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Professional paper

GENOTOXICITY OF METAL NANOPARTICLES: FOCUS ON *IN VIVO* STUDIES*

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With increasing production and application of a variety of nanomaterials (NMs), research on their cytotoxic and genotoxic potential grows, as the exposure to these nano-sized materials may potentially result in adverse health effects. In large part, indications for potential DNA damaging effects of nanoparticles (NPs) originate from inconsistent *in vitro* studies. To clarify these effects, the implementation of *in vivo* studies has been emphasised. This paper summarises study results of genotoxic effects of NPs, which are available in the recent literature. They provide indications that some NP types cause both DNA strand breaks and chromosomal damages in experimental animals. Their genotoxic effects, however, do not depend only on particle size, surface modification (particle coating), and exposure route, but also on exposure duration. Currently available animal studies may suggest differing mechanisms (depending on the duration of exposure) by which living organisms react to NP contact. Nevertheless, due to considerable inconsistencies in the recent literature and the lack of standardised test methods - a reliable hazard assessment of NMs is still limited. Therefore, international organisations (e.g. NIOSH) suggest utmost caution when potential exposure of humans to NMs occurs, as long as evidence of their toxicological and genotoxic effect(s) is limited.

KEY WORDS: *adverse health effects, chromosomal damage, coating, DNA damage, nanomaterials, rodents*

Nanomaterials have always been released into air by various natural phenomena, e.g. volcano ashes or wild fires, and this is how they unintentionally come into contact with humans, animals, and the environment. Besides, anthropogenic NMs set free by diesel engine exhaust, combustions, welding or cigarette fume are part of the plausible exposure to nano-sized particles.

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
Bw	Body weight
MRI	Magnetic resonance imaging
NIOSH	National Institute for Occupational Safety and Health
NM	Nanomaterial
NP	Nanoparticle
OECD	Organisation for Economic Co-operation and Development
ROS	Reactive oxygen species
UV irradiation	Ultraviolett irradiation

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However, over the past years the production and application of a wide variety of NMs have aroused great interest due to their very unique and industrially favourable physico-chemical properties that differ remarkably from bulk materials of the same composition. Due to these properties, materials produced from (or with) nanoparticles boast durability, flexibility, electrical conductivity or numerous other promising features. Metal nanoparticles are the most frequently produced NMs, as they are already widely applied in nanotechnology. Incorporated metal NPs not only improve consumer products like cosmetics and sport goods, but also positively affect contrast agents for magnetic resonance imaging. Another great field of interest is their future application in diagnostic and therapeutic medicine for drug delivery and hyperthermia treatments (1,2).

NMs, which is the umbrella term for other nano-sized morphologies such as NPs, nanofibers, and nanotubes, are defined as very small materials having at least one dimension below 100 nm in size. They can be synthesised by two primary strategies: the top-down fabrication, which crushes bulk material into smaller particles, and the bottom-up method, which uses chemical reactions to originate NPs from atoms or molecules (3).

The smaller the particles are, the bigger their total surface area per unit mass (the surface area of particles involved in biological interactions) (4) becomes. In fact, when the size of (nano-)particles diminishes, their number per unit of mass increases (5). The abovementioned increase in the surface area also increases the number of atoms on the particle surface, which leads to an increased biological reactivity and an extremely different behaviour compared to bigger particles consisting of the same material (6). For instance, while 5 nm gold particles absorb light strongly at 520 nm, bulk gold in turn reflects the light (7). Titanium dioxide (TiO_2) particles, on the other hand, lose their white colour when downsized below 50 nm (4). These remarkably different and favourable behaviours of small particles have brought about a concern that intentionally engineered NMs may at the same time cause adverse health effects when they come in contact with living organisms. Persons primarily exposed to engineered NMs are not only consumers of nanoproducts, but also employees in the field of nanotechnology who may potentially come in contact with NMs during the synthesis or further treatment and application of such materials. Nanomaterials may also be released during transportation and cleaning of production equipment where the primary exposure route is inhalation (8).

Toxicological information on the frequently engineered (metal) NMs is of pivotal importance in terms of risk assessment and management, as these are either already in use (e.g. contrast agents for MRI, cosmetics, and textiles) or may in future be applied in numerous further fields of interest. Nanotoxicological research on the potential adverse health effects of NMs, and especially NPs, has investigated only some of the numerous available NP types.

However, most of the cytotoxic and genotoxic effects of NPs have been documented in *in vitro* studies only. Titanium dioxide NPs (21 nm), which are approved as UV-absorbent substances in sunscreens (9), induced genotoxic effects in mouse lymphoma cells after simultaneous UV irradiation (10). Yet, further *in vitro* studies have provided evidence that UV irradiation is not necessarily required for the DNA damaging potential of TiO_2 NPs. Wang et al. (11) exposed human B-cell lymphoblastoid cells to TiO_2 NPs with a particle diameter of 6.57 nm, which resulted in DNA and chromosomal damages after an exposure duration of 24 and six hours, respectively. The negative size effect of NPs was clearly demonstrated by Gurr et al. (12) who exposed human bronchial epithelial cells to various sizes of TiO_2 NPs (10 nm and 20 nm, ≥200 nm, respectively). Interestingly, the two smaller TiO_2 NPs, sized 10 nm and 20 nm, at a concentration of 10 $\mu g\ ml^{-1}$ induced significant oxidative DNA and chromosomal damages, while the bigger (200 nm or above) TiO_2 NPs with the same composition and concentration did not. This size effect was confirmed by another recent study: on the one hand, it showed comparable DNA damages of nano-sized anatase (<25 nm) and fine rutile (<5 μm) TiO_2 particles in human bronchial epithelial cells, but on the other hand, significant chromosomal damages were only caused by the smaller nano-sized anatase TiO_2 particles (13).

In addition to the size, coating of an NP appears to be crucial in terms of both cellular toxicity and genotoxicity. NPs are coated with various coverings (i.e. polymers, amino acids) in order to obtain improved solubility in fluids, higher biocompatibility, and lower toxicity (14,15). As these coatings modify the particle surface, they may also alter the particle's (geno-)toxicity or inflammatory effects depending on the coating material (14,16). The effect of coating was investigated by Hong et al. (17) who revealed that positively charged coatings of iron oxide NPs resulted

in increased DNA strand breaks of fibroblasts, while negatively charged coatings did not show any significant genotoxicity. As an explanation for this behaviour, the authors assumed that only positively charged particles penetrated the nucleus and interacted with the DNA.

Likewise, polysaccharide coated silver NPs (25 nm) elevated the amount of DNA damage repair proteins and upregulated the tumour suppressor protein p53 in mouse cells. On the contrary, uncoated silver NPs of the same size did not result in altered protein expression (18). The authors refer to the plausibility that the coating of NPs with polysaccharides prevents their tendency to agglomerate, which results in an increased surface area and facilitated contact with cell membranes (18).

Besides TiO_2 NPs, sunscreens and other nanotechnology-based cosmetics frequently contain zinc oxide (ZnO) NPs. Alike TiO_2 NPs, ZnO NPs seem to induce genotoxic effects *in vitro*. They were able to induce DNA strand breaks and downregulate mitochondrial activity in different cell lines. It was further possible to illustrate their concentration and time dependent cellular internalisation (19).

Moreover, cobalt chromium (CoCr) NPs appear to be genotoxic under *in vitro* conditions due to significantly increased single and double DNA strand breaks and chromosomal damages in human fibroblasts (20).

In large part, *in vitro* investigations conducted up to this moment have demonstrated direct interaction between the NM and the DNA *per se* but have not considered genotoxic mechanisms which originate from intercellular processes. Nonetheless, there are indications that genotoxicity might result from indirect DNA damage by the cellular production of reactive oxygen species (ROS), the depletion of antioxidants or the altered synthesis of DNA repair proteins (5). If the indirect DNA damaging mechanism were to be confirmed, NPs would not necessarily need to come into direct contact with their target cells. A very sophisticated cell model conducted by Bhabra et al. (21) showed this possible indirect way of genotoxicity. In this study, the target fibroblasts, which functioned as monitoring cells, were placed underneath an intact cell barrier consisting of inert placenta cells and lying on a microporous membrane. The intact cell barrier may be comparable with the intact human blood-brain barrier. Following the exposure of the cell barrier to CoCr NPs, a significant DNA damage was detected in fibroblasts, although they had never been in direct contact with the NPs. Due to further experiments, the authors postulate that these genotoxic effects were induced by an increased ATP-release of the barrier cells. This increased ATP-release damaged the fibroblasts' DNA, reaching them via gap junctions and hemichannels.

In summary, there is evidence that a variety of metal NP types may be genotoxic to cultured cells *in vitro,* even though there are clear inconsistencies in the recent literature. Nevertheless, results of *in vitro* experiments may not fully reflect the natural genotoxic potential of NMs. Under *in vivo* conditions, NPs and NMs in general may act considerably different than under *in vitro* conditions. Furthermore, *in vivo* concentrations of NMs may differ from those *in vitro*.

Thus, researchers have started to investigate potential adverse health effects of NMs *in vivo* in order to obtain information on their behaviour in living organisms. Additionally, information on the mechanisms by which they interact and possibly interfere with cellular components is of utmost importance.

To our knowledge, there is only one review about the genotoxicity of metal NPs, which was published in 2011. Numerous further investigations on nanoparticulate genotoxicity have since been conducted. In this paper, we will give an overview on *in vivo* genotoxicity research undertaken up to this point, with a focus on metal NPs.

METHODS

Papers were retrieved from the open literature by a systematic search of databases MEDLINE and SCOPUS. The keywords for search included *metal nanoparticles*, *DNA damage*, *genotoxiciy*, *comet assay*, *micronucleus*, in vivo, *mice*, *rats*, *inhalation,* and *instillation* until May 2012. Language was restricted to German and English. This resulted with 17 references which are included in this paper (Table 1).

In terms of quantifying cellular genotoxicity *in vitro* and *in vivo*, the comet assay and the micronulceus test are the most commonly applied methods in nanotoxicology. Under *in vivo* conditions, the (alkaline) comet assay measures single and double DNA strand breaks in cells of exposed animals, which can visually be quantified by electrophoresis and fluorescence microscopy in the form of comet-like

Table 1 In vivo *studies on the genotoxicity of metal nanoparticles (NPs). bw body weight, TiO_2 titanium dioxide, Au gold, Co cobalt, ZnO zinc oxide, Al_2O_3 aluminium oxide, 8-OHdG 8-hydroxydeoxyguanosine, RPE retinal pigment epithelium,*

Authors	Nanoparticle and size	Concentration and exposure duration	Exposure and species	Assays	Results
Naya et al., 2012 (28)	TiO_2 NPs 5 nm	1.0 mg kg^{-1} and 5.0 mg kg^{-1} bw (single instillation),	Intratracheal instillation, rats	Histopathology	Increase in alveolar macrophages and neutrophils at 5 mg kg^{-1}
				Alkaline comet assay	No increase in DNA strand breaks (% tail DNA)
		0.2 mg kg^{-1} and 1.0 mg kg^{-1} bw (repeated instillation, once per week for 5 weeks)		Histopathology	Significant increase in alveolar macrophages and neutrophils at 1.0 mg kg^{-1}
				Alkaline comet assay	No increase in DNA strand breaks (% tail DNA)
Sycheva et al., 2011 (25)	Microsized TiO_2 particles (TDM) 160 nm	40 mg kg^{-1}, 200 mg kg^{-1} and 1000 mg kg^{-1} bw, daily for 7 days	Oral gavage, mice	Comet assay	Increase in DNA strand breaks (% tail DNA) in bone marrow (TDM, TDN) and liver cells (TDN)
	Nanosized TiO_2 NPs (TDN) 33 nm			Karyological assay	Increase in micronuclei in bone marrow (TDM), increase in mitotic index and apoptosis in forestomach, colon, atypical nuclei of spermatids (TDM, TDN) and apoptosis in forestomach (TDN)
Trouiller et al., 2012 (24)	TiO_2 NPs 160 nm	50 mg kg^{-1}, 100 mg kg^{-1}, 250 mg kg^{-1} and 500 mg kg^{-1} bw for 5 days	Oral gavage, mice	Alkaline comet assay	Increase in DNA strand breaks (tail moment) at 500 mg kg^{-1} bw
				Micronucleus test	2.1 fold increase in micronuclei at 500 mg kg^{-1}
				γ-H2AX immunostaining	Increase of γ-H2AX formation at 50 mg kg^{-1}, 100 mg kg^{-1}, 250 mg kg^{-1} and 500 mg kg^{-1} bw
				8-OHdG	Increase in 8-OHdG at 500 mg kg^{-1} bw
	TiO_2 NPs 160 nm	500 mg kg^{-1} for 10 days for pregnant mouse dams	Oral gavage, pregnant mouse dams	DNA deletion assay	Fetuses: increase in eyespots per RPE
Saber et al., 2011 (45)	2 coated rutile TiO_2 NPs, one uncoated anatase TiO_2 NPs	54 μg, single dose	Intratracheal instillation, mice	Comet assay	Increase in DNA damage in lung lining fluid

Table 1 *Continued*

Saber et al., 2012 (26)	Coated rutile TiO_2 NPs ($NANOTiO_2$) 20.6 nm	18 µg, 54 µg, 162 µg, single dose	Intratracheal instillation, mice	Alkaline comet assay	No increase in DNA strand breaks (normalized tail length) of broncho-alveolar cells
	Sanding dust of paint with TiO_2 NPs (Indoor-TiO_2) 10 nm to 1.7 µm			Alkaline comet assay	Increase in DNA strand breaks (normalized tail length) of liver tissue by 162 µg of $NANOTiO_2$
Landsiedel et al., 2010 (27)	ZnO NPs 30 nm to 200 nm	15 mg kg^{-1}, 30 mg kg^{-1}, 60 mg kg^{-1} bw, single dose	Intraperitoneal administration, mice	Micronucleus test	No increase in micronuclei in bone marrow cells
	TiO_2 NPs 10 nmx50 nm	0.5 mg m^{-3}, 2 mg m^{-3}, 10 mg m^{-3}, 6h on 5 consecutive days	Head-nose inhalation, rats	Alkaline comet assay	No increase in DNA strand breaks in broncho-alveolar cells
Hwang do et al., 2012 (33)	Silica-coated and uncoated cobalt ferrite NPs 50 nm and 35 nm, respectively	500 µg, single dose	Intravenous injection, mice	RT-PCR	Uncoated cobalt ferrite NPs enhanced expression of 17 genes related to DNA damage or repair, apoptosis, carcinogenesis, inflammation, oxidative stress and growth arrest
Girgis et al., 2012 (34)	Au and Au-Co NPs 15 nm	80 mg kg^{-1}, 160 mg kg^{-1}, 320 mg kg^{-1} bw, once daily for 7 and 14 days, respectively	Oral gavage, mice	RNA extraction	Alteration in tumor-initiating genes (CYP3A, p27, p53) by gold-cobalt NPs (160 mg kg^{-1} and 320 mg kg^{-1} bw) and Au-NPs (320 mg kg^{-1} bw)
				Micronucleus test	Increase in MN formation of bone marrow cells by Au-Co NPs (160 mg kg^{-1} and 320 mg kg^{-1} bw) and Au-NPs (320 mg kg^{-1} bw)
				Glutathione peroxidase activity	Decrease in glutathione peroxidase activity by Au-Co NPs (320 mg kg^{-1} bw) and Au-NPs (320 mg kg^{-1} bw)
				8-OHdG	Increase in 8-OHdG of hepatic mice genome by Au NPs and Au-Co NPs
Schulz et al., 2011 (41)	Gold NPs 2 nm, 20 nm, and 200 nm	18 µg, single dose	Intratracheal instillation, rats	Alkaline comet assay	No increase in relative tail intensity
				Micronucleus test	No increase in MN formation

Table 1 *Continued*

Sharma et al., 2012 (29)	ZnO NPs 30 nm	50 mg kg^{-1} and 300 mg kg^{-1} bw, 14 days	Oral administration	Fpg-Comet assay	Increase in % tail DNA and Olive tail moment by 300 mg kg^{-1} ZnO NPs
Tiwari et al., 2011 (36)	Ag NPs 15 nm to 40 nm	4 mg kg^{-1}, 10 mg kg^{-1}, 20 and 40 mg kg^{-1} bw, 5-day interval for 32 days	Repeated intravenous injection, rats	Alkaline Comet assay	Increase in DNA strand breaks (tail migration) by 40 mg kg^{-1}
Choi et al., 2010 (37)	Ag NPs 5 nm to 20 nm	30 mg L^{-1} Ag, 60 mg L^{-1} Ag and 120 mg L^{-1} Ag, 24h	Oral gavage, zebrafish	Western blot	Increase in γ-H2AX and dose-dependent increase in p53 mRNA
Ahamed et al., 2010 (38)	Polysaccharide-coated Ag NPs 10 nm	50 μg mL^{-1} and 100 μg mL^{-1}, 24h and 48h	Oral gavage, Drosophila melanogaster	Western blot	Increase in p53 and p38 proteins by 50 μg mL^{-1} and 100 μg mL^{-1} and after 24 h and 48 h exposure
Kim et al., 2008 (39)	Ag NPs 60 nm	30 mg kg^{-1}, 300 mg kg^{-1} and 1000 mg kg^{-1} bw, 28 days	Oral gavage, rats	Micronucleus test	No increase in MN formation of erythrocytes
Kang et al., 2011 (44)	Nickel hydroxide NPs 5 nm	79 μg m^{-3} Ni, 5h/day for 1 week or 5 days/week for 5 months	Whole-body inhalation, (ApoE-/-) mice	Long PCR assay	Increase in damaged mitochondrial DNA of the aorta only after 5 months of exposure
Balasubraman-yam et al., 2009 (42)	Al_2O_3 NPs 30 nm and 40 nm, bulk Al_2O_3 particles 50 μm to 200 μm	500 mg kg^{-1}, 1000 mg kg^{-1} and 2000 mg kg^{-1} bw, single dose	Oral gavage, female Wistar rats	Micronucleus test Chromosomal aberrations analysis	Significant increase in MN formation of bone marrow erythrocytes by Al_2O_3 NPs (1000 mg kg^{-1} and 2000 mg kg^{-1}) Significant increase in chromosome aberrations of bone marrow cells by 30 nm-Al_2O_3 (1000 mg kg^{-1} and 2000 mg kg^{-1}) and 40 nm-Al_2O_3 (2000 mg kg^{-1})

Table 1 *Continued*

Balasub-raman-yam et al., 2009 (43)	Al_2O_3 NPs 30 nm and 40 nm, bulk Al_2O_3 particles 50 μm to 200 μm	500 mg kg^{-1}, 1000 mg kg^{-1} and 2000 mg kg^{-1} bw, single dose	Oral gavage, female Wister rats	Micronucleus test	Significant dose-dependent increase in MN of bone marrow erythrocytes by 30 nm - Al_2O_3 and 40 nm - Al_2O_3 (1000 mg kg^{-1} and 2000 mg kg^{-1})
				Alkaline comet assay	Significant dose-related increase in DNA breakage (% tail DNA) by 30 nm - Al_2O_3 and 40 nm - Al_2O_3 (1000 mg kg^{-1} and 2000 mg kg^{-1})

cell nuclei (22). The micronucleus test measures general chromosomal damages. The detected micronuclei represent chromosomal fragments, which arise in daughter cells during mitosis (23).

RESULTS

Genotoxic potential of titanium dioxide NPs

The *in vivo* genotoxicity of TiO_2 NPs has been investigated in six studies which treated both rats and mice by intratracheal instillation and oral gavage. Four of these animal studies were positive. Trouiller et al. (24) determined several genotoxic endpoints after the oral treatment of male mice with TiO_2 NPs. At very high mass fractions (500 mg kg^{-1} bw), 160 nm TiO_2 NPs caused DNA strand breaks, a 2.1 fold increase in micronuclei, and inflammatory reactions in respect of changes in cytokine expression. Likewise, oxidatively induced DNA damage was significantly increased. *In utero* treatment of mouse fetuses showed a significant increase in DNA deletion frequency suggesting possible genome rearrangements. Due to these study results, it is likely that TiO_2 NPs may cause both direct and indirect DNA damage, the latter being due to oxidative stress.

The second positive animal study with similar TiO_2 NPs and the same particle size (160 nm) confirmed the increase of DNA strand breaks and micronuclei formation in bone marrow cells of treated mice. However, animals were exposed by daily oral gavage for seven days. Smaller TiO_2 NPs with a size of 33 nm showed similar genotoxic results, even if they additionally increased DNA strand breaks of mouse liver cells and apoptosis in forestomach cells (25).

Saber et al. (26) compared the inflammatory and genotoxic effects of pure TiO_2 NPs and TiO_2 NPs added to paints. While pure TiO_2 NPs induced significant inflammatory response in broncho-alveolar fluid cells of intratracheally instilled mice, TiO_2 NPs incorporated in paint matrix did not. Only pure TiO_2 NPs additionally caused an increase in DNA damage of liver cells. Authors assume that a relevant exposure to nanoparticulate TiO_2 incorporated in paints does not occur during the use of product, as single NPs are not released.

Nevertheless, two studies were clearly negative regarding genotoxicity *in vivo*. Although single intratracheal instillation of 5 nm TiO_2 NPs caused inflammatory responses in rats, these NPs did not enhance DNA damage neither after single, nor after repeated exposure. Landsiedel et al. (27) considered the recommended OECD test methods for NMs by including the Ames test with Salmonella, the micronucleus test, and comet assay *in vitro* and *in vivo*. Unexpectedly, parallel *in vitro* and *in vivo* (inhalatory exposure to mice) studies showed that zinc oxide and TiO_2 NPs were not genotoxic. These findings corroborate the above-mentioned absent genotoxicity in rats after intratracheal administration of TiO_2 NPs (28).

Genotoxic potential of zinc oxide NPs

Besides TiO_2 NPs, ZnO NPs are applied in cosmetics, UV-absorbent sunscreens, and food packaging. Despite its progressive use, the DNA damaging potential of ZnO NPs has so far been

investigated *in vivo* only once. Sharma et al. (29) orally exposed mice for 14 consecutive days. The exposures resulted in elevated liver enzymes, and oxidatively induced DNA breakage.

Genotoxic potential of cobalt NPs

Cobalt-based NPs may, on the one hand, embody a realistic (future) exposure hazard for humans because they are released by mechanical wear of orthopedic implants (30) and by further medical applications. On the other hand, they may also be dangerous because of their application in technical devices such as data storage and catalysts (31, 32).

Still, the DNA damaging potential of Co NPs has rarely been considered *in vivo*. One recent rodent study investigated genotoxicity of Co NPs dependent on surface coating. The authors showed that both silica-coated and uncoated cobalt ferrite ($CoFe_2O_4$) NPs, intravenously injected into mice, accumulated in liver tissue, while only uncoated $CoFe_2O_4$ NPs resulted in enhanced expression of genes related to DNA damage and repair, carcinogenesis, cell death, growth arrest, oxidative stress, and inflammation. In comparison with the coated NPs, uncoated $CoFe_2O_4$ NPs even induced a 45-fold expression ratio of cyp4a10 - a gene related to oxidative stress (33).

Similar results, partly overlapping, have been achieved in a study where mice were orally treated with 15 nm gold NPs and gold-cobalt (Au-Co) NPs for seven and 14 days. Both NP types caused alterations in tumour-initiating genes, micronucleus formation, and oxidative DNA adducts. Nevertheless, Au-Co NPs showed a much higher effect at already lower concentrations compared to Au NPs. These greater effects of Au-Co NPs regarding genotoxicity may be explicable by the fact that Co-based Au NPs are able to induce greater oxidative stress. A possible size and concentration effect can widely be excluded due to equal experimental conditions as described by the authors (34).

Genotoxic potential of silver NPs

The antibacterial property of silver (Ag) NPs has frequently been used for numerous applications such as wound dressings, other medical devices, textiles or plastics as they fight both Gram positive and Gram negative bacteria, as well as fungi and viruses (35). The actual mechanism of their bactericide property has not been fully clarified yet. This uncertainty and the high number of applications yield studies on the possible cytotoxic and genotoxic effects of Ag NPs.

Three studies regarding genotoxicity of Ag NPs showed positive results. Tiwari et al. (36) assessed increased single and double DNA breakage in rats after intravenous injection of 40 mg kg^{-1} bw of Ag NPs. In the second *in vivo* study, zebrafish were treated with oral Ag NPs (5 nm to 20 nm), which resulted in high levels of γ-H2AX - a marker for double DNA strand breaks. Moreover, the exposure to Ag NPs resulted in a non-significant dose-dependent increase in hepatic p53 mRNA - the precursor of the tumour suppressor protein and an indirect DNA damage marker (37). Likewise, polysaccharide-coated Ag NPs (10 nm) heightened the level of DNA damage markers (p53 and p38 proteins) in *Drosophila melanogaster* (38).

So far, there is one study which obsevered the effect of extended exposure periods in rats that were orally treated with various levels (maximum 1000 mg kg^{-1}) of 60 nm Ag NPs. This, however, resulted in slight liver damage but did not show a significant increase in genotoxicity (39).

Genotoxic potential of gold NPs

Bulk gold is considered biologically inert, whereas nanoparticulate Au particles seem to be genotoxic under *in vitro* conditions (40). *In vivo* genotoxicity however has only once been investigated. Single intratracheal instillation of Au NPs was not genotoxic in rats - as assessed by the comet assay and the micronucleus test. Genotoxicity could not be identified after the treatment with three different particle sizes: 200 nm, 20 nm, and 2 nm (41).

Genotoxic potential of aluminium NPs

Engineered aluminium (Al)-based NPs were investigated *in vivo* by Balasubramanyam et al (42, 43). In orally exposed rats, the authors observed significant dose related DNA breakage, dose dependent micronuclei formation, and chromosome aberrations by 30 nm and 40 nm aluminium oxide (Al_2O_3) NPs. In contrast, these genotoxic effects were not observed by bulk Al_2O_3 particles with a size of 50 µm to 200 µm.

Genotoxic potential of nickel NPs

The DNA damaging potential of nickel (Ni) NPs was a small part of a whole-body inhalation study. The experimental animals were hyperlipidemic and apoprotein E-deficient (ApoE-/-) mice which received treatment with 5 nm nickel hydroxide (NH) NPs.

Besides pulmonary and systemic inflammatory reactions, and atherosclerosis as a long-term effect, mice showed heightened levels of mitochondrial DNA damage in the aorta. Interestingly, mitochondrial DNA damage was (similar to atherosclerosis) only detected after long-term exposure of five months (44). As oxidative stress was induced simultaneously with mitochondrial genotoxicity, a potential relation between these two effects has to be taken into consideration.

DISCUSSION

Currently available data on NP genotoxicity *in vivo* indicate a potential for DNA damaging effect of various NP types, primarily in mice and rats. However, these *in vivo* investigations are rare and inconsistent. While some of the present *in vivo* studies on nanoparticulate genotoxicity are positive, others do not confirm genotoxic effects. Some NP types such as titanium dioxide, cobalt, zinc oxide, silver, aluminium, and nickel NPs indicate a possible DNA damaging potential in rodents.

So far, TiO_2 NPs are the most frequently investigated NPs in terms of genotoxicity. Still, some studies have revealed genotoxic effects by TiO_2 NPs, while others regard them as being non-genotoxic in particular animals. One reason for the above-mentioned negative study results may be the exposure route which differed from all other studies. Additionally, authors adhered to the recently published OECD recommendations on genotoxicity testing of soluble materials. In the negative study by Landsiedel et al. (27), animals were exposed by inhalation, whereas other studies primarily treated animals by oral gavage - which could have resulted in much higher incorporated NP concentrations. Indeed, inhalation is the most natural and relevant way of human exposure. So far, there are insufficient *in vivo* studies involving inhalatory exposure.

Contrary effects were also observed with surface coatings. Polysaccharide-coated Ag NPs increased the expression of tumour suppressor proteins but uncoated Ag NPs did not (18, 38). The opposite effect was seen with cobalt-based NPs. While silica-coated cobalt ferrite NPs were non-genotoxic, uncoated cobalt ferrite NPs significantly increased the expression of genes associated with DNA damage and repair (33). As mentioned above, the increased genotoxic effect of polysaccharide-coated NPs might be due to the inhibition of particle agglomeration (18). Coatings based on silica might prevent the oxidatively damaging capability of cobalt ferrite NPs. Uncoated cobalt ferrite NPs might release metal ions due to direct interaction with cell membranes, which in turn could result in oxidative stress and consequent DNA damage. Another possibility might be a genotoxic mechanism that depends on the electrical charge of the particle surface. Therefore, further investigations on particle coating and surface modification are needed.

Another determining fact in genotoxicity testing might be the varying exposure durations. Short exposure durations ranging between one and two days caused DNA breakage by Ag NPs. Surprisingly, long-term exposure (28 days) to Ag NPs was not genotoxic to rats. However, the differences in methods have to be considered, as the studies with shorter exposure durations assessed genotoxicity by potentially reversible DNA breakage, while the long-term exposure study determined irreversible chromosome breakage.

These inconsistent results on NP genotoxicity indicate that there are great challenges regarding risk assessment, as *in vivo* studies are, unfortunately, comparable among each other only to a limited extent. The problematic interpretation of nanotoxicological results arises out of the following circumstances:

First, a considerable number of various NMs and NP types are being produced. Thus, nanomaterials greatly differ one from the other by either core material, size, surface area, shape, stability, coating or electrical charge. Hence, these characteristics have a great impact on possible interactions with living cells or tissues and determine cytotoxicity and damage to DNA. Second, *in vitro* and *in vivo* studies may or may not reflect the actual effect of NMs in living organisms when spontaneous contact occurs. Hence, these studies may or may not be relevant for humans. Applied exposure dosages under experimental conditions might exceed the potential (occupational) exposure of humans. Within the framework of animal experiments, exposure routes such as oral gavage may be administered. Gastrointestinal intake of NMs may indeed occur through nanotechnological food, packaging or medical applications. Still, inhalation accounts for the majority of NM exposure routes in humans, as NMs can be released into air in occupational settings. Third, the comparison across different nanotoxicological studies remains questionable due to different dose metrics (i.e. $\mu g\ m^{-3}$ and $mg\ kg^{-1}$ bw) applied. Some authors additionally do not mention the concentration which study animals indeed received.

Finally, the applied NMs are frequently not sufficiently characterised in their chemical composition, physico-chemical properties, agglomeration status in the cell medium, and surface charge. For that reason, the OECD recently developed the "Guidance Manual for the Testing of Manufactured Nanomaterials" aiming at "high science-based, internationally harmonised standards" and the validation of test methods. Following these recommendations, researchers are requested, beyond other standardised procedures, to give detailed information on applied NMs, including their physico-chemical properties, as well as to evaluate their environmental fate and toxicity in mammals. In the end they should provide an explicit study report (46).

Thus, at present there is a lack of *in vivo* studies corresponding to *in vitro* studies with identical NMs, identical methods, and identical endpoints, which are necessary to gain knowledge of possible cytotoxic, genotoxic, and inflammatory effects of NMs on living cells and organisms. Due to the current uncertainties regarding adverse health effects of NMs, NIOSH (National Institute for Occupational Safety and Health) and other international organisations suggest caution when there is an imminent risk of potential exposure of humans to NMs , as long as there is limited evidence available (47). Thus, for the present moment, international organisations recommend a precautionary occupational safety approach, which regards NPs as potentially genotoxic to humans (47, 48). Furthermore, it is recommended to establish exposure registries for workers who handle NMs in order to systemically monitor those who are potentially exposed and who are possibly at risk. As the use of nanotechnology has increased, such exposure registries have gained importance and have been attributed high priority because conventional epidemiological studies on potential health effects of NMs are difficult to conduct (49).

CONCLUSIONS

Several metal NPs may have genotoxic potential *in vivo*. However, inconsistencies in the literature on nanotoxicology do exist; while TiO_2 and Ag NPs have been found to be genotoxic to rodents in large part, other metal NPs, which have rarely been studied *in vitro* and *in vivo*, showed diverging genotoxic effects. Nanoparticulate coatings seem to have a relevant impact on genotoxicity, as they may not only alter the particles' surface charge, but also their agglomeration status by which they gain total surface area. The currently available animal studies may also suggest differing genotoxic mechanisms depending on the duration of exposure.

REFERENCES

1. Sosnovik DE, Nahrendorf M, Weissleder R. Magnetic nanoparticles for MR imaging: agents, techniques and cardiovascular applications. Basic Res Cardiol 2008;103:122-30.
2. Corchero J, Villaverde A. Biomedical applications of distally controlled magnetic nanoparticles. Trends Biotechnol 2009;27:468-76.
3. Luther W. Industrial application of nanomaterials - chances and risks. Future Technologies 2004:54;1-112.
4. European Agency for Safety and Health at Work (EU-OSHA). Workplace exposure to nanoparticles 2009 [displayed 29 Jan 2012]. Available at http://osha.europa.eu/en/publications/literature_reviews/workplace_exposure_to_nanoparticles
5. Singh N, Manshian B, Jenkins G, Griffiths S, Williams P, Maffeis T, Wright C, Doak S. NanoGenotoxicology: the DNA damaging potential of engineered nanomaterials. Biomaterials 2009;30:3891-914.
6. Oberdörster G, Maynard A, Donaldson K, Castranova V, Fitzpatrick J, Ausman K, Carter J, Karn B, Kreyling W, Lai D, Olin S, Monteiro-Riviere N, Warheit D, Yang H; ILSI Research Foundation/Risk Science Institute Nanomaterial Toxicity Screening Working Group. Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy. Part Fibre Toxicol 2005;2:8.
7. Schatz G. Using theory and computation to model nanoscale properties. Proc Natl Acad Sci USA 2007;104:6885-92.
8. Brouwer D. Exposure to manufactured nanoparticles in different workplaces. Toxicology 2010;269:120-7.
9. Food and drug administration (FDA). Sunscreen Drug Products for Over-the-Counter Human Use. Amendment to the Tentative Final Monograph; Enforcement Policy. Federal Register /Vol. 63, No. 204/1998 [displayed 29 Jan 2012]. Available at http://www.fda.gov/ohrms/dockets/98fr/102298b.pdf
10. Nakagawa Y, Wakuri S, Sakamoto K, Tanaka N. The photogenotoxicity of titanium dioxide particles. Mutat Res 1997;394:125-32.
11. Wang JJ, Sanderson BJ, Wang H. Cyto- and genotoxicity of ultrafine TiO_2 particles in cultured human lymphoblastoid cells. Mutat Res 2007;628:99-106.
12. Gurr JR, Wang AS, Chen CH, Jan KY. Ultrafine titanium dioxide particles in the absence of photoactivation can induce oxidative damage to human bronchial epithelial cells. Toxicology 2005;213:66-73.
13. Falck G, Lindberg H, Suhonen S, Vippola M, Vanhala E, Catalán J, Savolainen K, Norppa H. Genotoxic effects of nanosized and fine TiO2. Hum Exp Toxicol 2009;28:339-52.
14. Borm P, Robbins D, Haubold S, Kuhlbusch T, Fissan H, Donaldson K, Schins R, Stone V, Kreyling W, Lademann J,

Krutmann J, Warheit D, Oberdorster E. The potential risks of nanomaterials: a review carried out for ECETOC. Part Fibre Toxicol 2006;3:11.
15. Snyder M, Lee J, Davis T, Scriven L, Tsapatsis M. Silica nanoparticle crystals and ordered coatings using lys-sil and a novel coating device. Langmuir 2007;23:9924-8.
16. Ostiguy C, Lapointe G, Ménard L, Cloutier Y, Trottier M, Boutin M, Antoun M, Normand C. Nanoparticles. Actual knowledge about occupational health and saftey risks and prevention measures. Studies and Research Projects. Report R-470. Montréal: IRSST; 2006.
17. Hong SC, Lee JH, Lee J, Kim HY, Park JY, Cho J, Han DW. Subtle cytotoxicity and genotoxicity differences in superparamagnetic iron oxide nanoparticles coated with various functional groups. Int J Nanomedicine 2011;6:3219-31.
18. Ahamed M, Karns M, Goodson M, Rowe J, Hussain SM, Schlager JJ, Hong Y. DNA damage response to different surface chemistry of silver nanoparticles in mammalian cells. Toxicol Appl Pharmacol 2008;233:404-10.
19. Sharma V, Singh SK, Anderson D, Tobin DJ, Dhawan A. Zinc oxide nanoparticle induced genotoxicity in primary human epidermal keratinocytes. J Nanosci Nanotechnol 2011;11:3782-8.
20. Papageorgiou I, Brown C, Schins R, Singh S, Newson R, Davis S, Fisher J, Ingham E, Case CP. The effect of nano- and micron-sized particles of cobalt-chromium alloy on human fibroblasts *in vitro*. Biomaterials 2007;28:2946-58.
21. Bhabra G, Sood A, Fisher B, Cartwright L, Saunders M, Evans WH, Surprenant A, Lopez-Castejon G, Mann S, Davis SA, Hails LA, Ingham E, Verkade P, Lane J, Heesom K, Newson R, Case CP. Nanoparticles can cause DNA damage across a cellular barrier. Nat Nanotechnol 2009;4:876-83.
22. Collins AR. The comet assay for DNA damage and repair: principles, applications, and limitations. Mol Biotechnol 2004;26:249-61.
23. Fenech M. The micronucleus assay determination of chromosomal level DNA damage. Methods Mol Biol 2008;410:185-216.
24. Trouiller B, Reliene R, Westbrook A, Solaimani P, Schiestl RH. Titanium dioxide nanoparticles induce DNA damage and genetic instability *in vivo* in mice. Cancer Res 2009;69:8784-9.
25. Sycheva LP, Zhurkov VS, Iurchenko VV, Daugel-Dauge NO, Kovalenko MA, Krivtsova EK, Durnev AD. Investigation of genotoxic and cytotoxic effects of micro- and nanosized titanium dioxide in six organs of mice *in vivo*. Mutat Res 2011;726:8-14.
26. Saber AT, Jacobsen NR, Mortensen A, Szarek J, Jackson P, Madsen AM, Jensen KA, Koponen IK, Brunborg G, Gützkow KB, Vogel U, Wallin H. Nanotitanium dioxide toxicity in mouse lung is reduced in sanding dust from paint. Part Fibre Toxicol 2012;9:4.
27. Landsiedel R, Ma-Hock L, Van Ravenzwaay B, Schulz M, Wiench K, Champ S, Schulte S, Wohlleben W, Oesch F. Gene toxicity studies on titanium dioxide and zinc oxide nanomaterials used for UV-protection in cosmetic formulations. Nanotoxicology 2010;4:364-81.
28. Naya M, Kobayashi N, Ema M, Kasamoto S, Fukumuro M, Takami S, Nakajima M, Hayashi M, Nakanishi J. *In vivo* genotoxicity study of titanium dioxide nanoparticles using comet assay following intratracheal instillation in rats. Regul Toxicol Pharmacol 2011;62:1-6.
29. Sharma V, Singh P, Pandey AK, Dhawan A. Induction of oxidative stress, DNA damage and apoptosis in mouse liver after sub-acute oral exposure to zinc oxide nanoparticles. Mutat Res 2012;745:84-91.
30. Jiang H, Liu F, Yang H, Li Y. Effects of cobalt nanoparticles on human T cells *in vitro*. Biol Trace Elem Res 2012;146:23-9.
31. Puntes VF, Krishnan KM, Alivisatos AP. Colloidal nanocrystal shape and size control: the case of cobalt. Science 2001;291:2115-7.
32. Skumryev V, Stoyanov S, Zhang Y, Hadjipanayis G, Givord D, Nogués J. Beating the superparamagnetic limit with exchange bias. Nature 2003;423:850-3.
33. Hwang dW, Lee DS, Kim S. Gene expression profiles for genotoxic effects of silica-free and silica-coated cobalt ferrite nanoparticles. J Nucl Med 2012;53:106-12.
34. Girgis E, Khalil WK, Emam AN, Mohamed MB, Rao KV. Nanotoxicity of gold and gold-cobalt nanoalloy. Chem Res Toxicol 2012;25:1086-98.
35. Li WR, Xie XB, Shi QS, Duan SS, Ouyang YS, Chen YB. Antibacterial effect of silver nanoparticles on *Staphylococcus aureus*. Biometals 2011;24:135-41.
36. Tiwari DK, Jin T, Behari J. Dose-dependent *in vivo* toxicity assessment of silver nanoparticle in Wistar rats. Toxicol Mech Methods 2011;21:13-24.
37. Choi JE, Kim S, Ahn JH, Youn P, Kang JS, Park K, Yi J, Ryu DY. Induction of oxidative stress and apoptosis by silver nanoparticles in the liver of adult zebrafish. Aquat Toxicol 2010;100:151-9.
38. Ahamed M, Posgai R, Gorey TJ, Nielsen M, Hussain SM, Rowe JJ. Silver nanoparticles induced heat shock protein 70, oxidative stress and apoptosis in Drosophila melanogaster. Toxicol Appl Pharmacol 2010;242:263-9.
39. Kim YS, Kim JS, Cho HS, Rha DS, Kim JM, Park JD, Choi BS, Lim R, Chang HK, Chung YH, Kwon IH, Jeong J, Han BS, Yu IJ. Twenty-eight-day oral toxicity, genotoxicity, and gender-related tissue distribution of silver nanoparticles in Sprague-Dawley rats. Inhal Toxicol 2008;20:575-83.
40. Johnston HJ, Hutchison G, Christensen FM, Peters S, Hankin S, Stone V. A review of the *in vivo* and *in vitro* toxicity of silver and gold particulates: particle attributes and biological mechanisms responsible for the observed toxicity. Crit Rev Toxicol 2010;40:328-46.
41. Schulz M, Ma-Hock L, Brill S, Strauss V, Treumann S, Gröters S, van Ravenzwaay B, Landsiedel R. Investigation on the genotoxicity of different sizes of gold nanoparticles administered to the lungs of rats. Mutat Res 2011;745:51-7.
42. Balasubramanyam A, Sailaja N, Mahboob M, Rahman MF, Misra S, Hussain SM, Grover P. Evaluation of genotoxic effects of oral exposure to aluminum oxide nanomaterials in rat bone marrow. Mutat Res 2009;676:41-7.
43. Balasubramanyam A, Sailaja N, Mahboob M, Rahman MF, Hussain SM, Grover P. *In vivo* genotoxicity assessment of aluminium oxide nanomaterials in rat peripheral blood cells using the comet assay and micronucleus test. Mutagenesis 2009;24:245-51.
44. Kang GS, Gillespie PA, Gunnison A, Moreira AL, Tchou-Wong KM, Chen LC. Long-term inhalation exposure to nickel nanoparticles exacerbated atherosclerosis in a

susceptible mouse model. Environ Health Perspect 2011;119:176-81.

45. Saber AT, Jensen KA, Jacobsen NR, Birkedal R, Mikkelsen L, Møller P, Loft S, Wallin H, Vogel U. Inflammatory and genotoxic effects of nanoparticles designed for inclusion in paints and lacquers. Nanotoxicology 2011 [Epub ahead of print]
46. Organisation for Economics Co-operation and Development (OECD). Guidance Manual for the Testing of Manufactured Nanomaterials. 2010 [displayed 29 Jan 2012]. Available at http://www.oecd.org/officialdocuments/displaydocumentpdf/?cote=ENV/JM/MONO(2009)20/REV&doclanguage=en
47. National Institute for Occupational Safety and Health (NIOSH). Approaches to Safe Nanotechnology. Managing the Health and Safety Concerns Associated with Engineered Nanomaterials. 2009 [displayed 29 Jan 2012]. Available at http://www.cdc.gov/niosh/docs/2009-125/pdfs/2009-125.pdf
48. Institut de recherche Robert-Sauvé en santé et en sécurité du travail (IRSST). Chemical Substances and Biological Agents, Studies and Research Projects, Report R-599. Best Practices Guide to Synthetic Nanoparticle Risk Management. 2009 [displayed 30 Jan 2012]. Available at http://www.irsst.qc.ca/media/documents/pubirsst/r-599.pdf
49. Schulte PA, Mundt DJ, Nasterlack M, Mulloy KB, Mundt KA. Exposure registries: overview and utility for nanomaterial workers. J Occup Environ Med 2011;53(Suppl 6):S42-7.

Sažetak

GENOTOKSIČNOST METALNIH NANOČESTICA: OSVRT NA PODATKE ISTRAŽIVANJA *IN VIVO*

S povećanjem proizvodnje i primjene niza različitih nanomaterijala (NM) raste i potreba istraživanja njihovih mogućih citotoksičnih i genotoksičnih učinaka kao i drugih štetnih učinaka na zdravlje u uvjetima profesionalne ili opće izloženost ljudi. Indikacije potencijanog oštećenja DNA kojeg uzrokuju nanočestice u velikoj mjeri proizlaze iz nedosljednih *in vitro* ispitivanja. Kako bi se razjasnili ti učinci, naglašena je potreba provedbe *in vivo* ispitivanja. Ovaj pregledni rad sažima rezultate procjene genotoksičnih učinaka nanočestica koji su objavljeni u novijoj stručnoj i znanstvenoj literaturi. Navedeni rezultati pokazuju da određene nanočestice uzrokuju lomove u molekuli DNA i oštećuju kromosome u eksperimentalnim životinjama. Njihovi genotoksični učinci ne ovise samo o veličini čestice, modifikaciji površine (oblaganje čestice) i načinu izlaganja, već i o trajanju izloženosti nanočesticama. Postojeća istraživanja provedena na životinjama upućuju na različite mehanizme koji ovise o trajanju izlaganja i pomoću kojih živi organizmi reagiraju na doticaj s nanočesticama. Međutim postoje brojne nedosljednosti u novijoj literaturi, a standardne testne metode nisu dostupne pa je stoga pouzdanija procjena opasnosti od izlaganja nanomaterijalima u ljudi još uvijek veoma ograničena. Stoga se u međunarodnim dokumentima savjetuje oprez prilikom svakog izlaganja ljudi nanomaterijalima kako bi se spriječili mogući opći toksični genotoksični učinci.

KLJUČNE RIJEČI: *eksperimentalne životinje, nanomaterijali, neželjni učinci na zdravlje, oblaganje čestice, oštećenje DNA, oštećenje kromosoma*

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