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REVIEW

Toxicological effect of engineered nanomaterials on the liver

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The liver has a *crucial* role in metabolic homeostasis, as it is responsible for the storage, synthesis, metabolism and redistribution of carbohydrates, fats and vitamins, and numerous essential proteins. It is also the principal detoxification centre of the body, removing xenobiotics and waste products by metabolism or biliary excretion. An increasing number of studies have shown that some nanomaterials (NMs) are capable of distributing from the site of exposure (e.g. lungs, gut) to a number of secondary organs, including the liver. As a secondary exposure site the liver has been shown to preferentially accumulate NMs (>90% of translocated NMs compared with other organs), and alongside the kidneys may be responsible for the clearance of NMs from the blood. Research into the toxicity posed by NMs to the liver is expanding due to the realization that NMs accumulate in this organ following exposure via a variety of routes (e.g. ingestion, injection and inhalation). Thus it is critical to consider what advances have been made in the investigation of NM hepatotoxicity, as well as appraising the quality of the information available and gaps in the knowledge that still exist. The overall aim of this review is to outline what data are available in the literature for the toxicity elicited by NMs to the liver in order to establish a weight of evidence approach (for risk assessors) to inform on the potential hazards posed by NMs to the liver.

LINKED ARTICLES

This article is part of a themed section on Nanomedicine. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2014.171.issue-17

Abbreviations

DC, dendritic cell; ENPRA, risk assessment of engineered nanoparticles; KC, Kupffer cells; LC_{50}, concentration of the chemical that killed 50% of the cells; MIP-2, macrophage inflammatory protein 2-alpha; MWCNT, multi-walled carbon nanotube; NM, nanomaterial; ROS, reactive oxygen species; SWCNT, single-walled carbon nanotube; TEM, transmission electron microscopy

The rapid expansion of technological, scientific and commercial uses of atomic- or molecular-scale materials, their assembly and their unique properties, has led to an escalating interest in the fields of nanoscience, nanotechnology and nanomedicine (Maynard *et al*., 2006). In 2013, there were over 1300 consumer products on the market that claim to contain aspects of nanotechnology. These include a wide range of electronics, optics, and consumer products for soil and water remediation, or for medical uses such as therapeutics, diagnostics and drug delivery (Project on Emerging Nanotechnologies, 2013). However, due to their unique chemical and physical properties (size, shape, composition, charge, crystal structure, solubility, electrical conductance, etc.) there is concern that some nanomaterials (NMs) could be hazardous for people living and working with these materials (Hoet *et al*., 2004; Johnston *et al*., 2012). The small size of particulate NMs results in a high surface area to volume ratio, which potentially offers a greater biological activity per given mass compared with larger-size materials (Oberdorster *et al*., 2005). In addition to this, the surface reactivity per unit surface area can be greater at the nanoscale due to higher curvature of surface (Bhattacharya *et al*., 2012).

The prefix 'nano' has been specifically coined for materials containing tens to thousands of atoms, with dimensions in the scale of less than 100 nm (Buzea *et al*., 2007). It is this small size that is fundamental to the field of nanotechnology, although other particle parameters also determine their physical, biological and toxicological properties (Jin *et al*., 2008).

As the potential for public and occupational exposure is likely to rise with increasing production of NMs, there is an urgent need to consider the possibility of any detrimental health consequences of all the different types of NMs produced. Health risk is assessed based upon the level of exposure to the manufactured NM, toxicity of the material in question, route of exposure and the persistence in the organism of the particular material.

The lungs and the gastrointestinal tract are in constant contact with the external environment so it is not surprising to find that these systems are primary exposure sites for NMs (Chen *et al.*, 1999; Sadauskas *et al.*, 2009). It has been shown that different types of NMs can translocate from these primary exposure sites (Sadauskas *et al.*, 2009). As a secondary exposure site, the liver is extremely important, as it has been shown to accumulate NMs at much higher quantities compared with other organs (Semmler-Behnke *et al.*, 2008; Sadauskas *et al.*, 2009).

Hepatocytes constitute the major cellular compartment of the liver (approximately 65% of total liver volume). These cells are polyhedral multifaceted parenchymal cells with eight or more faces, and range between 25 and 30 μm in diameter (Kmiec, 2001). Hepatocytes participate in almost all functions that are attributed to the liver. They play a substantial role in the metabolism of exogenous and endogenous lipids and catabolism of blood-derived cholesterol-enriched proteins (Kmiec, 2001). These cells are also responsible for the manufacture of important serum proteins such as complement components and acute-phase proteins crucial in the mammalian innate immune system (Kmiec, 2001). Moreover, hepatocytes are capable of synthesizing numerous hormones and cytokines (Stadnyk *et al.*, 1990; Dong *et al.*, 1998; Kermanizadeh *et al.*, 2013b,c; Gaiser *et al.*, 2013).

Kupffer cells (KCs; 20% of total cell numbers in a healthy liver) are the resident macrophages of the liver. These cells represent the largest number of macrophages in the mammalian body. KCs eliminate both soluble and particulate antigens from the portal circulation and are responsible for the clearance of gut-derived bacteria and potential bacterial toxins such as endotoxins and peptidoglycans (Kmiec, 2001). These cells resemble other macrophages in the body – characterized by numerous microvillus projections, blebs and lamellipodia (Tiegs and Lohse, 2009). KCs are generally concentrated in the periportal region of the liver, which allows them to monitor the blood entering the organ (Kmiec, 2001). It is hypothesized that due to the constant exposure to low levels of gut-derived bacterial products, KCs are in a permanent semi-activated state (Tiegs and Lohse, 2009). Under pathological conditions, bacteria that bypass the intestinal barrier are the most important activators of KCs. These macrophages have an extremely large array of surface receptors designed for identification of most gut-derived antigens. In addition, similar to other macrophages, once activated they are capable of producing an array of mediators involved in a wide range of functions including: protein degradation, modulation of cell function and defence mechanisms and cytotoxicity (Baffy, 2009). Although KCs have the ability to initiate and sustain an immune response to eliminate pathogenic antigens, under normal circumstances they are extremely important in the maintenance of liver tolerance (in which the organ does not mount an immune response to the antigen; Tiegs and Lohse, 2009). It is understood that following the initial activation and production of a pro-inflammatory response, KCs release IL-10, which down-regulates the production of TNF-α and IL-6 and other potentially damaging pro-inflammatory cytokines (Tiegs and Lohse, 2009).

Although hepatocytes and KCs make up the large majority of the cell population of the liver, the organ also contains other cell types that are crucial to its normal function. These cells could also potentially be involved in the overall response of the liver to NMs and include sinusoidal endothelial cells (Fainboim *et al.*, 2007), hepatic stellate cells, CD1d – restricted T-cells, natural killer T-cells, αβ T-cells, γδ T-cells, pit cells (natural killer cells – $CD3^{-}CD56^{+}$) and small numbers of B lymphocytes (Tiegs and Lohse, 2009). In addition, there is a subset of professional antigen presenting cells resident in the liver, the dendritic cells (DC) responsible for processing and presenting antigens to lymphocytes. These consist of both myeloid and plasmacytoid DCs (Baffy, 2009).

Effects of engineered NMs on the liver – a summary of *in vitro* studies

Numerous studies have investigated the nanotoxicological effects of a wide range of engineered NMs in the liver utilizing *in vitro* models. This section will summarize some of these studies while attempting to form conclusions from the data available in the literature. This can be used to establish future testing strategies aimed at assessing the hepatic toxicity of NMs.

In a set of recent studies [FP7-funded risk assessment of engineered nanoparticles (ENPRA) project] the acute toxicological effects of a large panel of engineered NMs to C3A (a human hepatocellular carcinoma cell line) and primary human hepatocytes were investigated. The panel of NMs included two zinc oxide materials (ZnO; uncoated 100 nm and triethoxycaprylylsilane coated 130 nm), two different multi-walled carbon nanotubes (MWCNTs; D: 5–35 L: 700–3000; D: 6–20 L: 700–4000), Ag (<20 nm), a 7 nm TiO_2 anatase, two rutile TiO_2 NMs (10 and 94 nm) and two derivatives of the 10 nm rutile with positive and negative covalent functionalization (Kermanizadeh *et al.*, 2013a,b,c). Doses were expressed as $\mu g{\cdot}cm^{-2}$ because many of the NMs in this study settled relatively rapidly (80–0.16 $\mu g{\cdot}cm^{-2}$ equating to 256–0.5 $\mu g{\cdot}mL^{-1}$). The authors noted that the Ag NMs elicited the greatest level of cytotoxicity [24 h lethal concentration 50 (LC_{50}) – 2 $\mu g{\cdot}cm^{-2}$], followed by the uncoated ZnO (24 h LC_{50} – 7.5 $\mu g{\cdot}cm^{-2}$) and coated ZnO (24 h LC_{50} – 15 $\mu g{\cdot}cm^{-2}$) materials. The ZnO NMs were found to be about 40–50% soluble, which could account for their toxicity. In contrast the Ag NM was <1% soluble suggesting that solubility was less important for driving the Ag NM-induced effects. The LC_{50} was not attained in the presence of any of the other engineered NMs (up to 80 $\mu g{\cdot}cm^{-2}$).

All NMs significantly increased IL-8 protein production at 24 h post exposure. Meanwhile no significant change in TNF-α, IL-6 or C-reactive protein was detected. Urea and albumin production were measured as indicators of hepatic function. These markers were only altered by the coated and uncoated ZnO, which significantly decreased albumin production (Kermanizadeh *et al.*, 2013c). Furthermore, a dose-dependent decrease in the cellular glutathione content following exposure of the C3A cells to Ag, the ZnO and the MWCNTs was observed suggesting oxidative stress (Kermanizadeh *et al.*, 2012). Intracellular reactive oxygen species (ROS) levels were also measured and shown to increase significantly following exposure of the C3A to the NMs with relatively low toxicity (MWCNT and TiO_2). The antioxidant Trolox in part prevented the detrimental effect of NMs on cell viability, and suppressed the NM-induced IL-8 production after exposure to all materials with the exception of the Ag NM (Kermanizadeh *et al.*, 2012). In contrast, following a 4 h exposure of C3A cells to sub-lethal doses of the NMs, the largest amount of DNA damage was induced by two of the TiO_2 samples with relatively low toxicity (7 nm and the positively charged 10 nm materials; Kermanizadeh *et al.*, 2012). These findings indicate that cytotoxicity alone is not sufficient to rank the hazard of NMs *in vitro* and that they vary in their mechanism of toxicity. Therefore a battery of tests may be required for a comprehensive *in vitro* toxicity analysis.

The use of hepatocyte cell lines as a replacement for animal models has been heavily criticized mainly due to low expression of metabolic enzymes. The same authors compared the response of primary human hepatocytes to the C3A cell line with respect to their toxicological response to six of the NMs mentioned earlier (two ZnO, two MWCNTs, one Ag and one positively functionalized TiO_2; Kermanizadeh *et al.*, 2013b). The cell line was comparable to the primary hepatocytes with regards to the cytotoxic effects of the NMs with the same rating of toxicity being generated in both primary hepatocytes and the C3A cell line (Ag > uncoated ZnO > coated ZnO). The LC_{50} was not attained in the presence of the MWCNTs and the TiO_2 NMs (Kermanizadeh *et al.*, 2013b). All NMs significantly increased IL-8 production, with no change in levels of TNF-α and IL-6 (Kermanizadeh *et al.*, 2013b). Furthermore NM uptake was similar for both the primary hepatocytes and C3A cells as investigated by transmission electron microscopy (TEM; Kermanizadeh *et al.*, 2013b). This study demonstrated that the C3A cell line is a good model for investigating NM-induced hepatocyte responses with respect to uptake, cytotoxicity, pro-inflammatory effects and cytokine secretion.

The majority of *in vitro* nanotoxicological experiments only utilize a single-cell type. However, it is difficult to gain a realistic understanding of how NMs affect an organ when investigating one single-cell type alone. In a recent set of trials, primary rat KCs were incorporated into a primary rat hepatocyte culture (C. Filippi, A. Kermanizadeh and V. Stone, in preparation). The cells were then exposed to Ag (<20 nm) and positively charged anatase TiO_2 NMs (10 nm) for a 24 h period. The data suggest that KCs are important in the overall liver response to NMs and play an important role in the orchestration of the response. This was highlighted by the up-regulation of TNF-α and IL-6 observed when KCs were present when compared with a hepatocyte only *in vitro* system.

Numerous studies have identified Ag NMs as being relatively toxic to hepatocytes. For instance, in a recent study investigating the toxicological effects of a 24 h exposure to Ag (35 nm) and cerium dioxide (CeO_2 < 25 nm) NMs on the human hepatocyte cell line (C3A) and primary trout hepatocytes, the Ag NMs were found to be more toxic than the CeO_2 NMs to the hepatocytes with an LC_{50} of 50 $\mu g \cdot mL^{-1}$ for the C3A cells and 1000 $\mu g \cdot mL^{-1}$ for the trout hepatocytes (Gaiser *et al.*, 2011). Additionally confocal microscopy confirmed that agglomerates of both Ag and CeO_2 NM were internalized by the hepatocytes (Gaiser *et al.*, 2011).

In a similar study in the human hepatoma cell line HepG2, Ag NMs (5–10 nm) were again shown to be highly toxic to the cells as demonstrated by the MTT, Alamarblue and lactate dehydrogenase (LDH) assays, with an LC_{50} of 1.95–3.38 $\mu g \cdot mL^{-1}$ depending on the assay utilized (24 h exposure; Kim *et al.*, 2009). In this study, the toxicity observed was not associated with the release of Ag^+ ions. Furthermore it was suggested that the mechanism of toxicity was oxidative as the Ag NM toxicity was reduced following pretreatment with the hydrophilic antioxidant N-acetylcysteine (Kim *et al.*, 2009).

Similarly, exposure of human chang liver cells to Ag NMs (28–35 nm) resulted in a reduction in cell viability (with an LC_{50} of 4 $\mu g \cdot mL^{-1}$), ROS production and reduced glutathione depletion (Piao *et al.*, 2011). The authors suggest that Ag induced a decrease in cell viability via a mechanism involving apoptosis and DNA fragmentation.

In another study, C3A cells were exposed to Ag (<20 nm) NMs for 24 h. The Ag NMs were again shown to be highly cytotoxic (LC_{50} – 5 $\mu g \cdot cm^{-2}$; Gaiser *et al.*, 2013). These NMs were detected within the cytoplasm and the nucleus of hepatocytes. There was also an increased secretion of the neutrophil chemo-attractant IL-8 from the hepatocytes following exposure to sub-lethal concentrations of the Ag NMs (Gaiser *et al.*, 2013).

Treatment of Wistar primary liver cells with 40 and 80 nm Ag NMs for 24 h resulted in a significant decrease in mitochondrial membrane potential and an ADP-induced depolarization of the mitochondria. Hence, the authors suggested that the Ag NMs have detrimental effects on liver mitochondrial function (Teodoro *et al.*, 2011).

Looking at the literature available the data seem to indicate that Ag NMs (with seemingly different physiochemical properties) are relatively, highly cytotoxic to hepatocytes *in vitro*. Another important point worth mentioning is that in most studies oxidative stress has been suggested as the mechanism for the toxicity observed in these liver models.

Exposure of the HepG2 hepatocyte cell line to single-walled carbon nanotubes (SWCNTs; 1000 nm with diameter rage of 1–6 nm) induced oxidative stress and increased the proportion of apoptotic cells (Yuan *et al.*, 2012). In the same study, the hepatocytes were exposed to graphene oxide (GO) nanosheets (lateral dimension of 100 nm and a height of 1 nm). The authors observed that the GO NMs were less cytotoxic and suggested that these materials are more biocompatible with the hepatocytes *in vitro* (Yuan *et al.*, 2012).

It is extremely difficult to come to a definitive conclusion about the adverse effects of carbon nanotubes on the liver due to the sparse number of studies that have been carried out. However the data available seem to suggest that the nanotubes only induce low level toxic effects to hepatocytes *in vitro*.

In order to establish the adverse effect of a 4 nm TiO_2 NM a number of human and rat hepatocyte cell lines including the human hepatocellular carcinoma cell line (SMMC-7721), human liver cell line (HL-7702), rat hepatocarcinoma cell line (CBRH-7919) and rat liver cell line (BRL-3A) were utilized. Despite the varying degrees of cytotoxicity between the different cell types, an LC_{50} was not obtained at concentrations up to 100 μg·mL^{-1} suggesting the relatively low cytotoxicity of these NMs (Sha *et al.*, 2011). However, exposure of these cells of TiO_2 NM was associated with increased cellular ROS and decreased intracellular glutathione (GSH) levels (Sha *et al.*, 2011).

Exposure of primary trout hepatocytes to 5 nm anatase TiO_2 for 96 h resulted in an LC_{40} at 30 μg·mL^{-1}. Furthermore, the authors did not observe any increased ROS formation above the control levels (Thomas *et al.*, 2011). In a similar study exposure of HepG2 cells to 30–70 nm anatase TiO_2 for 24 h resulted in significant oxidative DNA damage and apoptosis, as demonstrated by an up-regulation in the expression of p53, Bax, Apaf-1 and cyto-1 within the cells (Shukla *et al.*, 2013).

Currently there are conflicting views on the adverse effects of TiO_2 NMs *in vitro*. However, the majority of data suggest that these NMs are of relatively low toxicity. This being said many authors have reported adverse effects at sub-lethal doses of these NMs particularly with regards to genotoxicity.

Exposure of rat liver slices to 5 nm Au NMs for 24 h did not result in any cytotoxic effects as assessed utilizing the LDH and MTT assays, despite the uptake of NMs by the hepatocyte (this seems to indicate that uptake is not necessarily equated with cytotoxicity; Dragoni *et al.*, 2012). In addition there was no reduction in intracellular glutathione levels within the cells up to concentrations of 500 μM (Dragoni *et al.*, 2012).

It is difficult to summarize and compare all the nanotoxicological *in vitro* data available in the liver as all the experiments have a number of different variables. Differences exist between NMs (even if they are the same type), in the concentrations utilized, preparation of NMs, exposure times, the use of cell lines or primary cells, species and the media and the serum protein used in each experiment. However, there are some recognizable patterns among most if not all of the available literature. Firstly, different NMs can be roughly classified according to their cytotoxicity to liver cells *in vitro* in the order of Ag (Table 1 demonstrates the toxic nature of different Ag NMs in different studies) > ZnO > SWCNT > Al_2O_3 > TiO_2 > MWCNT > Ce_2O_3 and Au. It appears that in most instances the highly soluble NMs are more toxic than their insoluble counterparts (however, exceptions to this do exist i.e. Kim *et al.*, 2009; Kermanizadeh *et al.*, 2013c). A note of caution is therefore advised, as some or most of the toxicity mentioned earlier might be due to release of metal ions. Despite this some studies have shown that there is a clear 'nano-effect', which exceeds the toxicity of the equivalent amount of soluble metals. One suggested mechanism of soluble NM toxicity is the transport of materials into the cells and dissolution in the acidic environment of the lysosomes, with large amounts of ions being released (Stern *et al.*, 2012). In addition NMs, such as carbon nanotubes, are known to have metal impurities, which may cause oxidative stress when released inside the cell. In addition enzymatic biodegradation of carbon nanotubes could contribute to their overall toxicity.

In the majority of experiments oxidative stress has been suggested as the main mechanism of toxicity and sub-lethal changes to the liver cells *in vitro*. The recent abundant evidence suggesting the involvement of oxidative stress in the pathogenesis of various disorders and diseases has attracted much attention both in the scientific community and general public. ROS and other free radicals are critical intermediates in the normal physiology and pathophysiology of the liver in particular with regards to the hepatocytes (Diesen and Kuo, 2009). ROS are essential for many normal physiological functions. They are implicated in cell signalling and are considered to be the second messengers that can trigger cytokine, hormone and growth factor release from certain cells (Diesen and Kuo, 2009). ROS can also affect gene expression, as well as playing a role in the normal induction of apoptosis, although the exact mechanisms are currently unclear (Diesen and Kuo, 2009). Since ROS are ubiquitous in the normal physiology of so many processes, it is not surprising that when excess ROS are produced they subsequently affect many normal functions of a healthy cell. ROS are important in the creation of oxidative stimuli required for normal physiological homeostasis of hepatocytes. However, the equilibrium between ROS generation and the antioxidant defence within a cell can be disrupted resulting in an overall net oxidative stress (Kang, 2002). In the liver, free radicals triggered by ROS are created by the neutrophils, KCs, mitochondria and cytochrome P450 (Kang, 2002). The damage created by oxidative stress can affect all cells within the liver by inducing inflammation, ischaemia, apoptosis and necrosis (Lieber, 1997). It is believed that ROS also affect signal transduction pathways that when unbalanced may

Table 1

The toxicity of Ag NMs in a selected number of studies

Publication	Ag NM utilized	Model(s)	24 h – LC_{50}
Gaiser *et al.*, 2011	Ag 35 nm	C3A cells	50 μg·mL^{-1}
		Primary trout hepatocytes	1000 μg·mL^{-1}
Kim *et al.*, 2009	Ag 5–10 nm	HepG2 cells	1.95–3.38 μg·mL^{-1}
Piao *et al.*, 2011	Ag 28–35 nm	Chang liver cells	4 μg·mL^{-1}
Kermanizadeh *et al.*, 2012; 2013b,c	Ag <20 nm	C3A cells	1.25 μg·cm^{-2}
		Primary human hepatocytes	2.5 μg·cm^{-2}
		Primary rat Kupffer cells and hepatocytes	1.25 μg·cm^{-2}

lead to hepatic inflammation, necrosis, fibrosis and/or apoptosis (Diesen and Kuo, 2009).

The data from the literature indicate that the use of sub-lethal NM concentrations is critical with regards to mechanistic studies, otherwise the formation of any meaningful conclusions about the cause and effect and the mechanism of action is almost impossible. Finally, it seems that NMs with relatively low toxicity may still possess sub-lethal effects with toxicological consequences.

Effects of engineered NMs on the liver – a summary of *in vivo* studies

Exposure via the i.v. route

A bio-distribution study in which adult Wister rats were exposed to 20 nm Au NMs via an i.v. route, and in which NM localization was examined after 1 day, 1 week and 2 months showed that the NMs accumulated very rapidly in the liver (1 day – 49.4 ± 50.4 $ng \cdot g^{-1}$; 1 week 64.8 ± 39.7 $ng \cdot g^{-1}$; 2 months 72.2 ± 40.5 $ng \cdot g^{-1}$; Balasubramanian *et al.*, 2010). The authors also showed changes in the expression of genes in the organ related to detoxification, lipid metabolism and the cell cycle.

In another set of experiments i.v. treatment of male Wistar rats with 15 and 55 nm amorphous silica NMs (50 $mg \cdot kg^{-1}$) and 2, 20 and 200 nm Au NMs (6 μg of Au NMs per animal) resulted in DNA damage in the liver by the silica NMs only (no genotoxicity was observed following exposure to any of the Au NMs; Downs *et al.*, 2012). An influx of leukocytes as well as increased necrosis and apoptosis was observed following exposure to the silica NMs in the liver (Downs *et al.*, 2012).

A recent study investigated the effects of i.v. administration of a panel of NMs, consisting of two ZnO materials (coated 100 nm and uncoated 130 nm), two MWCNTs, one Ag (<20 nm) and one 10 nm positively charged rutile TiO_2, on the liver of C57/BL6 mice (Kermanizadeh *et al.*, 2013a). The animals were injected with either a single dose of NM (12.8 μg per animal) or three doses (6.4 μg per animal) every 24 h. Animals were killed 6, 24, 48 and 72 h after the single i.v. injection or 72 h after the triple injection regime. A wide array of NMs induced a neutrophil influx into the liver as early as 6 h post i.v. injection. However, the neutrophils were only involved in the initial phases of the immune response against the NMs as the leukocyte numbers had returned to control levels after 48 h. Furthermore the authors investigated whether Ag and the TiO_2 NMs depleted glutathione in the liver and found no significant effect on total GSH following exposure to the chosen NMs after a 24 h exposure (Kermanizadeh *et al.*, 2013a). Finally, the authors noted an up-regulation of IL-10, CXCL2 and ICAM-1 mRNA as well as a decrease in C3 and IL-6 in the livers of animals treated with the NMs (Kermanizadeh *et al.*, 2013a).

In female Wistar rats injected with 20 nm Ag NMs i.v., an up-regulation of a number of pro-inflammatory genes was observed in the liver after 24 h, including MIP-2, IL1R-1 and TNF-α (Gaiser *et al.*, 2013). Particles were detected by TEM in hepatocytes and KCs in both the cytoplasm and nuclei. Reduced glutathione levels in the liver were unaltered (Gaiser *et al.*, 2013).

Changes in the expression of genes have also been observed in the livers of BALB/c mice 30 min following i.v. injections of to 4 and 100 nm PEG Au-coated NMs; microarray analysis showed the changes occurred in genes associated with apoptosis, cell cycle, inflammation and metabolic processes (Cho *et al.*, 2009). The authors did not notice any significant differences between the effects of 4 and 100 nm Au NMs (Cho *et al.*, 2009).

KCs were identified as being very important for the removal of 2 and 40 nm Au NMs following their i.v. administration to C57/BL6 mice (Sadauskas *et al.*, 2007). Also i.v. injections of these NMs resulted in their rapid accumulation in the resident liver macrophages (Sadauskas *et al.*, 2007).

Intratracheal instillation (i.t.) route

In a study in which female Wistar rats were exposed to 1.4 and 18 nm Au NMs i.t., the labelled NMs translocated (up to 8% of total dose) from the lungs to secondary organs, one of which was the liver (1% of total administered Au NMs; Semmler-Behnke *et al.*, 2008). It is not surprising to note that the authors suggest that the route of the exposure is crucial in the proportion of NMs ending up in secondary organs (Semmler-Behnke *et al.*, 2008). In another study in which C57/BL6 mice were treated with 2, 40 and 100 nm Au NMs administered i.t., the 2 nm Au materials translocated to the liver (Sadauskas *et al.*, 2009).

A recent study in which male Sprague-Dawley rats were exposed to 20 nm CeO_2 NMs via the i.t. route resulted in the accumulation of NMs in the liver, elevations in serum alanine transaminase levels and a reduction in albumin levels (Nalabotu *et al.*, 2012). The authors also showed that the animals exposed to the NMs had a reduced overall liver weight, enlarged hepatocytes, sinusoidal dilatations and an accumulation of granular materials. The authors suggest that exposure to CeO_2 via the lungs (i.t.) can result in detrimental effects in the liver (Nalabotu *et al.*, 2012).

In another study the oxidative effect (glutathione depletion) and gene expression response of C57/BL6 mice liver tissue 24 h following the i.t. administration of NMs (via the lungs) was assessed. The mice were exposed to two ZnO materials (uncoated 100 nm and triethoxycaprylylsilane coated 130 nm), two MWCNTs (D: 5–35 L: 700–3000; D: 6–20 L: 700–4000), Ag (<20 nm), 7 nm TiO_2 anatase, two rutile TiO_2 NMs (10 and 94 nm) and two derivatives of the 10 nm rutile with positive and negative covalent functionalization (1, 4, 8, 16, 32, 64 and 128 μg of different NMs per animal; I. Gosens *et al.*, submitted). The study showed that the i.t. instilled Ag, ZnO and positively charged TiO_2 resulted in acute distal effects on the liver that involved glutathione depletion, while exposure to all NMs, with the exception of the MWCNTs, resulted in changes in gene expression in the liver most of which were anti-inflammatory genes. The authors did not associate these changes with toxicity.

In a recent study, exposure of C57/BL6 BomTac pregnant mice to carbon black Printex 90 via the i.t. route resulted in detrimental effects on the newborn (Jackson *et al.*, 2012). The newborn mice showed changes in the levels of expression of mRNA of hepatic genes associated with inflammation, cell cycle and lipid metabolism. Furthermore the authors concluded that the effects were more pronounced in the female offspring (Jackson *et al.*, 2012).

Oral route

The oral route exposure of male Swiss albino mice to 30 nm ZnO NMs resulted in the accumulation of materials within the liver. This accumulation was associated with an elevation in the serum levels of alanine aminotransferase and alkaline phosphatase, as well as pathological lesions in the liver (Sharma *et al.*, 2012). ZnO NM exposure resulted in oxidative stress, significant DNA damage and the induction of apoptosis in the liver compared with control animals (Sharma *et al.*, 2012). It is important to state that there are very few ingestion studies in which the effects of NMs on the liver have been investigated.

In summarizing the liver-related *in vivo* experiments, a few comparable outcomes are notable. Firstly, the route of exposure is extremely important in determining the proportion of the NM dose that reaches the liver. The largest proportion of NM dose reaching the liver occurred following an i.v. exposure. The size of the NM itself also seems to be important as smaller NMs reach the liver in higher quantities (Sadauskas *et al.*, 2009) especially following translocation from the lungs (in reality, this is due to the levels of NM penetrating through the lung rather than a specific liver effect). It has also been suggested that the route of exposure is extremely important in determining which proteins are acquired on the surface of NMs (Johnston *et al.*, 2012). This protein corona can and does influence the toxicity of the NM in the liver (Elbakry *et al.*, 2012; Johnston *et al.*, 2012). Therefore the overall toxicity of NM is not only dependent on its physico-chemical characteristics, but also on surface proteins recognized by the cells (i.e. how a NM is coated; Fadeel, 2012).

In vitro versus *in vivo* systems comparisons and limitations

As it has been shown that the potential of NMs for translocation to the liver is a realistic prospect and this organ accumulates a large proportion of the total translocated dose. Therefore, it is essential that the dangerous effects of NM exposure on normal liver function are thoroughly investigated. Although huge advances have been made in identifying the potential nanotoxicological effects on the liver, there are still gaps in the literature.

Very few studies have attempted to make a direct comparison between *in vitro* and *in vivo* liver models. However, two recent sets of trials conducted as part of European funded projects – ENPRA and InLiveTox have attempted to make this comparison. In these studies the adverse effects of a panel NMs on the liver were assessed using a hepatocyte cell line, primary human hepatocytes and liver tissues from exposed animals to determine if the response observed in the *in vitro* systems was indeed mirrored and representative of cells *in vivo*. The results show that there are some promising comparisons between certain NM-induced end points using the cell line (C3A) and primary liver cells (primary human hepatocytes), primary mice and rat liver tissue (Table 2). The data from these two recent studies suggest that simple *in vitro* test models can be extremely valuable in predicting the potential liver response *in vivo*. However, *in vitro* studies have some major limitations that need to be discussed.

It is often very difficult to make a direct comparison between cells *in vitro* and tissue responses *in vivo*. At best, *in vitro* findings can act as an indicator of possible *in vivo* responses. One principle reason for this is that the comparisons between the systems are rarely like for like, that is cytotoxicity in an *in vitro* system is not inflammation *in vivo*, and the utilization of doses that would cause liver cells to die *in vivo* would be unrealistic and unethical. In addition, these high doses would mask any sub-lethal effects. The limitations continue as an organ is never comprised of only a single cell type, and crosstalk between different cell types and different organs is essential in the overall response to a toxic challenge. This being said increasing numbers of new sophisticated *in vitro* models such as three-dimensional culture, tissue slices and fluidic models, as used in the InLiveTox project, are being developed in order to improve *in vitro* risk assessment, with a view to reducing, refining and replacing animal studies.

Table 2

General similarities for certain investigated end points between the ENPRA and InLiveTox projects (table offers a simplified summary)

End point	C3A cells	Primary human hepatocytes	Rat liver	Mice liver
Cytotoxicity	YES	YES	NA	NA
Antioxidant depletion	YES	NA	YES	NO (i.v. exposure) / YES (i.t. exposure)
Changes in gene expression	YES	NA	YES	YES
DNA damage	YES	NA	NA	NO
Cytokine production	YES	YES	NA	YES
NM Uptake	YES	YES	YES	YES
Functional markers	YES	YES	NO	NA

NA, not tested or not relevant.

In an *in vitro* system, soluble NMs remain trapped in the well, whereas soluble material constituents can disperse in an *in vivo* model. Likewise, many particle types will be removed from the site of deposition by phagocytes and eventually excreted. As already mentioned it is very unlikely that any NM will reach the liver without a protein coating, which may influence its overall toxicity to the organ. It is often very difficult to reproduce the exact protein corona in an *in vitro* study; however, the route of exposure and translocation can be used to improve the preparation and dispersion of NMs.

Although some attempts have been made to improve *in vitro* testing systems, the use of animal models is still in all probability the most reliable representation of a whole organ/body response to foreign materials such as NMs. However, the high cost and ethical implications of any *in vivo* study must be fully appraised.

The future

With the advances in the fields of nanotechnology and nanomedicine, the potential for public and occupational exposure is likely to increase, so there is an urgent need to consider the possibility of any detrimental health effects associated with this increased exposure to NMs. Hence it is crucial to identify the dangers associated with NM exposure both *in vitro* and *in vivo*, consequently assembling a knowledge base of the human health effects associated with NM exposure (Lin *et al*., 2012). Engineered NMs are manufactured from a diverse group of substances and can have very diverse physicochemical characteristics such as size, shape, surface charge, surface reactivity, crystalline phase, polarity, solubility or impurities. Hence, a range of materials with different characteristics needs to be evaluated for a comprehensive toxicity profile, which would allow structure activity relationships to be generated. Likewise, standardization of methods such as particle preparation and exposure conditions are essential to be able to compare studies carried out in different laboratories – ensuring that any differences in toxicological responses are due to the materials and not the methodology.

Therefore one of the most important reasons for conducting nanotoxicology studies is to provide a knowledge base towards assessing the risks associated with realistic NM exposures. The findings obtained in such studies should provide hazard data for the NMs that will be used for risk assessment purposes to determine any health implications associated with these NMs. For this purpose there are certain areas in liver nanotoxicology in which knowledge is severely lacking.

To our knowledge there have been very few studies if any that have investigated the effects of NMs on the liver following inhalation exposure (in all reality the most prominent route of NM exposure). It is important to note that inhalation and instillation are not always comparable – for example the deposition patterns of the particles in the lung can vary between the two methods meaning that potential adverse effects observed following intratracheal administration might not necessarily be seen following inhalation.

One of the most important paradigms of risk assessment is exposure. From the literature it is evident that there is a clear lack of studies in which low realistic relevant exposure scenarios have been employed that can be used for risk assessment purposes concentrating on the liver. Hence, there is a real need for long-term studies in which animals are exposed to low doses of NMs via different routes (i.e. inhalation, ingestion and i.v. routes). This will be the only means by which a realistic and reliable liver risk assessment model can be formulated.

Conflict of interest

The authors declare that they are no competing interests.

References

Baffy G (2009). Kupffer cells in non-alcoholic fatty liver disease: the emerging view. J Hepatol 51: 212–223.

Balasubramanian SK, Jittiwat J, Manikandan J, Ong CN, Yu LE, Ong WY (2010). Biodistribution of gold nanoparticles and gene expression changes in the liver and spleen after intravenous administration in rats. Biomaterials 13: 2034–2042.

Bhattacharya K, Hoffmann E, Schins RF, Prantl EM, Alink GM, Byrne HJ *et al*. (2012). Comparison of micro- and nanoscale Fe^{3+} containing (hematite) particles for their toxicological properties in human lung cells *in vitro*. Toxicol Sci 126: 173–182.

Buzea C, Pacheco II, Robbie K (2007). Nanomaterials and nanoparticles: sources and toxicity. Biointerphases 2: 18–67.

Chen C, Zhang P, Hou X, Chai Z (1999). Sub-cellular distribution of selenium and Se-containing proteins in human liver. Biochim Biophys Acta 1427: 205–215.

Cho WS, Kim S, Han BS, Son WC, Jeong J (2009). Comparison of gene expression profiles in mice liver following intravenous injection of 4 and 100 nm sized PEG coated gold nanoparticles. Toxicol Lett 191: 96–102.

Diesen DL, Kuo PC (2009). Nitric oxide and redox regulation in the liver: Part I. General considerations and redox biology in hepatitis. J Surg Res 162: 95–109.

Dong BW, Liang P, Yu XL, Zeng XQ, Wang PJ, Su L *et al*. (1998). Sonographically guided microwave coagulation treatment of liver cancer: an experimental and clinical study. Am J Roentgenol 171: 449–454.

Downs TR, Crosby ME, Hu T, Kumar S, Sullivan A, Sarlo K *et al*. (2012). Silica nanoparticles administrated at the maximum tolerated dose induce genotoxic effects through an inflammatory reaction while gold nanoparticles do not. Mutat Res 745: 38–50.

Dragoni S, Franco G, Regoli M, Bracciali M, Morandi V, Sgaragli G *et al*. (2012). Gold nanoparticles uptake and cytotoxicity assessed on rat liver precision cut slices. Toxicol Sci 128: 186–197.

Elbakry A, Wurster EC, Zaky A, Liebl R, Schindler E, Baur-Kreisel P *et al*. (2012). Layer-by-layer coated gold nanoparticles: size-dependant delivery of DNA into cells. Small 8: 3847–3856.

Fadeel B (2012). Clear and present danger? Engineered nanoparticles and the immune response. Swiss Med Wkly 142: w13609. doi: 10.4414/smw.2012.13609.

Fainboim L, Chernavsky A, Paladino N, Flores AC, Arruvito L (2007). Cytokines and chronic liver disease. Cytokine Growth Factor Rev 18: 143–157.

Gaiser BK, Fernandes TF, Jepson MA, Lead JR, Tyler CR, Baalousha M *et al*. (2011). Interspecies comparisons on the uptake and toxicity of silver and cerium dioxide nanoparticles. Environ Toxicol Chem 31: 144–154.

Gaiser BK, Hirn S, Kermanizadeh A, Kanase N, Fytianos K, Wenk A *et al*. (2013). Effects of silver nanoparticles on the liver and hepatocytes *in vitro*. Toxicol Sci 131: 537–547.

Hoet PHM, Hohlfeld IB, Salata O (2004). Nanoparticles – known and unknown health risks. J Nanobiotechnology 2: 12–27.

Jackson P, Hougaard KS, Vogel U, Wu D, Casavant L, Williams A *et al*. (2012). Exposure of pregnant mice to carbon black by intratracheal instillation: toxicogenomic effects in dams and offspring. Mutat Res 745: 73–83.

Jin CY, Zhu BS, Wang XF, Lu QH (2008). Cytotoxicity of Titanium dioxide nanoparticles in mouse fibroblast cells. Chem Res Toxicol 21: 1871–1877.

Johnston H, Brown D, Kermanizadeh A, Gubbins E, Stone V (2012). Investigating the relationship between nanomaterial hazard and physicochemical properties: informing the exploitation of nanomaterials with therapeutic and diagnosis applications. J Control Release 164: 307–313.

Kang KJ (2002). Mechanism of hepatic ischemia/reperfusion injury and protection against reperfusion injury. Transplant Proc 34: 2659–2661.

Kermanizadeh A, Gaiser BK, Hutchison GR, Stone V (2012). An *in vitro* liver model – assessing oxidative stress and genotoxicity following exposure of hepatocytes to a panel of engineered nanoparticles. Part Fibre Toxicol 9: 28. doi: 10.1186/1743-8977-9-28.

Kermanizadeh A, Brown DM, Hutchison G, Stone V (2013a). Engineered nanomaterial impact in the liver following exposure via an intravenous route – the role of polymorphonuclear leukocytes and gene expression in the organ. Nanomedicine Nanotechnol 4: 157. doi:10.4172/2157-7439.1000157.

Kermanizadeh A, Gaiser BK, Ward MB, Stone V (2013b). Primary human hepatocytes vs. hepatic cell line – assessing their suitability for *in vitro* nanotoxicology. Nanotoxicology 7: 1255–1271. doi: 10.3109/17435390.2012.734341.

Kermanizadeh A, Pojana G, Gaiser BK, Birkedal R, Bilaničová D, Wallin H *et al*. (2013c). *In vitro* assessment of engineered nanomaterials using C3A cells: Cytotoxicity, pro-inflammatory cytokines and function markers. Nanotoxicology 7: 301–313. doi: 10.3109/17435390.2011.653416.

Kim S, Choi JE, Choi J, Chung KH, Park K, Yi J *et al*. (2009). Oxidative stress-dependant toxicity of silver nanoparticles in human hepatoma cells. Toxicol in Vitro 23: 1076–1084.

Kmiec Z (2001). Cooperation of liver cells in health and disease. Adv Anat Embryol Cell Biol 161: 1–151.

Lieber CS (1997). Role of oxidative stress and antioxidant therapy in alcoholic and non alcoholic liver diseases. Adv Pharmacol 38: 601–628.

Lin HJ, Bu Q, Cen XB, Zhao YL (2012). Current methods and research progress in nanomaterials risk assessment. Curr Drug Metab 13: 354–363.

Maynard AD, Aitken RJ, Butz T, Colvin V, Donaldson K, Oberdorster G *et al*. (2006). Safe handling of nanotechnology. Nature 444: 267–269.

Nalabotu SK, Kolli MB, Triest WE, Ma JY, Manne NDPK, Katta A *et al*. (2012). Intratracheal instillation of cerium oxide nanoparticles induces hepatic toxicity in male Sprague-Dawley rats. Int J Nanomedicine 6: 2327–2335.

Oberdorster G, Maynard A, Donaldson K, Castronova V, Fitzpatrick J, Ausman K *et al*. (2005). Principles for characterising the potential human health effects from exposure to nanomaterials: elements of a screening strategy. Part Fibre Toxicol 2: 8. doi: 10.1186/1743-8977-2-8.

Piao MJ, Kang KA, Lee IK, Kim HS, Kim S, Choi JY *et al*. (2011). Silver nanoparticles induce oxidative cell damage in human liver cells through inhibition of reduced glutathione and induction of mitochondria-involved apoptosis. Toxicol Lett 201: 92–100.

Project on Emerging Nanotechnologies (2013). Consumer products inventory. Available at: http://www.nanotechproject.org/cpi (accessed 10/2/2013).

Sadauskas E, Wallin H, Stoltenberg M, Vogel U, Doering P, Larsen A *et al*. (2007). Kupffer cells are central in the removal of nanoparticles from the organism. Part Fibre Toxicol 4: 10. doi: 10.1186/1743-8977-4-10.

Sadauskas E, Jacobsen NR, Danscher G, Stoltenberg M, Vogel U, Larsen A *et al*. (2009). Biodistribution of gold nanoparticles in mouse lung following intratracheal instillation. Chem Cent J 3: 16. doi: 10.1186/1752-153X-3-16.

Semmler-Behnke M, Kreyling WG, Lipka J, Fertsch S, Wenk A, Takenaka S *et al*. (2008). Biodistribution of 1.4- and 18 nm gold particles in rats. Small 12: 2108–2111.

Sha BY, Gao W, Wang SQ, Feng X, Lu T (2011). Cytotoxicity of titanium dioxide nanoparticles in four liver cells from human and rat. Composites 42: 2136–2144.

Sharma V, Singh P, Pandey AK, Dhawan A (2012). Induction of oxidative stress, DNA damage and apoptosis in mouse liver after sub-acute exposure to zinc oxide nanoparticles. Mutat Res 745: 84–91.

Shukla RK, Kumar A, Gurbani D, Pandey AK, Singh S, Dhawan A (2013). TiO_2 nanoparticles induce oxidative DNA damage and apoptosis in human liver cells. Nanotoxicology 7: 48–60.

Stadnyk AW, Baumann H, Gauldie J (1990). The acute-phase protein response in parasite infection. *Nippostrongylus brasiliensis* and *Trachinella spiralis* in the rat. Immunology 69: 588–595.

Stern ST, Adiseshaiah PP, Crist RM (2012). Autophagy and lysosomal dysfunction as emerging mechanisms of nanomaterial toxicity. Part Fibre Toxicol 9: 20. doi: 10.1186/1743-8977-9-20.

Teodoro JS, Simoes AM, Duarte FV, Rolo AP, Murdoch RC, Hussain SM *et al*. (2011). Assessment of toxicity of silver nanoparticles *in vitro*: a mitochondrial perspective. Toxicol in Vitro 2: 664–670.

Thomas KV, Farkas J, Farman E, Christian P, Langford K, Wu Q *et al*. (2011). Effects of dispersed aggregates of carbon and titanium dioxide engineered nanoparticles on rainbow trout hepatocytes. J Toxicol Environ Health 74: 466–477.

Tiegs G, Lohse AW (2009). Immune tolerance: what is unique about the liver. J Autoimmun 34: 1–6.

Yuan J, Gao H, Sui J, Duan H, Chen WN, Ching CB (2012). Cytotoxicity evaluation of oxidized single-walled carbon nanotubes and grapheme oxide on human hepatoma HepG2 cells: an iTRAQ-coupled 2D LC-MS/MS proteome analysis. Toxicol Sci 12: 149–161.