INVESTIGATIONS OF CERTAIN ORGAN SPECIFIC TOXIC EFFECTS OF TITANIUM DIOXIDE NANOPARTICLES BY *IN VIVO* AND *IN VITRO* METHODS

Summary of PhD Thesis

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INTRODUCTION AND AIMS

The world of nanoparticles (NPs) has gained a great deal of attention in the last ca. 30 years in various fields of research, including life and health sciences. By the generally accepted definition, NPs are particles with not more than 100 nm typical diameter at least in one dimension. Within that, not only approximately cubical or spherical grains but elongated structures such as nano-rods, -wires, -tubes etc. exist, and nano-sized surface patterns on larger objects are also known. This size, together with high surface-to-volume ratio and surface reactivity, lends some peculiar characteristics to the NPs, so properties and behavior of the same chemical substance in nano vs. more conventional states can be different; resulting in biological, and hence, toxicological, interactions not seen with traditional materials, and possibly in novel health risks. The relationship between elevated level of ambient airborne NPs and human morbidity and mortality has been generally accepted by now. Aerosol concentration (which necessarily includes NPs) is an important measure of ambient air quality. The role of NPs in causing various non-communicable diseases (asthma, COPD, ischemic heart disease) has been repeatedly analyzed, and particulate air pollution has been recognized as an environmental carcinogenic agent.

Natural phenomena generating NPs are, among others, forest and bush fires, volcanoes, sea spray, as well as erosion of rocks and soil. As to man-made NPs, by-products of various technological procedures, acting as pollutants, and purposefully produced nanomaterials are to be distinguished. Nanotechnology has been gaining much importance in the last two decades which brought about certain novel occupational hazards, namely exposure to nanomaterials like carbon nanotubes and nanofibres, or metal oxide NPs. Engineered nanomaterials are also becoming part of everyday life, in form of NP-containing consumers' products. The broad spectrum of application with concomitant human exposure raises the question of health risk. Here, a major problem is the lack of toxicity data for most manufactured NPs – and this is why nanotoxicology, an interdisciplinary field related to both environmental hygiene and nanotechnology, is today an important field of research.

Exposure by NPs (first of all in case of pollutants, but partly also for nanotechnological materials) takes place most likely by inhalation of air containing a nano-aerosol. NPs, deposited either in the nasopharynx or in the alveoli, are not held back by barriers like the alveolar and capillary wall, and reach other target organs by being phagocyted and transferred along the

lymphatic system, or by different mechanisms including transcytosis, and finally are distributed throughout the body via the circulation. In case of consumers' products, gastrointestinal and dermal uptake may also be of interest. In the aqueous microenvironment of living tissues, surface reactivity of NPs leads to generation of reactive oxygen species (ROS). The resulting oxidative stress can induce damage to biomolecules, primarily lipids and proteins, and finally induce inflammation and/or cytotoxicity.

Titanium dioxide (TiO_2) is a white, odorless, water insoluble solid of high chemical stability and high melting temperature. It major crystal forms are anatase and rutile. TiO_2 as white pigment (grains of mostly 0.2-0.4 μm size but nano-sized fraction present) is used in the food and pharmaceutical industry. TiO_2 NPs of various shapes (grains, rods, wires, tubes) have a broad range of application, including skin-protective products against solar UV radiation, as well as self-cleaning windows and building facades, or self-disinfecting fabrics and surfaces.
Human exposure to TiO_2 NPs may occur during manufacturing, processing, and use; in form of aerosols, suspensions or emulsions. The most health relevant routes of exposure in workplaces are inhalation and dermal exposure, while non-occupational exposure is most likely dermal or oral. Ill effects – increased oxidative stress, inflammation, lung damage – due to airborne TiO_2 in humans suffering occupational exposure have been in fact documented and successfully modeled in animals. In rat lungs, even a single intratracheal application of TiO_2 NPs evoked transient inflammation with activation of cytokines and antioxidant enzymes. The reaction was stronger with smaller NPs, and the majority of the applied nano-TiO_2 remained in the lungs with 10 to 100 times lower levels in other organs.

The possible effects of NPs on the nervous system are of especial interest for two reasons, one being the role of an intact nervous system in the healthy life of animals, and even more of humans; and the other, the sensitivity of the nervous system to the biological effects of NPs. However, human data to nervous effects of nano-TiO_2 are scarce. Animal experiments, done predominantly in mice and less in rats, showed various central nervous alterations including cell damage, altered synaptic transmission, plasticity and transmitter turnover, memory impairment, and increased anxiety. The NPs were found to cross the blood-brain barrier and also to migrate to the brain along the olfactory and other nerves. Other organs, including the kidneys, also showed signs of damage – activation of inflammatory mediators, histological damage, decreased function – in rats and mice on TiO_2 NP administration. These effects could

be deduced to cellular uptake of nano-TiO_2, followed by ROS generation, engulfment by lysosomes, and release of enzymes from the latter initiating inflammation and cell death.
Exposure of humans to TiO_2 NPs can take place both in occupational settings, in the residential environment, or during free-time activities, and certain properties of the TiO_2 NPs suggest harmful effects on various organs and organ systems of humans or animals but the available reports on such effects are not yet conclusive.

Based on the experience gained at the Department of Public Health in examining the toxicity of metal oxide NPs for over 10 years, and on the properties and possible effects of TiO_2 NPs outlined above, it was decided to investigate in the present PhD work the alterations evoked by TiO_2 NPs with a complex approach. By treating rats subacutely via the airways, and applying pathomorphological, chemical, biochemical as well as behavioral and electrophysiological methods, answer to the following questions was sought:

- Is it possible to create effective internal TiO_2 NP load in the treated rats by intratracheal instillation?
- Do the TiO_2 NPs cross biological barriers, reach the circulation and/or distant organs (including essential targets such as brain, liver, kidneys), and show deposition in the latter?
- What alterations – morphological and/or biochemical-molecular – are elicited in the gate organ, the lungs?
- Is the set of behavioral and electrophysiological methods, applied previously to study the effects of other metal oxide NPs, suitable to examine the neurotoxic effects of TiO_2 NPs?
- What are the main functional alterations detectable at the level of cognitive behavioral and electrophysiological phenomena?
- How are the kidneys, as possible organs of excretion, affected by the internal TiO_2 NP load?
- What is the relationship between the observed morphological (cellular or subcellular), as well as biochemical and neuro-functional, alterations and Ti or TiO_2 NP load detected in the corresponding organs?
- What implications have the results, presented in this thesis, to human, primarily occupational, health risk?

MATERIALS and METHODS

Rod-shaped TiO_2 NPs of 15x65 nm size were suspended in phosphate-buffered saline containing 1% polyacrylic acid. For *in vivo* experimentation, the NPs were given to young adult male SPF Wistar rats (Crl:WIBr; 6 weeks old, 170±20 g body weight at start) by intratracheal instillation for altogether 28 days in a 5 treatment days per week scheme, in 5 (low dose, *LD*), 10 (medium dose, *MD*), and 18 (high dose, *HD*) mg/kg b.w. dose. There was an untreated control (*C*) and a vehicle control (*VC*) group also (see table below). The behavioral examinations applied were, on the one hand, climb test, and on the other hand, cognitive behavioral tests: elevated plus maze, open field, as well as acoustic startle response and pre-pulse inhibition. In electrophysiology, cortical spontaneous and evoked activity, and peripheral nerve action potential were recorded under urethane anesthesia and analyzed.

Scheme of the *in vivo* experiment

Duration and treatment scheme:	5 days per week, altogether 28 treatment days, resulting in 6 weeks	
Rat groups and treatments:	Untreated control; *C*	Saline, 1 mL/kg b.w.
	Vehicle control; *VC*	PBS + 1% PAA, 1mL/kg b.w.
	Treated, low dose; *LD*	TiO_2 nanorods, 5 mg/kg b.w.
	Treated, medium dose; *MD*	TiO_2 nanorods,10 mg/kg b.w.
	Treated, high dose; *HD*	TiO_2 nanorods,18 mg/kg b.w.
Investigations:	General toxicology:	Body weight gain Organ weights
	Cognitive behavior:	Climb test Elevated plus-maze (EPM) Open field (OF) Acoustic startle response and pre-pulse inhibition
	Electrophysiology:	Electrocorticogram Cortical evoked potentials Nerve compound action potential
	Chemistry, biochemistry:	Tissue Ti levels Oxidative stress: thiobarbiturate reaction, catalase activity Detection of cytokines: Array kit
	Pathomorphology:	Visualizing NPs and NP-laden macrophages: Light microscopy and TEM Verifying Ti in tissue slices: EDS

Abbreviations: PBS, Phosphate-buffered saline; PAA, polyacrylic acid; TEM, Transmission electron microscopy; EDS, Energy dispersive spectroscopy.

Pathomorphological analysis was done by light and electron microscopy on lung and kidney tissue sections. Ti levels were determined in blood, brain, lung and kidney samples; oxidative stress markers in brain and lung; and cytokines in lung samples. The doses, and the majority of methods, were based on a previous pilot study (described in the applicant's papers No. III and IV). In the *in vitro* work, A549 cells (of human alveolar origin) were exposed by the TiO_2 nanosuspension, and cell viability and ROS generation was measured.

Depending on the normality of data distribution, checked by Shapiro-Wilk test, body/organ weights, as well as behavioral and histopathological data, were analyzed by one-way ANOVA and post hoc Tukey test, while for electrophysiological and chemical-biochemical data nonparametric Kruskal-Wallis ANOVA and post hoc Mann-Whitney U-test was used. Changes of the open field activity, recorded three times during the experimental period, were tested by repeated measures ANOVA and post hoc Tukey test. The limit of significance was $p<0.05$ for all measurements.

RESULTS and DISCUSSION

The subacute nano-TiO_2 exposure had no effect on body weight but the body weight-related relative weight of the lungs and kidneys increased significantly in the *HD* group vs. *C* and *VC*. Measured Ti content of the *HD* treated rats' lungs increased massively, and presence of TiO_2 NPs was verified by light microscopy (macrophages packed with dark particles) and TEM (clusters of TiO_2 nanorods in the phagolysosomes of the macrophages). These organelles were likely damaged, and released enzymes, such as cathepsin B, leading to activation of caspases and further elements of the inflammatory cascade. The spectrum of interleukin activation, detected in the lung tissue of *HD* rats, indicated that the inflammation was acute but started to turn to chronic and systemic during the 6 weeks of treatment. In the *in vitro* test, the lung-derived A 549 cells showed dose- and time dependent viability decrease and ROS increase. NPs were seen in and on the exposed cells, and presence of Ti was directly verified by energy-dispersive X-ray spectroscopy.

The treated rats' behavior indicated increased anxiety and fear. They spent significantly more time in the corner zones of the open field box, and tended to avoid open arms of the elevated plus maze. This open field behavior developed in a time-and dose-dependent manner. Acoustic startle response of the treated rats showed delayed reaction. Besides, the treated rats appeared to have impaired muscle force or motor coordination in the climb test. Exploration of a novel place can be regarded as a product of the combined influences of curiosity and fear, and the

shift of the exploration–anxiety balance to the latter has been linked to dopaminergic hypofunction. Dopaminergic neurons are especially sensitive to oxidative stress due to the auto-oxidizing tendency of dopamine and the presence of monoamine oxidase producing hydrogen peroxide. This, together with the ROS-generating effect of TiO_2 NPs, may provide the mechanistic basis of the behavioral effects in the OF and EPM tests.

The general trend of the electrophysiological results showed delayed and increasingly fatigable evoked activity, both cortical and peripheral. These changes were dose-dependent and mostly significant. The tested corticostriatal connection was also weaker.

TiO_2 NPs could cause the observed functional alterations by several mechanisms. Oxidative stress may have played a major role. Nano-TiO_2 used in our experiments had anatase crystal structure which is the chemically more active one. The whole nervous system is sensitive to oxidative stress: neurons show both intense mitochondrial energy production because of the high energy demand, and abundance of (unsaturated) structural lipids, but their antioxidant defense capacity is low (certain neurons are especially sensitive as mentioned above). ROS have been regarded as “final common pathway” in the action of several neurotoxic agents. Increased lipid peroxidation could be measured in both brain and lung samples of the treated rats. Oxidative damage to membrane lipids is likely to result in changes of membrane fluidity and this way in alterations of membrane-bound events of pulse propagation and synaptic transmission, reflected in lengthened latencies of cortical evoked potentials and motor behavioral responses (ASR) on external sensory stimuli. Oxidative damage to mitochondria and energy shortage could affect ion pumps and transmitter turnover. Excess glutamate and abnormal ion gradients lead to disturbed synaptic transmission and spike propagation. Beside dopaminergic hypofunction, excess glutamate also might have contributed to elevated anxiety.

The chain of causal relationships from Ti levels in organs through oxidative stress up to functional alteration was supported by the correlations observed between electrophysiological and behavioral signs of functional damage, and the level of Ti and TBARS in the brain. For the detected presence of Ti in brain samples, the NPs must have crossed the BBB; but NPs may have migrated to the brain also along afferent nerves.

The Ti content in the kidneys was significantly elevated in the treated vs. control rats but the increase was not fully proportional to the dose. By light microscopy, necrotic glomerular and

tubular epithelial cells were seen in the kidney sections of *MD* and *HD* treated rats, together with dark, dense objects – possibly aggregates of NPs phagocyted by the tubular epithelial cells. TEM images also showed NPs and their intracellular aggregates.

The real determinant of a toxic effect is internal dose at the site or sites of action. In the present work, the highest levels of chemically detected Ti and visualized TiO_2 NPs were found in the lungs, but elevated Ti levels were found also in samples of blood as well as of organs including brain and kidneys. Translocation of NPs by means of phagosomes contained in alveolar macrophages was verified, and presence of NPs in the kidneys was also shown. This, together with detected Ti levels, outlined the complete fate of TiO_2 NPs and their metal content from uptake through sites of action up to excretion.

One further consequence of oxidative stress caused by TiO_2 NPs is inflammation. In our work, inflammation markers were detected in the exposed rats' lungs. From the lysosomes of macrophages, unable to break down the phagocyted NPs, enzymes are released that initiate the cascade of cytokine activation. Some of the detected mediators suggested that inflammation, developing first in the directly affected lungs, was becoming systemic; including neuroinflammation and kidney damage.

Conclusion: Nanoparticulate forms of TiO_2, including non-spherical shapes, are used in numerous applications, and can cause human exposure from several, purposeful or accidental, sources. The physicochemical properties of TiO_2 NPs suggest health damaging effects via several possible mechanisms. Experimental results of the PhD work, described and discussed in the thesis, showed that the rod-shaped TiO_2 NPs with anatase crystal structure, when applied to the airways, remained partly in the lungs but partly caused systemic exposure and reached distant organs like the CNS and the kidneys. Damages caused by the NPs were observed in the lungs, kidneys and the nervous system, with oxidative stress as most likely common element. Up to now, such effects were documented in the literature mostly for spherical TiO_2 NPs. The damages observed in our experimental work, especially those in the nervous system, point to the human health relevance of such studies.

Based on the results presented in this thesis, the points of aims, listed above, can be answered as follows:

- By intratracheal instillation of suspended TiO_2 nanorods, effective internal load could be achieved.
- TiO_2 nanorods and/or their metal content were detected in samples of the lungs, CNS, and kidneys – indicating that the NPs crossed biological barriers, and outlining their complete fate from uptake through sites of action up to excretion.
- In the lungs, significant deposition of nano-TiO_2 and signs of oxidative stress were observed. Cytokine activation indicated acute and chronic inflammation.
- It was possible to detect and quantify neuro-functional alterations in the treated rats by the behavioral and electrophysiological methods applied.
- The behavioral alterations in the rats treated with TiO_2 nanorods were mostly related to increased anxiety and fear, suggesting damage to the dopaminergic, and partly glutamatergic, regulation. Changes in the cortical evoked response underlined glutamatergic damage.
- In the treated rats' kidneys, elevated Ti levels were measured. Presence of NPs, and tissue alterations suggesting functional damage were observed.
- In several cases we could demonstrate that the alterations were proportional to the locally determined Ti load.
- Regarding mass production and widespread application of nano-TiO_2, including nanorods, the results presented in this thesis have human health relevance, first of all for those suffering occupational exposure.

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The Applicant's Relevant Publications

I. Horváth T, Papp A, Kiricsi M, Igaz N, Trenka V, Kozma G, Tiszlavicz L, Rázga Zs, Vezér T: Titán-dioxid-nanopálcikák tüdőre kifejtett hatásának állatkísérletes vizsgálata szubakut patkány modellben.
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