IN VIVO AND *IN VITRO* GENOTOXIC EFFECTS OF ENVIRONMENTAL PARTICLE EXPOSURE

PhD thesis

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1. Introduction

1.1 General introduction

Particle and fibre toxicology have been investigated for a long time. The exposure of particles and fibres of environmental origin can be found in different places: e.g. manufacturing, use of industrial products, mining and accidental exposures by human activity, resulting in various diseases like asthma, chronic obstructive pulmonary diseases, cardiovascular diseases or even fibrosis and different tumours. The new challenge of the century is the broad spectrum of particulate matter involving fibres and especially the nanotech products.

Specific genotoxicity of fibres and particles was investigated. Experiments were based on three types of particles: the carcinogenic asbestos fibres; carbon nanotubes being very similar in shape and size to asbestos fibres but presenting different biological activity; and medicinal muds applied all in balneotherapy and in areas of wellness facilities.

Particles and fibres form a special group compared to traditional chemicals from genotoxicity testing and risk assessment aspects. While chemical substances follow the classic toxicokinetics (distribution, biotransformation, elimination); it is completely different in case of particles. (The specific particle kinetics involves deposition, clearance, durability, overload, persistence of poorly soluble particles, etc.)

Their most important common feature can be the carrier function. Particles and fibres having large surface properties can adsorb several possible mutagens [1].

1.2 In vivo studies on 1-nitropyrene and asbestos

1-Nitropyrene (1-NP) is a carcinogenic polycyclic aromatic hydrocarbon of predominantly traffic origin and is a by-product of incomplete combustion [2]. Another important atmospheric pollutant is asbestos, which may serve as carrier of inhaled 1-NP [3].

Asbestos is widely used for its favourable properties (flexibility, resistance). Highly persistent asbestos fibres and fibrils will be present in the human environment due to erosion of asbestos products, even after they have been completely banned. Long-term exposure to asbestos fibres can initiate asbestosis, bronchial carcinoma, and pleural or peritoneal mesothelioma [3].

Inhalation is considered the main route of exposure, however, the particle-associated molecules of traffic origin are often finally ingested. Vast majority of inhaled fibres are coughed up and ingested. That is, oral exposure may be the typical route in the whole population. [4, 5].

In animal studies asbestos fibres showed accumulation of multiple sites, mainly in the omentum. [6, 7]. Human autopsy data also support the extremely high accumulation property of the omentum, which is probably related to peritoneal tumours [8].

1.3 Studies on potential genotoxicity and mesothelioma-induction of carbon nanotubes

Carbon nanotubes represent new members of carbon allotropes similar to fullerenes and graphite, forming single- (SWCNT) or multi-walled carbon nanotubes (MWCNT) with a diameter of <1 nm but a length of several micrometres (length/diameter ratio: ≥ 3:1) (Fig. 1).

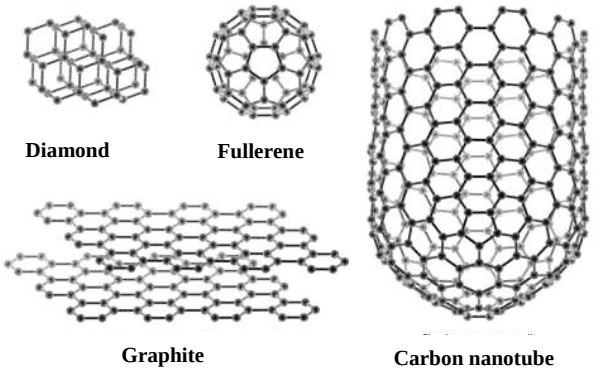

Figure 1: Carbon allotropes [9]

Their unique mechanical, electric and thermal properties predestine them for use in the electronics, aerospace and computer industries [9, 10]. Hundreds of metric tons are produced annually nowadays [11], however, only limited information has been published on their environmental and human health effects including specific toxicological features (*e.g.* genotoxicity and carcinogenicity) [12, 13]. The characteristics of nanotubes are very similar to those of asbestos fibrils, the well-known carcinogens [3].

Carbon nanotubes can cause apoptosis, cytotoxic effects, pulmonary toxicity [14-17], oxidative stress, dermal toxicity [18], granuloma induction [14, 17] and mesothelioma [19, 20] and can be involved in atherogenesis [15, 21, 22]. Monitoring the environmental and health effects of nanomaterials is an important step in order to control the balance of advanced technology.

1.4 Mutagenic activity of peloids in the Salmonella Ames test

Mud treatment is a very popular part of spa therapy. Medical muds (peloids) are fine granular materials having good water binding and heat retaining capacity, making them suitable for preparation of peloid wraps, mud baths and packs. They are widely used in treatment of rheumatic disorders [23, 24].

Although these treatments are frequently used in therapy or prevention, the side-effects of spa waters and peloids are mostly unknown. We also have limited evidence of the positive, healing effects of muds. High temperature and pressure are important in the formation of these natural resources. Under these conditions not only therapeutically effective compounds can be found, but also toxic ones. During the last decades several organic compounds were detected using gas chromatography, high pressure liquid chromatography (HPLC) and mass spectrometry. We have, however, limited information on their presence in medical muds, interactions or biological activity involving carcinogenicity and other specific toxicity [25].

2. Aims of the study

In vivo studies on 1-nitropyrene and asbestos

- To understand of the excretion kinetics and mode of action of the potentially mutagenic metabolites, data collection for risk assessment
- To develop an *in vivo* test for research on possible mesothelioma-inducing effect on long-term, direct contact of mesothelial cells and asbestos fibres
- To model environmental exposure with fibres pre-treated with 1-nitropyrene, and to study co-mutagenicity and other possible chemical and physical interactions

Studies on potential genotoxicity and mesothelioma-induction of carbon nanotubes

- To collect primary data on the potentially toxic and genotoxic effects of carbon nanotubes, or exclude them
- To confirm or reject the hypothesis of carcinogenicity of the two types of CNTs, to model environmental exposure of 1-NP pre-treated CNTs in our specific mesothelioma model involving long-term and direct contact between the test materials and the target (mesothelial) cells under *in vivo* conditions

Mutagenic activity of peloids in the Salmonella Ames test

- To collect data on the potential toxic and genotoxic effects of medical muds for risk assessment
- To develop new experimental methods for studying the possible complex genotoxic effects of peloid samples without any pre-treatment

Table I: Summary table of the methods described in the dissertation

Types of experiments	Methods		Samples	Subject	Duration	Treatment
in vitro	Standard Salmonella Ames test		MWCNT, SWCNT	*S. typhimurium* TA98, TA100	48 h	100, 50, 25 µl/plate cc. 0,01 mg/µl MWCNT cc. 0,002 mg/µl SWCNT
			peloid solvent extraction		48 h	100, 66, 33 µl/plate
	Preincubation Ames test		MWCNT		1 h preincub. + 48 h	5, 10 mg/plate
			peloids		30-60-120 perc preincub. + 48 h	20, 40, 80 mg/plate
	Cytogenetic assays	MN	MWCNT, SWCNT	human lymphocytes	72 h	10 mg/tube cc. 1 mg/ml
		SCE			72 h	
combined *in vitro/in vivo*	Urinary mutagenicity in Ames test		1-NP	rat n=6 animal/group urine sample *S. typhimurium* TA98, TA100	24 h, 48 h	po., ip. 30 µmol/bwkg
			MWCNT, SWCNT		24 h	po. 50 mg/bwkg
in vivo	Chronic, contact exposure model		crocidolite, chrysotile, +/- 1-NP	rat n=6 animal/group	12 month	25 mg/bwkg
			MWCNT, SWCNT, +/- 1-NP			

3.1 Salmonella Ames mutagenicity tests [26]

3.1.1 Plate incorporation test

Genetically modified, histidine auxotrophic mutant *Salmonella typhimurium* TA98 and 100 strains were used. If the sample is mutagenic, reverse mutation occurs and revertants grow on the minimal glucose medium containing minimal amount of histidine.

3.1.2 Preincubation Ames test

We used a modification of the standard procedure, the more sensitive preincubation test. Test materials and *S. typhimurium* bacteria incubate together in higher concentration than in the conventional method.

3.1.3 Urinary mutagenicity in Ames test [27]

To study the potential mutagenic effects of animal urine samples conventional plate incorporation Salmonella Ames test was used.

3.2 Cytogenetic assays on human cells

3.2.1 Micronucleus test [28]

It is suitable for detecting mutagenicity of chemicals which cause the formation of interphase chromosome fragments appearing in the cytoplasm of cells. Frequency of micronuclei increases on clastogenic or aneugenic effect.

3.2.2 Sister Chromatid Exchange analysis [29]

The *in vitro* cultures of peripheral lymphocytes incorporate the added nucleoside analogue (bromodeoxyuridine) into the DNA. Due to the semiconservative replication sister chromatids are differentially stained in the second metaphase. Genotoxic effects increase the frequency of exchanges between homologous compartments.

3.3 Chronic, contact exposure model for studying fibres related mutagenicity and co-mutagenicity

A novel animal model was developed to study the direct exposure of mesothelial cells to fibres [30]. The model involves long-term and direct contact between the test materials and the target (mesothelial) cells under *in vivo* conditions to detect potential mesothelioma-inducing effect. Pharmaceutical hard gelatine capsules were filled with pure and pre-treated fibres, respectively, and implanted into the peritoneal envelope lined with mesothelial cells (*Kertai's fold*, lig. hepatogastricum). Histological examinations were performed after 12 months.

3.4 Peloid solvent extraction study (31)

Conventional Salmonella plate incorporation test was employed to study possible mutagenicity of medical muds. Four types of samples were prepared from peloids using distilled water, 0.1 N hydrochloric acid, methanol and toluene, respectively.

4. Results

4.1 In vivo studies on 1-nitropyrene and asbestos

4.1.1 Urinary mutagenicity in Ames test

Statistically significant ($p<0.05$) mutagenicity in the TA98 strain was only detected in urine samples collected in the first 24 h, both in the *i.p.* and *p.o.* exposed groups. In the first case, no statistical difference could be observed between the enzymatically-treated and non-treated samples. However, urine of the group administered *p.o.* showed mutagenicity only in the presence of deconjugating enzymes. Only the *i.p.* treatment induced highly significant mutagenicity in the first 24 h urine samples studied using the TA100 tester strain. Mutagenicity was detected, both in the presence and absence of the enzymes, but deconjugation produced a further increase in mutagenicity in a statistically significant manner. Neither the *i.p.* 48 h-samples nor the *p.o.* 24/48 h-samples showed increased mutagenicity, as tested with TA100.

4.1.2 ***Chronic, contact exposure model***

Upon autopsy of animals exposed with pure and 1-NP pre-treated asbestos samples, gray/blue bulks of asbestos fibers were detected in the peritoneal cavity: in the Kertai's fold and on the surface of near organs (omentum, peritoneum). The histological evaluation of tissue samples showed only unchanged structure of the liver tissue and granulomatous reaction of foreign body type with multinucleated giant cells. Signs of mesothelioma were not detected either macroscopically or microscopically in either the exposed or control groups.

4.2 Studies on potential genotoxicity and mesothelioma-induction of carbon nanotubes

4.2.1 Salmonella Ames mutagenicity tests (*plate incorporation test, preincubation Ames test, urinary mutagenicity in Ames test)*

No statistically significant mutagenicity ($p<0.05$) was observed either with SWCNT or MWCNT.

4.2.2 Cytogenetic assays (*micronucleus test and SCE-analysis)*

In the micronucleus test MWCNT-exposure did not affect frequency of binucleated cells, reflecting the lack of cytotoxic effects. No statistically significant differences among paired control and exposed samples were detected ($p<0.05$). The SWCNT treatment at the same dose, however, indicated practical mitotic inhibition which was manifested in a considerable reduction of the binucleated cell number. Due to the insufficient cell quantity, we could not practically perform the MN analyses. Prior to the SCE analysis, cell kinetics of the control and exposed cultures were compared, based on the BrdU-incorporation pattern. Upon the MWCNT exposure, no significant differences were detected in the cell kinetics. This was also true for the SCE frequency analysis which showed no significant differences at $p<0.05$. In the case of SWCNT treatment, however, a complete shift in cell kinetics (towards M1) was observed, so the analysis of SCEs could not be performed.

4.2.3 Chronic, contact exposure model

Signs of mesothelioma were not observed either macroscopically or microscopically in either the exposed or control groups. The histological examination of tissue samples showed the structure of the liver tissue to be unchanged and the presence of granulomatous reaction of foreign body type, with multinucleated giant cells, in animals of groups treated with both types of nanotubes.

4.3 Mutagenic activity of peloids in the Salmonella Ames test

4.3.1 Peloids without pre-treatment

There was no statistically significant Ames mutagenicity ($p<0.05$) either in case of Hévíz or Kolop peloid samples.

4.3.2 Solvent extraction study

4.3.2.1 Experiment #1

After metabolic activation (S9+) inorganic solvent extracts of medical mud – HCl in TA98 strain and distilled water and HCl in TA100 strain – from Hévíz indicated statistically significant ($p<0.05$) mutagenicity. Organic solvent extracts from Hévíz – toluene in TA98 strain after metabolic activation and methanol in TA100 strain both in presence and absence of S9 mix (S9+/−) – showed statistically significant difference compared to the particular solvent control.
In case of distilled water and HCl extracts of medical mud from Kolop using TA98 strain there was no alteration. However, after metabolic activation of distilled water and HCl extracts statistically significant mutagenicity in TA100 strain was detected. Methanol and toluene extracts proved to be mutagenic after enzymatic activation in TA100 strain. Using TA98 strain we did not find alteration in the organic solvent extracts.

4.3.2.2 Experiment #2

In extracts of Hévíz peloid, HCl in TA98 strain indicated significant ($p<0.05$) mutagenicity without metabolic activation (S9-). Using TA100 strain there was no statistically significant alteration.
In case of Kolop distilled water (S9-), methanol (S9+) extracts in TA98 strain, and in TA100 strain HCl (S9+) extracts showed statistically significant difference compared to the particular solvent control.

5. Discussion and conclusion

In the dissertation specific genotoxicity of three completely different types of particles were investigated in an increasing size range. The nanometre scale carbon nanotubes having very similar shape and size distribution like asbestos fibres and fibrils, but different biological activity; the electron microscopic scale carcinogenic asbestos fibres, and medicinal muds with grain size distribution of less than 200 μm. Their carrier function seems to be their most significant common property and it is essential for the biological activity.
Where environmental samples are tested the observed consequences are supposed to be related to chemicals adsorbed to their surface. Previous HPLC results [32, 33] confirm the presence of surface-bound materials on asbestos fibres followed by consecutive outcomes of genotoxicity tests. These experiences were used in researching carbon nanotubes. The fractions of chemicals attached to the surface of peloids also support this hypothesis.
We started our investigations with mutagenicity tests of carcinogenic 1-NP, and an *in vivo* model of a potential asbestos exposure was developed. In our recent experiments, upon the single treatment of the same 1-NP dose used in the cytogenetic and gene expression studies [34-36], we demonstrated detectable mutagenicity in the collected urine samples. Using the TA100 strain, which is sensitive to base-pair substitution, mutagenicity was detected only in the first 24 h after ***i.p.*** treatment. This type of mutagenicity may involve conjugated substances since it could be enhanced

significantly with deconjugation. The *p.o.* administration of the compound can exclusively produce frame-shift mutagens observable only in the presence of deconjugating enzymes in the first 24 h. In the second 24 h, the urine samples did not show significant mutagenicity, that is, mutagenic metabolites were excreted within 24 h. These results indicate that biotransformation patterns may be completely different depending on the treatment and absorption. *Per os* administration is important from the point of view of risk assessment and is a possible route of environmental exposure to 1-NP, together with inhalation. The situation is quite similar to that of carcinogenic asbestos fibres [4].

The results of our *in vivo* mutagenicity studies on 1-NP were used to our newly developed *in vivo* chronic, contact exposure model, where pure and 1-NP pre-treated asbestos fibres modelling environmental exposure were investigated.

Even the long-term direct exposure of mesothelial cells to asbestos fibres was not able to induce peritoneal mesothelioma. This was completely unexpected, especially in the case of pre-treated fibres showing cogenotoxicity in separate studies [33, 37, 38]. This observation suggests the possibility that the mechanism of mesothelioma formation is independent of the physical contact among fibres and mesothelial cells.

Based on the information collected by genotoxicity research on asbestos fibres mesothelioma induction of carbon nanotubes was investigated considering their more or less similarity to asbestos fibrils.

As in the *in vitro* Ames test, clear negative results were also observed in the urinary mutagenicity studies upon exposure to both types of CNTs. Although, our preliminary *in vitro* studies did not detect any cytogenetic alteration (clastogenic, aneugenic or SCE-inducing effect) caused by MWCNTs, a specific cytotoxic effect, mitotic inhibition, was clearly observed at the same dose of SWCNTs. Oxidative or radical-inducing agents are frequently genotoxic but the tests applied might not be highly sensitive to this type of genotoxicity. Therefore, use of higher resolution cytogenetic and other assays (involving *in vivo* comet assay [38] on target tissues or testing further strains of *Salmonella typhimurium*) can yield mechanistic information. The wide size-scale of the two main CNT types, their large surface-to-mass ratio and great adsorptive capacity may also be crucial in any assessment.

In our *in vivo* study, related to carcinogenesis, no mesothelioma-induction was detected either with chemically pure or with 1-NP pre-treated carbon nanotubes.

Even the long-term direct exposure of mesothelial cells to carbon nanotubes was not able to induce peritoneal mesothelioma. The present results, therefore, do not indicate considerable risk, especially compared to the case of asbestos fibres. However, the potential cancer-causing effects of carbon nanotubes cannot be excluded. In previous studies physiologically relevant route of exposure assessment and studies of carcinogenic effects related to humans are not yet available. To have accurate results on exposure and risk assessment more data are required.

In case of the even larger scale environmental samples, peloids our main idea was also the potential presence and cogenotoxic effects of chemicals on the surface of peloids. The objective of our new experimental method was to study peloids without any pre-treatment (*in toto*), whether they can induce any mutation in bacterium strains. In spite of the usage of a more sensitive modification of Salmonella Ames test, the preincubation test, no statistically significant results were detected in both peloid samples. Even though the previous method has greater sensitivity, we could not reach sufficient sample concentrations. To eliminate this problem the organic and inorganic extracts of peloids were fractionated. For the two consecutive experimental sets we prepared organic and inorganic peloid extracts by standard soil extraction procedure. The examined organic and inorganic

extracts both in the presence and absence of metabolic activation (S9+/−) represented statistically significant mutagenicity in several fractions. In both solvent extraction studies we found more statistically significant alterations when applying metabolic activation (S9+) (14 vs. 4), which means that the number of indirectly acting mutagens in both peloid samples may be represented in significantly higher amount. We experienced more statistically significant mutagenicity in TA100 strains with metabolic activation (S9+) than in other cases, in both peloids. So we can assume that the peloids may contain mostly promutagens inducing base-pair substitution type of mutagenesis. In the first solvent extraction study we found more mutagenic fractions compared to the consecutive one. In the latter set the mutagenic pattern was almost completely different there was only minimal overlapping. Microorganisms having biological–metabolic activity and living in the maturating muds [39] might explain these results. According to Veniale et al. (2007) [39], the living microorganisms in peloids inducing maturation process may influence the biological– metabolic activity of organic materials of peloids. Therefore, our further examinations will be focused on the detection of microbiological activities and their role in mutagenesis, together with radioactivity measurements — because possible radioactive elements in medical muds can produce ionising radiation. Due to the mentioned circumstances mutagenic fractions also need more sophisticated chemical analyses with high performance analytical tools for isolation and identification of the suspicious toxic compounds.

None of the investigated fibres and particles showed effects related to their physical properties, the results were based only on presence of chemicals.

The human relevance of the experiments is summarized below:

Modelling the human exposure of the wide-spread human carcinogen asbestos fibres possessing excellent physical properties but also found in our environment, we contributed to a better understanding of their mechanism of action. Based on our results we concluded that the mechanism of mesothelioma-formation may be independent from physical contact of mesothelial cells and fibres.

The potential adverse effects of carbon nanotubes are still controversial. The various parameters of several comprehensive studies make the appropriate interpretation of results difficult. There are lack of epidemiological studies investigating carcinogenic effects. Consequently environmental and public health risks of carbon nanotubes cannot be adequately assessed. In our *in vivo* study the results refute the hypothesis that carbon nanotubes would induce mesothelioma, further we need to focus on other concerns of human risk assessment.

In the solvent extraction studies statistically significant mutagenicity was found in several fractions. Therefore, we have to consider the possibility that the medical muds may contain potentially harmful substances. On the other hand, since Ames test is only a bacterial screening test for potential mutagens, it does not provide the possibility of a direct extrapolation to humans. In addition, it is assumed that organic and inorganic compounds were provided in higher concentrations with the mud extracts than they are present in the human tissues during mud therapy. Metabolic activation represents potential or indirect hazard, because skin does not play as important role in metabolism as liver enzymes. However, adsorbed promutagens can easily reach metabolic organs, and we are not aware how skin can metabolise some specific organics of muds [40, 41]. However, cosmetic and paramedical products prepared from muds are very popular nowadays, which leads to other questions like the household application where original peloid (with possibly high density of microorganisms) has to be mixed with water before treatment, thus providing opportunity for maturation [39]. These circumstances could play an important role in toxicity.

Quick pre-tests as well as contemporary qualification are really important before use in order to determine the actual toxicity state of the particular mud specimen. That would be essential for developing household products of peloids to avoid toxic side effects.

6. Summary of novel findings

My experiments on specific genotoxicity of fibres and particles led to the following novel findings:

- Urinary mutagenicity of 1-NP treated animals was confirmed
- After *p.o.* or *i.p.* treatment significant mutagenicity of urine samples occurs only in the first 24 hours, consequently the mutagenic metabolites are excreted in 24 hours
- An animal model was developed for the risk assessment of chemicals absorbed to solid phase and affecting via oral route of exposure
- We have developed an *in vivo* chronic, contact exposure model for studying solid-phase substances, and confirmed that direct exposure of untreated- and 1-NP pre-treated asbestos fibres did not induce peritoneal mesothelioma

- Studying two subtypes of carbon nanotubes no mutagenic and other genotoxic effects, only cytotoxic and mitosis-inhibiting effects were found (regarding SWCNT)
- In chronic, direct exposure of untreated and 1-NP pre-treated carbon nanotubes to mesothelial cells mesothelioma formation was not observed, but foreign body-type granulomatous reaction, epithelioid and multinucleated giant cells were detected

- Hévíz and Kolop peloid samples induce no bacterial point mutation in Ames test *in toto*
- Only certain chemical fractions are considered to be mutagenic, mutagenicity varies over time
- Indirect- and base-pair substitution mutagenicity pattern is typical in case of peloid fractions

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List of publications related to this thesis

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