Effects of CoCr metal wear debris generated from metal-on-metal hip implants and Co ions on human monocyte-like U937 cells

Olga M Posada[a1], Rothwelle J. Tate[b] and M. Helen Grant[ac]

[a]Biomedical Engineering Department, University of Strathclyde, Wolfson Centre,Glasgow G4 0NW, UK. [1]O.M.PosadaEstefan@leeds.ac.uk,

[c]Corresponding author: m.h.grant@strath.ac.uk. TEL: +44 (0)141 548 3438.

[b]Strathclyde Institute for Pharmacy & Biomedical Sciences, University of Strathclyde, Glasgow G4 0RE, UK. r.j.tate@strath.ac.uk

Abstract

Hip resurfacing with cobalt-chromium (CoCr) alloy was developed as a surgical alternative to total hip replacement. However, the biological effects of nanoparticles generated by wear at the metal-on-metal articulating surfaces has limited the success of such implants. The aim of this study was to investigate the effects of the combined exposure to CoCr nanoparticles and cobalt ions released from a resurfacing implant on monocytes (U937 cells) and whether these resulted in morphology changes, proliferation alterations, toxicity and cytokine release. The interaction between prior exposure to Co ions and the cellular response to nanoparticulate debris was determined to simulate the situation in patients with metal-on-metal implants receiving a second implant. Effects on U937 cells were mainly seen after 120h of treatment. Prior exposure to Co ions increased the toxic effects induced by the debris, and by Co ions themselves, suggesting the potential for interaction *in vivo*. Increased TNF-α secretion by resting cells exposed to nanoparticles could contribute to osteolysis processes *in vivo*, while increased IFN-γ production by activated cells could represent cellular protection against

[1] Present address: LICAMM laboratories, University of Leeds, Leeds LS2 9JT, UK.

tissue damage. Data suggest that interactions between Co ions and CoCr nanoparticles would occur *in vivo*, and could threaten the survival of a CoCr metal implant.

Keywords: metal wear debris; hip replacements; nanoparticles; metal ions; cobalt-chrome alloy; monocytes

1. Introduction

The most common cause of failure of total hip arthroplasty is aseptic loosening of the implant initiated by adverse tissue response to prostheses wear particles (Luo *et al.*, 2005). Current evidence indicates that the size of wear particles generated by CoCr alloy metal-on-metal (MoM) articulations is in the nanometre size range (Hosman *et al.*, 2010). The large surface area enhances release of metal ions, predominantly Co and Cr ions, into the circulation (Lucarelli *et al.*, 2004). Wear particles from articular surfaces are phagocytosed mainly by macrophages. When particles are phagocytosed in sufficient amounts, the macrophages enter an active state of metabolism, releasing an array of cytokines, chemokines, and growth factors inducing inflammation, which accelerates osteoclast formation and bone resorption resulting in periprosthetic osteolysis (Germain *et al.*, 2003; Yagil-Kelmer *et al.*, 2004).

Circulating physiological levels of Co and Cr are normally <0.25µg/l (0.005µM) (Andrews *et al.*, 2011). Elevated levels of Co and Cr ions occur in both the hip synovial fluid and in peripheral blood after MoM hip replacement, and there is concern about the toxicity and biological effects of such ions both locally and systemically (Bisseling *et al.*, 2011; Friesenbichler *et al.*, 2012; Penny *et al.*, 2013).

48 Co corrodes faster than Cr under physiological conditions (Xia *et al.*, 2011) and, in contrast
49 to Cr, Co ions tend to remain mobile, which is reflected in the higher levels measured in
50 blood, allowing the ions to reach and enter remote organs (Afolaranmi *et al.*, 2012). Elevated
51 Co concentrations in patients with MoM implants are a concern, since increased Co levels in
52 blood have also been reported to be associated with neurological (hand tremor,
53 incoordination, cognitive decline, depression, vertigo, hearing loss, and visual changes)
54 (Oldenburg *et al.*, 2009; Tower, 2010), cardiac (myocardiopathy) (Dadda *et al.*, 1994;
55 Seghizzi *et al.*, 1994; Gilbert *et al.*, 2013) and endocrine (aberrant oestrogen signalling,
56 altered the production or circulation of sex hormones, and altered thyroid metabolism)
57 (Keegan *et al.*, 2007; Oldenburg *et al.*, 2009) symptoms.
58
59 In addition to the above, data from the seventh annual report of the National Joint Registry
60 for England and Wales showed high failure rates for MoM hip prostheses
61 (http://www.njrcentre.org.uk/njrcentre/portals/0/njr%207th%20annual%20report%202010.pd
62 f), which led to the market recall of the DePuy ASR™, both the Resurfacing and XL Systems
63 in August 2010 (DePuy International Ltd, Leeds, UK) (MDA/2010/069). Following this, the
64 Medicines and Healthcare products Regulatory Agency (MHRA) safety alert in September
65 2010 drew attention to the long term biological safety of all types of MoM hip implants. In
66 this document (MDA/2010/069; http://www.mhra.gov.uk/home/groups/dts-
67 bs/documents/medicaldevicealert/con093791.pdf) the MHRA explained the details behind the
68 safety alert and included four situations in which measurements of blood metal ions in
69 patients were recommended: 1) in patients who have symptoms associated with loose MoM
70 bearings; 2) in patients showing radiological features associated with adverse outcomes
71 including component position or small component size; 3) if the patient or surgeon are
72 concerned regarding the MoM bearing; and 4) if there is concern about patients with higher

73 than expected rates of failure. The MHRA have suggested that combined whole blood Co and
74 Cr levels of greater than 7ppb (7µg/l or 0.1µM of the combined ions) are associated with
75 significant soft-tissue reactions and failed MoM hips.

76

77 Aseptic loosening usually leads to revision surgery where the implant is removed and
78 replaced with an alternative bearing (Maezawa *et al.*, 2009; Naal *et al.*, 2011; Sehatzadeh *et*
79 *al.*, 2012). At the time of revision these patients may have high circulating metal ion
80 concentrations (particularly cobalt), and these may alter the response of the patient to the new
81 device. To investigate the effects of the metal ions already present in these patients in terms
82 of the biological response to the new device, cells were pre-treated *in vitro* with 0.1µM Co in
83 the present study for 4 days before being treated with the metal wear debris. Continued
84 exposure to Co ion release from an existing implant may also influence the responses to wear
85 debris and for this reason the combined effect of exposure to Co ions and wear debris was
86 also investigated. The concentration of Co ions used in the study was chosen to reflect the
87 maximum circulating concentration recommended in patients with MoM implants by MHRA
88 in 2010 (MDA/2010/069; http://www.mhra.gov.uk/home/groups/dts-
89 bs/documents/medicaldevicealert/con093791.pdf).

90

91 U937 cells are a human macrophage-like cell line derived from human leukemic monocyte
92 lymphoma (Yagil-Kelmer *et al.*, 2004). This cell line has been used previously as the cell
93 culture model to study the biological effects of different kinds of particles and ions, and it has
94 been demonstrated that U937 cells have comparable responses to polyethylene particles
95 (Matthews *et al.*, 2001) and metal ions (Wang *et al.*, 1996) as do primary macrophages in
96 terms of cytokine release.

97

The aim of this study was to find out if exposure to CoCr nanoparticles released from a resurfacing implant could activate monocytes, and whether this resulted in cytotoxicity and cytokine release. The interactions between prior exposure to Co ions and the nanoparticulate debris, and between combined exposure to wear debris and Co ions, were determined in order to simulate the *in vivo* situation in patients with MoM implants.

2. Methods

2.1. Preparation of wear debris

CoCr wear debris was a gift from DePuy International (Leeds, UK). A high-carbon (≥ 0.2%) content CoCr alloy (ISO 5832-12: Co Balance, Cr 26.0–30.0%, Mo 5.0–7.0%, Ni 1.0% max., Si 1.0% max., Mn 1.0% max., Fe 0.75% max., C 0.35% max., N 0.25% max.) hip resurfacing implant was worn on a multi-station hip joint simulator using the following protocol. The wear debris was produced over 250000 cycles using distilled water as the lubricating fluid. The use of only distilled water (instead of the more usual bovine serum (25% v/v) in distilled water) resulted in a more rapid and aggressive wear regime which produced a greater volume of wear debris of similar morphology and size for testing purposes to that produced under similar conditions in 25 per cent serum but in a more conducive time-frame (personal communication, Dr C. Hardaker, DePuy International, Leeds, UK). Wear debris produced by hip simulator under different conditions has previously been shown to be of similar size and morphology (Brown *et al.*, 2007).

Once produced, the wear debris was centrifuged at 3500g for 20 minutes. The majority of the water was then aspirated. The remaining suspension was heat-treated (180°C for 5h, 60kPa) in a vacuum oven to eliminate the remaining water and destroy any endotoxin. The dry debris was then suspended in sterile phosphate buffered saline (PBS; Invitrogen; Paisley, UK). The

123 sterility of the treated wear debris was tested as described by Akbar et al. (2012) by exposing
124 dendritic cells (isolated from bone marrow of male BALB/c (Harlan, UK) mouse femurs and
125 tibias (Lutz *et al.*, 1999)) to the debris for 24h, *in vitro*, and then assessing the expression of
126 surface activation markers via flow cytometry. The debris was found not to increase the
127 surface expression of CD40, CD86, or MHC II on these cells, and, therefore, the suspended
128 debris was deemed sterile and endotoxin-free (data not shown).
129
130 Heat treated wear debris was imaged with a Field Emission Scanning Electron Microscope
131 (FE-SEM) (Hitachi SU-6600, Hitachi; Germany) at magnifications of 100-1000x. Energy
132 Dispersive X-ray Spectroscopy (EDS) was used for quantitative analysis of elemental
133 composition. Hitachi TM-1000 and EDSwift-TM software was used to obtain the images and
134 chemical spectra of the wear debris. Metal ion release from the debris was determined by
135 incubating 156.25µg debris/cm^2 for 24h at 37°C in an atmosphere of 5% (v/v) CO_2 in air in
136 1ml of complete RPMI-1640 medium in the presence of 10% (v/v) foetal bovine serum (FBS)
137 as described below. The medium was collected and stored at -80°C until ICP-MS analysis
138 using an Agilent 7700x octopole collision system in helium gas mode using scandium as
139 internal standard. Quantification was based on the maximum signal for a particular isotope,
140 and five readings were taken, with the result taken as the mean value.
141
142 2.2. Cell culture
143 U937 (Human leukemic monocyte lymphoma cell line; European Collection of Cell Cultures)
144 cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS, 50U/ml
145 penicillin and 50µg/ml streptomycin. These cells were routinely split every three days at a
146 ratio of 1:10 from a starting passage number of 4 and for no more than 20 passages. This was
147 achieved by taking 3ml of cell suspension into a fresh 75cm^2 culture flask containing 27ml of

148 fresh complete RPMI-1640 medium. Cells were activated by incubating them with 10nM
149 phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich; Dorset, UK) for 72h (Lucarelli *et al.*,
150 2004). Activation was confirmed by monitoring cell morphology, adhesion, and aggregation
151 on a Zeiss Imager.Z1 microscope using wet lenses (20X) every 24h. For cobalt pre-treatment
152 of cells, Co^{2+} solutions were freshly prepared using cobalt chloride ($CoCl_2$) (Alfa Aesar;
153 Lancashire, UK) and diluted to 0.1μM in growth medium under sterile conditions. Resting
154 and activated cells were incubated with 0.1μM Co^{2+} for 72h.

155

156 2.3. Cell number and viability

157 In order to assess cell viability, U937 cells were exposed to metal debris and Co^{2+} in resting
158 and activated states for 24 and 120h. For both resting and activated U937 cells, the cells were
159 cultured ($1x10^4$ cells/well) in 96-well plates with 156.25μg debris/cm^2 (5mg
160 debris/$1x10^6$cells), 0.1μM of Co^{2+}, or the combination of 156.25μg debris/cm^2 plus 0.1μM of
161 Co^{2+} in complete RPMI-1640 for 24 and 120h at 37°C under 5% (v/v) CO_2. Culture medium
162 was not changed during these incubation periods. At each culture endpoint, cells were
163 washed twice with PBS to remove the debris layer before cell viability was assessed by the
164 neutral red (NR) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)
165 assays, as described by Repetto et al. (2008) and Mosmann (1983), respectively. The cells
166 were incubated with NR for 3h and MTT for 4h, and absorbance of the both NR and MTT
167 assay samples were then measured at 540nm using a Bio-Rad Model 450 microplate reader
168 (Bio-Rad, Hertfordshire, UK).

169

170 Viability was also monitored microscopically by double staining with Acridine Orange (AO;
171 Sigma-Aldrich; Dorset, UK) and Propidium Iodide (PI; Life Technologies; Paisley, UK) as
172 previously described by Bank (1988). Cells were viewed using a Carl Zeiss Axio Imager

173 microscope under a 40X water immersion lens with a numeric aperture of 0.80. Fluorescence
174 was excited using a mercury lamp and emission recorded using a fluorescein isothiocyanate
175 (FITC)/Rhodamine filter block (485/515-530nm; 546/580-563nm) for AO and PI. Cells
176 fluorescing green were scored as viable, and those fluorescing red as nonviable.
177

178 2.4. Cell proliferation

179 Resting and activated cells were cultured ($1x10^4$cells/well) in 96-well plates with 156.25µg
180 debris/cm^2, 0.1µM of Co^{2+}, or the combination of 156.25µg debris/cm^2 plus 0.1µM of Co^{2+} in
181 complete RPMI-1640 for 24h at 37°C under 5% (v/v) CO_2. U937 cell proliferation was then
182 determined using a BrdU Cell Proliferation Immunoassay kit (kit number QIA58, Merck
183 Chemicals; Nottingham, UK), as suggested by the manufacturer. Briefly, the BrdU label was
184 diluted 1:2000 into fresh complete RPMI-1640 medium. To label the DNA, 20µl of this
185 working solution were added to cells in 96-well plates during the final 4 hours of the
186 experiment. Plates were then centrifuged (300xg for 10min) and the supernatant aspirated.
187 Cells were then fixed, permeabilized, and the DNA denatured. Plates were incubated for
188 30min at room temperature and the fixative/denaturing solution was then aspirated. 100µl of
189 anti-BrdU antibody diluted in antibody dilution buffer were added to each well and incubated
190 with the cells for 1h at room temperature. Cells were washed 3 times with 1X wash buffer,
191 before 100µl of peroxidase goat anti-mouse IgG HRP conjugate diluted in conjugate diluent
192 were incubated with the cells for 30min at room temperature. Plates were then flooded with
193 dH_2O, emptied, and incubated with 100µl of substrate solution in the dark at room
194 temperature for 15min. Following the addition of 100µl of stop solution the absorbance was
195 measured using a Thermo Scientific Multiskan Ascent spectrophotometer plate reader at dual
196 wavelengths of 450-540nm.
197

198 2.5. Cytokine production: ELISA

199 Cytokine levels were determined by collecting the supernatants from the cell cultures at each
200 end point. The concentrations of tumour necrosis factor-alpha (TNF-α), interferon-γ (IFNγ)
201 and interleukin-6 (IL-6) in the culture media were determined from aliquots of cell-free
202 isolates using Ready-Set-Go! ELISA kits (eBioscience; Hatfield, UK) in accordance with the
203 manufacturer's instructions. Each of the kits had a sensitivity level of 4pg/ml, and linear
204 standard curves were generated between 0-500pg/ml for TNFα and IFNγ, and 0-200pg/ml for
205 IL-6. The presence of metal ions in the medium did not influence the measurement of the
206 cytokines.

207

208 2.6. Statistics

209 Statistical analyses were carried out by a one-way analysis of variance (ANOVA), followed
210 by a Dunnett's multiple comparison test, and a two-sample *t*-test. Significance was assigned
211 where p values were found to be <0.05.

212

213 3. Results

214 3.1. Characterisation of wear debris and release of metal ions

215 Wear particles generated by the hip simulator process have been reported with a mean size of
216 50nm (Brown et al, 2007). SEM images show irregular shapes and sizes varying from the
217 nano to the micro scale (from 150nm to 6.5μm). The larger irregular shaped particles suggest
218 that the debris aggregates (Image 1), and this has been reported previously by Akbar and co-
219 workers (2012). Doorn et al (1998) isolated particles from MoM retrieval tissues that varied
220 in size (51-116nm particles to micrometre sized aggregates) and shape. Moreover, metal
221 particles (0.1-3 microns in size) have also been found in tissues post-mortem (Brown *et al.*,
222 2013). EDS analysis indicated that the wear debris is primarily composed of Co and Cr

223 (which is in agreement with the alloy composition (Singh and Dahotre, 2007)). Analysis of
224 25 different particles indicated a mean composition of 59.57 per cent Co, 40.43 per cent Cr
225 and a small amount of Mo which was below the limit of quantification. The CoCr wear debris
226 released metal ions into culture medium as shown on Table 1. Co was the predominant ion
227 released after 24h incubation. The percentage dissolution of the debris over the 24h period
228 was negligible, 0.000042%, although the cobalt concentration released was 1259.41+/- 39.58
229 ng/ml.

230

231 3.2. Cell viability

232 In this study, the 156.25µg debris/cm^2 (5mg/1x10^6cells) concentration was chosen to mimic
233 the local metallosis environment surrounding an implant. The viability of the cells was
234 assessed after exposure to Co ions, to wear debris, and to a combination of the wear debris
235 and Co ions. Some of the cultures from each type of exposure had been pre-treated with Co
236 ions for 3 days prior to the experiment. Changes in cell number were measured by both NR
237 and MTT assays. The reduction of MTT is influenced by both reductase enzyme activities
238 and the supply of NADH/NADPH (the redox status) in the cells and is therefore, a measure
239 of the metabolic activity and redox status of the cells. In contrast, the NR assay is not
240 dependent on these factors. The latter may, however, be altered by changes in the number of
241 lysosomes in the cells. Resting and activated U937 cell numbers were assessed after 24 and
242 120h of treatment with the wear debris and/or ions. Figure 2 and Figure 3 summarise the
243 120h results, there being no significant changes in cell numbers for either resting or activated
244 U937 cells after 24h of exposure to the different treatments (data not shown).

245

246 After 120h there was a significant decrease in the absorbances measured by NR and MTT
247 assays respectively in both resting and activated cells, where the metal debris was present. In

248 the resting cells, exposure to Co ions for 120h caused an increase in the cell number
249 compared to controls, which was apparent when measured by both MTT and NR (Figure 2).
250 Additionally, cells exposed only for 3 days to Co and then the Co ions kept for 120h in
251 culture medium (PreCo U937+no debris) did not show any differences when compared to
252 untreated cells. The effect observed on resting cells was not as marked after activation of the
253 cells by PMA (Figure 3), although increased absorbances in activated cells were detected
254 where pre-treatment with Co ions had been carried out. When comparing the NR results of
255 resting cells exposed to the combination of wear debris and Co ions, Co pre-treated cell
256 numbers were significantly lower than those of non Co pre-treated cells. In fact, all the results
257 obtained in resting cells were exacerbated to some extent by the pre-treatment with Co ions.
258 Interestingly, such an effect was not seen after cell activation by PMA. Generally, the
259 magnitude of responses to both nanoparticles and to Co ions was greater in the resting cells
260 than in activated cells.
261
262 Viability was assessed by AO/PI double staining. PI is impermeable to intact plasma
263 membranes, but it easily penetrates the plasma membrane of dead or dying cells and
264 intercalates with DNA or RNA forming a bright red fluorescent complex. AO is a membrane-
265 permeable, monovalent, cationic dye which binds to nucleic acids in the cells to produce a
266 fluorescent green product (Bank, 1988). Images 2 and 3 summarise the 120h results, with
267 there being no significant changes in cell viability for either resting or activated U937 cells
268 after 24h of exposure to the different treatments (data not shown). After 120h treatment, there
269 was a decrease in cell number of both resting and activated cells in the presence of debris but
270 not Co ions. Furthermore, the few cells observed appeared to be mainly apoptotic.
271
272

273 3.3 Cell proliferation

274 Effects on cell proliferation were assessed with the BrdU cell proliferation assay after 24h of
275 exposure to the different treatments, at a time when control cells were in the log phase of
276 growth. The results are summarized in Figure 3.

277

278 Resting cells in the presence of metal debris had a lower rate of proliferation compared with
279 control cells, although this was not a significant difference. Additionally, the effects on
280 resting U937 and Co pre-treated resting U937 cells were compared in order to establish if the
281 pre-treatment with Co influenced the proliferative cell response to the treatments. Co pre-
282 treated resting cells proliferated significantly less than non Co pre-treated resting cells when
283 exposed to 0.1μM Co alone, suggesting a potential role of Co ions in the inhibition of resting
284 U937 cell proliferation. The exposure to 0.1μM Co also caused a significant decrease in the
285 proliferation of activated U937 cells. Moreover, the effects on activated U937 cells and Co
286 pre-treated activated U937 cells were compared in order to establish if the pre-treatment with
287 Co influenced the proliferative cell response to the treatments. Co pre-treated activated cells
288 proliferated significantly more than non Co pre-treated activated cells in the presence of
289 metal wear debris alone. However, the same effect was observed when cells were exposed to
290 the combination of metal wear debris and Co ions, showing a significantly higher
291 proliferation of Co pre-treated activated cells compared to non Co pre-treated activated cells.
292 No difference was observed in the presence of Co ions alone. These findings differ from the
293 results of resting cells, which indicates that the cellular activation state may influence the
294 biological response to metal particles and ions. Although not statistically significant, Co ions
295 alone increased the rate of proliferation of resting cells, and while at different endpoints,
296 analogous to the results found with MTT and NR.

297

298 3.4 Cytokine production measured by ELISA

299 Levels of IL-6, TNF-α, and IFN-γ released were determined in the culture medium after 120h
300 of exposure to the different treatments. The results are summarised in Figure 4 and 5.

301

302 Both untreated resting cells and PMA-activated cells (controls on both graphs) secreted the
303 three cytokines at the 120h culture end point. There was no statistical difference between the
304 levels of cytokines secreted by treated and untreated resting cells. The Co pre-treatment did
305 not cause a significant difference to the levels of cytokine secretion by resting U937 cells.
306 Activated cells exposed to the combination of metal debris and Co ions secreted significantly
307 more IL-6 and IFN-γ than control cells. Contrary to this, treatments caused a decrease in the
308 secretion of TNF-α by activated cells. Co pre-treated activated cells exposed to 0.1μM Co
309 secreted significantly less IFN-γ than non Co pre-treated cells.

310

311 4. Discussion

312 MoM hip implants are composed of CoCr alloys, and as such, during wear, both CoCr
313 nanoparticulate debris and Co and Cr ions are released. In this study the effects of a high
314 wear debris concentration and Co ions on U937 cells were studied. After release from MoM
315 implants, Cr ions tend to bind to the local tissue whereas Co ions tend to remain mobile and
316 enter the circulation (Simonsen *et al.*, 2012). The Cr forms stable complexes which deposit
317 around the implant, whereas Co concentrations in patients' blood are generally higher, and
318 thus the ions interact with distant tissues and cells to a greater extent.

319

320 The wear debris used in this study was produced from a 39mm ASR™ prosthesis, and
321 previous work emulating natural gait has shown that approximately 8 mm^3 of debris is
322 produced per million cycles on a simulator from this prosthesis (Leslie *et al.*, 2008). The

323 density of the CoCr alloy used was 8.32mg/mm^3 (Medley *et al.*, 1996), so 8mm^3 of wear
324 would be equivalent to 66.56mg debris. An active person might walk 3.5 million cycles per
325 year, so 232.96mg debris/year would be produced locally in the environment of the
326 prosthesis. The 156.25µg/cm^2 metal debris concentration used in this investigation was
327 chosen to mimic metallosis, a situation where metallic debris infiltrates into the periprosthetic
328 tissues, with resulting severe adverse effects. The range of debris and ion concentrations
329 measured locally varies hugely in the literature, for example, in catastrophic failure of a
330 prosthesis amounts of wear debris up to 67mg have been reported (Matziolis *et al.*, 2003).
331 The high metal ion concentrations released from debris in the current experiments simulates
332 this local situation.
333
334 Exposure to 156.25µg debris/cm^2 proved to be toxic for both resting and activated U937 cells
335 in the presence and absence of Co ions, which could be in part due to the high concentration
336 of ions released from the particles. However, there was no evidence of toxicity observed after
337 cell exposure to 0.1µM Co ions alone. In fact, the cell numbers of resting cultures increased
338 after exposure to the Co ions alone, and this type of response has been reported previously
339 (Zijlstra *et al.*, 2012), and is thought to represent a protective response to a non-toxic insult.
340 The statistically significant difference in proliferation between Co pre-treated and non-Co
341 pre-treated resting cells exposed to 0.1µM Co ions alone, suggests that longer exposure to Co
342 ions (>4 days) may result in a toxic response. These results seem to indicate once more that
343 Co ions, particularly at high concentrations, are important in the adverse tissue response to
344 metal wear debris and their effect may differ between cell activation states.
345
346 In the current investigation, a pre-treatment with cobalt ions was carried out to find out if
347 such tissue pre-exposure and ion dissemination *in vivo* would have an effect on cell responses

348 to subsequent exposure to metal wear debris and ions. Such an effect would be important if a
349 patient received a second MoM implant or indeed had a revision procedure to replace a
350 failing implant. Many patients with failing MoM implants have shown high circulating Co
351 ions in their blood (Antoniou *et al.*, 2008; Tkaczyk *et al.*, 2010b; Bisseling *et al.*, 2011;
352 Lavigne *et al.*, 2011; Friesenbichler *et al.*, 2012; Penny *et al.*, 2013), and recently the
353 importance of cobalt release in the inflammatory response to CoCr debris has been
354 demonstrated (Caicedo *et al.*, 2009; Hart *et al.*, 2012). Results from the present study indicate
355 that exposure to wear debris had a pronounced detrimental effect on cell number and
356 metabolic activity of cells pre-treated with Co after an incubation period of 120h. Even
357 though effects observed in resting cells were exacerbated to some extent by the pre-treatment
358 with Co ions, such an effect was not seen after cell activation by PMA. PMA exerts its
359 biologic effects by altering gene expression through the activation of protein kinase C (PKC)
360 and modulating the activity of transcriptional factors such as nuclear factor kappa-light-
361 chain-enhancer of activated B cells (NFkB) and activator protein 1 (AP1) (Garcia *et al.*,
362 1999; Daigneault *et al.*, 2010). Thus, it seems unlikely that PKC was directly involved in the
363 pathways leading to the decrease in cell numbers. Despite the changes in proliferation at 24h
364 by BrdU assay, no changes in cell number or metabolic activity were observed at this same
365 endpoint by NR and MTT. Due to the intrinsic characteristics of the assays themselves and
366 complex dynamics of the balance of cell proliferation and cell death, it is unlikely that the
367 changes in cell proliferation (cell division) measured at 24h would be simultaneously
368 apparent as an increase in cell numbers at the same time point. On retrospect, such changes in
369 terms of cell numbers would have been detectable had we measured this at later time points.
370 Cells were treated with the Co ion concentration that has been advised by MHRA as the
371 recommended maximum safe circulating blood level for patients with MoM implants
372 ((MDA/2010/069); http://www.mhra.gov.uk/home/groups/dts-

373 bs/documents/medicaldevicealert/con093791.pdf). It should be noted that cells pre-treated
374 with Co ions at a 120h end point would have been exposed to Co for 9 days in total. In
375 patients, the effects of Co ions and nanoparticles released from metal implants may be
376 additive in terms of adverse effects. Contrary to Cr, Co ions tend to remain mobile and as a
377 result greater Co concentration (compared to Cr) in blood and remote organs have been
378 reported (Afolaranmi *et al.*, 2012) and will be more likely to affect the responses of distant
379 organs to metal nanoparticles than the Cr ions. The importance of Co ions in the
380 inflammatory responses to Co-Cr particles has been recognised, and chronic exposure to
381 circulating levels of ions, plus high local concentrations may act synergistically *in vivo* to
382 trigger and promote implant loosening (Hallab *et al.*, 2001; Caicedo *et al.*, 2010; Hart *et al.*,
383 2012) . Although circulating systemic Cr levels are generally lower *in vivo*, it may be
384 interesting in the future to investigate the influence of prior exposure to Cr ions in the CoCr
385 wear debris *in vitro* exposure experiments. The presence of wear debris in the peri-implant
386 area leads to macrophage phagocytosis of particulate debris and activation, the release of a
387 variety of mediators, such as free radicals and nitric oxide, and a myriad of proinflammatory
388 cytokines and chemokines (Sethi *et al.*, 2003). The uptake of the CoCr particles into the cells
389 was not measured due to technical difficulties in detecting the particles in the cells, but in a
390 preliminary study phagocytosis by the activated cells was demonstrated using 1µm size
391 FITC-labelled dextran beads (results not shown). Differences in the uptake of the CoCr
392 particles could have occurred in resting and/or activated cells after the different treatments,
393 and a contribution towards any differences in response cannot be excluded. Generally, the
394 magnitude of the response to both the particles and the Co ions was greater in the resting cells
395 than in the activated cells, and so the role of phagocytosis in the toxic responses is not clear.
396 It has been reported that local acidification may develop during acute and chronic
397 inflammation (Rajamaki *et al.*, 2013) and high hydrogen ion concentrations down to pH 5.4

398 have been found in inflamed tissue (Steen *et al.*, 1995). In turn, such an acidic environment
399 created by actively metabolizing immune cells may enhance the corrosion process of the
400 nanoparticles increasing the amount of metal ions being released (Afolaranmi *et al.*, 2011).
401
402 A number of studies have shown that metal wear particles and high levels of metal ions,
403 particularly Co and Cr, have a cytotoxic effect on a variety of cells such as human osteoblast-
404 like cell lines (SaOS-2 and MG-63), and human monocytic-like U937 cells *in vitro* (Allen *et*
405 *al.*, 1997; Fleury *et al.*, 2006; Petit *et al.*, 2006). Most of these studies have been focused
406 mainly on the short-term exposure, acute cell response, or have been limited to evaluation of
407 one parameter like cell viability or cytokine levels. Studies carried out with U937 cells have
408 been performed either with resting U937 cells (Ingham *et al.*, 2000; Howling *et al.*, 2003;
409 Tkaczyk *et al.*, 2010a) or activated U937 cells (Lucarelli *et al.*, 2004; Yagil-Kelmer *et al.*,
410 2004; Wang *et al.*, 2011), and have investigated the effects of different kinds of metals,
411 particles, and ions on U937 cells. Papageorgiou et al. (2007) compared the cytotoxic and
412 genotoxic effects of nanoparticles and micron-sized particles of CoCr alloy using human
413 fibroblasts in tissue culture. Their results showed that exposure of human fibroblasts to
414 nanoparticles and micron-sized particles of cobalt chrome alloy, at the same particle mass per
415 cell, cause different types and amounts of cellular damage. In particular, they found
416 nanoparticles to be more cytotoxic and induce more DNA damage than micron-sized
417 particles. This difference in induction of toxicity will contribute to the adverse effects found
418 *in vivo* from CoCr nanoparticulate debris generated from MoM implants. Dalal et al. (2012)
419 compared the responses of human osteoblasts, fibroblasts, and macrophages exposed to
420 particles of different metal-based particles (i.e., cobalt-chromium (CoCr) alloy, titanium (Ti)
421 alloy, zirconium (Zr) oxide, and Zr alloy). They found that CoCr-alloy particles were by far
422 the most toxic and decreased viability and proliferation of human osteoblasts, fibroblasts, and

423 macrophages. Germain et al. (2003) studied the effects cobalt–chromium wear particles at
424 various doses on the viability of U937 cells. CoCr particles at 5μm^3 (0.042mg) and 50μm^3
425 (0.42mg) per cell reduced the viability of U937 cells by 42% and 97%, respectively.
426 Papageorgiou et al. (2007) and Germain et al. (2003) used high particle concentrations in
427 comparison to the concentration used in the present study, which indicates that even at lower
428 doses CoCr nanoparticles can exert cytotoxic effects. (Akbar *et al.*, 2011) investigated effects
429 of Cr^{6+} and Co^{2+} on primary human lymphocytes *in vitro*. Their results showed that exposure
430 to 10 and 100μM Cr^{6+} significantly decreased cell viability and increased apoptosis in both
431 resting and activated lymphocytes. The exposure of resting and activated lymphocytes to
432 100μM Co^{2+} also resulted in significant decreases in cell viability accompanied by a
433 significant increase in apoptosis and they showed that activated cells were significantly more
434 sensitive to Co^{2+} toxicity. The concentration of Co ions used in the present study was 1000
435 fold lower (0.1μM) than that used by Akbar et al. (2011), and no significant effect in cell
436 viability and proliferation was observed herein. From the literature there is little doubt that
437 the nanoparticulate debris and metal ions released from MoM implants have toxic effects and
438 the results in the present study point to the potential for interaction between them *in vivo*.
439

440 Although not statistically significant, there were higher levels of secretion of IL-6, TNF-α
441 and IFN-γ by resting cells after 120h of exposure to 156.25μg/cm^2 metal debris and the
442 combination of metal debris and Co ions. In the case of activated cells, there was
443 significantly higher secretion of IL-6 and IFN-γ by cells exposed to the combination of
444 156.25μg/cm^2 metal debris and Co ions after 120h. On the other hand, and although not
445 statistically significant, all treatments caused a reduction in the level of secretion of TNF-α.
446 Contrary to what was observed in resting cells, Co pre-treatment did not seem to cause a
447 difference in cytokine secretion by activated cells. It should be noted that the secretion of

448 cytokines has not been correlated for cell numbers in these experiments, and changes may
449 reflect cytotoxicity. These results again suggest a larger impact of a chronic exposure and a
450 key role of Co ions. They also suggest that different molecular pathways are affected in
451 activated U937 cells when compared to resting U937 cells.
452
453 Bone remodelling involves tight regulation of three proteins, receptor activator of NF-κB
454 ligand (RANKL), receptor activator of NF-κB (RANK), and osteoprotegerin (OPG). These
455 proteins are key determinants of osteoclastogenesis and regulate bone resorption
456 (Takayanagi, 2005). Pro-inflammatory cytokines such as IL-6, and TNF-α, can upregulate
457 RANKL expression on osteoblasts and accelerate RANKL signalling, and thus directly
458 contribute to bone destruction (Oishi *et al.*, 2012). In biomaterials research, TNF-α, IL-1, and
459 other pro-inflammatory cytokines are also known mediators of the foreign body reaction, an
460 inflammatory response that can cause both severe tissue damage and premature failure of
461 implanted materials (Mountziaris and Mikos, 2008). IFN-γ promotes innate immune
462 responses by activating macrophages. In parallel, IFN-γ exerts regulatory functions to limit
463 tissue damage associated with inflammation like suppressing osteoclastogenesis (Hu and
464 Ivashkiv, 2009). Results from this study suggest that high concentrations of metal wear
465 debris, particularly in the presence of Co ions, promote an immune response with the
466 secretion of pro-inflammatory cytokines, which can contribute to tissue damage and
467 ultimately result in aseptic loosening.
468
469 ## 5. Conclusions
470 The results from this study suggest that a high concentration of metal debris in combination
471 with Co ions not only have a direct effect on cell viability but also influence cell function.
472 Previous exposure to Co ions seems to sensitise U937 cells to the toxic effects of both Co

473 ions themselves and to nanoparticles, pointing to the potential for interaction *in vivo*. The
474 increase in TNF-α secretion by the resting U937 cells could be a factor contributing to the
475 osteolysis process, while the increase in IFN-γ production by the activated cells could be a
476 cellular effort to counteract tissue damage. This also suggests that cellular activation state
477 affects the biological response to wear debris and for this reason caution should be taken
478 when choosing *in vitro* models to study immune and molecular responses. Moreover, these
479 findings mean that the survival and well-functioning of a second implanted MoM device
480 could be compromised in patients undergoing revision surgery or receiving a second device,
481 due to the interactions between recirculating Co ions and CoCr nanoparticles.

482

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487

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Figure Captions (all figures are 2-column fitting images)

Image 1. Scanning Electron Microscopy image of simulator generated wear debris from an ASR™ hip implant. Image taken at 15kX with a FE-SEM Hitachi SU-6600.

Image 2. Fluorescence microscopy images (40X) following PI (Dead cells, red)/AO (Live cells, green) staining of resting U937 and Co pre-treated resting U937 cells exposed to 156.25μg debris/cm^2 (5mg debris/1x10^6 cells) **, 0.1μM Co and** 156.25μg debris/cm^2 (5mg

699 debris/1x10^6 cells) + **0.1µM Co for 120h.** Images are representative of 5 independent images

700 from each sample at each end point. “B” indicates cell blebbing, “S” indicates cell shrinkage,

701 and “N” indicates necrosis (colour reproduction **only** on the web).

702

703 **Image 3. Fluorescence microscopy images (40X) following PI (Dead cells, red)/AO (Live**

704 **cells, green) staining of activated U937 and Co pre-treated resting U937 cells exposed to**

705 156.25µg debris/cm^2 (5mg debris/1x10^6 cells) **, 0.1µM Co and** 156.25µg debris/cm^2 (5mg

706 debris/1x10^6 cells) + **0.1µM Co for 120h.** Images are representative of 5 independent images

707 from each sample at each end point. “B” indicates cell blebbing, and “S” indicates cell

708 shrinkage (colour reproduction **only** on the web).

709

710 **Figure 1. Neutral Red and MTT assays measured in resting cells at 120h.** Results are

711 percentage values (Mean ± SEM, n=9) where 100% corresponds to control values (dash

712 lines). PreCo-debris: cells pre-treated with Co ions and then the Co ions kept in culture

713 medium throughout the experiment. *Significantly different from control values ($p<0.05$) by

714 one-way ANOVA followed by Dunnett’s multiple comparison test. †Significantly different

715 from non Co pre-treated cell values ($p<0.05$) by 2 sample t-Test.

716

717 **Figure 2. Neutral Red and MTT assays measured in activated cells at 120h.** Results are

718 percentage values (Mean ± SEM, n=9) where 100% corresponds to control value (dash lines).

719 PreCo+debris: cells pre-treated with Co ions and then exposed to treatments. PreCo-debris:

720 cells pre-treated with Co ions and then the Co ions kept in culture medium throughout the

721 experiment. *Significantly different from control values ($p<0.05$) by one-way ANOVA

722 followed by Dunnett’s multiple comparison test. †Significantly different from non Co pre-

723 treated cell values ($p<0.05$) by 2 sample t-Test.

724

725 **Figure 3. Cell proliferation at 24h measured by BrdU.** Results are percentage values

726 (Mean±SEM, n=8) where 100% corresponds to control untreated cells (dash lines). PreCo:

727 cells pre-treated with Co ions. *Significantly different from control values ($p<0.05$) by one-

728 way ANOVA followed by Dunnett's multiple comparison test. †Significantly different from

729 non Co pre-treated cell values ($p<0.05$) by 2 sample t-Test.

730

731 **Figure 4. Resting U937 cell cytokine secretion measured by ELISA after 120h of**

732 **treatment.** Results are expressed as cytokine concentration values per 1000 cells (± SEM,

733 n=4). Untreated resting U937 cells were used as control. *Significantly different from control

734 values ($p<0.05$) by one-way ANOVA followed by Dunnett's multiple comparison test.

735 †Significantly different from non Co pre-treated resting cell values ($p<0.05$) by 2 sample t-

736 Test.

737

738 **Figure 5. Activated U937 cell cytokine secretion measured by ELISA after 120h of**

739 **treatment.** Results are expressed as cytokine concentration values per 1000 cells (± SEM,

740 n=5). Untreated activated U937 cells were used as control. *Significantly different from

741 control values ($p<0.05$) by one-way ANOVA followed by Dunnett's multiple comparison

742 test. †Significantly different from non Co pre-treated activated cell values ($p<0.05$) by 2

743 sample t-Test.

744

Table 1. Release of Co and Cr ions into culture medium from metal wear debris *in vitro*.

Incubation	**Cr release** (ng/ml)	**Co release** (ng/ml)	**Mo release** (ng/ml)
RPMI control	0.19 +/-0.06	0.09 +/- 0.09	6.32 +/- 0.16
RPMI plus CoCr wear debris	18.18 +/- 2.64	1259.41 +/- 39.58	124.60 +/- 2.70

5mg wear debris were incubated for 24h at 37 ^{o}C in 1ml complete RPMI-1640 medium in the presence of 10% foetal bovine serum. Metal ion release was measured by ICPMS Results are expressed as mean +/- SEM (n=3). Release of each ion (ng/ml) from wear debris was significantly different from the concentration in control RPMI medium by one-way ANOVA followed by Dunnett's multiple comparison test (P<0.05). The dissolution rate of the CoCr wear debris in terms of Co release was 0.000042% over the 24h period.

Figure 1

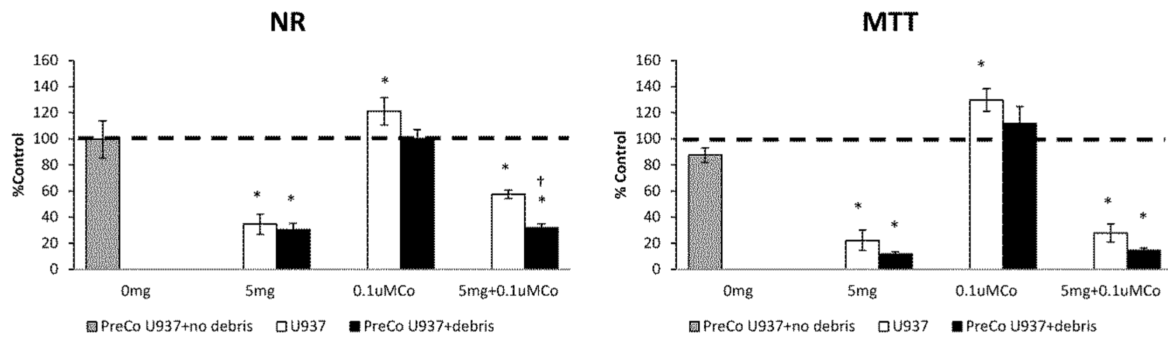


Figure 2

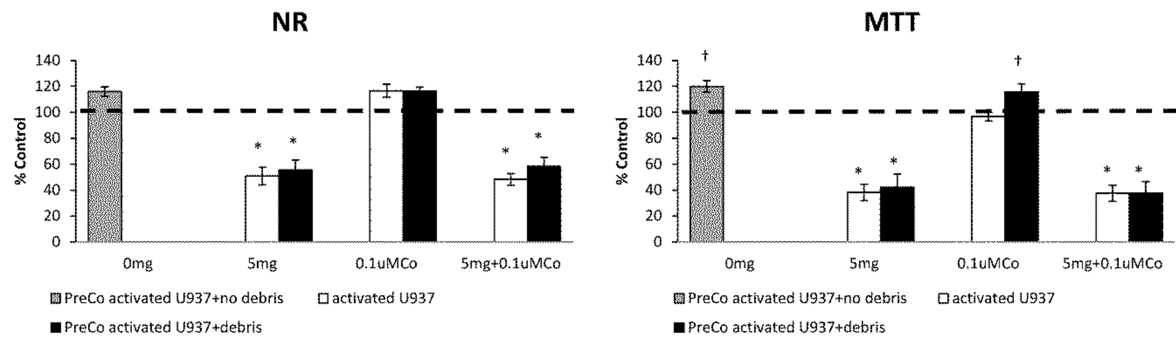


Figure 3

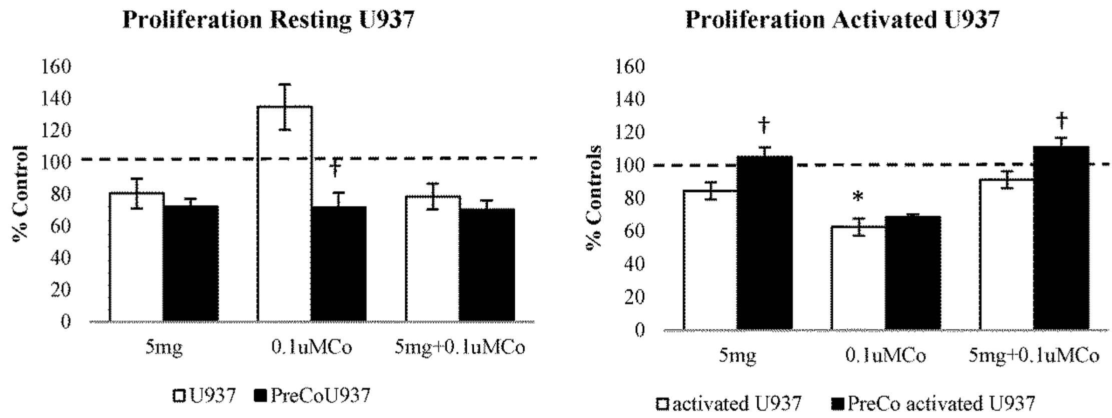


Figure 4

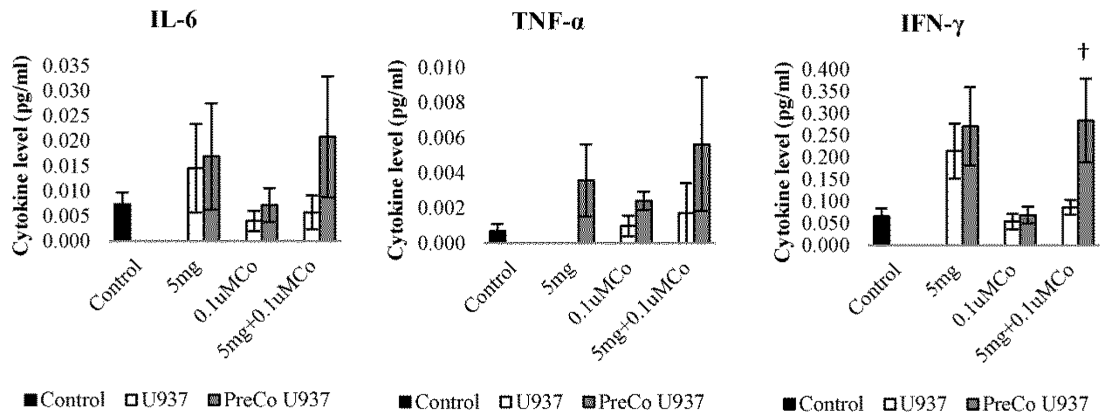


Figure 5

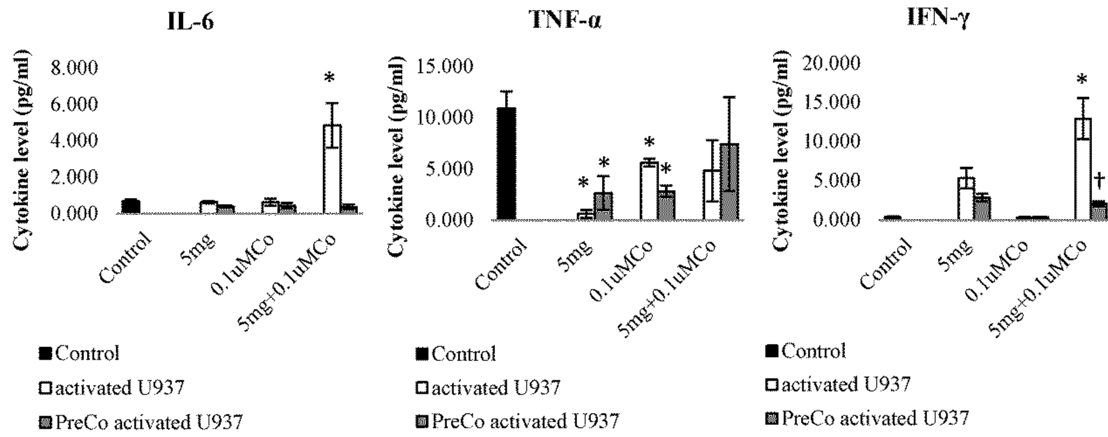


Image 1

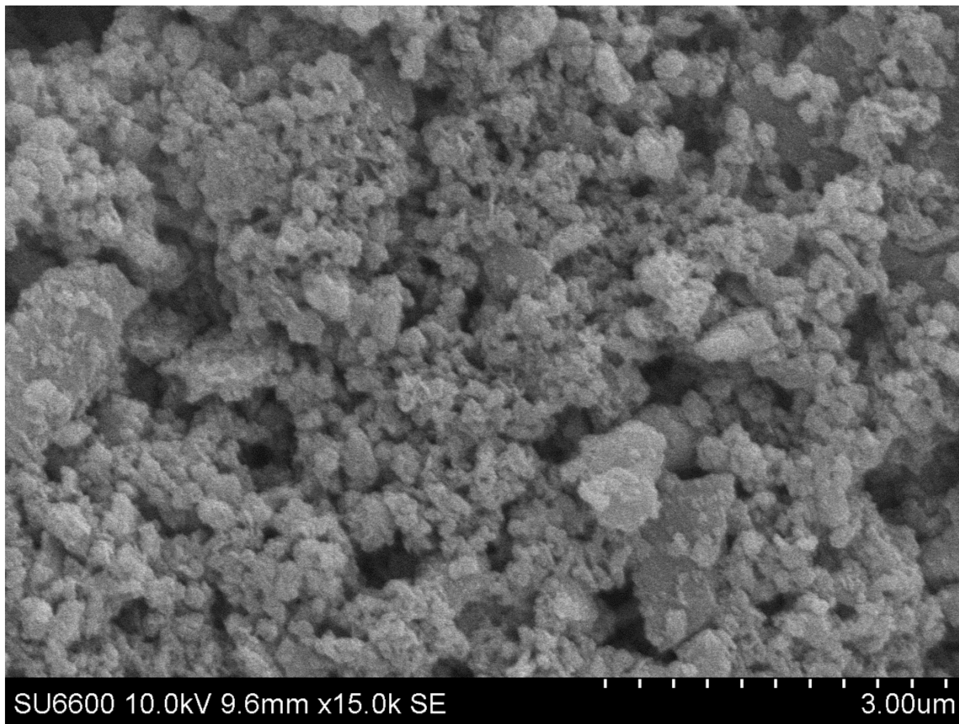


Image 2

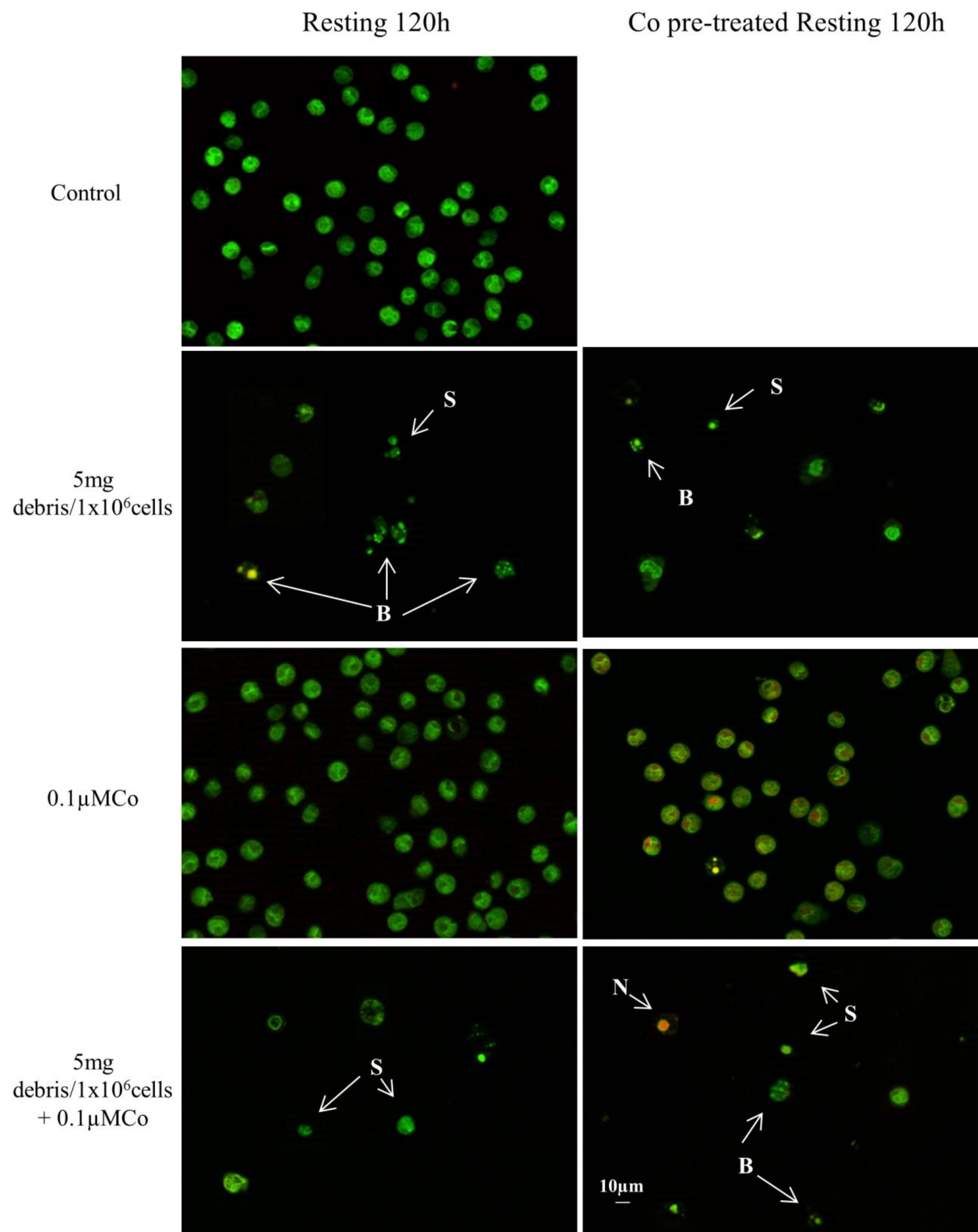
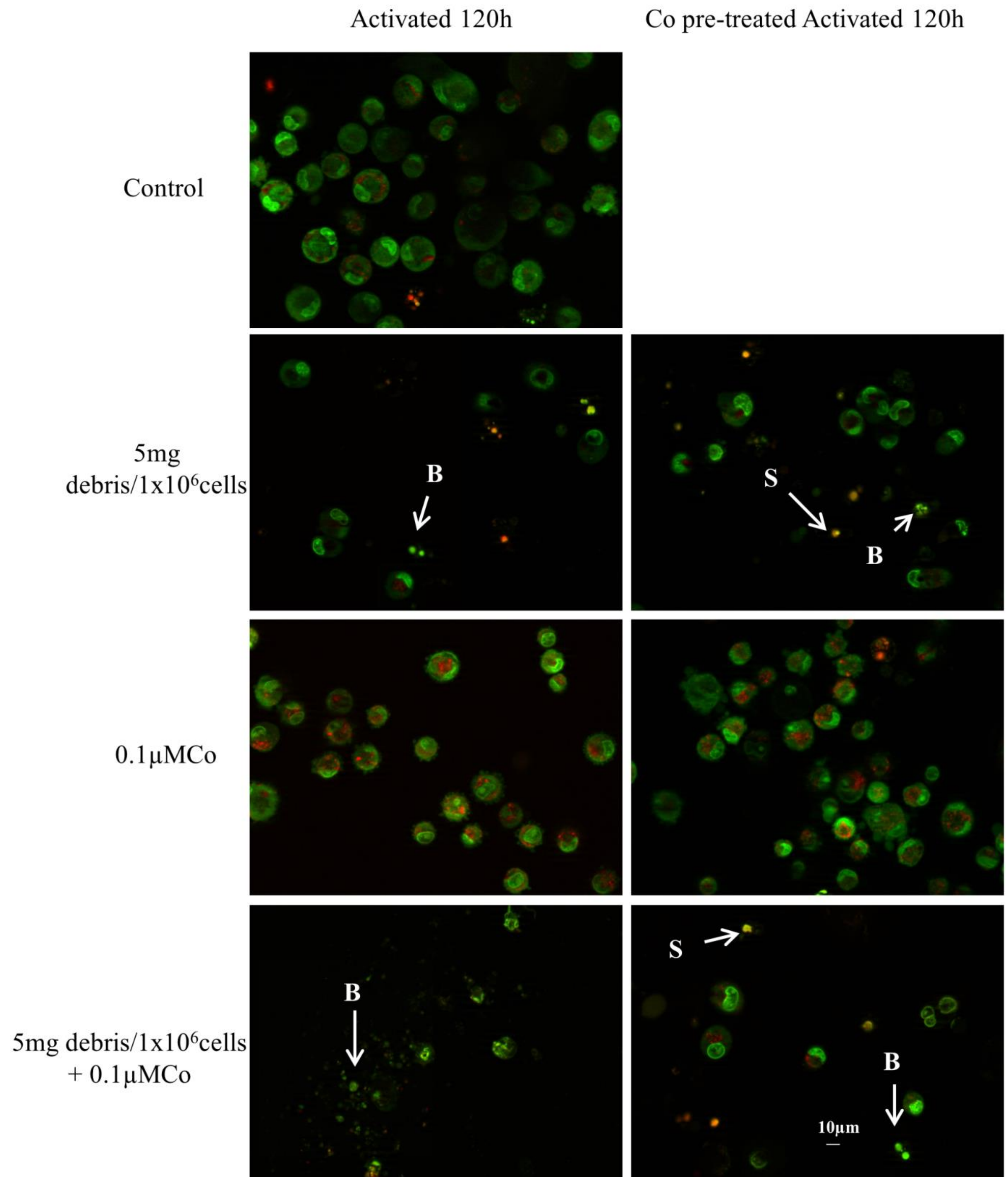


Image 3



745 **Highlights**

746 - Metal debris in combination with Co ions influence cell function
747 - Pre-exposure to Co ions seems to sensitise cells to the toxic effects particles
748 - Experimental conditions may not allow to discriminate between cytotoxic and cytostatic
749 - Cellular activation state affects the biological response to wear debris
750 - Interaction between circulating ions and particles may threaten MoM device survival

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