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Toxicity and oxidative stress responses induced by nano- and micro-CoCrMo particles

Andrea L. Armstead, PhD;[a,b] Thiago A. Simoes, PhD;[c] Xianfeng Wang, PhD;[a,d] Rik Brydson, PhD;[c] Andy Brown, PhD;[c] Bing-Hua Jiang, PhD;[e] Yon Rojanasakul, PhD[b,f] and Bingyun Li, PhD[a,b,f,*]

[a]Department of Orthopaedics, School of Medicine, West Virginia University, Morgantown, WV 26506, USA
[b]School of Pharmacy, West Virginia University, Morgantown, WV 26506, USA
[c]Institute for Materials Research, School of Chemical and Process Engineering, University of Leeds, LS2 9JT, UK
[d]Donghua University, Shanghai 201620, China
[e]Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, PA 19107, USA
[f]Mary Babb Randolph Cancer Center, Morgantown, WV 26506, USA

[*]Correspondence to:

Bingyun Li, PhD, Professor
Director, Biomaterials, Bioengineering & Nanotechnology Laboratory
Department of Orthopaedics
School of Medicine, West Virginia University
1 Medical Center Drive
Morgantown, WV 26506-9196, USA
Tel: 1-304-293-1075, Fax: 1-304-293-7070, Email: bili@hsc.wvu.edu
URL: http://medicine.hsc.wvu.edu/ortho-bli/

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ABSTRACT

Metal implants are used routinely during total hip and knee replacements and are typically composed of cobalt chromium molybdenum (CoCrMo) alloys. CoCrMo "wear particles", in the nano- and micro-size ranges, are generated in situ. Meanwhile, occupational exposure to CoCrMo particles may be associated with the development of industrial dental worker's pneumoconiosis. In this study, we report that both nano- and micro-CoCrMo particles induced a time and dose-dependent toxicity in various cell types (i.e. lung epithelial cells, osteoblasts, and macrophages), and the effects of particle size on cell viability and oxidative responses were interesting and cell specific. Our findings highlight the potential roles that nano- and micro-CoCrMo particles, whether exposure is due to inhalation or implant wear, and associated oxidative stress may play in the increasingly reported implant loosening, osteolysis, and systemic complications in orthopaedic patients, and may explain the risk of lung diseases in dental workers.

Keywords: Nanoparticle, implant wear, toxicity, oxidative stress, cobalt chromium molybdenum

1. Introduction

Over a million total hip replacement procedures are performed each year and cobalt chromium molybdenum (CoCrMo) alloys have been widely used as metal-on-metal or metal-on-polyethylene implant devices. While metal implant devices offer advantages, such as high strength, evidence emerges that metal (e.g. CoCrMo) implant devices may generate wear particles in situ, within the micro- and nano-size range, as a result of implant breakdown between the articulating joint surfaces.[1, 2] The generation of wear particles increases when the implant is improperly aligned, causing aseptic loosening of the joint, uneven wear and damage within the implant area.[2, 3] The specific role of CoCrMo particles in joint loosening or associated osteolysis remains unclear, although several sources suggest that the presence of wear particles within the joint cavity promotes a localized inflammatory response succeeded by resorptive bone loss.[4-7] Given this evidence and emerging concerns regarding the long term effects of CoCrMo particle exposure in joint replacement patients, the toxicity of CoCrMo wear particles has recently gained great interests both in vitro [8-12] and in vivo.[13-15]

In addition to "internal" and localized CoCrMo particle exposure due to implant wear, alternative routes of exposure such as inhalation or secondary exposure(s) due to particle translocation or migration from the initial site must be considered. For instance, CoCrMo particle inhalation may occur during the manufacturing and production in the medical device industry, thereby presenting an occupational exposure hazard. Although occupational exposure to CoCrMo particles has not been directly reported to date in orthopaedic implant manufacturing settings, pulmonary exposure to CoCrMo "dusts" with a similar composition to metal orthopaedic implant material have been reported previously in dental implant manufacturing settings.[16] Inhalation of CoCrMo particles might have been associated with the "dental technician's pneumoconiosis"

(DTP) in a number of cases.[17] In other industrial and manufacturing settings, inhalation of cobalt-containing metal "dusts", such as tungsten carbide cobalt (WC-Co), have been well-associated with the development of pneumoconiosis, occupational asthma and lung disease with increased risk of lung cancer.[18, 19] For DTP resulting from exposure to CoCrMo particles, patients develop lung disease with a similar clinical presentation to hard metal lung disease (HMLD) resulting from occupational inhalation of WC-Co particles; [3, 17, 20] therefore, we believe it is pertinent to examine the effects of CoCrMo particle exposure in a relevant in vitro pulmonary model.

There is also emerging evidence that particles within the nano-size range are capable of tissue translocation and migration to other organs, such as the liver, spleen or lungs,[21-23] where tissue deposition occurs and a secondary particle exposure is generated. This phenomenon may occur for CoCrMo particles generated internally at orthopaedic implant sites and the potential for secondary CoCrMo toxicity at sites distant from the initial exposure cannot be excluded. Therefore, it is critically important to understand the full range of effects of CoCrMo particle exposure on a variety of cell types which are potential targets for CoCrMo particle exposure, whether the initial exposure was due to internal particle generation from orthopaedic implants or from external sources such as inhalation in occupational settings. The goal of the current study was to examine the toxicity and oxidative stress response induced by nano- and micro-sized CoCrMo particles in various cell types using a nanotoxicity model recently developed in our lab.[24] We hypothesized that nano- and micro-CoCrMo particles would exert cell-specific, time and dose-dependent toxicity and oxidative stress response in lung epithelial cells, osteoblasts, and macrophages.

2. Methods

2.1. Materials and Reagents: CoCrMo microparticles (micro-CoCrMo) in the form of gas atomized powders from ASTM75 implants were used as received from Sandvik Osprey (Sandviken, Sweden); the chemical composition was 63.3±1.1 wt.% Co, 30.2±0.7 wt.% Cr and 6.5±1.2 wt.% Mo. Human lung bronchial epithelial BEAS-2B cells,[24] THP-1 (TIB-202) human monocyte/macrophage[25] and h.FOB1.19 (CRL-11372) human osteoblast cells[26-29] from our previous studies were from American Type Tissue Collection (ATCC; Manassas, VA). Dulbecco's Modified Eagle Media (DMEM), Ham's F12 Medium, sterile phosphate buffered saline (PBS), 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA), fetal bovine serum (FBS), G418 sulfate (geneticin) cell selection agent and penicillin/streptomycin were purchased from Lonza (Allendale, NJ). RPMI-1640 culture medium was purchased from ATCC. Isopropanol, hydrochloric acid, Triton-X-100, thiazolyl blue tetrazolinium bromide (MTT reagent), 2',7'-dichlorofluorescein diacetate (DCF), dihydroethidium (DHE) and phorbol-12-mystirate-13-acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Particle Preparation and Characterization: CoCrMo nanoparticles (nano-CoCrMo) were obtained via mechanical milling of the micro-CoCrMo particles (see Supplemental Materials). Dilute particle suspensions, ranging from 0.1 to 1000 μg/mL, were prepared in DMEM containing 10% FBS and used immediately on the day of each experiment. The particle size of nano-CoCrMo was analyzed using transmission electron microscopy (TEM). Average particle size was achieved by measuring Feret diameter of ca. 300 particles, which is defined as the distance between the most widely spaced nanoparticles in an agglomerate.[30] The particle size of micro-CoCrMo particles was characterized using scanning electron microscope (SEM). In addition, the average sizes of nano- and micro-CoCrMo particles in suspension in 10% FBS were determined using dynamic light scattering (DLS, Malvern Zetasizer version 7.01, Malvern Instruments). The

CoCrMo particles had a zeta potential of -25 mV and showed negligible aggregations in suspension in short time periods (e.g. 24 hr).[31]

2.3. Cell Culture and THP-1 Macrophage Differentiation: THP-1 monocytes were maintained in suspension culture and upon confluency, THP-1 cells were transferred and centrifuged to pellet. The cell pellet was re-suspended in RPMI containing PMA which induces THP-1 monocytes to undergo macrophage (M0) differentiation, and plated in a 96-well culture plate. More details of the cell culture of BEAS-2B, osteoblasts (OB), and macrophages (M0) are provided in the Supplemental Materials.

2.4. CoCrMo Particle Assay Interference: Prior to execution of the cell viability and oxidative stress assays, the potential interference of CoCrMo particles was examined under the experimental conditions (see Supplemental Materials).

2.5. CoCrMo Particle Exposure: Exposure to nano- and micro-CoCrMo particles was achieved by aspirating the media from each well and immediately replacing it with an equivalent volume of CoCrMo particle suspension at a concentration of 0.1-1000 μg/mL. Cell plates were then incubated at 37° C and 5% CO_2 for exposure periods of 6, 12, 24 and 48 hr.

2.6. Cell Viability Assay: For the viability assay, cells were exposed to either nano- or micro-CoCrMo particles at concentrations of 0.1, 1, 10, 100 and 1000 μg/mL for exposure periods of 6, 12, 24 and 48 hr. Following particle treatment, cells were rinsed once with sterile PBS to remove traces of media and excess particles. Then, 100 μL of un-supplemented DMEM was added to each well, followed by the addition of 10 μL MTT reagent to achieve a final concentration of 0.5 mg/mL MTT reagent per well. Cells were incubated for 2 hr at 37° C and 5% CO_2 to allow conversion of the soluble salt (yellow) to formazan crystals (purple). Crystal formation was confirmed using light microscopy. 100 μL of solubilization solution (0.1 M HCl in isopropanol with 10% Triton-X) was

then added to each well to dissolve the formazan crystals and the absorbance of each well was recorded at 570 nm using a Bio-Tek μQuant microplate reader (Winooski, VT). Blank values were subtracted from absorbance readings. Cell viability was calculated by dividing the absorbance of particle treated cells by the absorbance of control cells receiving media treatment only and converted to percentage; control cells represented 100% viability.

2.7. Oxidative Stress Assay: Oxidative stress was examined at the same CoCrMo particle concentrations and exposure range described for the viability assay (above). Following particle treatment, cells were rinsed once with sterile PBS to remove traces of media and excess particles. Oxidative stress was then determined by the addition of 10 μM DCF or DHE in PBS following particle treatment. Plates were incubated for 15 min in the dark and then fluorescence intensity of each well was quantified at 520 nm for DCF or 620 nm for DHE using a Bio-Tek Synergy H4 plate reader (Winooski, VT). The relative fluorescence of particle-treated cells was calculated as fold over control.

2.8. Statistical Analyses: All experiments were performed in triplicate and data are presented as mean ± standard deviation. Statistical analysis was carried out by two-way analysis of variance (ANOVA) using GraphPad Prism 6 software (La Jolla, CA). P values < 0.05 were considered significant.

3. Results

3.1. CoCrMo Particle Characterization and Assay Interference: TEM and SEM examinations showed that the nano- and micro-CoCrMo particles had average sizes of 35.4 ± 30.4 nm (**Figures. 1A** and **C**) and 4.8 ± 3.0 μm (**Figures 1B** and **D**), respectively. DLS analysis indicated that nano-CoCrMo averaged 54 nm and micro-CoCrMo particles averaged 5.0 μm in suspensions. EDX

confirmed that the composition of nano- and micro-CoCrMo particles were largely Co, Cr and Mo (**Figure S1**). We did not find any significant CoCrMo particle interference in our assays; no significant auto-reduction of the MTT dye was identified in the viability assay (**Figure S2**) and no significant changes in DCF/DHE fluorescence were observed due to CoCrMo particles under the assay conditions tested (**Figure S3**).

3.2. CoCrMo Effects on Cell Viability: BEAS-2B, OB and macrophages were exposed to nano- and micro-CoCrMo particles at concentrations of 0.1, 1, 10, 100 and 1000 μg/mL for durations of 6, 12, 24 and 48 hr. For BEAS-2B, the average cell viability was about 90-98% (vs. control of 100%) for cells exposed to nano- and micro-CoCrMo particles at concentrations of 0.1, 1 and 10 μg/mL for durations of 6-48 hr; the cell viability tended to decrease with increasing particle exposure time from 6 hr to 48 hr at concentrations of both 100 and 1000 μg/mL (**Figure 2**). In cells exposed to nano-CoCrMo particles (**Figure 2A**), a significant reduction in viability (compared to control) was observed at 100 μg/mL after 12, 24 and 48 hr of exposure and at the highest concentration of 1000 μg/mL after 6-48 hr of exposure. Similarly, in BEAS-2B cells exposed to micro-CoCrMo particles (**Figure 2B**), a significant reduction in viability (compared to control) was observed at 100 μg/mL after 12, 24 and 48 hr of exposure and at the highest concentration of 1000 μg/mL after 6-48 hr of exposure. When comparing the toxicity of nano- and micro-CoCrMo under identical conditions, nano-CoCrMo caused significantly less toxicity than micro-CoCrMo in BEAS-2B cells at 100 μg/mL after 24 and 48 hr of exposure and at 1000 μg/mL after 6 and 12 hr of exposure; toxicity was similar for 1000 μg/mL nano- and micro-CoCrMo after 24 and 48 hr of exposure.

For osteoblasts (OB), cell viability remained high (> 90%) over the exposure periods tested (6-48 hr) for 0.1-10 μg/mL nano- and micro-CoCrMo particles (**Figure 2C**). At 100 and 1000

μg/mL, a significant decrease in cell viability (compared to control) was observed after 6-48 hr exposure of nano- (**Figure 2C**) and micro-CoCrMo (**Figure 2D**) particles and the cell viability decreased with increasing exposure time. There were no significant differences in the toxicity of nano- and micro-CoCrMo particles over the concentration and exposure range studied, with the exception of 1000 μg/mL, where nano-CoCrMo caused significantly less toxicity than micro-CoCrMo in OB after 24 hr of exposure (~70 % vs. ~60 % remaining cell viability, respectively).

In macrophages (M0), cell viability remained > 90% for the lowest concentrations of 0.1 and 1 μg/mL over the 6-48 hr exposure period for both nano- and micro-CoCrMo (**Figure 2**). M0 exposed to nano-CoCrMo had significantly reduced viability (compared to control) after 24 and 48 hr exposure to 10 μg/mL (**Figure 2E**); no significant toxicity was observed between CoCrMo particles and controls at this concentration in either BEAS-2B or OB under these conditions. Significantly reduced cell viability was also observed for the micro-CoCrMo particles at 10 μg/mL after 48 hr of exposure (**Figure 2F**). Moreover, at 100 and 1000 μg/mL, a significant decrease in cell viability (compared to control) was observed for both nano- and micro-CoCrMo particles at the time exposures studied except at 6 hr of 100 μg/mL of micro-CoCrMo particles. When compared directly, M0 viability after exposure to 1000 μg/mL nano-CoCrMo for 24 and 48 hr was significantly lower than M0 exposed to micro-CoCrMo particles under identical conditions.

3.3. CoCrMo Effects on Oxidative Stress: Oxidative stress was measured in the form of DCF/DHE fluorescence after exposure to nano- and micro-CoCrMo particles under identical exposure conditions tested in the viability assay. Compared to control, there was a significant increase in DCF fluorescence in BEAS-2B cells exposed to 100 μg/mL nano-CoCrMo after 6, 12 and 24 hr of exposure and at 1000 μg/mL after 6, 12, 24 and 48 hr of exposure; a maximum 3.5 fold increase in DCF fluorescence was observed in BEAS-2B cells exposed to 1000 μg/mL nano-

CoCrMo after 6 hr of exposure, after which DCF fluorescence decreased with increasing exposure time (**Figure 3A**). In BEAS-2B cells exposed to micro-CoCrMo particles, a significant increase in DCF fluorescence was observed after 6 hr exposure to 10 and 100 μg/mL and after 6, 12, 24 and 48 hr exposure to 1000 μg/mL micro-CoCrMo; a maximum 2.3 fold increase in DCF fluorescence was observed in cells exposed to 1000 μg/mL micro-CoCrMo after 6 hr of exposure (**Figure 3B**). At 1000 μg/mL of both nano- and micro-CoCrMo particles, the DCF fluorescence decreased with increasing exposure time (**Figure 3**). In addition, nano-CoCrMo particles caused a significantly greater change in DCF fluorescence compared to micro-CoCrMo particles after 6, 12 and 24 hr exposure to 100 μg/mL and after 6, 12, 24 and 48 hr at 1000 μg/mL (**Figure 3**).

For dihydroethidium (DHE), no significant differences, compared to control, were observed in BEAS-2B fluorescence after exposure to nano-CoCrMo (**Figure 4A**) or micro-CoCrMo (**Figure 4B**) particles. The observed DHE fluorescence in BEAS-2B cells exposed to both nano- and micro-CoCrMo particles was about the same as the control cells at all concentrations (0.1-1000 μg/mL) and exposure times (6-48 hr) studied.

In osteoblasts (OB), nano-CoCrMo caused a significant increase in 2',7'-dichlorofluorescein diacetate (DCF) fluorescence, compared to control, at 0.1 μg/mL after 12 hr, at 100 μg/mL after 12 and 24 hr and a maximum increase in DCF fluorescence at 1000 μg/mL after 24 hr of exposure, about 1.5-fold higher than control (**Figure 5A**). Exposure to micro-CoCrMo caused significantly increased DCF fluorescence, compared to control, after 12 hr exposure to 0.1, 10, 100 and 1000 μg/mL and after 24 hr exposure to 1000 μg/mL (**Figure 5B**). Overall, nano-CoCrMo caused significantly higher DCF florescence than micro-CoCrMo in OB after 24 hr exposure to 100 and 1000 μg/mL (**Figure 5**).

A varied effect on dihydroethidium (DHE) fluorescence was observed in osteoblasts (OB) exposed to nano- and micro-CoCrMo particles (**Figure 6**). Compared to control, a significant increase in DHE fluorescence was observed in OB exposed to nano-CoCrMo at 0.1 μg/mL after 48 hr, at 1 μg/mL after 6, 24 and 48 hr, at 10 μg/mL after 12, 24 and 48 hr, at 100 μg/mL after 6 and 12 hr and at 1000 μg/mL after 6, 12, 24, and 48 hr of exposure (**Figure 6A**). For micro-CoCrMo particles, a significant increase in DHE, compared to control, was observed for 0.1-1000 μg/mL after 6 hr of exposure and for 1, 10, 100 and 1000 μg/mL after 12 hr of exposure (**Figure 6B**). Compared to micro-CoCrMo, nano-CoCrMo caused significantly less DHE fluorescence at 0.1 and 1 μg/mL after 6 hr and at 1, 10 and 100 μg/mL after 12 hr; however, at 1000 μg/mL, nano-CoCrMo caused significantly higher DHE fluorescence than micro-CoCrMo after 6, 24 and 48 hr of exposure (**Figure 6A**).

In macrophages (M0), nano- and micro-CoCrMo particles caused significant increases in 2',7'-dichlorofluorescein diacetate (DCF) fluorescence, compared to control, at all concentrations (0.1-1000 μg/mL) and exposure times tested (**Figure 7**). The maximum increase in DCF was observed at 1000 μg/mL after 6 and 12 hr exposure (**Figure 7**) for both nano- and micro-CoCrMo particles. Compared directly, nano-CoCrMo caused significantly less DCF fluorescence than micro-CoCrMo particles after 12 hr exposure to 0.1, 10 and 100 μg/mL; however, nano-CoCrMo caused significantly higher DCF fluorescence than micro-CoCrMo after 6 and 12 hr exposure to 100 μg/mL and after 24 and 48 hr exposure to 1000 μg/mL (**Figure 7**).

Significantly increased dihydroethidium (DHE) fluorescence, compared to control, was observed in macrophages (M0) exposed to nano-CoCrMo at all concentrations tested (0.1-1000 μg/mL) after 6, 12 and 24 hr of exposure; no changes in DHE were observed after 48 hr of exposure at any concentration (**Figure 8A**). In M0 exposed to micro-CoCrMo, a significant increase in DHE

fluorescence was observed after 6 and 12 hr exposure to 0.1-1000 μg/mL; DHE levels were similar to control at all concentrations after 24 and 48 hr of exposure to micro-CoCrMo (**Figure 8B**). Compared to micro-CoCrMo, nano-CoCrMo caused significantly higher DHE levels in M0 at all concentrations (0.1-1000 μg/mL) after 12 and 24 hr of exposure (**Figure 8**).

4. Discussion

Nanoparticles, due to their smaller size, have a higher capacity (compared to microparticles) to enter the circulatory system and deposit in tissues and organs such as liver, spleen, kidney, lymph node and lung,[3, 32-34] and the potential systemic effects of nanoparticle exposure could be of importance.[35] However, the role of nanoparticles and microparticles from orthopaedic implant wear in systemic responses is unknown although patients who undergo CoCrMo joint replacements have presented translocation and deposition of CoCrMo wear particles in lymph nodes, liver and spleen.[3, 36] Meanwhile, inhalation of cobalt-containing metal particles may be associated with dental technician's pneumoconiosis,[16, 17, 20, 37] and CoCrMo wear particles have also been a major concern of local toxicity and inflammation. Therefore, the goal of this study was to examine the toxic effects of nano- and micro-sized CoCrMo particles, originating from ASTM F75 orthopaedic implant materials, in a range of relevant cell types representing the potential routes of exposures, including lung epithelial cells, osteoblasts, and macrophages.

Our studies suggest that both nano- and micro-CoCrMo particles can induce toxicity in all cell types studied and the responses of cell viability and oxidative stress are dose, exposure time and cell type specific. Across the three cell types tested, at low concentrations (i.e. 0.1 and 1 μg/mL), nano- and micro-CoCrMo particles did not cause significant toxicity in our viability assay. Typically, in the presence of small amounts of foreign particles, cells may isolate the particles in

internal phagolysosomal compartments, which could prohibit them from further interacting with other cellular components thereby preventing extensive cellular toxicity.[11, 38] The similarity in low toxicity between the nano- and micro-CoCrMo particles reported here in lung epithelial cells, osteoblasts and macrophages at concentrations less than 10 μg/mL seems to support the high biocompatibility of CoCrMo alloys in orthopaedic settings;[39] CoCrMo has been used prevalently in orthopaedic surgeries.[3] At high concentrations (i.e. 100 and 1000 μg/mL for BEAS-2B and OB cells, and 10, 100 and 1000 μg/mL for M0 cells), both nano- and micro-CoCrMo particles could lead to significant decreases in viability in all cell types tested. It was reported that significant toxicity was observed in osteoblast-like cells exposed to ≥ 100 μg/mL micro-CoCr alloy particles after 24 and 48 hr exposure.[40] The current study provides direct evidence that nano- and micro-CoCrMo particles cause toxicity toward lung epithelial cells in vitro; although lung epithelial cells are not a direct site of exposure in the case of orthopaedic joint wear, we speculate that these data may help explain the risk of lung disease in dental workers[16, 17, 20, 37, 41, 42] and highlights the need for further examination of pulmonary toxicity caused by CoCrMo particles, whether exposure is due to inhalation (in the case of DTP) or tissue migration of implant wear particles to the lung.

One would normally expect that nanoparticles exert greater toxic effects than microparticles of the same chemical composition due to their smaller size and increased surface area.[31, 33, 43-45] However, in this study, no significant differences in cell viability were observed between nano- and micro-CoCrMo particle exposures in most of the concentrations and exposure times studied. Interestingly, compared to micro-CoCrMo particles, nano-CoCrMo particles led to significantly lower viability of macrophages and significantly higher viability of lung epithelial cells and osteoblasts at 1000 μg/mL. In macrophages, it was believed that nanoparticles, due to their smaller size and thereby faster degradation at a given pH, could lead to more impairment in

phagocytosis and be more toxic to macrophages compared to microparticles.[46-48] In this case, it possible that differences in the uptake of nano- and micro-CoCrMo could have contributed to the higher toxicity of nano-CoCrMo, as smaller particles may be more frequently and rapidly phagocytosed compared to the larger micro-CoCrMo particles. It is not clear why nano-CoCrMo was less toxic, compared to micro-CoCrMo, to lung epithelial cells and osteoblasts in this study and further investigations are much needed.

Oxidative stress has been implicated in age-related bone resorption and osteoporosis[49] and in toxicity of CoCrMo particles in fibroblasts,[50-52] and may also play a role in the progression of lung diseases,[53] such as those caused by cobalt-containing metal exposures.[54] Therefore, it is important to examine the capacity of nano- and micro-CoCrMo particles in causing oxidative stress in our cell models. In this case, we used a two-fold approach to assess the induction of oxidative stress using DCF, which serves as a 'generalized' marker for reactive oxygen species,[55] and DHE, which serves as a specific marker of superoxide anion.[56] It seems that the oxidative responses against nano- and micro-CoCrMo particles were cell specific: both nano- and micro-CoCrMo particles resulted in significantly higher DCF levels and DHE levels in OB and M0 cells; significantly higher DCF and DHE levels were observed in macrophages at all concentrations studied (0.1-1000 µg/mL). It seems that the OB cells behaved like the M0 immune cells, which are known to exhibit a "respiratory burst" upon phagocytosis of microbes, marked by significant increases in the production of hydrogen peroxide and superoxide anion via enzymatic pathways that are critical for initiating anti-microbial response and infection clearance.[57] Meanwhile, corrosion of metal in aqueous environment could contribute to oxidative stress. Low levels (e.g. 0.02 µg/mL) of Mo, Co, and Cr ions have been detected in CoCrMo particle solutions after short time exposures (e.g. 24 hr),[58] and substantial evidence has indicated that metals and metal ions,

including Co and Cr, cause oxidative stress in situ regardless of the means of exposure.[5, 7] In this study, the oxidative stress was likely attributed to the combined effects of nanoparticle exposure and the ions released.

The significantly increased oxidative stress of osteoblasts (OB) and macrophage (M0) cells may help explain the increased risks of implant loosening and osteolysis in orthopaedic implant patients,[4-7] as there is evidence suggesting that the presence of wear particles in the joint fluid stimulates a localized inflammatory response.[4] Localized inflammation promotes osteoclast activity, bone resorption and loosening of the implant.[59] By contrast, BEAS-2B cells had no significant DHE changes but had significantly increased DCF levels at relatively high particle concentrations (e.g. 100 and 1000 μg/mL). Moreover, nano-CoCrMo caused significantly higher levels of oxidative stress in lung epithelial cells compared to micro-CoCrMo particles at concentrations of 100 and 1000 μg/mL, which was consistent with the expected size-dependent effect due to the increased reactive surface area of nano-CoCrMo compared to micro-CoCrMo. No significant differences were found in the DHE assay, which suggests that CoCrMo particles cause oxidative stress via other species than superoxide anion. Additionally, we found these results were consistent with the fibroblast studies in the literature,[51, 60] which found high levels of oxidative stress, marked by increased levels of DCF fluorescence, after as little as 2 hr of exposure[60] and increased levels of 8-OHdG staining, a marker of oxidative stress induced DNA damage, after 24 hr of exposure to CoCrMo particles.[51] Increased levels of oxidative stress in lung epithelial cells could ultimately lead to downstream effects such as DNA damage and genotoxicity upon long term exposure[11, 51, 61] and may therefore be a contributing factor in the development of lung disease from pulmonary CoCrMo particle exposure in occupational settings.

5. Conclusions

This study examined the toxicity of nano- and micro-CoCrMo particles and determined whether their exposure induced oxidative stress in human lung epithelial cells, osteoblasts and macrophages. These in vitro findings suggest that both nano- and micro-CoCrMo particles can induce toxicity and the responses of cell viability and oxidative stress are dose, exposure time and cell type specific. In future studies, the mechanism of cellular uptake and the cellular distribution and excretion of CoCrMo particles will be investigated. The toxicity of these particles will be further examined in animal models which generally provides a better approximation of what may occur during a real-life exposure situation. For instance, CoCrMo nanoparticles may be injected in a bone implant rat model[62-64] or exposed to the lung in an intra-tracheal instillation rat model[65] to examine their local and systemic toxicity.

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LIST OF ABBREIVIATED TERMS

ATCC: American Type Tissue Collection

CoCrMo: cobalt chromium molybdenum

DCF: 2',7'-dichlorofluorescein diacetate

DHE: dihydroethidium

DLS: dynamic light scattering

DMEM: Dulbecco's Modified Eagle Media

DTP: dental technician's pneumoconiosis

EDTA: ethylenediaminetetraacetic acid

FBS: fetal bovine serum

HMLD: hard metal lung disease

micro-CoCrMo: CoCrMo microparticles

nano-CoCrMo: CoCrMo nanoparticles

OB: osteoblast

PBS: phosphate buffered saline

PMA: phorbol-12-mystirate-13-acetate

SEM: scanning electron microscope

TEM: transmission electron microscopy

WC-Co: tungsten carbide cobalt

FIGURE AND TABLE LEGEND

Figure 1. A,B) Images and C,D) particle size distribution of A,C) nano- and B, D) micro-CoCrMo particles.

Figure 2. Viability of (A, B) BEAS-2B lung epithelial cells, (C, D) osteoblasts, and (E, F) macrophages after exposure to (A, C, E) nano- and (B, D, F) micro-CoCrMo particles. (*$P < 0.05$, †$P < 0.01$ compared to control; ‡$P < 0.05$ vs. micro-CoCrMo)

Figure 3. BEAS-2B oxidative stress measured via fluorescence intensity of DCF after exposure to A) nano- and B) micro-CoCrMo particles. (*$P < 0.05$, †$P < 0.01$ compared to control; ‡$P < 0.05$ vs. micro-CoCrMo)

Figure 4. BEAS-2B oxidative stress measured via fluorescence intensity of DHE after exposure to A) nano- and B) micro-CoCrMo particles. (*$P < 0.05$, †$P < 0.01$ compared to control; ‡$P < 0.05$ vs. micro-CoCrMo)

Figure 5. Osteoblast oxidative stress measured via fluorescence intensity of DCF after exposure to A) nano- and B) micro-CoCrMo particles. (*$P < 0.05$, †$P < 0.01$ compared to control; ‡$P < 0.05$ vs. micro-CoCrMo)

Figure 6. Osteoblast oxidative stress measured via fluorescence intensity of DHE after exposure to A) nano- and B) micro-CoCrMo particles. (*$P < 0.05$, †$P < 0.01$ compared to control; ‡$P < 0.05$ vs. micro-CoCrMo)

Figure 7. Macrophage oxidative stress measured via fluorescence intensity of DCF after exposure to A) nano- and B) micro-CoCrMo particles. (*$P < 0.05$, †$P < 0.01$ compared to control; ‡$P < 0.05$ vs. micro-CoCrMo)

Figure 8. Macrophage oxidative stress measured via fluorescence intensity of DHE after exposure to A) nano- and B) micro-CoCrMo particles. (*$P < 0.05$, †$P < 0.01$ compared to control; ‡$P < 0.05$ vs. micro-CoCrMo)

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