IRON OXIDE NANOPARTICLES AND THEIR TOXICOLOGICAL EFFECTS: *IN VIVO* AND *IN VITRO* STUDIES

PhD Thesis

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INTRODUCTION

Nanotechnology has large potential benefits to a wide range of disciplines, such as environment, human health and medicine. Nanoparticles (NPs) are defined as particles with at least one dimension less than 100 nm. NPs have been designed and made as part of the recent advances in nanotechnology.

NPs are emitted into the environment by primary sources such as natural phenomena, combustion processes or industrial activities (e.g. welding) or are released during generation and handling of engineered NPs. Because the lung is considered by far the most important portal of entry for NPs into the human body, this overview will mainly focus on the lung as a potential barrier for inhaled NPs. The deposition of particles in the lung is size dependent. 90% of inhaled 1 nm particles are deposited in the nasopharyngeal region, only 10% in the trachea-bronchial region, and essentially none in the alveolar region. 5 nm particles show about equal deposition of approximately 30% of the inhaled particles in all three regions. 20 nm particles have the highest deposition efficiency in the alveolar region (~ 50%), whereas in trachea-bronchial and nasopharyngeal regions this particle size deposits with approximately 15% efficiency. Deposited NPs overcome the tissue barrier as well as the cellular membranes and translocate to extrapulmonary sites and reach other target organs by transcytosis across epithelia of the respiratory tract into the interstitium and access to the blood circulation directly or via lymphatics, resulting in distribution throughout the body. They reach first the central compartment of the body, and finally the central nervous system after damaging and penetrating the blood brain

barrier. The toxicokinetics of NPs has not been elucidated yet, and the most of evidence is based on animal models.

Investigations of metal and metal oxide NPs are currently an area of intense scientific research, due to a wide variety of potential applications in medical diagnostics, cosmetics industry, optical and electronic fields. Iron oxide nanoparticles (IONPs) with their unique magnetic properties have a high potential for use in several biomedical applications, including magnetic drug targeting, magnetic detection, hyperthermia and magnetic resonance imaging. Toxicological examination of NPs is essential before we can fully exploit their advantages in practical applications and ensure that potential adverse consequences are minimized.
IONPs are found naturally in the environment as particulate matter in air pollution and in volcanic eruptions. Either magnetite or maghemite – the two most commonly studied iron oxides − particles can be generated as emissions from traffic, industry and power stations but can also be specifically synthesised chemically for a wide variety of applications (so-called engineered nanoparticles).

The aim of this study was determination the potential, and up to now not completely examined, adverse effects associated with iron oxide NPs following airways exposure, including general and specific toxicological actions (on the respiratory system, immune system and on certain macromolecules). Both *in vivo* animal tests and *in vitro* examinations have notable advantages and disadvantages; but by combining these in a complex experimental model, early detection of possible toxicological effects can be achieved, and new relationships

between the primary outcomes can be revealed. This thesis comprises both *in vivo* and *in vitro* tests for toxicological examination of iron oxide NPs. We looked for answers to the following questions:

- Does intratracheal application of iron oxide nanoparticles cause any general toxic effect and general and or specific histopathological changes in rat organs?

- Does it cause any specific toxic effect on rat respiratory system?
    - Does it change production of any immunoglobulin in the early stage of exposure in BAL and the whole blood?
    - Does it cause changes of some components of pulmonary redox system?

- Does it cause any harmful effect in in vitro cell lines?
    - Does it cause any damage in the primary culture of rat lung cells and human A549 cell line?
    - If yes, is there any relationship between cell damage and production of pro-inflammatory proteins?
    - Does it induce cytotoxic effect in another mammalian cell line?

- Can mutagenic activity be detected in bacterial cells exposed to iron oxide nanoparticles?

METHODS

In *in vivo* experiments male rats were treated once intratracheally with 1 and/or 5 mg/ml iron oxide nanoparticles. The instilled volume was 1 ml/kg body weight. An untreated control (UnC, neither anesthesia nor intratracheal instillation) and a vehicle control group (Con, anesthetized and vehicle treated) was used. In Experiment I each group contained 30 animals at start and 6 of the 30 rats per group were sacrificed after 1, 3, 7, 14 and 30 days, respectively. In Experiment II each group contained 24 animals at start and 6 of the 24 rats per group were sacrificed after 1, 3, 7 and 14 days. In Experiment III each group contained 12 animals at start and 6 of the 12 rats per group were sacrificed after 7 and 30 days, respectively.

In Experiment I, body and organ weights (brain, liver, lungs, heart, kidneys, spleen, thymus and adrenals) were monitored, and histopathological analysis was undertaken.

In Experiment II, immunological studies were performed. Blood samples were taken from the abdominal aorta and bronchoalveolar lavage (BAL) was carried out with physiological saline. IgA, IgG, and IgM concentrations were determined by the sandwich ELISA (enzyme-linked immunosorbent) assay.

In Experiment III, effect of iron oxide NPs on pulmonary redox system were examined. The treated animals' lungs were frozen in liquid nitrogen and placed in a freezer. On the day of examination, after homogenisation of the lungs, samples were centrifuged. Total gluthatione (GSH) estimation was determined using the GSH reductase method. Extracellular Cu,Zn/superoxide dismutase (EC-SOD) was estimated with the use of a Randox kit.

In *in vitro* studies effect of iron oxide NPs on primary culture of rat lung cells (alveolar macrophages and type II pneumocytes isolated from male rats) and human A549 cell line, and potential relationship between cell damage and proinflammatory proteins was assessed. Finally, cytotoxic and genotoxic effects of these nanoparticles were studied on Mammalian Vero cell line and bacterial strains, respectively.

In Experiment IV, animal alveolar macrophages, type II pneumocytes and human A549 lung cells were examined by lectin histochemistry assay (for detection of oligosaccharide molecules bound to protein molecules in the cell membranes) and chemokines levels were measured. Lung cells were exposed to iron oxide NPs at doses 1, 5, and 10 μg/ml.

In Experiment V potential relationship between cell damage and production of chemokines were examined. The primary culture of rat lung cells were exposed to iron oxide NPs at doses 1, 5, and 10 μg/ml. After 24 h incubation the supernatants of the cells were collected and sandwich ELISA was used to measure chemokines (MCP-1 and MIP-1α) levels.

In Experiment VI, Vero cells were grown in 96-well plates (3000 cells/well) until subconfluent. IONPs were then added to the cells at 78, 156, 313, 625, 1250, 2500, 5000 and 10000 μg/ml concentration, and incubated for 4 and 24 hours. Percentage viability of the cells was calculated as the ratio of mean absorbance of triplicate readings of sample wells (I_{sample}) compared to the mean absorbance of control wells ($I_{control}$): Cell viability (%) = ($I_{sample}/I_{control}$) × 100.

In Experiment VII, Ames test was used for assess the genotoxic potential of chemicals and pharmaceuticals. The test employs histidine dependent mutant (*Salmonella typhimurium*) and tryptophan dependent (*Escherichia col*ii) strains.

All values were expressed as Mean±S.D. From the general toxicological data, group means (±SD) were calculated. All data were tested for significance with one-way analysis of variance (ANOVA), and significant difference between the groups was tested using a two-way paired Student's *t*-test, the MTT analyses were performed using Student's *t*-test for unpaired data. *P* values < 0.05 were considered statistically significant. The results of immunoglobulins were calculated on the basis of Bazin's results. Ames test data were processed using standard statistical software COLONY with recommendation of UKEMS (U.K. Environmental Mutagen Society).

RESULTS

Intratracheal instillation of iron oxide NPs caused a significantly slowed body weight gain compared to the control groups. Among the relative organ weights, weight of the lungs decreased significantly with increasing dose and time. Histopathological examination revealed no abnormalities in the exposed rats' organs (lungs, liver, kidneys and spleen) except in the lungs, where the interstitium was widened and a weak pulmonary fibrosis developed by the end of examination period.

Immunological examination showed that 1 mg/ml iron oxide NPs decreased the IgA level in the blood but not in BAL. IgG and IgM (immunoglobulins of peripherial airways) showed significant decrease in BAL whereas they did not alter in the blood.
In biochemical examination GSH and EC-SOD were estimated. At 7 and 30 days after the exposure by IONPs neither GSH content nor EC-SOD activity could be measured.

According to *in vitro* lectin histochemistry assay at lower concentrations, the membranes of alveolar macrophages and type II pneumocytes proved to be intact similarly to control, while A549 cells showed incomplete membranes already at 1 µg/ml concentration. At 10 µg/ml concentration the membranes of both animal and human lung cells became irregular and lost continuity, finally the cells were fragmented.
Iron oxide nanoparticles significantly increased the expression of MCP-1 and MIP-1α in AM. In PII cells, production of MCP-1 significantly increased while MIP-1α significantly decreased.
In cytotoxicity assay, cell viability changed from ca. 100% to approximately 13% with increasing iron oxide NP concentrations.
In Ames test, the average number of revertant colonies and change in the background growth were similar in the nanoparticle-treated groups and the negative control. None of the revertant rates was greater than or equal to the twofold of the negative controls, and no concentration-dependent increase was observed.

EXPERIMENT	AIM	METHOD	RESULT
I	general toxic effect: body and organ weight analyses	single intratracheal instillation 1, 5 mg/ml IONPs	significantly slowed body weight gain; weight of the lungs decreased
	histopathological examination: lungs, liver, kidneys, spleen	HE, Berlin blue, Giemsa, Gömöri's and van Gieson stains	no abnormalities in the exposed rats' organs except in the lungs
II	immunological examination: Ig level changes	single intratracheal instillation 1 mg/ml IONPs; blood and broncho-alveolar lavage (BAL) samples	IgA (blood): ↓ IgA (BAL): − IgG (blood): − IgG (BAL): ↓ IgM (blood): − IgM (BAL): ↓
III	biochemical examination: effect of IONPs on pulmonary redox sytem	single intratracheal instillation 1 mg/ml IONPs; homogenisated lungs	GSH and EC-SOD changes: −

Table 1 summarizes the *in vivo* experiments iron oxide nanoparticles (IONPs) toxicology with focus on aims, methods and results.

EXPERIMENT	AIM	METHOD	RESULT
IV	effect of IONPs on primary culture of rat lung cells and human A549 cell line	AM, PII and A549 cells: exposition to 1-10μg/ml IONPs (24h) lectin histochemistry assay	IONPs caused the injury of cell membranes; human cells were more sensitive
V	relationship between cell damage and proinflammatory proteins	AM, PII and A549 cells: exposition to 1-10μg/ml IONPs (24h) chemokine detection	AM cells - MCP-1: ↑; AM cells - MIP-1α : ↑ PII cells - MCP-1: ↑; PII cells - MIP-1a: −
VI	cytotoxic effect of IONPs	Vero cells: exposition to 78 - 10000 μg/ml IONPs (4 and 24h)	time- and concentration dependent cytotoxicity
VII	genotoxic effect of IONPs	Ames test with *S. typhimurium* and *E. coli* strains 6.9 - 5000 μg/ml IONPs	no mutagenic effect

Table 2 summarizes the *in vitro* experiments iron oxide nanoparticles (IONPs) toxicology with focus on aims, methods and results.

CONCLUSION

In conclusion, the results of this work and the questions pointed out as aims of the study can be answered as follows:

- Acute intratracheal application of iron oxide nanoparticles had evident general toxic effect (altered body and lung weights) and caused morphological changes in the treated rats' lungs.

- Nanoparticles which reached the lower airways proved to be immunosuppressive: there was decreased immunoglobulin level (IgM and IgG) in the peripherial bronchioles. However, two components of pulmonary redox system (GSH and EC-SOD) did not change, therefore further examination is required.

- The nanoparticles caused irreversible injury to the membranes of alveolar cells. Human A549 cells were more sensitive than animal cells. Our results showed connection between damage of lung cells and production of chemokines (significantly elevated MCP-1 and MIP-1α levels were measured from the supernatant of treated lung cells). The alveolar epithelial cells could produce chemokines through which they may regulate inflammatory and immune responses in the alveolar microenvironment. The iron oxide NPs had moderate cytotoxic effect on Vero cell line.

- No mutagenic activity could be observed in the bacterial reverse mutation (Ames) test.

- The new, complex experimental model, comprising both *in vivo* and *in vitro* investigations, proved to be suitable for early detection of (previously unknown or partially documented) toxic effects of iron oxide NPs, and for revealing certain connections between the individual toxic effects.

From the research presented in this study, the need for more toxicological examination on nanoparticles is clear. In addition to animal experiments, there is a need to develop and use better and rapid screening tests. *In vitro* studies are likely to provide initial data on nanoparticles, with the findings having to be followed up by *in vivo* studies in animal.

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