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

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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 \textit{in vivo}. Increased TNF-\textalpha{} secretion by resting cells exposed to nanoparticles could contribute to osteolysis processes \textit{in vivo}, while increased IFN-\gamma{} production by activated cells could represent cellular protection against

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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 prosthesis 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).
Co corrodes faster than Cr under physiological conditions (Xia et al., 2011) and, in contrast to Cr, Co ions tend to remain mobile, which is reflected in the higher levels measured in blood, allowing the ions to reach and enter remote organs (Afolaranmi et al., 2012). Elevated Co concentrations in patients with MoM implants are a concern, since increased Co levels in blood have also been reported to be associated with neurological (hand tremor, incoordination, cognitive decline, depression, vertigo, hearing loss, and visual changes) (Oldenburg et al., 2009; Tower, 2010), cardiac (myocardiopathy) (Dadda et al., 1994; Seghizzi et al., 1994; Gilbert et al., 2013) and endocrine (aberrant oestrogen signalling, altered the production or circulation of sex hormones, and altered thyroid metabolism) (Keegan et al., 2007; Oldenburg et al., 2009) symptoms.

In addition to the above, data from the seventh annual report of the National Joint Registry for England and Wales showed high failure rates for MoM hip prostheses (http://www.njrcentre.org.uk/njrcentre/portals/0/njr%207th%20annual%20report%202010.pdf), which led to the market recall of the DePuy ASR™, both the Resurfacing and XL Systems in August 2010 (DePuy International Ltd, Leeds, UK) (MDA/2010/069). Following this, the Medicines and Healthcare products Regulatory Agency (MHRA) safety alert in September 2010 drew attention to the long term biological safety of all types of MoM hip implants. In this document (MDA/2010/069; http://www.mhra.gov.uk/home/groups/dts-bs/documents/medicaldevicealert/con093791.pdf) the MHRA explained the details behind the safety alert and included four situations in which measurements of blood metal ions in patients were recommended: 1) in patients who have symptoms associated with loose MoM bearings; 2) in patients showing radiological features associated with adverse outcomes including component position or small component size; 3) if the patient or surgeon are concerned regarding the MoM bearing; and 4) if there is concern about patients with higher
than expected rates of failure. The MHRA have suggested that combined whole blood Co and Cr levels of greater than 7ppb (7µg/l or 0.1µM of the combined ions) are associated with significant soft-tissue reactions and failed MoM hips.

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

U937 cells are a human macrophage-like cell line derived from human leukemic monocyte lymphoma (Yagil-Kelmer et al., 2004). This cell line has been used previously as the cell culture model to study the biological effects of different kinds of particles and ions, and it has been demonstrated that U937 cells have comparable responses to polyethylene particles (Matthews et al., 2001) and metal ions (Wang et al., 1996) as do primary macrophages in terms of cytokine release.
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
sterility of the treated wear debris was tested as described by Akbar et al. (2012) by exposing dendritic cells (isolated from bone marrow of male BALB/c (Harlan, UK) mouse femurs and tibias (Lutz et al., 1999)) to the debris for 24h, in vitro, and then assessing the expression of surface activation markers via flow cytometry. The debris was found not to increase the surface expression of CD40, CD86, or MHC II on these cells, and, therefore, the suspended debris was deemed sterile and endotoxin-free (data not shown).

Heat treated wear debris was imaged with a Field Emission Scanning Electron Microscope (FE-SEM) (Hitachi SU-6600, Hitachi; Germany) at magnifications of 100-1000x. Energy Dispersive X-ray Spectroscopy (EDS) was used for quantitative analysis of elemental composition. Hitachi TM-1000 and EDSwift-TM software was used to obtain the images and chemical spectra of the wear debris. Metal ion release from the debris was determined by incubating 156.25μg debris/cm² for 24h at 37°C in an atmosphere of 5% (v/v) CO₂ in air in 1ml of complete RPMI-1640 medium in the presence of 10% (v/v) foetal bovine serum (FBS) as described below. The medium was collected and stored at -80°C until ICP-MS analysis using an Agilent 7700x octopole collision system in helium gas mode using scandium as internal standard. Quantification was based on the maximum signal for a particular isotope, and five readings were taken, with the result taken as the mean value.

2.2. Cell culture

U937 (Human leukemic monocyte lymphoma cell line; European Collection of Cell Cultures) cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS, 50U/ml penicillin and 50µg/ml streptomycin. These cells were routinely split every three days at a ratio of 1:10 from a starting passage number of 4 and for no more than 20 passages. This was achieved by taking 3ml of cell suspension into a fresh 75cm² culture flask containing 27ml of
fresh complete RPMI-1640 medium. Cells were activated by incubating them with 10nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich; Dorset, UK) for 72h (Lucarelli et al., 2004). Activation was confirmed by monitoring cell morphology, adhesion, and aggregation on a Zeiss Imager.Z1 microscope using wet lenses (20X) every 24h. For cobalt pre-treatment of cells, Co$^{2+}$ solutions were freshly prepared using cobalt chloride (CoCl$_2$) (Alfa Aesar; Lancashire, UK) and diluted to 0.1µM in growth medium under sterile conditions. Resting and activated cells were incubated with 0.1µM Co$^{2+}$ for 72h.

2.3. Cell number and viability

In order to assess cell viability, U937 cells were exposed to metal debris and Co$^{2+}$ in resting and activated states for 24 and 120h. For both resting and activated U937 cells, the cells were cultured (1x10$^4$ cells/well) in 96-well plates with 156.25µg debris/cm$^2$ (5mg debris/1x10$^6$ cells), 0.1µM of Co$^{2+}$, or the combination of 156.25µg debris/cm$^2$ plus 0.1µM of Co$^{2+}$ in complete RPMI-1640 for 24 and 120h at 37°C under 5% (v/v) CO$_2$. Culture medium was not changed during these incubation periods. At each culture endpoint, cells were washed twice with PBS to remove the debris layer before cell viability was assessed by the neutral red (NR) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, as described by Repetto et al. (2008) and Mosmann (1983), respectively. The cells were incubated with NR for 3h and MTT for 4h, and absorbance of the both NR and MTT assay samples were then measured at 540nm using a Bio-Rad Model 450 microplate reader (Bio-Rad, Hertfordshire, UK).

Viability was also monitored microscopically by double staining with Acridine Orange (AO; Sigma-Aldrich; Dorset, UK) and Propidium Iodide (PI; Life Technologies; Paisley, UK) as previously described by Bank (1988). Cells were viewed using a Carl Zeiss Axio Imager
microscope under a 40X water immersion lens with a numeric aperture of 0.80. Fluorescence
was excited using a mercury lamp and emission recorded using a fluorescein isothiocyanate
(FITC)/Rhodamine filter block (485/515-530nm; 546/580-563nm) for AO and PI. Cells
fluorescing green were scored as viable, and those fluorescing red as nonviable.

2.4. Cell proliferation
Resting and activated cells were cultured (1x10^4 cells/well) in 96-well plates with 156.25μg
debris/cm², 0.1μM of Co²⁺, or the combination of 156.25μg debris/cm² plus 0.1μM of Co²⁺ in
complete RPMI-1640 for 24h at 37°C under 5% (v/v) CO₂. U937 cell proliferation was then
determined using a BrdU Cell Proliferation Immunoassay kit (kit number QIA58, Merck
Chemicals; Nottingham, UK), as suggested by the manufacturer. Briefly, the BrdU label was
diluted 1:2000 into fresh complete RPMI-1640 medium. To label the DNA, 20μl of this
working solution were added to cells in 96-well plates during the final 4 hours of the
experiment. Plates were then centrifuged (300xg for 10min) and the supernatant aspirated.
Cells were then fixed, permeabilized, and the DNA denatured. Plates were incubated for
30min at room temperature and the fixative/denaturing solution was then aspirated. 100μl of
anti-BrdU antibody diluted in antibody dilution buffer were added to each well and incubated
with the cells for 1h at room temperature. Cells were washed 3 times with 1X wash buffer,
before 100μl of peroxidase goat anti-mouse IgG HRP conjugate diluted in conjugate diluent
were incubated with the cells for 30min at room temperature. Plates were then flooded with
dH₂O, emptied, and incubated with 100μl of substrate solution in the dark at room
temperature for 15min. Following the addition of 100μl of stop solution the absorbance was
measured using a Thermo Scientific Multiskan Ascent spectrophotometer plate reader at dual
wavelengths of 450-540nm.
2.5. Cytokine production: ELISA

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

2.6. Statistics

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

3. Results

3.1. Characterisation of wear debris and release of metal ions

Wear particles generated by the hip simulator process have been reported with a mean size of 50nm (Brown et al, 2007). SEM images show irregular shapes and sizes varying from the nano to the micro scale (from 150nm to 6.5µm). The larger irregular shaped particles suggest that the debris aggregates (Image 1), and this has been reported previously by Akbar and co-workers (2012). Doorn et al (1998) isolated particles from MoM retrieval tissues that varied in size (51-116nm particles to micrometre sized aggregates) and shape. Moreover, metal particles (0.1-3 microns in size) have also been found in tissues post-mortem (Brown et al., 2013). EDS analysis indicated that the wear debris is primarily composed of Co and Cr
(which is in agreement with the alloy composition (Singh and Dahotre, 2007)). Analysis of
25 different particles indicated a mean composition of 59.57 per cent Co, 40.43 per cent Cr
and a small amount of Mo which was below the limit of quantification. The CoCr wear debris
released metal ions into culture medium as shown on Table 1. Co was the predominant ion
released after 24h incubation. The percentage dissolution of the debris over the 24h period
was negligible, 0.000042%, although the cobalt concentration released was 1259.41 +/- 39.58
ng/ml.

3.2. Cell viability
In this study, the 156.25μg debris/cm² (5mg/1x10⁶ cells) concentration was chosen to mimic
the local metallosis environment surrounding an implant. The viability of the cells was
assessed after exposure to Co ions, to wear debris, and to a combination of the wear debris
and Co ions. Some of the cultures from each type of exposure had been pre-treated with Co
ions for 3 days prior to the experiment. Changes in cell number were measured by both NR
and MTT assays. The reduction of MTT is influenced by both reductase enzyme activities
and the supply of NADH/NADPH (the redox status) in the cells and is therefore, a measure
of the metabolic activity and redox status of the cells. In contrast, the NR assay is not
dependent on these factors. The latter may, however, be altered by changes in the number of
lysosomes in the cells. Resting and activated U937 cell numbers were assessed after 24 and
120h of treatment with the wear debris and/or ions. Figure 2 and Figure 3 summarise the
120h results, there being no significant changes in cell numbers for either resting or activated
U937 cells after 24h of exposure to the different treatments (data not shown).

After 120h there was a significant decrease in the absorbances measured by NR and MTT
assays respectively in both resting and activated cells, where the metal debris was present. In
the resting cells, exposure to Co ions for 120h caused an increase in the cell number compared to controls, which was apparent when measured by both MTT and NR (Figure 2). Additionally, cells exposed only for 3 days to Co and then the Co ions kept for 120h in culture medium (PreCo U937+no debris) did not show any differences when compared to untreated cells. The effect observed on resting cells was not as marked after activation of the cells by PMA (Figure 3), although increased absorbances in activated cells were detected where pre-treatment with Co ions had been carried out. When comparing the NR results of resting cells exposed to the combination of wear debris and Co ions, Co pre-treated cell numbers were significantly lower than those of non Co pre-treated cells. In fact, all the results obtained in resting cells were exacerbated to some extent by the pre-treatment with Co ions. Interestingly, such an effect was not seen after cell activation by PMA. Generally, the magnitude of responses to both nanoparticles and to Co ions was greater in the resting cells than in activated cells.

Viability was assessed by AO/PI double staining. PI is impermeable to intact plasma membranes, but it easily penetrates the plasma membrane of dead or dying cells and intercalates with DNA or RNA forming a bright red fluorescent complex. AO is a membrane-permeable, monovalent, cationic dye which binds to nucleic acids in the cells to produce a fluorescent green product (Bank, 1988). Images 2 and 3 summarise the 120h results, with there being no significant changes in cell viability for either resting or activated U937 cells after 24h of exposure to the different treatments (data not shown). After 120h treatment, there was a decrease in cell number of both resting and activated cells in the presence of debris but not Co ions. Furthermore, the few cells observed appeared to be mainly apoptotic.
3.3 Cell proliferation

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

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

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

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

4. Discussion

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

The wear debris used in this study was produced from a 39mm ASR™ prosthesis, and previous work emulating natural gait has shown that approximately 8 mm³ of debris is produced per million cycles on a simulator from this prosthesis (Leslie et al., 2008). The
density of the CoCr alloy used was 8.32mg/mm$^3$ (Medley et al., 1996), so 8mm$^3$ of wear would be equivalent to 66.56mg debris. An active person might walk 3.5 million cycles per year, so 232.96mg debris/year would be produced locally in the environment of the prosthesis. The 156.25μg/cm$^2$ metal debris concentration used in this investigation was chosen to mimic metallosis, a situation where metallic debris infiltrates into the periprosthetic tissues, with resulting severe adverse effects. The range of debris and ion concentrations measured locally varies hugely in the literature, for example, in catastrophic failure of a prosthesis amounts of wear debris up to 67mg have been reported (Matziolis et al., 2003). The high metal ion concentrations released from debris in the current experiments simulates this local situation.

Exposure to 156.25μg debris/cm$^2$ proved to be toxic for both resting and activated U937 cells in the presence and absence of Co ions, which could be in part due to the high concentration of ions released from the particles. However, there was no evidence of toxicity observed after cell exposure to 0.1μM Co ions alone. In fact, the cell numbers of resting cultures increased after exposure to the Co ions alone, and this type of response has been reported previously (Zijlstra et al., 2012), and is thought to represent a protective response to a non-toxic insult. The statistically significant difference in proliferation between Co pre-treated and non-Co pre-treated resting cells exposed to 0.1μM Co ions alone, suggests that longer exposure to Co ions ( >4 days) may result in a toxic response. These results seem to indicate once more that Co ions, particularly at high concentrations, are important in the adverse tissue response to metal wear debris and their effect may differ between cell activation states.

In the current investigation, a pre-treatment with cobalt ions was carried out to find out if such tissue pre-exposure and ion dissemination in vivo would have an effect on cell responses
to subsequent exposure to metal wear debris and ions. Such an effect would be important if a
patient received a second MoM implant or indeed had a revision procedure to replace a
failing implant. Many patients with failing MoM implants have shown high circulating Co
ions in their blood (Antoniou et al., 2008; Tkaczyk et al., 2010b; Bisseling et al., 2011; Lavigne et al., 2011; Friesenbichler et al., 2012; Penny et al., 2013), and recently the
importance of cobalt release in the inflammatory response to CoCr debris has been
demonstrated (Caicedo et al., 2009; Hart et al., 2012). Results from the present study indicate
that exposure to wear debris had a pronounced detrimental effect on cell number and
metabolic activity of cells pre-treated with Co after an incubation period of 120h. Even
though effects observed in resting cells were exacerbated to some extent by the pre-treatment
with Co ions, such an effect was not seen after cell activation by PMA. PMA exerts its
biologic effects by altering gene expression through the activation of protein kinase C (PKC)
and modulating the activity of transcriptional factors such as nuclear factor kappa-light-
chain-enhancer of activated B cells (NFkB) and activator protein 1 (AP1) (Garcia et al.,
1999; Daigneault et al., 2010). Thus, it seems unlikely that PKC was directly involved in the
pathways leading to the decrease in cell numbers. Despite the changes in proliferation at 24h
by BrdU assay, no changes in cell number or metabolic activity were observed at this same
endpoint by NR and MTT. Due to the intrinsic characteristics of the assays themselves and
complex dynamics of the balance of cell proliferation and cell death, it is unlikely that the
changes in cell proliferation (cell division) measured at 24h would be simultaneously
apparent as an increase in cell numbers at the same time point. On retrospect, such changes in
terms of cell numbers would have been detectable had we measured this at later time points.
Cells were treated with the Co ion concentration that has been advised by MHRA as the
recommended maximum safe circulating blood level for patients with MoM implants
((MDA/2010/069); http://www.mhra.gov.uk/home/groups/dts-
bs/documents/medicaldevicealert/con093791.pdf). It should be noted that cells pre-treated with Co ions at a 120h end point would have been exposed to Co for 9 days in total. In patients, the effects of Co ions and nanoparticles released from metal implants may be additive in terms of adverse effects. Contrary to Cr, Co ions tend to remain mobile and as a result greater Co concentration (compared to Cr) in blood and remote organs have been reported (Afolaranmi et al., 2012) and will be more likely to affect the responses of distant organs to metal nanoparticles than the Cr ions. The importance of Co ions in the inflammatory responses to Co-Cr particles has been recognised, and chronic exposure to circulating levels of ions, plus high local concentrations may act synergistically in vivo to trigger and promote implant loosening (Hallab et al., 2001; Caicedo et al., 2010; Hart et al., 2012). Although circulating systemic Cr levels are generally lower in vivo, it may be interesting in the future to investigate the influence of prior exposure to Cr ions in the CoCr wear debris in vitro exposure experiments. The presence of wear debris in the peri-implant area leads to macrophage phagocytosis of particulate debris and activation, the release of a variety of mediators, such as free radicals and nitric oxide, and a myriad of proinflammatory cytokines and chemokines (Sethi et al., 2003). The uptake of the CoCr particles into the cells was not measured due to technical difficulties in detecting the particles in the cells, but in a preliminary study phagocytosis by the activated cells was demonstrated using 1μm size FITC-labelled dextran beads (results not shown). Differences in the uptake of the CoCr particles could have occurred in resting and/or activated cells after the different treatments, and a contribution towards any differences in response cannot be excluded. Generally, the magnitude of the response to both the particles and the Co ions was greater in the resting cells than in the activated cells, and so the role of phagocytosis in the toxic responses is not clear. It has been reported that local acidification may develop during acute and chronic inflammation (Rajamaki et al., 2013) and high hydrogen ion concentrations down to pH 5.4
have been found in inflamed tissue (Steen et al., 1995). In turn, such an acidic environment created by actively metabolizing immune cells may enhance the corrosion process of the nanoparticles increasing the amount of metal ions being released (Afolaranmi et al., 2011).

A number of studies have shown that metal wear particles and high levels of metal ions, particularly Co and Cr, have a cytotoxic effect on a variety of cells such as human osteoblast-like cell lines (SaOS-2 and MG-63), and human monocytic-like U937 cells in vitro (Allen et al., 1997; Fleury et al., 2006; Petit et al., 2006). Most of these studies have been focused mainly on the short-term exposure, acute cell response, or have been limited to evaluation of one parameter like cell viability or cytokine levels. Studies carried out with U937 cells have been performed either with resting U937 cells (Ingham et al., 2000; Howling et al., 2003; Tkaczyk et al., 2010a) or activated U937 cells (Lucarelli et al., 2004; Yagil-Kelmer et al., 2004; Wang et al., 2011), and have investigated the effects of different kinds of metals, particles, and ions on U937 cells. Papageorgiou et al. (2007) compared the cytotoxic and genotoxic effects of nanoparticles and micron-sized particles of CoCr alloy using human fibroblasts in tissue culture. Their results showed that exposure of human fibroblasts to nanoparticles and micron-sized particles of cobalt chrome alloy, at the same particle mass per cell, cause different types and amounts of cellular damage. In particular, they found nanoparticles to be more cytotoxic and induce more DNA damage than micron-sized particles. This difference in induction of toxicity will contribute to the adverse effects found in vivo from CoCr nanoparticulate debris generated from MoM implants. Dalal et al. (2012) compared the responses of human osteoblasts, fibroblasts, and macrophages exposed to particles of different metal-based particles (i.e., cobalt-chromium (CoCr) alloy, titanium (Ti) alloy, zirconium (Zr) oxide, and Zr alloy). They found that CoCr-alloy particles were by far the most toxic and decreased viability and proliferation of human osteoblasts, fibroblasts, and
macrophages. Germain et al. (2003) studied the effects cobalt–chromium wear particles at various doses on the viability of U937 cells. CoCr particles at 5µm$^3$ (0.042mg) and 50µm$^3$ (0.42mg) per cell reduced the viability of U937 cells by 42% and 97%, respectively. Papageorgiou et al. (2007) and Germain et al. (2003) used high particle concentrations in comparison to the concentration used in the present study, which indicates that even at lower doses CoCr nanoparticles can exert cytotoxic effects. (Akbar et al., 2011) investigated effects of Cr$^{6+}$ and Co$^{2+}$ on primary human lymphocytes in vitro. Their results showed that exposure to 10 and 100µM Cr$^{6+}$ significantly decreased cell viability and increased apoptosis in both resting and activated lymphocytes. The exposure of resting and activated lymphocytes to 100µM Co$^{2+}$ also resulted in significant decreases in cell viability accompanied by a significant increase in apoptosis and they showed that activated cells were significantly more sensitive to Co$^{2+}$ toxicity. The concentration of Co ions used in the present study was 1000 fold lower (0.1µM) than that used by Akbar et al. (2011), and no significant effect in cell viability and proliferation was observed herein. From the literature there is little doubt that the nanoparticulate debris and metal ions released from MoM implants have toxic effects and the results in the present study point to the potential for interaction between them in vivo.

Although not statistically significant, there were higher levels of secretion of IL-6, TNF-α and IFN-γ by resting cells after 120h of exposure to 156.25µg/cm$^2$ metal debris and the combination of metal debris and Co ions. In the case of activated cells, there was significantly higher secretion of IL-6 and IFN-γ by cells exposed to the combination of 156.25µg/cm$^2$ metal debris and Co ions after 120h. On the other hand, and although not statistically significant, all treatments caused a reduction in the level of secretion of TNF-α. Contrary to what was observed in resting cells, Co pre-treatment did not seem to cause a difference in cytokine secretion by activated cells. It should be noted that the secretion of
cytokines has not been correlated for cell numbers in these experiments, and changes may reflect cytotoxicity. These results again suggest a larger impact of a chronic exposure and a key role of Co ions. They also suggest that different molecular pathways are affected in activated U937 cells when compared to resting U937 cells.

Bone remodelling involves tight regulation of three proteins, receptor activator of NF-κB ligand (RANKL), receptor activator of NF-κB (RANK), and osteoprotegerin (OPG). These proteins are key determinants of osteoclastogenesis and regulate bone resorption (Takayanagi, 2005). Pro-inflammatory cytokines such as IL-6, and TNF-α, can upregulate RANKL expression on osteoblasts and accelerate RANKL signalling, and thus directly contribute to bone destruction (Oishi et al., 2012). In biomaterials research, TNF-α, IL-1, and other pro-inflammatory cytokines are also known mediators of the foreign body reaction, an inflammatory response that can cause both severe tissue damage and premature failure of implanted materials (Mountziaris and Mikos, 2008). IFN-γ promotes innate immune responses by activating macrophages. In parallel, IFN-γ exerts regulatory functions to limit tissue damage associated with inflammation like suppressing osteoclastogenesis (Hu and Ivashkiv, 2009). Results from this study suggest that high concentrations of metal wear debris, particularly in the presence of Co ions, promote an immune response with the secretion of pro-inflammatory cytokines, which can contribute to tissue damage and ultimately result in aseptic loosening.

5. Conclusions

The results from this study suggest that a high concentration of metal debris in combination with Co ions not only have a direct effect on cell viability but also influence cell function. Previous exposure to Co ions seems to sensitise U937 cells to the toxic effects of both Co
ions themselves and to nanoparticles, pointing to the potential for interaction in vivo. The
increase in TNF-α secretion by the resting U937 cells could be a factor contributing to the
osteolysis process, while the increase in IFN-γ production by the activated cells could be a
cellular effort to counteract tissue damage. This also suggests that cellular activation state
affects the biological response to wear debris and for this reason caution should be taken
when choosing in vitro models to study immune and molecular responses. Moreover, these
findings mean that the survival and well-functioning of a second implanted MoM device
could be compromised in patients undergoing revision surgery or receiving a second device,
due to the interactions between recirculating Co ions and CoCr nanoparticles.

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6. References

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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/cm² (5mg debris/1x10⁶ cells), 0.1µM Co and 156.25μg debris/cm² (5mg
debris/1x10^6 cells) + 0.1μM Co for 120h. Images are representative of 5 independent images from each sample at each end point. “B” indicates cell blebbing, “S” indicates cell shrinkage, and “N” indicates necrosis (colour reproduction only on the web).

**Image 3. Fluorescence microscopy images (40X) following PI (Dead cells, red)/AO (Live cells, green) staining of activated U937 and Co pre-treated resting U937 cells exposed to 156.25μg debris/cm² (5mg debris/1x10^6 cells) , 0.1μM Co and 156.25μg debris/cm² (5mg debris/1x10^6 cells) + 0.1μM Co for 120h.** Images are representative of 5 independent images from each sample at each end point. “B” indicates cell blebbing, and “S” indicates cell shrinkage (colour reproduction only on the web).

**Figure 1. Neutral Red and MTT assays measured in resting cells at 120h.** Results are percentage values (Mean ± SEM, n=9) where 100% corresponds to control values (dash lines). PreCo-debris: cells pre-treated with Co ions and then the Co ions kept in culture medium throughout the experiment. *Significantly different from control values (p<0.05) by one-way ANOVA followed by Dunnett’s multiple comparison test. †Significantly different from non Co pre-treated cell values (p<0.05) by 2 sample t-Test.

**Figure 2. Neutral Red and MTT assays measured in activated cells at 120h.** Results are percentage values (Mean ± SEM, n=9) where 100% corresponds to control value (dash lines). PreCo+debris: cells pre-treated with Co ions and then exposed to treatments. PreCo-debris: cells pre-treated with Co ions and then the Co ions kept in culture medium throughout the experiment. *Significantly different from control values (p<0.05) by one-way ANOVA followed by Dunnett’s multiple comparison test. †Significantly different from non Co pre-treated cell values (p<0.05) by 2 sample t-Test.
Figure 3. Cell proliferation at 24h measured by BrdU. Results are percentage values (Mean±SEM, n=8) where 100% corresponds to control untreated cells (dash lines). PreCo: cells pre-treated with Co ions. *Significantly different from control values (p<0.05) by one-way ANOVA followed by Dunnett’s multiple comparison test. †Significantly different from non Co pre-treated cell values (p<0.05) by 2 sample t-Test.

Figure 4. Resting U937 cell cytokine secretion measured by ELISA after 120h of treatment. Results are expressed as cytokine concentration values per 1000 cells (± SEM, n=4). Untreated resting U937 cells were used as control. *Significantly different from control values (p<0.05) by one-way ANOVA followed by Dunnett’s multiple comparison test. †Significantly different from non Co pre-treated resting cell values (p<0.05) by 2 sample t-Test.

Figure 5. Activated U937 cell cytokine secretion measured by ELISA after 120h of treatment. Results are expressed as cytokine concentration values per 1000 cells (± SEM, n=5). Untreated activated U937 cells were used as control. *Significantly different from control values (p<0.05) by one-way ANOVA followed by Dunnett’s multiple comparison test. †Significantly different from non Co pre-treated activated cell values (p<0.05) by 2 sample t-Test.
Table 1. Release of Co and Cr ions into culture medium from metal wear debris in vitro.

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Cr release (ng/ml)</th>
<th>Co release (ng/ml)</th>
<th>Mo release (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI control</td>
<td>0.19 +/- 0.06</td>
<td>0.09 +/- 0.09</td>
<td>6.32 +/- 0.16</td>
</tr>
<tr>
<td>RPMI plus CoCr wear debris</td>
<td>18.18 +/- 2.64</td>
<td>1259.41 +/- 39.58</td>
<td>124.60 +/- 2.70</td>
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</tbody>
</table>

5mg wear debris were incubated for 24h at 37 °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 2
Figure 3

Proliferation Resting U937

Proliferation Activated U937

% Control

5mg  0.1uMCo  5mg+0.1uMCo

U937  PreCoU937

activated U937  PreCo activated U937

\[ \text{% Control} \]

\[ \text{5mg} \quad 0.1\mu\text{MCu} \quad 5\text{mg}+0.1\mu\text{MCu} \]

\[ \text{U937} \quad \text{PreCoU937} \]

\[ \text{activated U937} \quad \text{PreCo activated U937} \]
Figure 4
Figure 5

![Graph showing cytokine levels of IL-6, TNF-α, and IFN-γ for different conditions: Control, activated U937, and PreCo activated U937.](image-url)
Image 2

<table>
<thead>
<tr>
<th>Control</th>
<th>Co pre-treated Resting 120h</th>
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</thead>
<tbody>
<tr>
<td>Resting 120h</td>
<td></td>
</tr>
<tr>
<td>5mg debris/1x10^6 cells</td>
<td></td>
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<tr>
<td>0.1μM Co</td>
<td></td>
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<tr>
<td>5mg debris/1x10^6 cells</td>
<td></td>
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<tr>
<td>+ 0.1μM Co</td>
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</table>

10μm
Highlights

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