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An *in vitro* assessment of panel of engineered nanomaterials using a human renal cell line: cytotoxicity, pro-inflammatory response, oxidative stress and genotoxicity

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Background: It has been shown that nanomaterials (NMs) are able to translocate to secondary tissues one of the important being the kidneys. Oxidative stress has been implicated as a possible mechanism for NM toxicity, hence effects on the human renal proximal tubule epithelial cells (HK-2) treated with a panel of engineered nanomaterials (NMs) consisting of two zinc oxide particles (ZnO - coated - NM 110 and uncoated - NM 111), two multi walled carbon nanotubes (MWCNT) (NM 400 and NM 402), one silver (NM 300) and five TiO₂ NMs (NM 101, NRCWE 001, 002, 003 and 004) were evaluated.

Methods: In order to assess the toxicological impact of the engineered NMs on HK-2 cells - WST-1 cytotoxicity assay, FACSArray, HE oxidation and the comet assays were utilised. For statistical analysis, the experimental values were compared to their corresponding controls using an ANOVA with Tukey's multiple comparison.

Results: We found the two ZnO NMs (24 hr LC₅₀ - 2.5 µg/cm²) and silver NM (24 hr LC₅₀ - 10 µg/cm²) were highly cytotoxic to the cells. The LC₅₀ was not attained in the presence of any of the other engineered nanomaterials (up to 80 µg/cm²). All nanomaterials significantly increased IL8 and IL6 production. Meanwhile no significant change in TNF-α or MCP-1 was detectable. The most notable increase in ROS was noted following treatment with the Ag and the two ZnO NMs. Finally, genotoxicity was measured at sub-lethal concentrations. We found a small but significant increase in DNA damage following exposure to seven of the ten NMs investigated (NM 111, NRCWE 001 and NRCWE 003 being the exception) with this increase being most visible following exposure to Ag and the positively charged TiO₂.

Conclusions: While the NMs could be categorised as low and highly cytotoxic, sub-lethal effects such as cytokine production and genotoxicity were observed with some of the low toxicity materials.

Keywords: Kidneys, Nanotoxicity, Inflammation, ROS, DNA damage

Background

The prefix “nano” was specifically coined for particles containing tens or hundreds of atoms, with dimensions at the scale of less than 100 nm [1]. It is this small size which is fundamental to the field of nanotechnology, although other physicochemical properties influence the physical, biological and toxicological properties of these manufactured materials [2]. The principal reason for

producing and exploiting nanomaterials (NMs) is that their behaviour is fundamentally different from the bulk form of the same compound [2]. Accordingly, previously unexploited beneficial properties of a given material can be explored in its nano size range [2]. As a result the amount of interest in nanotechnology has risen exponentially across many fields aiming to exploit a wealth of opportunities. These applications include medicine, cosmetics, textiles, electronics and engineering. However, there is concern that the increased release and exposure to these engineered NMs could be potentially hazardous

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in an occupational and consumer setting [3-5]. A thorough risk evaluation is urgently required to provide an improved understanding of the implication of exposure to different NMs and any potential threat to humans or the environment [6]. A very effective strategy for achieving this is in the form of a critical risk assessment. Risks are assessed based upon the level of exposure to the manufactured NM, toxicology (hazard) of the material in question, route of exposure and its bio-persistence. Hence, it is necessary to identify the hazards associated with NM exposure both *in vitro* and *in vivo*, to assemble a knowledge base on the potential toxicity associated with NM exploitation on human health [4].

It is widely accepted that the skin, lungs and the gastrointestinal tract (GIT) are in constant contact with the external environment, and it is thus not surprising to find all three systems being primary exposure sites for NMs [5]. However following exposure some NMs are able to translocate to secondary target organs located at a distance from the original point of entry, which includes the kidneys [7,8].

The kidneys are principally responsible for the removal of metabolic waste such as urea and ammonia. However it is believed that other waste products and toxic substances such as NMs could also be excreted through urine [9]. The kidneys receive blood from the renal arteries which branch directly from the dorsal aorta. Despite their relatively small size, the kidneys receive approximately 20% of the entire cardiac output [10], making the organs highly susceptible to xenobiotics such as NMs [11]. In theory both the glomerular structures (during plasma filtration) and tubular epithelial cells may be exposed to NMs. Since the major function of the kidneys is to eliminate a variety of potentially harmful substances (including the potential excretion of NMs), these organs are extremely important targets for investigation with regards to nanomaterial exposure and hazard.

Previous experiments have demonstrated that mice exposed to copper NMs had severe damage to the proximal tubular cells [11], while Wang and colleagues observed glomerulonephritis and pathological degeneration of the renal proximal convoluted tubules following oral TiO₂ administration [12]. Proximal tubule cells exhibit well developed basal infolding and an apical brush border, enabling intense pinocytotic activity and variable transport and co-transport of particles [13], therefore these cells would be an important target for a nanotoxicological investigation. Hence, this *in vitro* study conducted as part of the European funded project ENPRA (Risk assessment of engineered nanoparticles) investigated the adverse effects of a panel of widely utilised engineered NMs (five TiO₂, two ZnO, two MWCNTs and one Ag) on the immortalized adult human renal proximal tubule epithelial cells (HK-2) with

respect to cytotoxicity, oxidative stress, cytokine secretion and DNA damage.

Methods

Nanomaterials

Nanomaterials were purchased as stated: NM 101 (Hombikat UV100; rutile with minor anatase; 7 nm), NM 110 (BASF Z-Cote; zincite, uncoated, 100 nm), NM 111 (BASF Z-Cote; zincite coated with triethoxycaprylylsilane, 130 nm), NM 300 (RAS GmbH; Ag capped with polyoxylaurat Tween 20 - < 20 nm), NM 400 (Nanocyl; entangled MWCNT, diameter 30 nm, length 5 µm), NM 402 (Arkema Graphistrength C100; entangled MWCNT, diameter 30 nm, length 20 µm). The above mentioned nanomaterials were sub-sampled under Good Laboratory Practice conditions and preserved under argon in the dark until use. These NMs were received from the European Commission Joint Research Centre (Ispra, Italy). The NRCWE samples were procured by the National Research Centre for the Working Environment. Sub-sampling was completed into 20 ml Scint-Burk glass pp-lock with Alu-Foil (WHEA986581; Wheaton Industries Inc.) after pooling and mixing of the material. NRCWE 001, TiO₂ rutile 10 nm was purchased from NanoAmor (Houston, USA) and also used for production of NRCWE 002 (TiO₂ rutile 10 nm with positive charge) and NRCWE 003 (TiO₂ rutile 10 nm with negative charge) using the procedures described previously [14]. NRCWE 004 (TiO₂ rutile 94 nm) was purchased from NaBond.

Characterisation of the panel of engineered nanomaterials

All the nanomaterials used in this study were characterised by a combination of analytical techniques in order to infer primary physical and chemical properties useful to understand their toxicological behaviour. A comprehensive list of the main physical and chemical properties of the panel NMs has been shown (Table 1) [Reproduced and modified from 14]. Furthermore the hydrodynamic size distributions and zeta potential of the NMs dispersed in the complete renal cell medium (K-SFM) and RPMI with 10% FCS (RPMI-FCS) were determined in the 1–128 µg/ml concentration range by Dynamic Light Scattering (DLS) using a Malvern Metasizer nano series – Nano ZS (USA) (Table 1).

Cell culture and treatment with nanomaterials

The immortalized adult human renal proximal tubule epithelial cells (HK-2) were obtained from the American Type Culture Collection (ATCC, USA). The cells were maintained in Keratinocyte Serum Free Medium (Gibco, UK) containing 25 µl of bovine (pituitary) extract, 0.2 ng/ml human recombinant epidermal growth factor and 100 U/ml penicillin/streptomycin (termed K-SFM) or RPMI (Sigma, France) with 10% FCS and 100 U/ml

Table 1 Main physicochemical characteristics of engineered nanomaterials investigated - reproduced from [14]

ENM code	ENM type	Phase	XRD Size [nm]	TEM Size	Primary characteristics by TEM analysis	Surface area (BET) [m ² /g]	Known coating	Size in K-SFM (DLS) Ψ	Size in RPMI-FCS (DLS) Ψ	Zeta potential K-SFM	Zeta potential RPMI-FCS
NM101	TiO ₂	Anatase ^ε	9	4-8/50-100	Two structures found; type 1 show agglomerates in the 50–1500 nm range	322	none	221	358	-11.4	-17.7
NM110	ZnO	Zincite	70 to > 100	20-250/50-350	Mainly 2 euhedral morphologies: 1) aspect ratio close to 1 (20–250 nm range and few particles of approx. 400 nm) 2) ratio 2 to 7.5 (50–350 nm). Minor amounts of particles with irregular morphologies observed.	14	none	393	453.6	-11.5	-13
NM111	ZnO	Zincite	58-93	20-200/10-450	As NM110, but with different size distributions. 1) particles with aspect ratio close to 1 (~90% in the 20–200 nm range); 2) particles with aspect ratio 2 to 8.5 (~90% in the 10–450 nm ratio).	18	Tri-ethoxy-capyrylsilane 130	332	362.4	-11.4	-12.6
NM300	Ag	Ag	7 ⁵ 14 ^ε <18/15/> 100 [#]	8-47 (av.: 17.5)	Mainly euhedral NM; minor fractions have either elongated (aspect ratio up to ~5) or sub-spherical morphology	NA	none	87	51.59	-10.2	-13.3
NM400	MWCNT	-	-	D: 5-35 L: 700-3000	Irregular entangled kinked and mostly bent MWCNT (10–20 walls). Some CNTs were capped and some cases multiple caps were found due to overgrowth. Fe/Co catalysts (6–9 nm, average 7.5 nm) were found inside the tubes.	298	none	*	*	*	*
NM402	MWCNT	-	-	D: 6-20 L: 700-4000	Entangled irregular, mostly bent MWCNT (6–14 walls). Some tubes were capped by unknown material. Some nano-onions (5–10 nm) and amorphous carbon structures mixed with Fe (5–20 nm). Residual catalyst was observed. Individual catalyst particles up to 150 nm were also detected.	225	none	*	*	*	*
NRCWE001	TiO ₂	Rutile ^δ	10	80-400	Irregular euhedral particles detected by TEM	99	none	349	337.5	-11.6	-14.7
NRCWE002	TiO ₂	Rutile	10	80-400	Irregular euhedral particles detected by TEM	84	Positive charged	314	378.8	-13.2	-12.7
NRCWE003	TiO ₂	Rutile	10	80-400	Irregular euhedral particles detected by TEM	84	Negative charged	384	423.6	-15.3	-13.2

Table 1 Main physicochemical characteristics of engineered nanomaterials investigated - reproduced from [14] (Continued)

NRCWE004	TiO ₂	Rutile	App. 100	1-4/10-100/ 100-200/1000- 2000	Five different particle types were identified: 1) irregular spheres, 1–4 nm (av. Diameter); 2) irregular euhedral particles, 10–100 nm (longest dimension); 3) fractal-like structures in long chains, 100–200 nm (longest dimension); 4) big irregular polyhedral particles, 1-2 μm (longest dimension); 5) large irregular particles with jagged boundaries, 1–2 μm (longest dimension).	396	482.6	-11.3	-12.4
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€ 1 percent rutile found in one of two samples analyzed.

§ wet XRD in capillary tube.

£ dried samples.

sample with deposits.

§ ca. 6% anatase was observed in one of two samples analyzed.

* Not detectable by DLS due to the very large aspect ratio.

Ψ Intensity based size average in biological media after 15 mins.

Zincite –mineral form of ZnO.

PDI (polydispersity index) values were under 0.45 for all given DLI values.

Abbreviations:

D, Diameter.

DLS, Dynamic Light Scattering.

ENM, Engineered nanomaterial.

L, Length.

XRD, X ray diffraction.

penicillin/streptomycin (termed RPMI-FCS), at 37°C and 5% CO₂. Two different media with varying amounts of protein (K-SFM less than 2% proteins, RPMI-FCS 10% protein) were utilised to allow comparisons of the *in vitro* cytotoxicity to HK-2 cells.

The Ag was supplied in de-ionised water (85%) with 7% stabilizing agent (ammonium nitrate) and 8% emulsifiers (4% each of Polyoxyethylene Glycerol Trioleate and Tween 20). All other materials were supplied as dry powders. NMs were dispersed in MilliQ deionised water with 2% FCS. For the coated ZnO, the particles were wetted with 0.5% vol ethanol before the addition of the dispersion media. The nanomaterials were sonicated for 16 mins without pause following the protocol developed for ENPRA [15]. Following the sonication step, all samples were immediately transferred to ice.

To ascertain the toxicity of NMs to the HK-2 cells, ten concentrations between 0.16 and 80 µg/cm² were used (corresponding to 0.5 to 256 µg/ml).

WST-1 cell viability assay

The renal cell lines were seeded in 96 well plates 10⁴ cells per well in 100 µl of the cell culture medium and incubated for 24 hr in K-SFM or 48 hr with comp RPMI-FCS at 37°C and 5% CO₂. The following day the cells were exposed to the materials or controls for 24 hr at 37°C, 5% CO₂. Subsequent to NM treatment, cell supernatants were collected and frozen at -80°C and later used for FACS array analysis. Plates were washed twice with phosphate buffered saline (PBS), followed by the addition of 10 µl of the WST-1 cell proliferation reagent and 90 µl of fresh medium. Plates were then incubated for 1 hr at 37°C, 5% CO₂. The supernatant was transferred to a fresh plate and the absorbance measured by dual wavelength spectrophotometry at 450 nm and 630 nm using a micro-plate reader.

Production of IL6, IL8, TNF-α and MCP-1

The levels of human interleukin 6 (IL 6), IL8, tumour necrosis factor α (TNF-α) and monocyte chemo-attractant protein 1 (MCP-1) from HK-2 supernatants was measured using the BDTM Cytometric Bead Array cytokine Flex sets (bead based immunoassay) according to the manufacturer's instructions. A BDTM FACSArray, USA was utilised for cytokine measurement.

HE oxidation

The renal cells were seeded in 12 well plates (10⁴ cells per cm² in 1 ml of medium) and incubated for 48 hr at 37°C and 5% CO₂. Cells were exposed to the materials or controls for 4 hr at 37°C, 5% CO₂. The treatment was removed and the cells were washed with complete medium. Cells were then harvested with 0.05% trypsin/EDTA and centrifuged at 300 g for 5 min before being

re-suspended in phenol red free media containing 1 µM of dihydroethidium (DHE) (specific for superoxide production) (Sigma, France) for 30 min at 37°C in the dark and analysed by CyAn ADP LX DakoCytomation cytometer (Beckman Coulter, France). Excitation and emission wavelengths were 488 and 620 nm respectively. Minimum of 10000 cells was analyzed after exclusion of cellular debris and NMs from the analysis by gating on the 620 nm Log versus FS area graph.

Detection of DNA strand breaks in HK-2 cells

The FPG (formamidopyrimidine [fapy] – DNA glycosylase) modified Comet assay was used to measure DNA strand breaks and specific oxidative DNA damage such as 7, 8-dihydro-8-oxoguanine, 8-oxoadenine, fapy-guanine etc., as previously described [16]. In this study the tail moment [17] was measured using an automatic image analyser (Comet Assay IV; Perceptive Instruments, UK) connected to a fluorescence microscope. Images were captured using a stingray (F-033B/C) black and white video camera.

Briefly, after a 4 hr NM treatment (or positive control - 60 µM of H₂O₂), the HK-2 cells were rinsed twice with PBS and detached using trypsin before being suspended in 5 ml of culture medium. Cells were centrifuged for 10 mins at 250 g, 4°C and re-suspended at a concentration of 1.5 × 10⁶ cells/ml in complete medium. A 20 µl volume of calculated cell suspension was added to 240 µl of 0.5% low melting point agarose. Next, 125 µl of the mixture was added to pre-coated slides (1.5% agarose) in triplicate. Following 10 mins of solidification on ice, slides were lyzed overnight at 4°C in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, pH 10, containing 10% DMSO and 1% TritonX-100). The slides were washed three times for 5 mins with FPG enzyme buffer (40 mM HEPES, 100 mM KCl, 0.5 mM EDTA, 0.2 mg/ml BSA - pH 8), covered with 100 µl of either buffer or FPG in buffer (1:30), sealed with a cover slip and incubated for 30 mins at 37°C. FPG cleaves DNA at locations of oxidation leading to a greater tail for cells exhibiting oxidative DNA damage [18]. All slides were then transferred into a black chilled electrophoresis tank. After alkaline unwinding (pH 13) for 20 mins, electrophoresis was performed for 15 mins at 270 mA, 24 V. Slides were neutralized three times for 5 mins using a neutralization buffer (0.4 M TrisBase, pH 7.5). Before analysis, slides were dried for 10 mins and stained with GelRed (2 in 10000, 40 µl per slide). A total of 50 cells were analyzed per slide per experiment.

Statistical analysis

All data are expressed as mean ± standard error of the mean. For statistical analysis, the experimental results were compared to their corresponding control values using an ANOVA with Tukey's multiple comparison. All

statistical analysis was carried out utilizing Minitab 15. A p value of < 0.05 was considered to be significant. All experiments were repeated a minimum of three times.

Results

Characteristics of nanomaterials and exposure media

Investigated nanomaterials were characterised by a combination of analytical techniques in order to infer primary physical and chemical properties useful to understand their toxicological behaviour. A list of the measured physical and chemical properties is described (Table 1). In order to investigate if the nanomaterials behaved differently in K-SFM or RPMI-FCS, the hydrodynamic size distributions and zeta potential of the NMs dispersed in the two media was measured between a 1–128 µg/ml concentration range by DLS (Table 1). It is widely accepted that DLS is not a suitable method of ascertaining the size of carbon nanotubes (due to the fact that the machine measures size based on the principle that particles are spherical and well dispersed), hence we examined how the two MWCNTs behave in our two chosen media utilising light microscopy. We found that the MWCNTs agglomerated into larger clusters in the complete RPMI which contained higher levels of FCS in comparison to the K-SFM (data not shown). Our zeta potential data seems to demonstrate that these NMs have the tendency to aggregate in the media utilised in this study (Table 1).

Impact of the selected panel of NMs on HK-2 cell viability

Our toxicity data show a dose dependent decrease in cell viability at 24 hr across the entire nanomaterial panel (Table 2). The Ag and the two ZnO NMs were shown to be highly cytotoxic (NM 111 LC₅₀ 2.5 µg/cm² – K-SFM, LC₅₀ 0.9 µg/cm² – RPMI-FCS; NM 110 LC₅₀ 2.5 µg/cm² – K-SFM, LC₅₀ 1 µg/cm² – RPMI-FCS; NM 300 10 µg/cm² –

K-SFM, LC₅₀ 4.5 µg/cm² – RPMI-FCS) after a 24 hr exposure. All of the TiO₂ and MWCNT NMs were considered to be low toxicity materials as the LC₅₀ was not reached after a 24 hr exposure to the HK-2 cells at the range investigated. We observed slightly higher cytotoxicity to the cells in RPMI-FCS exposed to the Ag and ZnO NMs. We also investigated the toxicity of the ENPRA dispersants namely NM 300 dispersant termed (NM 300 DIS) and 0.5% ethanol in complete medium. We found no cytotoxicity of any of these dispersants to the HK-2 cells (data not shown) so we conclude that all observed toxicity is due to exposure to the NMs investigated. LC₂₀ are shown as these values were utilised for the FPG modified comet assay (Table 2).

Cytokine secretion by HK-2 cells following exposure to the panel of NMs

Changes in cytokine production as a consequence of NM exposure were assessed within the supernatant of exposed renal cells and quantified via FACSArray. We found a dose dependent (exposure to sub-lethal concentrations of the NMs) increase in the levels of IL8 and IL6 from the kidney cells with statistical significance being reached at the higher concentrations following exposure to nine of the ten nanomaterials (TiO₂ - NM 101 being the exception) (Figure 1J). Additionally we found no change in the levels of MCP-1 or TNF-α secreted from the HK-2 cells following the 24 hr exposure to any of the selected NMs (data not shown).

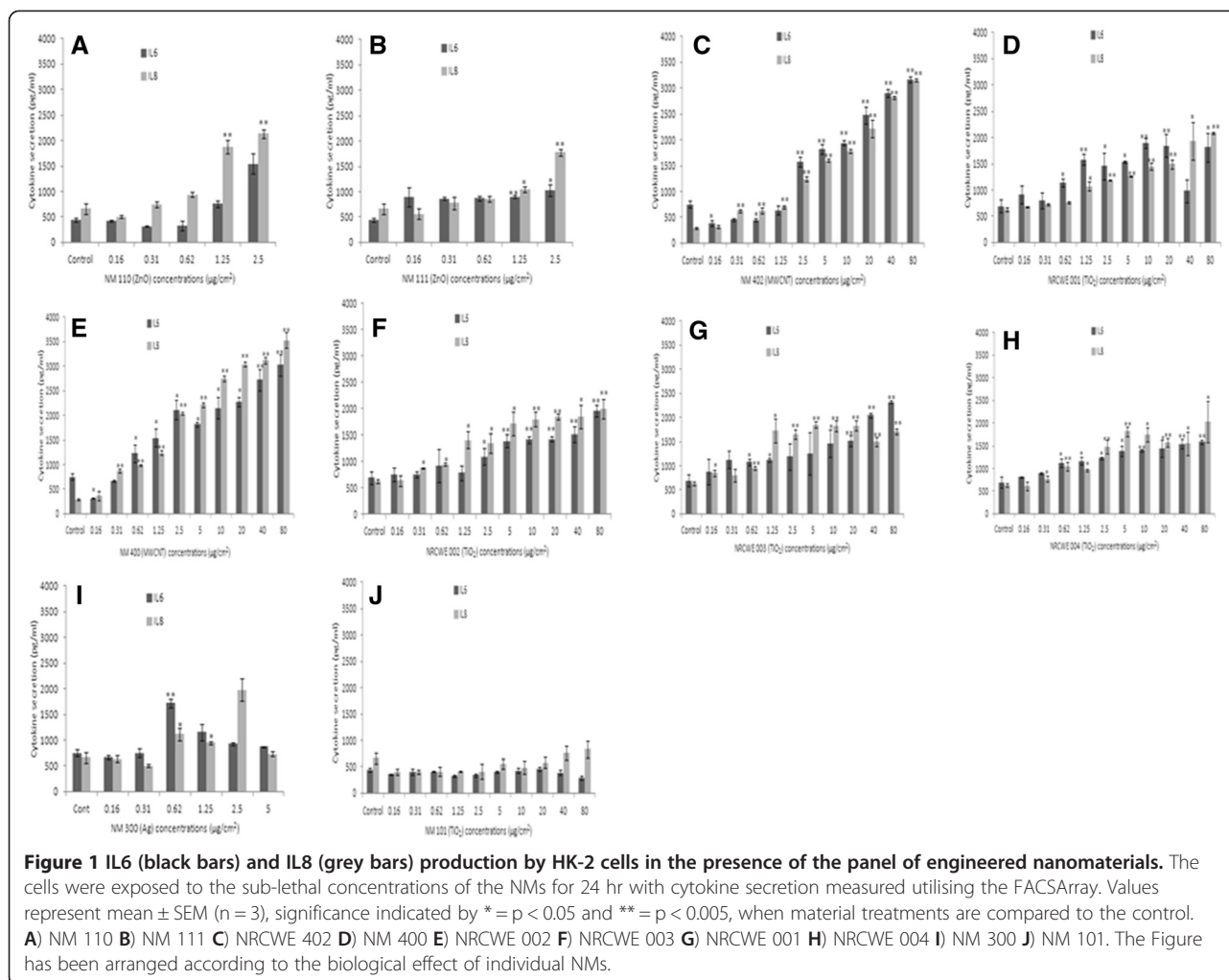
HE Oxidation

Hydroethidium is the chemically reduced form of the commonly used DNA intercalating dye ethidium bromide [19]. This reduced dye is therefore useful for detection of oxidative activities in viable intact cells. Only once it is internalized and dehydrogenated (oxidized) to

Table 2 Cytotoxicity following exposure of the HK-2 cells in two different media to a panel of engineered nanomaterials

NM	K-SFM		RPMI-FCS	
	LC ₂₀	LC ₅₀	LC ₂₀	LC ₅₀
NM 111	0.8 µg/cm ²	2.5 µg/cm ²	0.5 µg/cm ²	0.9 µg/cm ²
NM 110	0.64 µg/cm ²	2.5 µg/cm ²	0.64 µg/cm ²	1 µg/cm ²
NM 300	1.25 µg/cm ²	10 µg/cm ²	1.25 µg/cm ²	4.5 µg/cm ²
NM 400	2.5 µg/cm ²	Not reached up to 80 µg/cm ²	20 µg/cm ²	Not reached up to 80 µg/cm ²
NM 402	4.5 µg/cm ²	Not reached up to 80 µg/cm ²	20 µg/cm ²	Not reached up to 80 µg/cm ²
NRCWE 002	20 µg/cm ²	Not reached up to 80 µg/cm ²	Not reached up to 80 µg/cm ²	Not reached up to 80 µg/cm ²
NRCWE 001	40 µg/cm ²	Not reached up to 80 µg/cm ²	Not reached up to 80 µg/cm ²	Not reached up to 80 µg/cm ²
NRCWE 004	40 µg/cm ²	Not reached up to 80 µg/cm ²	Not reached up to 80 µg/cm ²	Not reached up to 80 µg/cm ²
NRCWE 003	60 µg/cm ²	Not reached up to 80 µg/cm ²	Not reached up to 80 µg/cm ²	Not reached up to 80 µg/cm ²
NM 101	Not reached up to 80 µg/cm ²	Not reached up to 80 µg/cm ²	Not reached up to 80 µg/cm ²	80 µg/cm ²

The cells were exposed to the NM for 24 hr with cytotoxicity measured via WST-1 (NMs ranked in order of cytotoxicity to HK-2 cells – high to low).



ethidium, it can intercalate into DNA. Normally due to their compromised membranes, only dead cells are typically labelled by ethidium bromide when it selectively binds to DNA. However HE is a neutral probe and is able to penetrate the cell membrane of live cells, staining their cytoplasm blue as well as the chromatin/nucleus of living cells red. We noted a significant increase in HE positive cells following exposure to Ag and ZnO NMs (Figure 2A and B). A very small yet significant increase was also observed to all the TiO₂ NM with the exception of NRCWE 003 (Figure 2C). However no change in intracellular reactive oxygen species was noted following exposure to either of the MWCNTs (Figure 2D).

DNA damage in the HK-2 cells

In order to investigate the possible DNA damage caused by the panel of nanomaterials, HK-2 cells were exposed to the NMs for 4 hr. In this study we chose the LC₂₀ value for each individual NM plus one concentration above (2× LC₂₀) and one below (0.5× LC₂₀). For the TiO₂ NMs (NM 101, NRCWE 001 and NRCWE 002)

were an LC₂₀ was not reached the three highest concentrations were utilised. We observed that DNA damage was most evident following exposure to NM 300 (Ag) and NRCWE 002 (positively charged TiO₂) (Figure 3A, B). We also noted a small but significant increase in the percentage of tail DNA following exposure to five of the other eight NMs investigated (ZnO - NM 111, TiO₂ - NRCWE 001 and 003 TiO₂ NMs being the exception) (Figure 3H, I and J).

Discussion

This study was conducted as part of a large consortium (FP7 project – ENPRA) to investigate the hazards associated with a wide range of nanomaterials on a variety of target systems for risk assessment. For this reason, the wide dose response range was used in order to allow calculation of values such as LC₅₀ for comparisons between different materials and cell target types both *in vitro* and *in vivo*. For this particular manuscript kidneys were the target of interest.

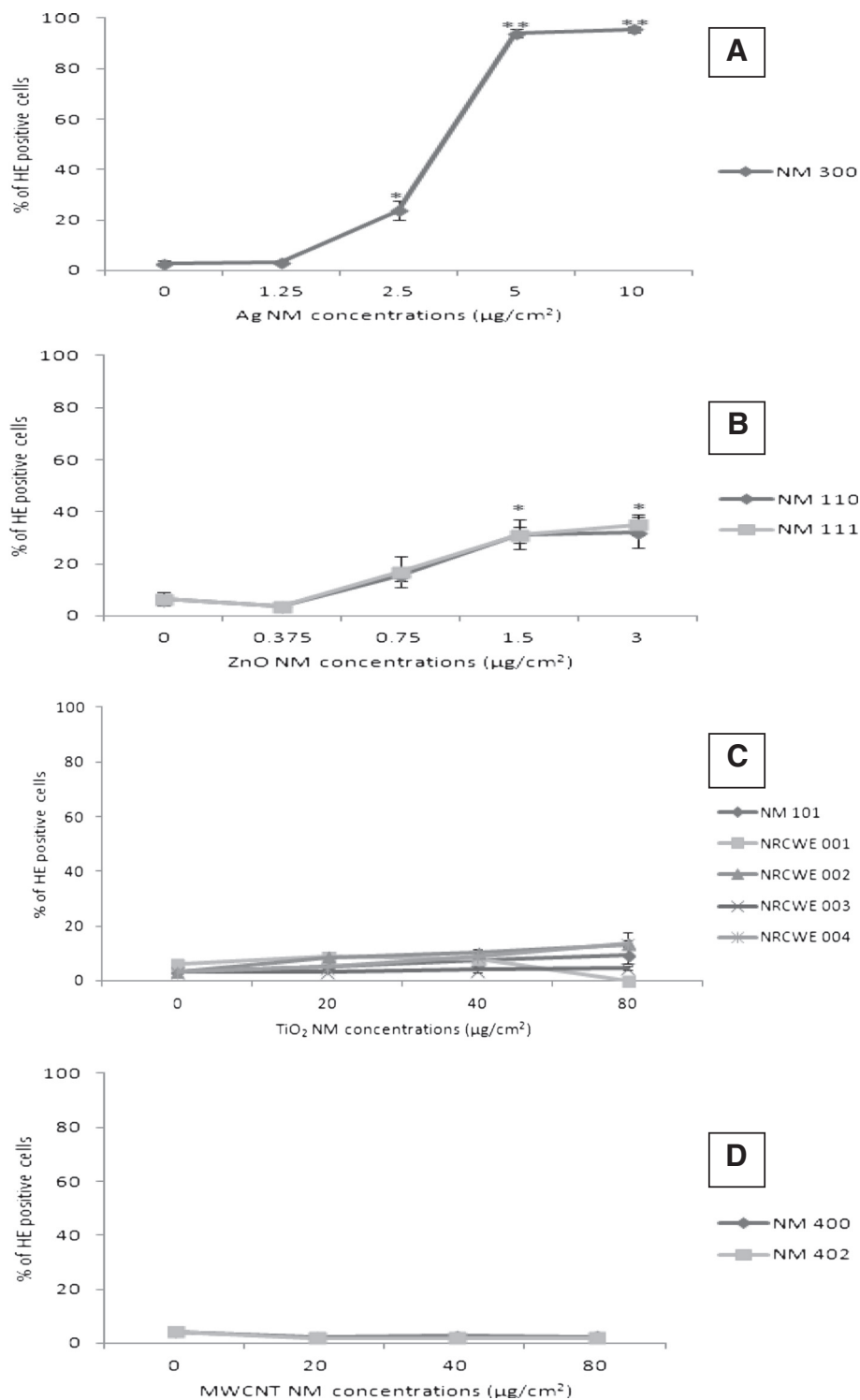
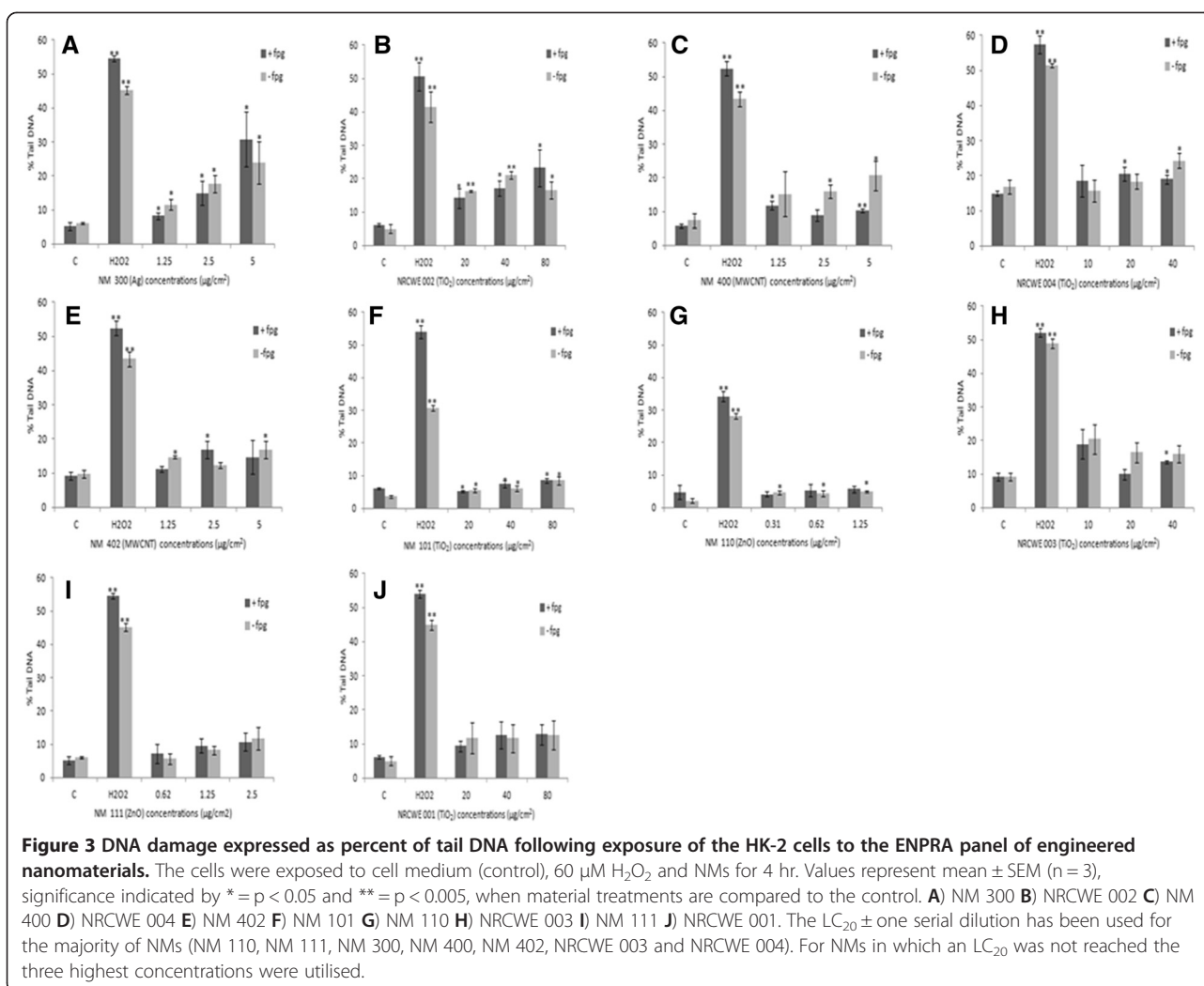


Figure 2 Intracellular ROS production following exposure of the HK-2 cells to the panel of engineered nanomaterials. The cells were exposed to the NM for 4 hr with oxidative stress measured via HE oxidation by flow cytometry. Values represent mean \pm SEM (n = 6) **A**) Ag - NM 300 **B**) ZnO - NM 110 and NM 111 **C**) TiO₂ - NM 101, NRCWE 001, 002, 003 and 004 **D**) MWCNT - NM 400 and NM 402.



To date human kidneys have received relatively little attention in terms of a nanotoxicological studies. Due to high blood supply level and ability to concentrate toxins, this organ could be potentially affected by any nanomaterial that reaches the circulatory system. During active and passive transport of molecules during re-absorption across the nephral proximal tubule, this part of the nephron may be at high risk of potential damage from NMs. It has been shown previously that the proximal renal tubule is more susceptible to gold NM toxicity than the distal tubule [20]. In a recent study it was shown that a high concentration of orally administrated Ag NMs accumulated in kidney tissues at a higher wet mass than any other organ [21]. This observation suggests that kidneys are important organs in clearing the NMs from the mammalian system and at potential risk of being damaged by NM exposure. Hence we have chosen an immortalized proximal tubule epithelial cell line from adult human kidney (HK-2) as a well differentiated cell line representing an alternative to primary kidney cells [22]. This study has

focused on the impacts of the investigated panel of nanomaterials on cell cytotoxicity, pro-inflammatory cytokine production, intracellular ROS and DNA damage.

Here we have shown that following acute *in vitro* exposure of the cells to the panel of the NMs, they can be segregated into a low (TiO₂ and MWCNT) and a high toxicity group (Ag, and ZnO) (Table 1). This complements our previous data for a hepatocyte cell line [14] and primary human hepatocytes [23]. We also observed slightly higher cytotoxicity (not significant) to the cells in RPMI-FCS exposed to the Ag and ZnO NMs (higher amounts of protein within the media compared to the K-SFM) utilised in this study. One theory offering an explanation for this could be that the serum interaction with the NMs might make the materials more bio-available to the cells. It is also possible that the cells might be healthier in the K-SFM compared to RPMI with FCS under the conditions in this study.

We noted that the Ag NM (NM 300) was highly toxic to the HK-2 cells (LC₅₀ between 4.5 - 10 µg/cm²). To

our knowledge no study has investigated the toxicity of Ag nanomaterials and human kidney cells *in vitro* however a number of studies have demonstrated that Ag NMs can be highly toxic in other organs and systems both *in vivo* and *in vitro* [14,24,25]. Oxidative stress is often proposed as a mechanism of toxicity [25,26]. Our HE oxidation data show an increase in intracellular ROS levels following exposure to the NM 300. However a note caution is required as this increase in HE positive cells could be due to cell death. We do not believe this to be the case predominately for two reasons. Firstly, only sub-lethal doses were utilised in these experiments. Secondly, the cells were only exposed to NMs for 4 hr in these experiments with the aim of identifying the mechanisms underlying the cytotoxicity. Furthermore we noted that there was an increase in IL6 and IL8 levels following exposure of the cells to the Ag NMs (highest release at intermediated doses – suggesting cytotoxicity (cell death) inhibits cytokine production at the highest doses), as well as a significant increase in genotoxicity following exposure to sub-lethal concentrations of the nanomaterial. This indicates that Ag NMs are highly genotoxic to human kidney cells. So far a number of other studies have confirmed dose dependent DNA damage after Ag NM treatment to a liver cell line [27] or genotoxicity in testicular cells [28].

Next we have shown that the two ZnO NMs were highly toxic (with the LC_{50} around 0.9 - 2.5 $\mu\text{g}/\text{cm}^2$) to the renal cells. Our findings are similar to a previous study in which HK-2 cells were shown to be highly susceptible to exposure to ZnO NMs (LC_{50} - 2.4 $\mu\text{g}/\text{cm}^2$) [29]. The toxicity was associated with increased intracellular ROS production which is very similar to our findings here [29]. Notably, exposure to the two zinc oxide NMs showed only a small increase in percentage of tail damage. It is possible that the 4 hr exposure time for genotoxicity was not sufficient to cause larger effects from the two ZnO NMs in this study. Next, we noted a dose dependant increase in IL6 and IL8 secretion from the cells following NM exposure. It has been shown previously that there is an increase of these pro-inflammatory mediators from the kidney following exposure to various antigens [30,31]. In a similar theme exposure of human embryonic kidney cell line HEK 293 to 100 nm ZnO NMs resulted in up-regulation of IL6 and IL8 genes from the cells *in vitro* [32].

We had previously shown that the two ZnO NMs used in this study are highly soluble (40-50%) while less than 1% of Ag (NM 300) was soluble after 24 hr of incubation in complete medium [14]. Therefore there is a real possibility that the high cytotoxicity of the two ZnO materials are in part due to the release of ions, with this scenario being less likely following exposure to the Ag NMs.

It has been shown previously that in sufficient doses TiO_2 can cause damage to cells and tissue [14,33,34]. Furthermore other studies have shown that after translocation from the primary site of exposure, the NMs can induce oxidative stress - mediated toxicity in many cell types by producing large amounts of free radicals [12,35,36]. In this study we found all five TiO_2 were of relatively low cytotoxicity to the HK-2 cells (LC_{50} was not reached in the presence of any of the NMs up to 80 $\mu\text{g}/\text{cm}^2$) with only small increases of oxidative stress presenting cells following exposure to four of the five TiO_2 NMs. Our findings are similar to a previous study in which exposure of HK-2 cells to a 12 nm TiO_2 resulted in low toxicity [29]. Similarly, in a study using Caco2 cells it was found that there was low cytotoxicity following 24 hr exposure to TiO_2 NMs [35]. In addition the data presented here indicate that relatively high TiO_2 exposure concentrations can induce production of the pro-inflammatory cytokine IL6 and IL8. In a recent study exposure of mice to 6 nm TiO_2 NM via an intragastric route promoted the expression of IL2, IL4, IL6, IL8, IL10, IL18, IL1- β , TGF- β and IFN- γ from the kidney [37]. Furthermore exposure of C3A cell line *in vitro* also resulted in IL8 secretion at high TiO_2 concentrations [14,38].

We show a significant increase in genotoxicity following a 4 hr exposure to two of the five TiO_2 NMs investigated in this study (NRCWE 002 and NRCWE 004 - most notable following exposure to the positively charged TiO_2 NMs). One possible explanation for this could be that the positively charged NMs enter cells utilising faster and more effective pathways (fast attachment to cell membrane and ingestion) than their neutral and negative counterparts [39-41]. Overall our findings are similar to a recent study in which exposure of Cos-1 monkey kidney fibroblasts to TiO_2 NMs resulted in significant DNA damage as measured via the comet assay [42].

We found that the MWCNTs tested were relatively non-toxic to the HK-2 cells at the times and concentrations tested. The toxicity of MWCNTs is widely documented, with adverse effects observed in pulmonary [34], hepatic [14], renal [43], dermal cells [44] as well as monocyte [45] and macrophage cells [46]. It has been shown that exposure of NRK-52E cells (an *in vitro* renal model) to MWCNT resulted in low toxicity (LC_{25} following 24 hr exposure) [47] which is similar to our findings in this study. Exposure of the HK-2 cells to the two MWCNTs in this study resulted in a dose dependant increase in both IL6 and IL8 following a 24 hr exposure. Our findings are similar to a recent study in which exposure of HEK 293 cells to two types of MWCNTs (80 and 150 nm) resulted in increased production of IL8 [48]. The HE oxidation assay showed no intracellular ROS following exposure to either of two MWCNTs. This is contradictory to findings from Reddy

and colleagues in which cytotoxicity was associated with oxidative stress [48]. Finally we show that short term (4 hr) exposure of the HK-2 cells to the two carbon nanotubes at sub-lethal concentrations resulted in significant DNA damage. Barillet *et al.* also witnessed small but significant genotoxicity following exposure of NRK-52E cells to 100 nm MWCNT which is similar to our findings in this study [43]. In another study exposure of lung A549 cells for 24 hr to -OH functionalized and pristine MWCNTs resulted in a concentration-dependent increase of direct DNA damage [49]. Similarly A549 cells exposed to MWCNTs for 24 hr resulted in an induction of direct DNA with statistical significance reached at 10 µg/ml [50].

No TNF-α or MCP-1 production was observed following exposure to the ENPRA panel of NMs. It has been shown that under certain disease models that kidney cells produce TNF-α [51] and MCP-1 [52,53]. Here we show that exposure to these particular nanomaterials even at very high (in some cases cytotoxic) doses are not sufficient for MCP-1 or TNF-α secretion from the cells.

Overall this study emphasises the importance of the use of sub-lethal NM concentrations in particular with regards to mechanistic studies - otherwise conclusions about cause and effect and the mechanism of action can be misleading or completely missed. It is very possible that low toxicity nanomaterials may still possess sub-lethal effects of toxicological consequence.

Conclusions

In conclusion, this *in vitro* renal model demonstrated that ZnO and Ag NMs were consistently more potent with respect to cytotoxicity, cytokine production (IL6 and IL8) and intracellular reactive oxygen species production. In comparison the MWCNT and TiO₂ nanomaterials investigated revealed relatively lower toxicity. We noted that short term sub-lethal exposure to seven of the ten nanomaterials resulted in DNA damage to the cells (most evident following exposure to the Ag and the positively charged TiO₂ NMs). Studies conducted by project partners are utilising other target cells such as macrophages, lung epithelial cells, fibroblasts, endothelial cells and hepatocytes. *In vivo* studies are also being conducted for comparison with *in vitro* models. All of this data will be combined into a database to be used in risk assessment.

Competing interests

The authors declare that they are no competing interests.

Authors' contributions

AK, SV, KM and LAA have carried out the experiments within this study. SB, BKG, ABS and VS have all been heavily involved in the preparation and revision of the manuscript. All authors have read and approved the final manuscript.

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