of the exposed animals. McCain et al. [31] suggested that 1-MN was accumulated in skin and muscle in flatfish exposed experimentally to oiled sediments. There are no other data in the open literature on the distribution of 1-MN metabolites in animal tissues.

The analysis of ALT and AST activities indicate that 1-MN may induce changes in liver function. Similar activities of transaminases were noted after 4-week exposure to 2-methylnaphthalene (1-MN isomer) at a dose of

Table 7. Effects of urine extracts from 1-methylnaphthalene (1-MN) exposed rats on the micronuclei frequency in binucleated V79 cells, the cytokinesis-block proliferation index and cytostasis (the 24-hr treatment in the absence of metabolic activation). Mitomycin C (MMC) was used as negative control/extracts of urine taken from unexposed rats, in the study conducted at the Nofer Institute of Occupational Medicine, Łódź, Poland, in 2014

Variable	Binucleated V79 cells [n/2000 cells]		Cytokinesis-block proliferation index	Cytostasis [%]
	with micronuclei	micronuclei		
Vehicle control (DMSO) [0.25%]	31	31	2.0	0
Positive control (CP) 0.05 µg/ml	233	365	2.0	0
Control group (M±SD)	64±7.4	67±8.5		
rat 1	60	61	2.0	0
rat 2	70	73	2.0	0
rat 3	71	75	2.0	0
rat 4	56	58	2.0	0
1-MN concentration				
50 mg/m^3				
exposure time: 6 h (M±SD)	80±3.8*	80±3.8*		
rat 1	83	83	1.65	29.5
rat 2	82	82	1.75	23.5
rat 3	n.a	n.a.	1.55	41.5
rat 4	76	76	1.75	22.0
exposure time: 5 days (M±SD)	81±16.1	83±14.7		
rat 1	n.a.	n.a.	1.55	43.0
rat 2	76	78	1.90	7.5
rat 3	68	72	1.60	38.0
rat 4	99	100	1.90	5.5
200 mg/m^3				
exposure time: 6 h (M±SD)	94±15.6*	96±13.4*		
rat 1	n.a.	n.a	1.30	72.0
rat 2	83	86	1.85	15.0
rat 3	n.a.	n.a.	1.20	78.5
rat 4	105	105	1.85	16.0
exposure time: 5 days (M±SD)	106±3.5**,#	106±3.5**,#		
rat 1	n.a.	n.a.	1.50	46.5
rat 2	n.a.	n.a.	1.20	78.0
rat 3	108	108	1.90	9.0
rat 4	103	103	1.85	10.5

Abbreviation as in Table 6.
n.a. – not analyzed.
* $p < 0.05$; ** $p < 0.01$ – significantly different from the control group.
# $p < 0.01$ significantly different from inhalation of the 50 mg/m^3 concentration (6 h) of 1-MN (t-test).

50 mg/m^3 and after 13 weeks (6 h daily, 5 days/week) exposure to 1-MN at a dose of 30 ppm (approx. 174 mg/m^3) in rats of both sexes [32,33]. Based on the results of histopathological examinations, it was found that 1-MN mainly affected the upper respiratory tract. The authors suggested that the no observed adverse effect level (NOAEL) is 4 ppm (approx. 23 mg/m^3) [32].

No epidemiology studies or case reports on the potential effects of human exposure to 1-MN are available. Occupational (inhalation or dermal route) exposure to 1-MN is the most probable in humans [34–36]. It is suggested that 1-MN and 1-NA in urine may be potential biomarkers of occupational and non-occupational exposure to 1-MN.

It was found that the 5-day exposure of rats to 1-MN vapors increased the activity of liver CYP1A1 and CY1A2. The increased activity of these cytochromes in animals exposed to 1-MN at a concentration of 50 mg/m^3 resulted in an increase in the concentration of the determined metabolite (1-NA) and a significant decrease in the concentration of 1-MN in rat urine [30]. Animals exposed to 1-MN at the concentration of 200 mg/m^3 had slightly higher urine 1-NA levels during the next 5 days of repeated inhalation exposure. Despite the increased activity of cytochromes, in animals exposed to 1-MN at the concentration of 200 mg/m^3, no decrease of 1-MN concentration and no increase in 1-NA concentration in urine during the 5-day exposure cycle [30] was found. This result is probably related to the maximum metabolic efficiency of the rat reached for the 1-MN at the concentration of 200 mg/m^3.

In the *in vitro* genotoxicity testing, it was found that 1-MN did not induce micronuclei frequency in V79 cells at concentrations up to 25 μg/ml. The concentrations of 1-MN tested *in vitro* were much higher than these determined in urine collected from rats exposed to 1-MN vapors at the higher concentration (200 mg/m^3) that were lower than 1 μg/ml of urine [30]. At the same time, urine extracts of rats exposed to 1-MN both at the low and high dose induced an increase in the frequency of micronuclei in V79 cells, suggesting that observed increase in mutagenicity of urine extracts may results from activity of 1-MN metabolites rather than on 1-MN itself.

Bao et al. [37] reported that the metabolic activation and transformation of the cytochrome P450 (CYP 1B1) play an important role in the potential carcinogenicity of naphthalene, a parental compound of 1-MN. The activation of CYP 1B1 generates naphthalene metabolites (naphthalene 1,2-oxide, 1,2-naphthoqinoline) which are able to interact with DNA. The results serve as a basis for future studies on activation and the carcinogenesis of naphthalene. They also indicate a need for future research on the metabolic activation and DNA reactivity of 1-MN.

CONCLUSIONS

In conclusion, the metabolism of 1-MN after the inhalation exposure of rats to 1-MN was rapid and leading to generation of 1-NA. Repeated exposure of animals to 1-MN vapors increased the activity of cytochrome P_{450} (isoforms: CYP1A1 and CYP1A2) in the liver of exposed rats therefore it may lead to development of adverse changes in this organ. The metabolites of 1-MN present in the urine of rats after repeated inhalation exposure to 1-MN may induce an increase in the frequency of micronuclei in V79 cells.

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