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

3
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31

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44 **ABSTRACT**

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

56

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

58 **1. Introduction**

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

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

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

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

102

103 **2. Methods**

104 **2.1. Materials and Reagents:** CoCrMo microparticles (micro-CoCrMo) in the form of gas
105 atomized powders from ASTM75 implants were used as received from Sandvik Osprey
106 (Sandviken, Sweden); the chemical composition was 63.3 ± 1.1 wt.% Co, 30.2 ± 0.7 wt.% Cr and
107 6.5 ± 1.2 wt.% Mo. Human lung bronchial epithelial BEAS-2B cells,²⁴ THP-1 (TIB-202) human
108 monocyte/macrophage²⁵ and h.FOB1.19 (CRL-11372) human osteoblast cells²⁶⁻²⁹ from our
109 previous studies were from American Type Tissue Collection (ATCC; Manassas, VA). Dulbecco's
110 Modified Eagle Media (DMEM), Ham's F12 Medium, sterile phosphate buffered saline (PBS),
111 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA), fetal bovine serum (FBS), G418 sulfate
112 (geneticin) cell selection agent and penicillin/streptomycin were purchased from Lonza (Allendale,
113 NJ). RPMI-1640 culture medium was purchased from ATCC. Isopropanol, hydrochloric acid,
114 Triton-X-100, thiazolyl blue tetrazolium bromide (MTT reagent), 2',7'-dichlorofluorescein
115 diacetate (DCF), dihydroethidium (DHE) and phorbol-12-myristate-13-acetate (PMA) were
116 purchased from Sigma-Aldrich (St. Louis, MO).

117 **2.2. Particle Preparation and Characterization:** CoCrMo nanoparticles (nano-CoCrMo) were
118 obtained via mechanical milling of the micro-CoCrMo particles (see Supplemental Materials).
119 Dilute particle suspensions, ranging from 0.1 to 1000 $\mu\text{g}/\text{mL}$, were prepared in DMEM containing
120 10% FBS and used immediately on the day of each experiment. The particle size of nano-CoCrMo
121 was analyzed using transmission electron microscopy (TEM). Average particle size was achieved
122 by measuring Feret diameter of ca. 300 particles, which is defined as the distance between the
123 most widely spaced nanoparticles in an agglomerate.³⁰ The particle size of micro-CoCrMo
124 particles was characterized using scanning electron microscope (SEM). In addition, the average
125 sizes of nano- and micro-CoCrMo particles in suspension in 10% FBS were determined using
126 dynamic light scattering (DLS, Malvern Zetasizer version 7.01, Malvern Instruments). The

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

129 **2.3. Cell Culture and THP-1 Macrophage Differentiation:** THP-1 monocytes were maintained
130 in suspension culture and upon confluency, THP-1 cells were transferred and centrifuged to pellet.
131 The cell pellet was re-suspended in RPMI containing PMA which induces THP-1 monocytes to
132 undergo macrophage (M0) differentiation, and plated in a 96-well culture plate. More details of
133 the cell culture of BEAS-2B, osteoblasts (OB), and macrophages (M0) are provided in the
134 Supplemental Materials.

135 **2.4. CoCrMo Particle Assay Interference:** Prior to execution of the cell viability and oxidative
136 stress assays, the potential interference of CoCrMo particles was examined under the experimental
137 conditions (see Supplemental Materials).

138 **2.5. CoCrMo Particle Exposure:** Exposure to nano- and micro-CoCrMo particles was achieved
139 by aspirating the media from each well and immediately replacing it with an equivalent volume of
140 CoCrMo particle suspension at a concentration of 0.1-1000 µg/mL. Cell plates were then incubated
141 at 37° C and 5% CO₂ for exposure periods of 6, 12, 24 and 48 hr.

142 **2.6. Cell Viability Assay:** For the viability assay, cells were exposed to either nano- or micro-
143 CoCrMo particles at concentrations of 0.1, 1, 10, 100 and 1000 µg/mL for exposure periods of 6,
144 12, 24 and 48 hr. Following particle treatment, cells were rinsed once with sterile PBS to remove
145 traces of media and excess particles. Then, 100 µL of un-supplemented DMEM was added to each
146 well, followed by the addition of 10 µL MTT reagent to achieve a final concentration of 0.5 mg/mL
147 MTT reagent per well. Cells were incubated for 2 hr at 37° C and 5% CO₂ to allow conversion of
148 the soluble salt (yellow) to formazan crystals (purple). Crystal formation was confirmed using light
149 microscopy. 100 µL of solubilization solution (0.1 M HCl in isopropanol with 10% Triton-X) was

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

155 **2.7. Oxidative Stress Assay:** Oxidative stress was examined at the same CoCrMo particle
156 concentrations and exposure range described for the viability assay (above). Following particle
157 treatment, cells were rinsed once with sterile PBS to remove traces of media and excess particles.
158 Oxidative stress was then determined by the addition of 10 μ M DCF or DHE in PBS following
159 particle treatment. Plates were incubated for 15 min in the dark and then fluorescence intensity of
160 each well was quantified at 520 nm for DCF or 620 nm for DHE using a Bio-Tek Synergy H4
161 plate reader (Winooski, VT). The relative fluorescence of particle-treated cells was calculated as
162 fold over control.

163 **2.8. Statistical Analyses:** All experiments were performed in triplicate and data are presented as
164 mean \pm standard deviation. Statistical analysis was carried out by two-way analysis of variance
165 (ANOVA) using GraphPad Prism 6 software (La Jolla, CA). P values $<$ 0.05 were considered
166 significant.

167

168 **3. Results**

169 **3.1. CoCrMo Particle Characterization and Assay Interference:** TEM and SEM examinations
170 showed that the nano- and micro-CoCrMo particles had average sizes of 35.4 ± 30.4 nm (**Figures**
171 **1A** and **C**) and 4.8 ± 3.0 μ m (**Figures 1B** and **D**), respectively. DLS analysis indicated that nano-
172 CoCrMo averaged 54 nm and micro-CoCrMo particles averaged 5.0 μ m in suspensions. EDX

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

178 **3.2. CoCrMo Effects on Cell Viability:** BEAS-2B, OB and macrophages were exposed to nano-
179 and micro-CoCrMo particles at concentrations of 0.1, 1, 10, 100 and 1000 $\mu\text{g}/\text{mL}$ for durations of
180 6, 12, 24 and 48 hr. For BEAS-2B, the average cell viability was about 90-98% (vs. control of
181 100%) for cells exposed to nano- and micro-CoCrMo particles at concentrations of 0.1, 1 and 10
182 $\mu\text{g}/\text{mL}$ for durations of 6-48 hr; the cell viability tended to decrease with increasing particle
183 exposure time from 6 hr to 48 hr at concentrations of both 100 and 1000 $\mu\text{g}/\text{mL}$ (**Figure 2**). In
184 cells exposed to nano-CoCrMo particles (**Figure 2A**), a significant reduction in viability
185 (compared to control) was observed at 100 $\mu\text{g}/\text{mL}$ after 12, 24 and 48 hr of exposure and at the
186 highest concentration of 1000 $\mu\text{g}/\text{mL}$ after 6-48 hr of exposure. Similarly, in BEAS-2B cells
187 exposed to micro-CoCrMo particles (**Figure 2B**), a significant reduction in viability (compared to
188 control) was observed at 100 $\mu\text{g}/\text{mL}$ after 12, 24 and 48 hr of exposure and at the highest
189 concentration of 1000 $\mu\text{g}/\text{mL}$ after 6-48 hr of exposure. When comparing the toxicity of nano- and
190 micro-CoCrMo under identical conditions, nano-CoCrMo caused significantly less toxicity than
191 micro-CoCrMo in BEAS-2B cells at 100 $\mu\text{g}/\text{mL}$ after 24 and 48 hr of exposure and at 1000 $\mu\text{g}/\text{mL}$
192 after 6 and 12 hr of exposure; toxicity was similar for 1000 $\mu\text{g}/\text{mL}$ nano- and micro-CoCrMo after
193 24 and 48 hr of exposure.

194 For osteoblasts (OB), cell viability remained high (> 90%) over the exposure periods tested
195 (6-48 hr) for 0.1-10 $\mu\text{g}/\text{mL}$ nano- and micro-CoCrMo particles (**Figure 2C**). At 100 and 1000

196 $\mu\text{g}/\text{mL}$, a significant decrease in cell viability (compared to control) was observed after 6-48 hr
197 exposure of nano- (**Figure 2C**) and micro-CoCrMo (**Figure 2D**) particles and the cell viability
198 decreased with increasing exposure time. There were no significant differences in the toxicity of
199 nano- and micro-CoCrMo particles over the concentration and exposure range studied, with the
200 exception of 1000 $\mu\text{g}/\text{mL}$, where nano-CoCrMo caused significantly less toxicity than micro-
201 CoCrMo in OB after 24 hr of exposure (~70 % vs. ~60 % remaining cell viability, respectively).

202 In macrophages (M0), cell viability remained > 90% for the lowest concentrations of 0.1
203 and 1 $\mu\text{g}/\text{mL}$ over the 6-48 hr exposure period for both nano- and micro-CoCrMo (**Figure 2**). M0
204 exposed to nano-CoCrMo had significantly reduced viability (compared to control) after 24 and
205 48 hr exposure to 10 $\mu\text{g}/\text{mL}$ (**Figure 2E**); no significant toxicity was observed between CoCrMo
206 particles and controls at this concentration in either BEAS-2B or OB under these conditions.
207 Significantly reduced cell viability was also observed for the micro-CoCrMo particles at 10 $\mu\text{g}/\text{mL}$
208 after 48 hr of exposure (**Figure 2F**). Moreover, at 100 and 1000 $\mu\text{g}/\text{mL}$, a significant decrease in
209 cell viability (compared to control) was observed for both nano- and micro-CoCrMo particles at
210 the time exposures studied except at 6 hr of 100 $\mu\text{g}/\text{mL}$ of micro-CoCrMo particles. When
211 compared directly, M0 viability after exposure to 1000 $\mu\text{g}/\text{mL}$ nano-CoCrMo for 24 and 48 hr was
212 significantly lower than M0 exposed to micro-CoCrMo particles under identical conditions.

213 **3.3. CoCrMo Effects on Oxidative Stress:** Oxidative stress was measured in the form of
214 DCF/DHE fluorescence after exposure to nano- and micro-CoCrMo particles under identical
215 exposure conditions tested in the viability assay. Compared to control, there was a significant
216 increase in DCF fluorescence in BEAS-2B cells exposed to 100 $\mu\text{g}/\text{mL}$ nano-CoCrMo after 6, 12
217 and 24 hr of exposure and at 1000 $\mu\text{g}/\text{mL}$ after 6, 12, 24 and 48 hr of exposure; a maximum 3.5
218 fold increase in DCF fluorescence was observed in BEAS-2B cells exposed to 1000 $\mu\text{g}/\text{mL}$ nano-

219 CoCrMo after 6 hr of exposure, after which DCF fluorescence decreased with increasing exposure
220 time (**Figure 3A**). In BEAS-2B cells exposed to micro-CoCrMo particles, a significant increase in
221 DCF fluorescence was observed after 6 hr exposure to 10 and 100 $\mu\text{g}/\text{mL}$ and after 6, 12, 24 and
222 48 hr exposure to 1000 $\mu\text{g}/\text{mL}$ micro-CoCrMo; a maximum 2.3 fold increase in DCF fluorescence
223 was observed in cells exposed to 1000 $\mu\text{g}/\text{mL}$ micro-CoCrMo after 6 hr of exposure (**Figure 3B**).
224 At 1000 $\mu\text{g}/\text{mL}$ of both nano- and micro-CoCrMo particles, the DCF fluorescence decreased with
225 increasing exposure time (**Figure 3**). In addition, nano-CoCrMo particles caused a significantly
226 greater change in DCF fluorescence compared to micro-CoCrMo particles after 6, 12 and 24 hr
227 exposure to 100 $\mu\text{g}/\text{mL}$ and after 6, 12, 24 and 48 hr at 1000 $\mu\text{g}/\text{mL}$ (**Figure 3**).

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

233 In osteoblasts (OB), nano-CoCrMo caused a significant increase in 2',7'-
234 dichlorofluorescein diacetate (DCF) fluorescence, compared to control, at 0.1 $\mu\text{g}/\text{mL}$ after 12 hr,
235 at 100 $\mu\text{g}/\text{mL}$ after 12 and 24 hr and a maximum increase in DCF fluorescence at 1000 $\mu\text{g}/\text{mL}$
236 after 24 hr of exposure, about 1.5-fold higher than control (**Figure 5A**). Exposure to micro-
237 CoCrMo caused significantly increased DCF fluorescence, compared to control, after 12 hr
238 exposure to 0.1, 10, 100 and 1000 $\mu\text{g}/\text{mL}$ and after 24 hr exposure to 1000 $\mu\text{g}/\text{mL}$ (**Figure 5B**).
239 Overall, nano-CoCrMo caused significantly higher DCF fluorescence than micro-CoCrMo in OB
240 after 24 hr exposure to 100 and 1000 $\mu\text{g}/\text{mL}$ (**Figure 5**).

241 A varied effect on dihydroethidium (DHE) fluorescence was observed in osteoblasts (OB)
242 exposed to nano- and micro-CoCrMo particles (**Figure 6**). Compared to control, a significant
243 increase in DHE fluorescence was observed in OB exposed to nano-CoCrMo at 0.1 $\mu\text{g}/\text{mL}$ after
244 48 hr, at 1 $\mu\text{g}/\text{mL}$ after 6, 24 and 48 hr, at 10 $\mu\text{g}/\text{mL}$ after 12, 24 and 48 hr, at 100 $\mu\text{g}/\text{mL}$ after 6
245 and 12 hr and at 1000 $\mu\text{g}/\text{mL}$ after 6, 12, 24, and 48 hr of exposure (**Figure 6A**). For micro-
246 CoCrMo particles, a significant increase in DHE, compared to control, was observed for 0.1-1000
247 $\mu\text{g}/\text{mL}$ after 6 hr of exposure and for 1, 10, 100 and 1000 $\mu\text{g}/\text{mL}$ after 12 hr of exposure (**Figure**
248 **6B**). Compared to micro-CoCrMo, nano-CoCrMo caused significantly less DHE fluorescence at
249 0.1 and 1 $\mu\text{g}/\text{mL}$ after 6 hr and at 1, 10 and 100 $\mu\text{g}/\text{mL}$ after 12 hr; however, at 1000 $\mu\text{g}/\text{mL}$, nano-
250 CoCrMo caused significantly higher DHE fluorescence than micro-CoCrMo after 6, 24 and 48 hr
251 of exposure (**Figure 6A**).

252 In macrophages (M0), nano- and micro-CoCrMo particles caused significant increases in
253 2',7'-dichlorofluorescein diacetate (DCF) fluorescence, compared to control, at all concentrations
254 (0.1-1000 $\mu\text{g}/\text{mL}$) and exposure times tested (**Figure 7**). The maximum increase in DCF was
255 observed at 1000 $\mu\text{g}/\text{mL}$ after 6 and 12 hr exposure (**Figure 7**) for both nano- and micro-CoCrMo
256 particles. Compared directly, nano-CoCrMo caused significantly less DCF fluorescence than
257 micro-CoCrMo particles after 12 hr exposure to 0.1, 10 and 100 $\mu\text{g}/\text{mL}$; however, nano-CoCrMo
258 caused significantly higher DCF fluorescence than micro-CoCrMo after 6 and 12 hr exposure to
259 100 $\mu\text{g}/\text{mL}$ and after 24 and 48 hr exposure to 1000 $\mu\text{g}/\text{mL}$ (**Figure 7**).

260 Significantly increased dihydroethidium (DHE) fluorescence, compared to control, was
261 observed in macrophages (M0) exposed to nano-CoCrMo at all concentrations tested (0.1-1000
262 $\mu\text{g}/\text{mL}$) after 6, 12 and 24 hr of exposure; no changes in DHE were observed after 48 hr of exposure
263 at any concentration (**Figure 8A**). In M0 exposed to micro-CoCrMo, a significant increase in DHE

264 fluorescence was observed after 6 and 12 hr exposure to 0.1-1000 $\mu\text{g}/\text{mL}$; DHE levels were similar
265 to control at all concentrations after 24 and 48 hr of exposure to micro-CoCrMo (**Figure 8B**).
266 Compared to micro-CoCrMo, nano-CoCrMo caused significantly higher DHE levels in M0 at all
267 concentrations (0.1-1000 $\mu\text{g}/\text{mL}$) after 12 and 24 hr of exposure (**Figure 8**).

268

269 **4. Discussion**

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

282 Our studies suggest that both nano- and micro-CoCrMo particles can induce toxicity in all
283 cell types studied and the responses of cell viability and oxidative stress are dose, exposure time
284 and cell type specific. Across the three cell types tested, at low concentrations (i.e. 0.1 and 1
285 $\mu\text{g}/\text{mL}$), nano- and micro-CoCrMo particles did not cause significant toxicity in our viability assay.
286 Typically, in the presence of small amounts of foreign particles, cells may isolate the particles in

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

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

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

316 Oxidative stress has been implicated in age-related bone resorption and osteoporosis⁴⁹ and
317 in toxicity of CoCrMo particles in fibroblasts,⁵⁰⁻⁵² and may also play a role in the progression of
318 lung diseases,⁵³ such as those caused by cobalt-containing metal exposures.⁵⁴ Therefore, it is
319 important to examine the capacity of nano- and micro-CoCrMo particles in causing oxidative stress
320 in our cell models. In this case, we used a two-fold approach to assess the induction of oxidative
321 stress using DCF, which serves as a ‘generalized’ marker for reactive oxygen species,⁵⁵ and DHE,
322 which serves as a specific marker of superoxide anion.⁵⁶ It seems that the oxidative responses
323 against nano- and micro-CoCrMo particles were cell specific: both nano- and micro-CoCrMo
324 particles resulted in significantly higher DCF levels and DHE levels in OB and M0 cells;
325 significantly higher DCF and DHE levels were observed in macrophages at all concentrations
326 studied (0.1-1000 µg/mL). It seems that the OB cells behaved like the M0 immune cells, which
327 are known to exhibit a “respiratory burst” upon phagocytosis of microbes, marked by significant
328 increases in the production of hydrogen peroxide and superoxide anion via enzymatic pathways
329 that are critical for initiating anti-microbial response and infection clearance.⁵⁷ Meanwhile,
330 corrosion of metal in aqueous environment could contribute to oxidative stress. Low levels (e.g.
331 0.02 µg/mL) of Mo, Co, and Cr ions have been detected in CoCrMo particle solutions after short
332 time exposures (e.g. 24 hr),⁵⁸ and substantial evidence has indicated that metals and metal ions,

333 including Co and Cr, cause oxidative stress *in situ* regardless of the means of exposure.^{5, 7} In this
334 study, the oxidative stress was likely attributed to the combined effects of nanoparticle exposure
335 and the ions released.

336 The significantly increased oxidative stress of osteoblasts (OB) and macrophage (M0) cells
337 may help explain the increased risks of implant loosening and osteolysis in orthopaedic implant
338 patients,⁴⁻⁷ as there is evidence suggesting that the presence of wear particles in the joint fluid
339 stimulates a localized inflammatory response.⁴ Localized inflammation promotes osteoclast
340 activity, bone resorption and loosening of the implant.⁵⁹ By contrast, BEAS-2B cells had no
341 significant DHE changes but had significantly increased DCF levels at relatively high particle
342 concentrations (e.g. 100 and 1000 µg/mL). Moreover, nano-CoCrMo caused significantly higher
343 levels of oxidative stress in lung epithelial cells compared to micro-CoCrMo particles at
344 concentrations of 100 and 1000 µg/mL, which was consistent with the expected size-dependent
345 effect due to the increased reactive surface area of nano-CoCrMo compared to micro-CoCrMo.
346 No significant differences were found in the DHE assay, which suggests that CoCrMo particles
347 cause oxidative stress via other species than superoxide anion. Additionally, we found these results
348 were consistent with the fibroblast studies in the literature,^{51, 60} which found high levels of
349 oxidative stress, marked by increased levels of DCF fluorescence, after as little as 2 hr of
350 exposure⁶⁰ and increased levels of 8-OHdG staining, a marker of oxidative stress induced DNA
351 damage, after 24 hr of exposure to CoCrMo particles.⁵¹ Increased levels of oxidative stress in lung
352 epithelial cells could ultimately lead to downstream effects such as DNA damage and genotoxicity
353 upon long term exposure^{11, 51, 61} and may therefore be a contributing factor in the development of
354 lung disease from pulmonary CoCrMo particle exposure in occupational settings.

355

356 **5. Conclusions**

357 This study examined the toxicity of nano- and micro-CoCrMo particles and determined
358 whether their exposure induced oxidative stress in human lung epithelial cells, osteoblasts and
359 macrophages. These *in vitro* findings suggest that both nano- and micro-CoCrMo particles can
360 induce toxicity and the responses of cell viability and oxidative stress are dose, exposure time and
361 cell type specific. In future studies, the mechanism of cellular uptake and the cellular distribution
362 and excretion of CoCrMo particles will be investigated. The toxicity of these particles will be
363 further examined in animal models which generally provides a better approximation of what may
364 occur during a real-life exposure situation. For instance, CoCrMo nanoparticles may be injected
365 in a bone implant rat model⁶²⁻⁶⁴ or exposed to the lung in an intra-tracheal instillation rat model⁶⁵
366 to examine their local and systemic toxicity.

367

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377

378 **LIST OF ABBREVIATED TERMS**

- 379 ATCC: American Type Tissue Collection
- 380 CoCrMo: cobalt chromium molybdenum
- 381 DCF: 2',7'-dichlorofluorescein diacetate
- 382 DHE: dihydroethidium
- 383 DLS: dynamic light scattering
- 384 DMEM: Dulbecco's Modified Eagle Media
- 385 DTP: dental technician's pneumoconiosis
- 386 EDTA: ethylenediaminetetraacetic acid
- 387 FBS: fetal bovine serum
- 388 HMLD: hard metal lung disease
- 389 micro-CoCrMo: CoCrMo microparticles
- 390 nano-CoCrMo: CoCrMo nanoparticles
- 391 OB: osteoblast
- 392 PBS: phosphate buffered saline
- 393 PMA: phorbol-12-myristate-13-acetate
- 394 SEM: scanning electron microscope
- 395 TEM: transmission electron microscopy
- 396 WC-Co: tungsten carbide cobalt
- 397
- 398

399 **FIGURE AND TABLE LEGEND**

400

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

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

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

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

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

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

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

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

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