

# Nanotoxicity: emerging concerns regarding nanomaterial safety and occupational hard metal (WC-Co) nanoparticle exposure

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**Abstract:** As the number of commercial and consumer products containing engineered nanomaterials (ENMs) continually rises, the increased use and production of these ENMs presents an important toxicological concern. Although ENMs offer a number of advantages over traditional materials, their extremely small size and associated characteristics may also greatly enhance their toxic potentials. ENM exposure can occur in various consumer and industrial settings through inhalation, ingestion, or dermal routes. Although the importance of accurate ENM characterization, effective dosage metrics, and selection of appropriate cell or animal-based models are universally agreed upon as important factors in ENM research, at present, there is no “standardized” approach used to assess ENM toxicity in the research community. Of particular interest is occupational exposure to tungsten carbide cobalt (WC-Co) “dusts,” composed of nano- and micro-sized particles, in hard metal manufacturing facilities and mining and drilling industries. Inhalation of WC-Co dust is known to cause “hard metal lung disease” and an increased risk of lung cancer; however, the mechanisms underlying WC-Co toxicity, the inflammatory disease state and progression to cancer are poorly understood. Herein, a discussion of ENM toxicity is followed by a review of the known literature regarding the effects of WC-Co particle exposure. The risk of WC-Co exposure in occupational settings and the updates of in vitro and in vivo studies of both micro- and nano-WC-Co particles are discussed.

**Keywords:** engineered nanomaterial, occupational exposure, lung disease, cancer, toxicity, particle

## Engineered nanomaterials (ENMs), nanotoxicity, and means of exposure

Because of recent technological and manufacturing advancements, the production and use of ENMs has increased at a rapid pace. The term ENM broadly encompasses a number of nano-sized materials that vary in shape, such as nanotubes, nanowires, or nanoparticles (NPs), which are generally defined as “any material having at least one dimension smaller than 100 nm.”<sup>1</sup> Because of their extremely small size and high surface area, NPs offer a number of advantages over traditional “bulk” materials and are suitable for a wide variety of applications in consumer goods,<sup>2,3</sup> medical devices and diagnostics,<sup>4-6</sup> pharmaceutical products,<sup>4,7-10</sup> fuel additives,<sup>11,12</sup> and other industrial uses.<sup>13-17</sup> As a result, the number of manufactured goods containing NPs is continually rising; in 2006, manufacturers reported that NPs were incorporated in over 600 consumer products worth \$50 billion in market value,<sup>18</sup> a number which has doubled to >1,600 NP-containing consumer products in 2013.<sup>19</sup>

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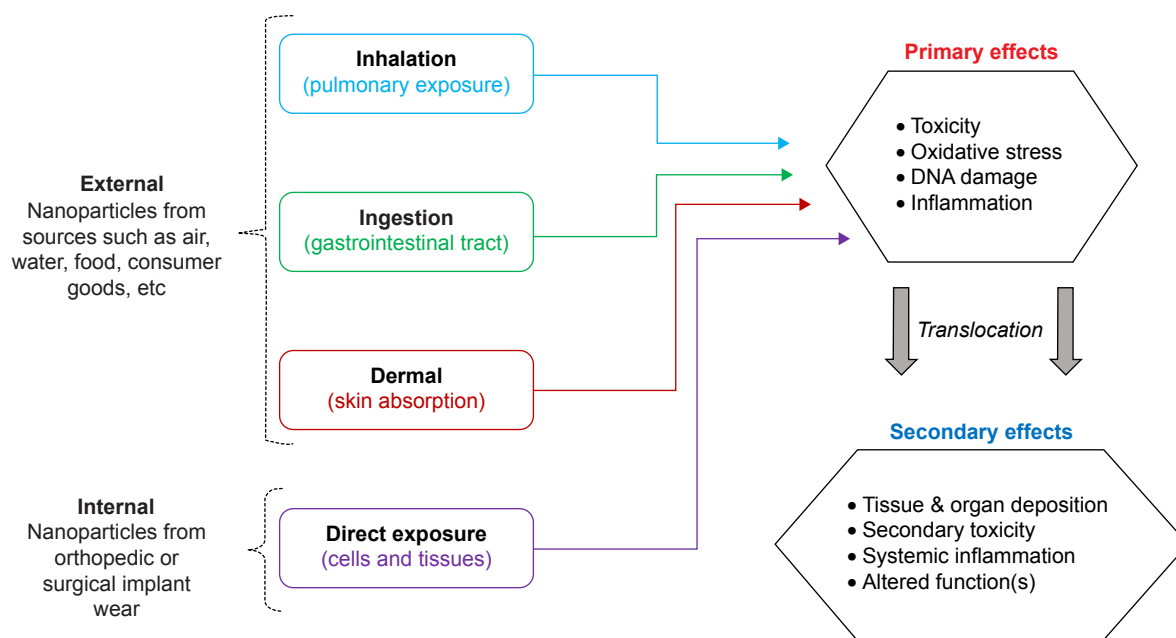
With the increased use of NPs in such diverse applications, a concomitant risk of exposure exists across consumer households and in commercial occupational settings. NPs exist in our daily environments and are often referred to as “contaminants” of important air, water, and soil resources.<sup>20,21</sup> Because these NPs are all around us, exposure can occur through mechanisms such as inhalation, ingestion, and dermal exposure.<sup>22</sup> Additionally, emerging evidence suggests that humans may also be exposed to NPs internally, which may be generated in situ due to orthopedic surgical implant wear.<sup>23,24</sup> These potential routes of NP exposure are summarized graphically in Figure 1. Importantly, the route of exposure ultimately determines which body system or specific tissues the NPs interact with which in turn determines the effects of NP exposure such as toxicity<sup>25,26</sup> or alterations in physiological function.<sup>27</sup>

The effects of NP exposure can be divided into primary and secondary categories (Figure 1), depending upon the extent of exposure. Primary effects resulting from direct cellular NP contact may include toxicity, oxidative stress, DNA damage, and inflammation.<sup>1,28,29</sup> Because of their small size, NPs may translocate through tissue barriers into the blood, where they can circulate and eventually deposit in other organs, thereby generating a secondary NP exposure. Secondary effects may include toxicity at the site of NP deposition, in organs such as the liver, spleen, or kidneys, stimulation of systemic inflammation or alterations in systemic function.<sup>1,28–30</sup> The first three routes of exposure

occur through external NP sources, but there is emerging evidence which indicates that humans may also be exposed internally, when orthopedic or surgical implant wear NPs are released locally from the implant site.<sup>23,31,32</sup> Most commonly, humans are exposed to NPs in their environments through the pulmonary route, by inhaling airborne NPs during normal breathing.<sup>27,33</sup>

Despite their potential toxic properties, it is worth mentioning that certain NPs have distinct advantages that outweigh the risk of use and have greatly improved consumer products on the market today, such as sunscreens and cosmetics.<sup>22</sup> For example, titanium dioxide NPs present in sunscreens and cosmetic products are highly beneficial, offering excellent protection from sun exposure, thus protecting the skin against UV damage and preventing sunburn. NPs have been approved for use under these conditions; however, the long-term effects of titanium dioxide NPs on aquatic life in contaminated water sources (lakes, oceans, etc) remain an important concern for future research.

As a direct result of the increased use of NPs and likelihood of exposure, the study of the acute and chronic effects of NP exposure has recently emerged as the field of “nanotoxicology”.<sup>34</sup> Currently, there is no standard approach for NP toxicity testing, and a number of arguments support the implementation of a standard testing procedure, so that results and outcomes can be directly comparable among all NPs tested. However, this remains as a difficult task because the route and realistic amount of exposure (dose) vary greatly



**Figure 1** Routes and potential detrimental effects of nanoparticle exposure.

**Table 1** Important components of NP toxicity testing

Characterization	Primary particle size
	Agglomerate size in suspension
	Size distribution range
	Elemental composition & contaminant levels
	Zeta potential
Dosage metrics	Particle shape/morphology & density
	Mass per volume (mg/mL)
	Surface area ( $\mu\text{m}^2$ )
	Particle number
Model system	Single or multi-dose & dose frequency
	In vitro: cell selection, mono- or co-culture model
	In vitro: assay selection (MTT, LDH, etc)
	In vivo: animal selection (rat, mouse, etc)
	In vivo: method of exposure (inhalation, intra-tracheal instillation, injection, internal wear debris, etc) & exposure duration
	In vivo: sample collection (fluid, tissue, organ, etc)

**Abbreviations:** LDH, lactate dehydrogenase; NP, nanoparticle.

depending on the particular NP in question.<sup>35</sup> In order to define the toxic potential of NPs, several key factors must be addressed (summarized in Table 1).<sup>1,29,34</sup> First, it is imperative to characterize NPs in terms of size, shape, and properties (ie, morphology, surface charge), so that the observed effects can be attributed to a particular property or characteristic. Next, an appropriate dosage metric must be selected for the NPs to be examined. In the literature, NP dosages have been reported as concentration per volume or mass (mg/mL or mg/kg), as reactive surface area ( $\text{cm}^2$ ) or as particle number in solution, calculated based on NP size.<sup>35</sup>

In addition to particle characterization and selection of the dosing metric, an appropriate model must be selected for toxicity testing. Typically, in vitro cell-based models are the starting point due to lower cost and relative ease of execution compared to in vivo animal-based model systems.<sup>36</sup> In vitro systems require small amounts of NPs for testing and allow for dose–response testing and sample collection over time in various systems, such as cells or tissues.<sup>36</sup> Primary

or commercial cells are commonly used for ENM toxicity testing and cell selection for a given assay is frequently based on the potential route of NP exposure; some examples of in vitro models are summarized in Table 2. Aside from the advantage of being “fast and cheap,” in vitro models can be used to estimate toxico-kinetic parameters, identify potential target organ toxicity, and help define appropriate NP dosages for in vivo studies, which help to reduce the number of experimental animals required for nanotoxicity research.

Although in vitro models and toxicity assays are in general reliable and used frequently, several groups have reported NP interference with commonly used cell-based assays,<sup>37,38</sup> bringing into question whether the results are accurate or whether they are simply artifacts generated because of NP interference with the assay mechanism.<sup>38,39</sup> To address this issue, a number of control experiments are required to identify any potential NP assay interference, and these effects must be considered while interpreting the final results.

Although they offer a number of advantages, cell-based systems are limited and generally demonstrate the effects of NPs within a very specific and small compartment compared to whole-animal in vivo models.<sup>40</sup> Animal models may offer more realistic insights as to the effects of NP exposure in humans because the body systems can be utilized as a whole, with all of its cells, tissues, and organs interacting with the NPs as would be encountered in a realistic exposure scenario.<sup>40</sup>

Since there are a number of NP exposure routes for humans, a number of in vivo exposure approaches must be used such that the effects of the NP on the appropriate tissue or organ system can be identified. Commonly used in vivo NP exposure routes are summarized Table 3, including NP inhalation, intra-tracheal instillation (IT), injection, and ingestion or gavage. As mentioned earlier, NP inhalation and pulmonary exposure are most common in humans and

**Table 2** Examples of in vitro models used in NP toxicity testing

Cell type	NPs studied	NP size	NP dose	References
Human lung carcinoma (A549)	Cerium dioxide ( $\text{CeO}_2$ )	10 nm	6.25, 25, or 100 $\mu\text{g}/\text{mL}$	105
Human hepatocyte (C3A), human colon adenocarcinoma (CaCo-2), primary trout hepatocytes	Gold (Au) Cerium dioxide ( $\text{CeO}_2$ )	35 nm 25 nm	0–1,000 $\mu\text{g}/\text{mL}$	21
Human fibroblasts, peripheral blood mononuclear cells, macrophages	Cobalt chromium (CoCr)	30 nm 3 $\mu\text{m}$	1–5,000 $\mu\text{m}^3/\text{well}$	106–108
Hepatocellular carcinoma (HepG2)	Silicon dioxide ( $\text{SiO}_2$ )	43 nm	25, 50, 100, 200 $\mu\text{g}/\text{mL}$	109
Human lung epithelial cells (BEAS-2B)	Titanium dioxide ( $\text{TiO}_2$ )	25 nm	5, 10, 20, 40 $\mu\text{g}/\text{mL}$	110
Rat alveolar macrophages (RAW 264.7)	Crystalline silica (DQ12)	960 nm	1, 5, 10, 40 $\mu\text{g}/\text{cm}^2$	111
	Zinc oxide (ZnO)	10 nm		
	Magnesium oxide (MgO)	8 nm		

**Abbreviation:** NP, nanoparticle.

**Table 3** Examples of in vivo exposure models used in NP toxicity testing

NP delivery method	Animal model	NPs studied	NP size	NP dose	References
Inhalation	Mouse	Cadmium oxide (CdO)	15 nm	250 $\mu\text{g}/\text{m}^3$ for 3 h/day $\times 7$ days	112
	Rat	Magnetite ( $\text{Fe}_3\text{O}_4$ )	1.3 $\mu\text{m}$	4.7, 16.6, 52.1 $\mu\text{g}/\text{m}^3$ for 6 h/day $\times 5$ days/week $\times 13$ weeks	113
Intra-tracheal instillation	Rat	Cerium dioxide ( $\text{CeO}_2$ )	191 nm	10, 100, 400 $\mu\text{g}$ per rat	114
	Mouse	Chitosan	633 nm	2 mg/kg	115
Injection	Mouse (periarticular injection)	Cobalt chromium (CoCr)	32 nm	$1.2 \times 10^6$ $\mu\text{m}^3/25$ g	116
	Rats (articular injection)	Cobalt chromium (CoCr)	60 nm	1 injection/week $\times 2$ weeks 0.05, 0.25, 1.25 $\mu\text{g}/\text{mL}$	117
Ingestion (via GI tract)	Daphnia magna, Cyprius carpo (fish species)	Gold (Au)	35 nm	0–10 $\mu\text{g}/\text{mL}$	21,118
		Cerium dioxide ( $\text{CeO}_2$ )	25 nm	1 injection/week $\times 10$ weeks	

**Abbreviations:** NP, nanoparticle; GI, gastrointestinal tract.

are therefore the most frequent routes by which experimental animals are exposed in nanotoxicity studies. However, not all NPs are suitable for aerosolization and for the purposes of an inhalation chamber, so alternatively, pulmonary NP exposure can be achieved via IT.<sup>41</sup> IT is a highly reproducible, direct method for depositing NP within the lungs, and NPs delivered in this manner have very similar lung distribution to NP inhalation;<sup>41</sup> therefore, IT is considered as an excellent approach for the pulmonary delivery of NP. In addition to pulmonary routes of exposure, animals may also be exposed to NP via injection for the purposes of systemic or local NP exposure. Intra-peritoneal (IP) or intravenous (IV) NP injections can be used to achieve systemic NP exposure, as the NPs circulate through the vasculature and may deposit in organs such as the liver, spleen, or kidneys. Injection may also be used to achieve localized NP exposure within articular locations, such as the knee or hip, or may be used to target subcutaneous tumors.

## Industrial hard metal (WC-Co) applications and exposure

Among the plethora of NPs gaining industrial use and popularity is tungsten carbide cobalt (WC-Co), which is a hard composite metal known for its extreme hardness, stability, and sharpness.<sup>42,43</sup> The use of WC-Co NPs as a spray coating on heavy machinery, drill bits, and saw blades substantially increases strength, durability, and resistance to wear, which drastically improves the operating lifetimes in industrial applications.<sup>42,43</sup> In particular, WC-Co coatings are popular in mining and drilling industries, where maintenance of sharp saw blades and drills is of critical importance.

Despite the improved durability and strength of WC-Co NP coatings compared to other conventional materials, these coatings do not last long and eventually wear out with extended use and over time. As a result, airborne WC-Co dusts containing particles of varying size can be generated during

use.<sup>44</sup> These airborne WC-Co “dusts” present an occupational exposure hazard not only in hard metal manufacturing facilities but also in mining and drilling industries where these WC-Co-coated implements are used extensively in enclosed environments. Typical WC-Co dusts encountered in industrial environments have a reported size range of several microns down to highly respirable particles in the nano-size range.<sup>45</sup>

Although exposure to WC-Co containing dusts has been identified as an occupational workplace hazard by the National Institutes of Occupational Safety and Health,<sup>46</sup> exposure limits for WC-Co dusts and powders remain undefined. Daily occupational exposures likely vary, as it is extremely difficult to predict an accurate exposure given the variability in dust generation and ventilation among worksites. Therefore, it is difficult to estimate what a relevant WC-Co dosage or concentration should be for experimental models. The existing exposure limits for cobalt metal and ionic cobalt<sup>47–49</sup> are difficult to translate to WC-Co exposure; ionic cobalt is soluble in physiological fluids and cell culture buffers, and exposure modality would experience different physiological effects than exposure to a solid (non-soluble) WC-Co composite NP.<sup>47–49</sup> Additionally, cobalt content can vary, depending on the application, and the dusts generated may not have uniform composition between multiple industrial sites.<sup>50</sup> More importantly, the combination of WC-Co is more toxic than Co, W, or WC particles alone (see section entitled Defining hard metal (WC-Co) toxicity: in vitro and in vivo studies). Therefore, understanding the toxic effects of hard metal WC-Co particles is crucial in order to develop occupational exposure guidelines.

## Risk of WC-Co exposure: hard metal lung disease (HMLD) prognosis and cancer risk

Exposure to “hard metal dust” containing WC-Co is well associated with the occurrence of occupational asthma and

is a known risk factor for the development of HMLD.<sup>44,51–59</sup> Diagnosis of HMLD is a challenge, as symptoms are general and often mistaken for other respiratory ailments: patients often report difficulty breathing and present with reduced lung capacity, progressive lung inflammation, and eventual fibrosis.<sup>57,60,61</sup> HMLD has been reported in hard metal manufacturing and oil and mining/drilling industries, where workers were exposed to WC-Co dusts or fumes on a daily basis for a number of years prior to diagnosis.<sup>44,51,62–65</sup> Currently, treatments are limited and no disease-specific therapy or diagnostic tool exists. There has been some success in treating HMLD with corticosteroids or immunosuppressive therapies,<sup>66,67</sup> typically resulting in moderate attenuation of breathing challenges and delayed onset of further symptoms (ie, fibrosis and fluid in the lungs), but in most cases, avoidance of further hard metal exposure is the recommended course of action.

It has recently been established that the defining characteristic of HMLD is the presence of “bizarre, cannibalistic, multinucleated giant cells” in lung biopsy specimens of workers exposed to WC-Co.<sup>57,61,63,65,68,69</sup> These giant cell complexes are thought to originate from macrophages which have engulfed WC-Co particles, which then stimulate inflammatory and fibrotic processes in the surrounding lung tissue.<sup>57,63,69</sup> In some cases, WC-Co particle “deposits” have been found in biopsy specimens, confirming causative exposure to WC-Co dust and HMLD diagnosis.<sup>62,63,69,70</sup> It has been hypothesized that there may be an allergic reaction or a genetic predisposition which may contribute to the development of HMLD in certain individuals.<sup>71</sup>

There is also accumulating evidence that patients with HMLD are at a twofold increased risk of developing lung cancer.<sup>54,56</sup> It has been argued that the generation of reactive oxygen species by WC-Co particles, which may directly cause DNA damage, along with the ability of cobalt ions to inhibit DNA repair mechanisms, may play a synergistic role in the development of lung cancer in HMLD patients.<sup>56</sup> However, this hypothesis is yet to be verified *in vivo* and the relationship between the inflammatory disease state and development of lung cancer remains unclear.

### Defining hard metal (WC-Co) toxicity: *in vitro* and *in vivo* studies

Early data concerning the effects of inhaled WC-Co dusts first emerged in the 1960s and continued through the 1980s, providing researchers the foundation to further explore the toxic effects of hard metal exposure using *in vitro*<sup>71–88</sup> and *in vivo*<sup>72,89–96</sup> models. Although cobalt itself was originally considered the causative agent of HMLD, several studies

demonstrated that this is not the case and the disease mainly develops due to the simultaneous presence of WC with Co.<sup>71,74,76–79,90,91</sup> It is currently understood that the combination of WC-Co is more toxic than Co, W, or WC particles alone, both *in vitro* and *in vivo*,<sup>71,72,74,78,79,81,85,90,91,93,94,97–99</sup> but the reason for this enhanced toxicity is still not well defined.

On the *in vitro* side, the effects of direct WC-Co particle exposure were examined by a number of research groups in terms of cell viability, apoptogenic potential, genotoxicity, oxidative stress, and DNA damage in multiple cell lines of various origins. The major outcomes of these early *in vitro* studies are summarized in Table 4, where the cell type, particle size, and dosage parameter is noted for each study. In alveolar macrophages, 2  $\mu\text{m}$  WC-Co particles caused significant toxicity after 24 h of exposure at concentrations ranging from 50 to 1,667  $\mu\text{g}/\text{mL}$ , but were less toxic toward type II pneumocytes under these conditions,<sup>72,99</sup> confirming pulmonary WC-Co toxicity in a relevant *in vitro* model. Toxicity has also been reported in mouse peritoneal macrophages, where WC-Co particles ranging from 2 to 4  $\mu\text{m}$  caused toxicity within 6 h of exposure at concentrations ranging from 50 to 300  $\mu\text{g}/\text{mL}$ .<sup>79,98,100</sup> In human peripheral blood mononuclear cells, exposure to micron-sized WC-Co particles caused apoptosis, DNA damage, genotoxicity, and alterations in gene expression after exposure times as short as 15 min and up to 6 h.<sup>81,82,92,101</sup> Additionally, a number of these studies compared the toxicity of WC-Co, WC, or Co particles and determined that the interaction of WC with Co significantly enhances the toxicity of the composite compared to any single component.<sup>71,74,79,101</sup>

There are fewer studies that have examined the effects of nano-WC-Co particles; however, nano-WC-Co toxicity has been reported in human keratinocytes, liver carcinoma cells, oligodendroglial precursor cells, and neurons, at concentrations ranging from 3 to 30  $\mu\text{g}/\text{mL}$  and exposure times from 1 h up to 3 days.<sup>74,75,78</sup> Internalization of nano-WC-Co has been reported in the keratinocytes (epidermal cells) after 2 days of exposure, which suggested that nano-WC-Co could potentially be absorbed through the skin.<sup>74,75</sup> Nano-WC-Co toxicity has also been reported in rainbow trout gill cells, murine epidermal cells, and fibroblasts at concentrations <100  $\mu\text{g}/\text{mL}$  for 3 h and up to 3 days.<sup>83,84</sup> Nano-WC-Co has been shown to exert genotoxic effects by affecting the expression of genes involved in cellular apoptosis and stress responses.<sup>75</sup> In addition, nano-WC-Co has been found to induce greater cellular toxicity and higher levels of oxidative stress than micro-WC-Co particles of the same composition under identical conditions (Figure 2).<sup>86</sup> These studies are consistent with the other reports where the

**Table 4** Summary of in vitro WC-Co toxicity studies

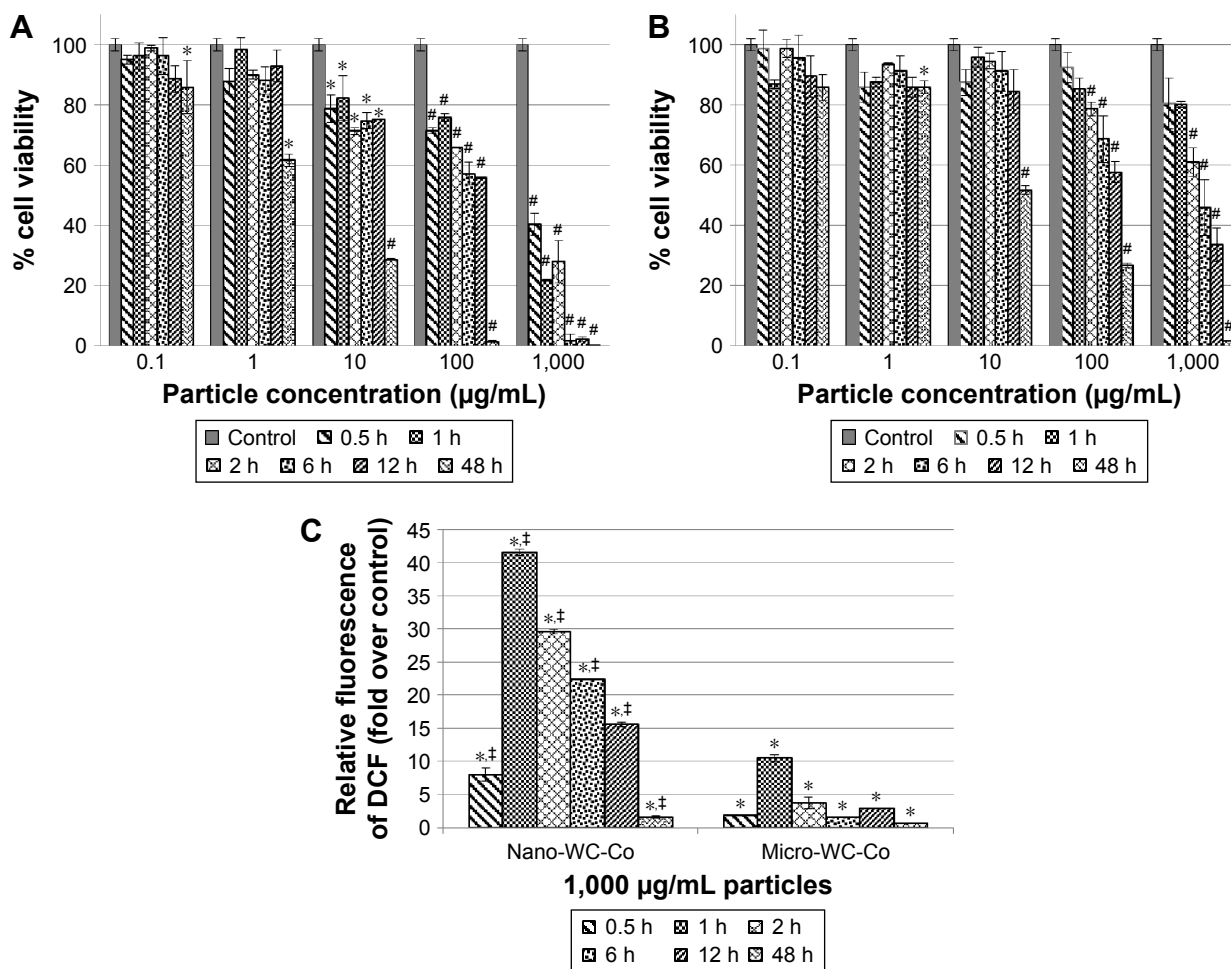
Cell type	Particle size & dosage	Major outcome(s)	References
Primary rat type II pneumocytes	2 µm 50 µg/mL WC-Co	No changes in the levels of TNFα, IL-1, fibronectin or cystatin-C (compared to control) were observed after WC-Co exposure for 12 or 24 h in isolated rat type II pneumocytes	72
Human colon adenocarcinoma (CaCo-2), human keratinocytes (HaCaT), human lung carcinoma (A549), OLN-93 oligodendro-glial precursor cells, rat neurons, astrocytes	145 nm 3.3, 6.6, 8.25, 11, 16.5, or 33 µg/mL WC-Co	WC-Co particles exhibited significant toxicity at a concentration of 33 µg/mL after 3 days of exposure to CaCo-2, HaCaT, and A549 cells. Significant toxicity was also observed in astrocytes after exposure to 3.3 µg/mL and higher WC-Co concentration after 1 and 3 days. Primary rat neurons were not sensitive to WC-Co toxicity. Additionally, WC-Co particles were internalized into the cytoplasm of HaCaT, A549, and OLN-93 cells after 2 days	74
Human peripheral blood mononucleated cells (PMBC)	1 µm 10, 50, or 100 µg/mL WC-Co or Co	After 15 min exposure to WC-Co, PMBC demonstrated 1.5-fold increase in DNA damage, marked by increased formation of micronuclei due to oxidative stress, compared to control and Co particle treatment alone	119
Rainbow trout gill cells (RTgill-WV1)	145 nm 8.25, 16.5, or 33 µg/mL WC-Co	WC-Co caused significant reduction in cell viability after 3 h and 3 days of exposure in RTgill cells. WC-Co particles were also found to be internalized into the cytoplasm after 2 days	120
Human keratinocyte (HaCaT) and hepato-cellular liver carcinoma (HepG2) cells	145 nm 7.5, 15, or 30 µg/mL WC-Co	WC-Co internalization in HaCaT was confirmed; however, WC-Co did not cause significant toxicity at the concentrations studied after 1 h, 3 h, or 3 days. WC-Co particles did not induce reactive oxygen species (ROS) or DNA micronuclei under the conditions tested	78
Mouse peritoneal macrophages	2 µm 50, 100, 200, or 300 µg/mL of WC-Co, Co, or WC	After 18–24 h of exposure, WC-Co caused significant toxicity, marked by increased LDH release and significant induction of oxidative stress compared to control. Activated oxygen species were implicated in associated DNA damage (micronuclei) in macrophages	79,80,98,121,122
Human keratinocytes (HaCaT)	62 nm 33 µg/mL WC-Co	WC-Co exposure caused significant changes in gene expression, such as <i>HIF1</i> , after 3 h and 3 days of exposure. WC-Co responsive genes were involved in cellular death and stress responses	75
Mouse peritoneal macrophages	2–4 µm 0–500 µg/mL of carbides: WC, TaC, SiC, NbC, Fe, TiC, Mo <sub>2</sub> C; all plus 6% Co	The addition of 6% Co particles to each of the “carbide” particles significantly enhanced the toxicity of WC, TiC, and NbC in macrophages after 18 h exposure, marked by increased levels of LDH release compared to control and the various carbide particles alone. Enhanced toxicity was attributed to the interaction of Co with the carbides	123
Human peripheral blood mononuclear cells (PMBC)	<1 µm 100 µg/mL WC-Co	Exposure to WC-Co particles for 15 min caused altered gene expression after 6 and up to 24 h post-exposure in PMBC, including activation of HIF-1α, p53, and altered expression of <i>HMOX1</i> which is involved in oxidative stress response mechanisms	81
Human PMBC	<1 µm 33.3, 45, or 100 µg/mL WC-Co or WC	WC-Co particles caused cellular apoptosis, marked by annexin-V staining, after 15 min and 6 h of exposure. Apoptosis was induced via the caspase-9 pathway and DNA fragmentation was significantly elevated in WC-Co exposed cells compared to WC alone	101
Human PMBC and human monocytes	<1 µm 100 µg/mL WC-Co or WC	A 24 h exposure to WC-Co caused significant up-regulation of apoptosis and stress response genes in both PMBC and monocytes, namely <i>BNIP3</i> , which is involved in mitochondrial mediated cell death	82
Human peripheral lymphocytes and mouse fibroblasts (3T3)	2 µm 100 µg/mL WC-Co, WC, or Co	In lymphocytes, WC-Co caused significant induction of DNA strand breaks after 15 min of exposure, attributed to oxidative stress damage, and caused extensive DNA damage in isolated 3T3 cellular DNA compared to WC or Co particles alone	83

(Continued)

**Table 4** (Continued)

Cell type	Particle size & dosage	Major outcome(s)	References
Mouse epidermal cells (JB6)	80 nm, 4 $\mu\text{m}$ 25, 37.5, 50, 75, or 150 $\mu\text{g}/\text{mL}$ WC-Co	Nano-WC-Co induced greater oxidative stress and hydroxyl radicals, marked by significantly decreased cellular GSH levels, in JB6 cells compared to micro-WC-Co. Nano-WC-Co also stimulated induction of <i>AP-1</i> and <i>NF-kappaB</i> and increased cellular proliferation in JB6 cells compared to micro-WC-Co under identical conditions	84
Rat alveolar macrophages (AM) and type II pneumocytes	2 $\mu\text{m}$ 83, 417, or 1,667 $\mu\text{g}/\text{mL}$ WC-Co or Co	After 24 h exposure to WC-Co, significant toxicity was observed in AM, but not in type II pneumocytes, compared to controls. However, type II cells were more sensitive toward Co toxicity than AM, in the absence of WC components	99
Lung epithelial cells	4 $\mu\text{m}$ and 80 nm of WC-Co 0.1–1,000 $\mu\text{g}/\text{mL}$ for 0.5–48 h	Nano-WC-Co was more toxic than micro-WC-Co	86
Lung epithelial cells, macrophages, and their co-culture	80 nm of WC-Co 1–1,000 $\mu\text{g}/\text{mL}$ for 2–48 h	Toxicity of nano-WC-Co was cell dependent, macrophages in the co-culture may play a protective role against nano-WC-Co-mediated toxicity, and nano-WC-Co exposure stimulated the M1 phenotype of macrophages	87

**Abbreviations:** TNF, tumor necrosis factor; IL, interleukin; LDH, lactate dehydrogenase; WC-Co, tungsten carbide cobalt.



**Figure 2** Cell viability after (A) nano-WC-Co and (B) micro-WC-Co particle exposure and (C) oxidative stress indicated by DCF fluorescence after exposure to 1,000  $\mu\text{g}/\text{mL}$  nano- and micro-WC-Co particles.<sup>86</sup> \* $P < 0.05$ , # $P < 0.001$  compared to control, † $P < 0.05$  compared to micro-WC-Co.

**Note:** Reproduced from Armstead AL, Arena CB, Li B. Exploring the potential role of tungsten carbide cobalt (WC-Co) nanoparticle internalization in observed toxicity toward lung epithelial cells in vitro. *Toxicol Appl Pharmacol.* 2014;278(1):1–8.<sup>86</sup>

**Abbreviations:** DCF, 2',7'-dichlorofluorescein; WC-Co, tungsten carbide cobalt.

enhanced toxicity of nano-sized over micro-sized particles has been clearly established.<sup>22,26,102,103</sup>

Although the effects of WC-Co particles have been established in multiple cell lines of varying origin, most of these studies used mono-culture systems in their examination of WC-Co particle toxicity, which may not provide an accurate assessment of what happens during/after WC-Co exposure since the local environment of the lung is highly dynamic and contains more than a single cell type. In a recent study,<sup>87</sup> the inflammatory response toward nano-WC-Co particles in macrophages and the toxicity of WC-Co have been examined using a co-culture model of lung epithelial cells and macrophages to more closely represent the dynamic tissue environment of the lung. Nano-WC-Co exposure has stimulated an inflammatory response in macrophages,

marked by high levels of interleukin-12 (IL-12) and IL-1 $\beta$  secretion.<sup>87</sup> In HMLD, lung inflammation and fibrosis occur in a progressive fashion; hence, it has been speculated that the induction of a pro-inflammatory response in macrophages may be an important factor in HMLD. This idea is supported by another study which has indicated that IL-1 in particular may play a role in pulmonary fibrosis,<sup>104</sup> and it seems reasonable to suggest that WC-Co may induce a similar type of pulmonary inflammatory response which promotes lung fibrosis after inhalation.

The large majority of previous WC-Co animal studies summarized in Table 5 employed the IT exposure model as the preferred means of delivery for hard metal particles and “dust” in vivo.<sup>5,72,89–92,94</sup> As noted in Table 5, the in vivo studies examined the effects of WC-Co particles in the

**Table 5** Summary of in vivo WC-Co toxicity studies

Method of delivery	Animal model & particle size	WC-Co dosage	Major outcome(s)	References
Intra-tracheal instillation (IT)	Rat Mixed WC-Co dusts ~0.1 to 6 $\mu$ m	Single IT bolus at high dose ( $\geq$ 1 mg/kg body weight)	At 6 months following single IT WC-Co exposure, rats presented with pulmonary edema, alveolar congestion, and lung fibrosis in regions of deposited WC-Co dusts	89
IT	Rat 5 $\mu$ m WC-Co	Single IT bolus at 1 mg/100 g body weight	WC-Co caused high mortality with massive pulmonary edema, increased macrophage counts, LDH, albumin and total protein content at 24 h post-exposure	90
IT	Rat 5 $\mu$ m WC-Co	Single and repeated IT bolus at 1, 5, or 10 mg/kg body weight	Single IT WC-Co exposure caused acute alveolitis which persisted for about 1 month following the IT bolus. Repeated weekly exposure (4x for 1 month) caused interstitial lung fibrosis and increased lung hydroxyproline levels in exposed rats	91
IT	Rat 2 $\mu$ m WC-Co	Single IT bolus at 16.6 mg/kg body weight	WC-Co exposure caused significant elevation of LDH, total protein & albumin in BAL fluids after 12 h and up to 72 h following exposure. In rat type II pneumocytes isolated after IT exposure, increased induction of micronuclei was observed, indicating genotoxicity and DNA damage	92
IT	Rat 2 $\mu$ m WC-Co	Single IT bolus at 1 mg/100 g body weight	24 h after single IT WC-Co exposure, significant increases in LDH, total protein, and albumin were found in BAL fluids. WC-Co did not exert any effects on the levels of IL-1, TNF $\alpha$ , fibronectin, or cystatin-C in BAL fluids of exposed animals	72
IT	Rat 3 $\mu$ m WC-Co	Single IT bolus at 2.5, 5, or 10 mg/100 g body weight	Pulmonary edema, fibrin formation, and increased number of inflammatory cells were observed in WC-Co-exposed rat lungs, along with decreased reactivity to methacholine, increased levels of nitric oxide synthase (NOS), LDH, total protein and albumin in BAL fluids	94
IT	Rat 1 $\mu$ m WC-Co	Single IT bolus at 1 or 3 mg per rat	A significant increase in LDH was observed after 1, 4, 7, and 30 days of WC-Co exposure and fibrosis alveolitis developed in rats after 30 days post-IT exposure	95
IT	Rat 80 nm WC-Co 190 nm CeO <sub>2</sub>	Single IT bolus at 0–500 $\mu$ g per rat 24 h exposure	A consistent lack of acute local pulmonary inflammation was observed in terms of the BAL fluid parameters examined in animals exposed to WC-Co NPs while significant acute pulmonary inflammation was observed in the CeO <sub>2</sub> NP group	96

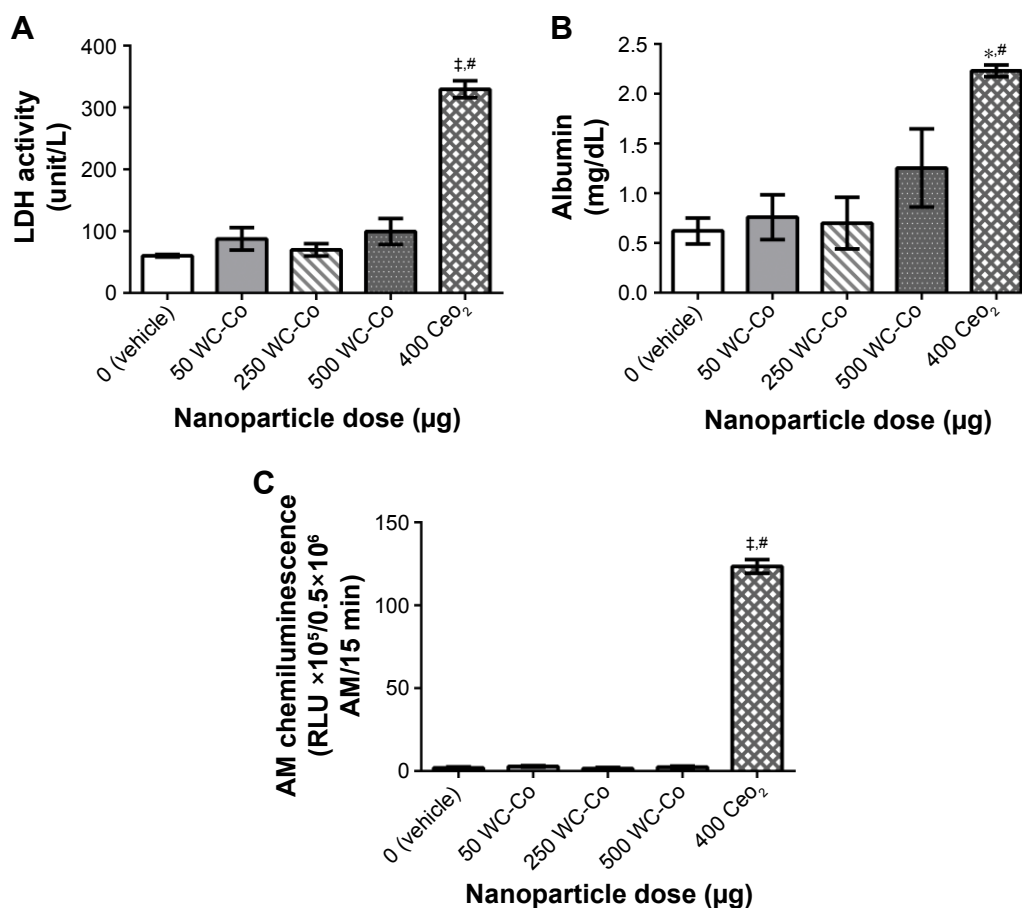
**Abbreviations:** BAL, bronchoalveolar lavage; NP, nanoparticle; LDH, lactate dehydrogenase; WC-Co, tungsten carbide cobalt.



micron-size range, using the mass-per-body weight dosing scheme (ie, mg/kg or mg/g), although some variation in particle size was noted in most cases (overall, WC-Co size ranging from 0.1 to 6  $\mu\text{m}$ ). Specifically, micro-sized WC-Co particle exposure caused significant pulmonary inflammation in rats, marked by increased bronchoalveolar lavage (BAL) fluid parameters such as lactate dehydrogenase (LDH) and albumin content, compared to control animals, in as little as 24 h after delivery the of WC-Co IT bolus.<sup>72,91,92,95</sup> Significant pulmonary edema, interstitial lung fibrosis, alveolar congestion, and alveolitis were also reported in micro-sized WC-Co-exposed animals at 1 and 6 months following a single IT exposure.<sup>89,90,95</sup> Given this body of literature, the local effects of micro-sized WC-Co following pulmonary exposure have been characterized relatively well, and it is understood that micro-sized WC-Co exerts both acute and chronic effects in vivo.

The local toxicity and potential systemic effects resulting from pulmonary nano-WC-Co exposure have recently

been investigated in rats, and surprisingly, an overall lack of toxicity and pulmonary inflammation after 24 h exposure to a single dose of nano-WC-Co (50, 250, or 500  $\mu\text{g}$ ) has been observed, while exposure to CeO<sub>2</sub> (400  $\mu\text{g}$ ) NPs has resulted in significant increases in the BAL fluid parameters examined (ie, LDH, albumin, and macrophage activation) (Figure 3).<sup>96</sup> This outcome seems to be perplexing, as most evidence regarding the effects of WC-Co exposure thus far has suggested that nano-WC-Co should cause acute toxicity both in vivo and in vitro, and the doses selected were based on the known literature for other NP generating pulmonary toxicity. The inflammatory response might have been missed since only a single exposure time (24 h) has been studied, and it is possible that a single exposure at these concentrations in vivo did not generate enough pulmonary toxicity/inflammation to be effectively detected through the BAL technique;<sup>96</sup> toxicity and inflammation may have occurred but not at detectable levels after the 24 h exposure time. It is also possible that the effects of nano-WC-Co exposure



**Figure 3** Pulmonary inflammation parameters assessed in the BAL fluid following 24 h exposure to WC-Co and CeO<sub>2</sub> NPs: (A) LDH activity, (B) albumin, and (C) AM chemiluminescence.<sup>96</sup> Values presented as mean  $\pm$  SD. <sup>\*</sup> $P < 0.05$ , <sup>‡</sup> $P < 0.001$  compared to the vehicle control, and <sup>#</sup> $P < 0.01$  compared to WC-Co NP exposed groups.

**Notes:** Reproduced from Armstead AL, Minarchick VC, Porter DW, Nurkiewicz TR, Li B. Acute inflammatory responses of nanoparticles in an intra-tracheal instillation rat model. *PLoS One*. 2015;10(3):e0118778.<sup>96</sup>

**Abbreviations:** BAL, bronchoalveolar lavage; LDH, lactate dehydrogenase; AM, alveolar macrophage; SD, standard deviation; NP, nanoparticle; WC-Co, tungsten carbide cobalt.

may occur in a cumulative fashion (ie, toxicity is observed after repeated or chronic exposures) *in vivo*; hence, multiple/repeated exposure may be a prerequisite for WC-Co toxicity in whole animals.

## Summary

NP exposure can occur in various consumer and industrial settings through various means such as inhalation, ingestion, or dermal routes. NP exposure, either via internal or external routes, can produce detrimental effects and has been studied both *in vitro* and *in vivo* using cell or animal-based models. Occupational exposure to hard metals such as WC-Co is an important concern in the field of nanotoxicology, since inhalation of WC-Co dusts and particles is known to cause HMLD, marked by progressive lung inflammation, fibrosis, and an increased risk of lung cancer. The current body of research clearly demonstrates that WC-Co particles are highly toxic and are capable of inducing oxidative stress and genotoxicity following exposure, and highlights the detrimental effects of WC-Co NP exposure in occupational settings, including hard metal manufacturing facilities and mining and drilling industries. It is important to define occupational WC-Co exposure limits so that future generations of workers are protected from this harmful disease. However, the pathology of HMLD and presence of the “hallmark” multinucleated giant cells have yet to be successfully reproduced in any animal models. Therefore, future studies should further investigate the mechanism underlying acute and chronic WC-Co toxicity and progression to HMLD in exposed workers.

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## Disclosure

The authors report no conflicts of interest in this work.

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