Toxicity of Cobalt-Chromium nanoparticles released from a resurfacing hip implant and Cobalt ions on primary human lymphocytes *in vitro*

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Running title: *CoCr nanoparticle toxicity in lymphocytes*

Abstract

Adverse tissue responses to prostheses wear particles and released ions are important contributors to hip implant failure. In implant-related adverse reactions T-lymphocytes play a prominent role in sustaining the chronic inflammatory response. To further understand the involvement of lymphocytes in metal-on-metal (MoM) implant failure, primary human lymphocytes were isolated and treated with CoCr wear debris and Co ions, individually, and in combination, for 24, 48, and 120h. There was a significant increase in cell number where debris was present, as measured by the Neutral Red assay. Interleukin 6 (IL-6), interferon γ (IFNγ), and tumour necrosis factor α (TNFα) secretion levels significantly decreased in the presence of metal particles, as measured by ELISA. Interleukin 2 (IL-2) secretion levels were significantly decreased by both debris and Co ions. Flow cytometry analysis showed that the metal nanoparticles induced a significant increase in apoptosis after 48h exposure. This investigation showed that prolonged exposure (120h) to metal debris induces lymphocyte proliferation, suggesting that activation of resting lymphocytes may have occurred. Although cytokine production was affected mainly by metal debris, cobalt toxicity may also modulate IL-2 secretion, and even Co ion concentrations below the MHRA guideline levels (7ppb) may contribute to the impairment of immune regulation *in vivo* in patients with MoM implants.

Short abstract

In implant-related adverse reactions T-lymphocytes play a prominent role in sustaining the chronic inflammatory response. Primary human lymphocytes were isolated and treated with CoCr wear debris and Co ions, individually, and in combination, for 24, 48, and 120h. Prolonged exposure to metal debris induced lymphocyte proliferation, suggesting that activation of resting lymphocytes may have occurred. Furthermore, cobalt toxicity may modulate IL-2 secretion, which may contribute to the impairment of immune regulation *in vivo* in patients with MoM implants.

Key words: nanoparticles; metal wear debris; metal-on-metal hip replacement; implant failure.

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Introduction

Modern day metal-on-metal (MoM) total hip resurfacings were introduced in the 1990s (Quesada *et al.*, 2008). They represented approximately 10% of all hip arthroplasties in developed countries between 1990 and 2010 (Corten and MacDonald, 2010; Jiang *et al.*,

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2011). MoM hip resurfacing bearings are made from high-carbon CoCr alloy (Amstutz and Le Duff, 2006; Mahendra *et al.*, 2009) .

The most common cause of failure of total hip arthroplasty is aseptic loosening of the implant due predominantly to adverse tissue responses to prostheses wear particles (Luo *et al.*, 2005). Host response to a prosthesis or prosthetic debris results in the formation of a fibrous synovial-like membrane surrounding the prosthesis (Wang *et al.*, 1996). It is believed that mononuclear phagocytic cells in the pseudomembrane surrounding the implant phagocytose wear particles and become activated. This activation results in the release of pro-inflammatory cytokines, such as IL-6 and TNF-α, and inflammatory mediators, such as PGE_2, which stimulate osteoclastic bone resorption (Ingham *et al.*, 2000). Lymphocytes are known to be important regulators of macrophage function (Arora *et al.*, 2003). T cells are recognised as modulators of immune response pathways as a result of stimulation of either the Th1 or Th2 pathway, which involves cell types and cytokines that may influence loosening of total hip replacements (Cachinho *et al.*, 2013). The Th1-cell response is crucial to the activation of macrophages and cytotoxic T-lymphocytes and is involved in the cell-mediated immune response. On the other hand, the Th2-cell response is the most effective activator of B-lymphocytes and is associated with humoral immunity (Cachinho *et al.*, 2013).

T lymphocytes also play a prominent role in cell mediated type IV hypersensitivity reactions sustaining the chronic inflammatory response. Cell-mediated type-IV hypersensitivity reactions are characterised in vivo by vasculitis with perivascular and intramural lymphocytic infiltration of the postcapillary venules, swelling of the vascular endothelium, recurrent localised bleeding, and necrosis which has been reported following MoM hip replacements (Willert *et al.*, 2005). Lymphocyte infiltrates have also been reported in soft-tissue masses, described as pseudo-tumours, following MoM resurfacing arthroplasty (Boardman *et al.*, 2006; Pandit *et al.*, 2008).

Metals modulate the activities of immunocompetent cells by a variety of mechanisms. The outcome of this modulation depends on the particular metal, its concentration and biological availability (Lawrence and McCabe, 2002). A variety of soluble metals, including Co^{2+} and Cr^{3+}, at a range of concentrations between 0.05 and 5mM were found to induce Jurkat T-lymphocyte DNA damage, apoptosis, and/or direct necrosis in a metal-, and concentration-dependent manner (Caicedo *et al.*, 2008).

Co corrodes faster than Cr under physiological conditions (Xia *et al.*, 2011) and, contrary to Cr, Co ions tend to remain mobile, which is reflected in the higher levels measured in blood, allowing them to reach remote organs (Afolaranmi *et al.*, 2012). Data from the seventh annual report of the National Joint Registry for England and Wales showed high failure rates for MoM hip prostheses, 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. The MHRA have suggested that combined whole blood Co and Cr levels of greater than 7ppb (7µg/l or 0.1µM) are associated with significant soft-tissue reactions and failed MoM hips (MDA/2010/069). However, there is still considerable debate about the existence of a safe threshold.

In the present study, the effects of CoCr alloy wear debris and Co ions on primary human lymphocytes were explored in terms of viability, proliferation, cytokine production, and

apoptosis. Release of Co and Cr ions from the CoCr debris was measured at physiological pH of 7.4, and at the pH estimated to exist in inflammatory conditions (Mansson *et al.*, 1990). Cells were pretreated with Co ions before exposure to the CoCr wear debris in order to detect any interactions between the ions and particle effects.

Methods

Preparation of wear debris

Co-Cr wear debris was donated by DePuy International (Leeds, UK). A high-carbon cast (≥ 0.2%) cobalt chrome (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. (Dearnley, 1999) hip resurfacing implant was worn on a multi-station hip joint simulator using a non-standard protocol (personal communication, Dr C. Hardaker, DePuy International, Leeds, UK). The wear debris was produced over 250000 cycles using distilled water as the lubricating fluid. Wear debris produced by hip simulator under different conditions has previously been shown to be of similar size and morphology to wear debris produced *in vivo* (Brown *et al.*, 2007), Once produced, the wear debris was centrifuged at 3500g for 20 minutes. The debris was heat-treated (180°C for 5h, 60kPa) in a vacuum oven to destroy any endotoxin. The dry debris was then suspended in sterile phosphate buffered saline (PBS; Invitrogen; Paisley, UK). Heat-treated wear debris was characterised with a Field Emission Scanning Electron Microscope (FE-SEM) (Hitachi SU-6600, Hitachi; Germany) at magnifications of 100-1000x. The sample was then transferred to a Scanning Electron Microscope (SEM) (Hitachi TM-1000, Hitachi; Germany). 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. The sterility of the treated wear debris was tested as described elsewhere (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 by 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).

ICP-MS analysis of metal ion release from CoCr nanoparticles

Experiments were carried out to determine the extent of metal ion release when wear debris was incubated with cultured cells *in vitro*. In order to assess the effects of foetal calf serum (FCS) and pH on the metal ion release, 2.5mg metal wear debris /1x10^6cells were incubated for 24h in RPMI-1640 medium in the presence and absence of FCS and complete RPMI-1640 medium, pH 4. Controls of each condition with no metal debris were also present. Standards were prepared by diluting Multielement Standard Solution 1 for ICP (Sigma-Aldrich (Fluka); Dorset, UK) in RPMI-1640. Samples were analysed using an Agilent 7700x octopole collision system ICP-MS (Agilent Technologies; Wokingham, UK) in helium gas mode using scandium as internal standard.

Human lymphocyte isolation

Human buffy coat samples were collected from the Scottish Blood Transfusion Service (SNBTS), Glasgow, UK) with ethical permission from the SNBTS Committee for the Governance of Blood and Tissue Samples for Non-Therapeutic Use. All samples had been donated by anonymous healthy donors no more than 5h before use. Peripheral blood mononuclear cells (PBMCs) were isolated under sterile conditions from 60 ml of Buffy Coat by density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich, Cambridge, UK),

and lymphocyte enrichment performed as previously described (Martin-Romero *et al.*, 2000). Briefly, PBMC (2.5×10^6cells/ml) were incubated in a 75cm^2 culture flask (TPP, Trasadingen, Switzerland) with complete RPMI-1640 for 1h at 37°C in a 5% (v/v) CO_2 chamber. The medium with the non-adherent cell suspension was then transferred to another culture flask and incubated for an additional 1h to further deplete the numbers of any monocytes present in the population. Lymphocyte viability ≥90% and a mean lymphocyte yield of $5.89\text{x}10^6$cells/ml (±0.61 SEM, n=3) was obtained.

Exposure of lymphocytes to wear debris and metal ions

Isolated peripheral human lymphocytes were exposed to metal wear debris and Co^{2+} in a resting state. Lymphocytes were cultured ($1\text{x}10^5$cells/well) in 96-well round-bottom plates (100µl/well) with 5mg wear debris/$1\text{x}10^6$cells, 0.1µM of Co^{2+} and 5mg wear debris/$1\text{x}10^6$cells combined with 0.1µM of Co^{2+} in complete RPMI-1640. Cultures were carried out for 24, 48, and 120h at 37°C under 5% (v/v) CO_2 air. For apoptosis analyses, debris concentration was 2.5mg wear debris/$1\text{x}10^6$ cells, a lower concentration than for cytotoxicity studies, in order to facilitate detection of early apoptosis.

Measurement of viability, proliferation and apoptosis

At 24 and 120h, cell viability was assessed by the neutral red (NR) and MTT assays as described previously (Akbar et al, 2011). Proliferation was determined after 48 and 120h of exposure to the treatments using a BrdU Cell Proliferation Immunoassay kit (kit number QIA58, Merck Chemicals; Nottingham, UK), as suggested by the manufacturer. The absorbance was measured using a Thermo Scientific Multiskan Ascent spectrophotometer plate reader (Thermo Scientific; Hampshire, UK) at dual wavelengths of 450–540nm. At 24 and 48h post treatment, lymphocytes cells were collected by centrifugation and incubated for 15min with phycoerythrin-labelled annexin V and 7-aminoactinomycin D in the dark. The samples were analysed by a FACSCanto flow cytometer (BD Bioscience, Oxford, UK), and all data were analysed using FACSDiva software.

Cytokine secretion measured by ELISA

Cytokine levels were determined by collecting the supernatants from cell cultures at 24 and 120h following exposure to the treatments. The concentrations of tumor necrosis factor-alpha (TNF-α), interferon-γ (IFNγ), interleukin-2 (IL-2), 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, 0-250pg/ml for IL-2, and 0-200pg/ml for IL-6. The presence of high concentrations of metal ions did not interfere with detection of cytokines.

Statistics

Statistical analyses were carried out by a one-way analysis of variance (ANOVA), followed by a Dunnett's multiple comparison test. Significance was assigned where p values were found to be < 0.05.

Results

The aim of this study was to assess the toxicity of CoCr nanoparticles released from a resurfacing hip implant and Co ions on primary human lymphocytes. In order to achieve this, viability, proliferation, cytokine production, and apoptosis were evaluated in lymphocytes exposed to the ions and the CoCr wear debris particles.

Characterisation of heat treated wear debris

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 coworkers (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% (±1.15%) Co and 40.43% (±1.25%) Cr, with a small content of Mo which was below the quantification limit.

Metal ion release into cell culture medium

Metal debris was incubated, in the absence of cells, under different conditions. Analysis of ICP-MS results found that CoCr wear debris releases metal ions into culture medium (Figure 1) was no significant difference ($p>0.05$) in ion release from metal debris in the presence and absence of 10% (v/v) FCS. This concentration of FCS was used as it was the concentration used when the Co-Cr wear debris was incubated with cells for up to 120h. In contrast to these data, the acidic pH 4.0 had a considerable effect as seen in the significant increase ($p<0.05$) in the levels of ion release compared with release in medium at normal physiological pH of 7.4. Even though Co was the ion predominantly released in all cases, the change in pH seemed to have a more pronounced effect on Cr ion release.

Effects of metal debris and ions on human primary lymphocyte cell viability, proliferation and apoptosis.

The viability of primary human lymphocytes was tested after 24 and 120h of exposure to 5mg wear debris/1x10^6cells; 0.1µM of Co^{2+}; and 5mg wear debris/1x10^6cells combined with 0.1µM of Co^{2+}. In this study, the 5mg/1x10^6cells debris concentration was chosen to mimic the local metallosis environment surrounding an implant. There was a significant increase in cell number as indicated by the NR assay, measured both at 24 and 120h in the presence of metal debris when compared to controls (Figure 2). Although there was an initial increase in cell number (24h), there was no significant difference in the reduction of MTT in cells in the presence of metal wear debris. Co ions on their own did not seem to have an effect on cell number or cell metabolic activity. These findings suggest that the effects of the debris on the lymphocytes are due to the synergistic action of the nanoparticles and the Co and Cr ions released from the particles.

Effects on cell proliferation were assessed with the BrdU cell proliferation assay after 48 and 120h of treatment with 5mg wear debris/1x10^6cells; 0.1µM of Co^{2+}; and 5mg wear debris/1x10^6cells combined with 0.1µM of Co^{2+}. At 48h, there is an initial decrease in cell proliferation followed by an increase by 120h of treatment (Figure 3). These results suggest an activation response of the cells to both debris and ions, where the cells overcome the initial growth arrest effect.

To determine the effects of metal ions on cell damage leading to apoptosis, flow cytometry following Annexin V and 7-AAD staining at 24 and 48h of exposure was performed. To avoid cytotoxic effects and the growth arrest effect observed after exposure to 5mg, a lower debris concentration (2.5mg metal debris/1x10^6 cells) was used for apoptosis analysis in order to facilitate the detection of the process at early stages (to measure both early apoptosis and detect any repair). Apoptosis was not observed within 24h of exposure, but was evident

after 48h (Figure 5) where the debris caused apoptosis whereas the Co ions did not, and the effect of the debris was unaltered by preincubation with Co ions.

Effects of metal debris and ions on cytokine release by human primary lymphocyte cells

Interleukins such as IL-2, IL-6, IFNγ, and TNFα are regarded as indicators of the inflammation evoked by particulate metals (Cachinho *et al.*, 2013). Levels of these four cytokines were determined in the supernatants of human primary lymphocyte cultures after 24 and 120h of treatment with cobalt ions and wear debris. There was a general decrease in cytokine production particularly when cells were in contact with metal particles (Figure 4). IFNγ and IL-6 levels decreased after 24h of exposure and continued to be low for 120h. TNFα levels were mainly decreased after 120h of exposure. Interestingly, IL-2 was the only cytokine to be affected by all treatments. Cobalt ion treatment did not have an effect on IL-6, IFNγ, and TNFα production.

Discussion

Several studies have described accumulation of perivascular lymphocytes in tissue membranes around failed MoM implants apparently not associated with infection, and these authors have interpreted this inflammation as an immunologic reaction against metal ions or metal particles associated with those articulations (Bohler *et al.*, 2002; Davies *et al.*, 2005; Willert *et al.*, 2005; Korovessis *et al.*, 2006; Campbell *et al.*, 2010). In the present study, primary human lymphocytes were exposed to Co ions and high concentrations of metal wear debris derived from a MoM hip resurfacing device in order to evaluate the cell response to these treatments and to assess whether or not CoCr particles and ions could activate primary cultures of human lymphocytes.

The wear debris used in this study was produced from a 39mm ASR^{TM} prosthesis, and previous work replicating natural gait on a simulator has shown that approximately 8 mm^3 of debris is produced per million cycles from this prosthesis (Leslie *et al.*, 2008). The density of the CoCr alloy used was 8.32mg/mm^3 (Medley *et al.*, 1996), so 66.56mg debris would be produced per million cycles. 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 5mg/1x10^{6}cells 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).

Cellular uptake of the wear debris by the lymphocytes was not demonstrated in the present study primarily due to technical difficulties in proving uptake by SEM. Showing specific uptake of the CoCr debris would have made a significant contribution to the study. However, uptake of dextran coated glass beads of similar size range was measured by fluorescent labelling proving that the lymphocytes were capable of phagocytosis.

T-lymphocytes are normally maintained in a quiescent state while remaining capable of rapid responses and effector function (Macintyre and Rathmell). T-cell proliferation occurs as the result of a precisely orchestrated set of events involving two distinct signals, namely recognition of an antigen followed by release of co-stimulatory molecules such as IL-2 cytokine (Deweck *et al.*, 1984; Habetswallner *et al.*, 1988). After activation, T cells undergo a transient period with little cell growth and then begin to rapidly grow and divide (Macintyre

and Rathmell). In this investigation, high concentrations of metal debris caused an increase in cell number despite the initial decrease in cell proliferation suggesting that the particles may exert a cell activation effect. It is proposed that this took place during the first 48h of treatment reflected by the initial lower proliferation rates (24-48h) followed by the rapid growth and division seen by 120h. In addition to this, a significant decrease in IL-2 production was observed after 24h of exposure to all the treatments. Since IL-2 is an important molecule for lymphocyte activation and proliferation, its diminished production could have contributed to the decrease in proliferation observed at 48h.

A state of reduced function in which a viable, antigen-specific T cell is unable to respond to an immunogenic stimulus has been referred to as anergy (Zheng *et al.*, 2008). Anergy can be induced under a number of circumstances that can be categorised as resulting from either a normal antigenic stimulus received in the absence of co-stimulation or from an altered and/or chronic T-cell receptor stimulus (Wells, 2009). To the authors' knowledge, anergy has not been described as part of the biological reaction to metal debris and ions, and most studies report implant-related hypersensitivity reactions, in particular type-IV delayed-type hypersensitivity mediated by T lymphocytes. Nevertheless, results from this investigation suggest that there may be an anergy-like response to high concentrations of metal debris. The significant decrease in IL-2 production and proliferation observed here are hallmarks of T-cell anergy (Chappert and Schwartz, 2010; Kuklina, 2013). Moreover, defective production of inflammatory cytokines such as IFNγ and TNFα is also a characteristic of anergy (Wells, 2009) and significant decreases in both cytokines were observed in the present study in the presence of metal debris.

Metals corrode *in vivo* releasing metal ions (Hanawa, 2004). Such ions can potentially bind to proteins, remain in solution, or disseminate into the surrounding tissues and bloodstream, and thus reach remote organs. The microenvironment conditions surrounding the debris can influence the rate of ion release (Cadosch *et al.*, 2009). It is generally presumed that metal ions facilitate cell activation and sensitization. However, depending on the concentration of metal ions present, they may also be cytotoxic and suppressive. It has been shown that production of TNFα and IL-6 by human peripheral blood mononuclear cells exposed to Cr (1, 5, and 10μM) significantly decreases (Villanueva *et al.*, 2000). In the current investigation, inhibition of cytokine production was observed in the presence of metal particles. IFNγ, TNFα, and IL-6 levels did not seem to be affected by Co ions alone, whereas IL-2 levels were decreased. Although the effects of Cr may be related to the regulation of TNFα and IL-6, IL-2 production is more likely to be modulated by Co. Additionally, high concentrations (5mg debris/1x10^6cells) of metal debris were not cytotoxic to primary lymphocytes. However, a marked increase in apoptosis was observed at a lower dose (2.5mg debris/1x10^6cells). These findings suggest a dose dependent effect on cell damage pathways. Akbar *et al.* (2011) exposed resting and activated lymphocytes to a range of Co and Cr ions. They found that exposure to higher concentrations of Cr^{6+} (10 and 100μM), and Co^{2+} (100μM) significantly decreased cell viability and increased apoptosis in both resting and activated lymphocytes at 24 and 48h of exposure. In their study metal ions were assessed independently. The effects observed in the current study are the results of the concerted action of the particles and both Co and Cr ions. It would have been interesting to pre-incubate the cells with Co ions and subsequently treat them with the particles to identify if sensitisation occurs. In fact, priming human monocyte-like U937 cells with Co ions for subsequent challenge with wear debris has been investigated in our laboratory (Posada *et al.*, 2014). Results from such investigation showed that metal debris was more effective as an inducer of apoptosis and gene expression when cells had been pre-treated with Co ions. However, this set of experiments could not

readily be performed with the primary lymphocytes due to difficulties in maintaining prolonged culture.

Released metal ions can activate the immune system by forming metal-protein complexes that are considered to be candidate antigens for eliciting hypersensitivity responses (Korovessis *et al.*, 2006). Upon recognition by lymphocytes, the metal-protein complexes induce the production of proinflammatory cytokines and chemokines by various cell types due to triggering of innate immune responses (Martin, 2004). According to this, it is thought that high local metal ion and nanoparticles concentrations facilitate a T-lymphocyte mediated inflammatory response resulting in the destruction seen around the prostheses (Davies *et al.*, 2005; Willert *et al.*, 2005; Boardman *et al.*, 2006; Kwon *et al.*, 2010). Three mechanisms have been proposed by which metal–protein complexes can activate lymphocytes: 1. antigen-independent, 2. antigen-dependent, and 3. superantigen-like, which is a synergistic combination of the first two mechanisms (Hallab *et al.*, 2001b). Metals may act with serum proteins to crosslink lymphocyte receptors (e.g., BV17 of CDR1 T cell receptor) without the presence of an antigen-presenting cell leading to a superantigen enhancement of T cell receptor-protein contact (Vollmer *et al.*, 1997; Vollmer *et al.*, 1999). In this circumstance, proteins or peptides that would not otherwise be antigenic are able to provoke a response (Hallab *et al.*, 2001b). The lymphocyte reactions in the current investigation seem to be consistent with such nonspecific mitogenic activation mechanisms, which could explain the increase in cell proliferation despite the significant decrease in IL-2 production.

Implant failure is largely caused by aseptic loosening and osteolysis (Huber *et al.*, 2010) in response to accumulation of metal particles in the periprosthetic tissues, which also generates inflammation, pain, and pseudotumours (Langton *et al.*, 2011). Evidence of systemic effects can also be found in multiple reports (Steens *et al.*, 2006; Oldenburg *et al.*, 2009; Rizzetti *et al.*, 2009; Ikeda *et al.*, 2010; Tower, 2010; Machado *et al.*, 2012; Pelclova *et al.*, 2012; Tower, 2012) describing patients with MoM implants who presented symptoms including neurological symptoms such as auditory impairment/deafness, visual impairment/blindness, peripheral neuropathy/dysesthesia of the extremities, poor concentration/cognitive decline, cardiomyopathy and hypothyroidism. All patients had elevated cobalt and/or chromium concentrations in their blood, serum, plasma, and/or urine, suggesting that these systemic symptoms may be due to metal toxicity as a result of excessive implant wear. Polyzois et al (2012), reviewed the evidence of local and systemic toxicity of wear debris from total hip arthroplasty. They found extensive evidence and experimental data supporting the fact that orthopaedic metals induce local immunological effects characterised by an unusual lymphocytic infiltration and cell-mediated hypersensitivity. In terms of systemic toxicity, there are *in vivo* and *in vitro* experimental, as well as a limited number of epidemiological studies, where the systems most commonly involved are haematopeietic, immune, hepatobiliary, renal, respiratory, nervous, cardiovascular, musculoskeletal, skin, and endocrine and reproductive. Concern has been raised regarding a potential link between metal wear debris and carcinogenesis. In an attempt to address this, Christian et al (2014) used quantitative methods to evaluate the relationship between CoCr-containing hip implants and increased cancer risk. They concluded that although the evidence suggests that such implants are unlikely to be associated with an increased risk of systemic cancers, additional research is warranted in this area.

The importance of Co ions in the inflammatory responses to CoCr 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.*, 2001a;

Caicedo *et al.*, 2010; Hart *et al.*, 2012). The present study has shown that high concentrations of wear debris, derived from a CoCr MoM hip resurfacing, induce lymphocyte proliferation and inhibit cytokine production after 120h exposure. The fact that IL-2 production was affected by 0.1µM Co (5.9ppb or 5.9µg/L) suggests that even circulating blood metal ion concentrations within the MHRA guideline levels of 7ppb or 7µg/L (MHRA) may contribute to the impairment of immune regulation in patients with MoM implants.

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Figure and Image legends

Image 1. Scanning Electron Microscopy images of simulator generated wear debris from an ASR hip implant. Image taken at 5000X with a FE-SEM Hitachi SU-6600.

Figure 1. Metal ions in RPMI-1640 in the presence and absence of metal wear debris. Results are expressed as mean values (±SEM, n=3). (a) Cr ion concentrations. (b) Co ion concentrations. *Significantly different from control values ($p<0.05$) by one-way ANOVA followed by Dunnett's multiple comparison test. ^{b}Significant difference between pH 7.4 and pH 4.0.

Figure 2. Cell number (NR) and metabolic activity (MTT) of human lymphocytes. Results are expressed as percentages (±SEM, n=12) where 100% represents control untreated cells. (a) Effects on cell number as measured by NR. (b) Cell metabolic activity as measured by MTT. *Significantly different from control values ($p<0.05$) by one-way ANOVA followed by Dunnett's multiple comparison test.

Figure 3. Proliferation of human lymphocytes measured by BrdU assay. Results are expressed as percentages (±SEM, n=6) where 100% represents control untreated cells. *Significantly different from control values ($p<0.05$) by one-way ANOVA followed by Dunnett's multiple comparison test.

Figure 4. Cytokine production by human lymphocytes. Results are expressed as mean values (±SEM, n=4). (a) TNF-α levels. (b) INF-γ levels. (c) IL-6 levels. (d) IL-2 levels. *Significantly different from control values ($p<0.05$) by one-way ANOVA followed by Dunnett's multiple comparison test.

Figure 5. Early and late apoptosis. Results are expressed as percentages (±SEM, n=12). (a) Percentage of early apoptosis after 24h of treatment. (b) Percentage of late apoptosis after 24h of treatment. (c) Percentage of early apoptosis after 48h of treatment. (d) Percentange of late apoptosis after 48h of treatment. *Significantly different from control values ($p<0.05$) by one-way ANOVA followed by Dunnett's multiple comparison test.

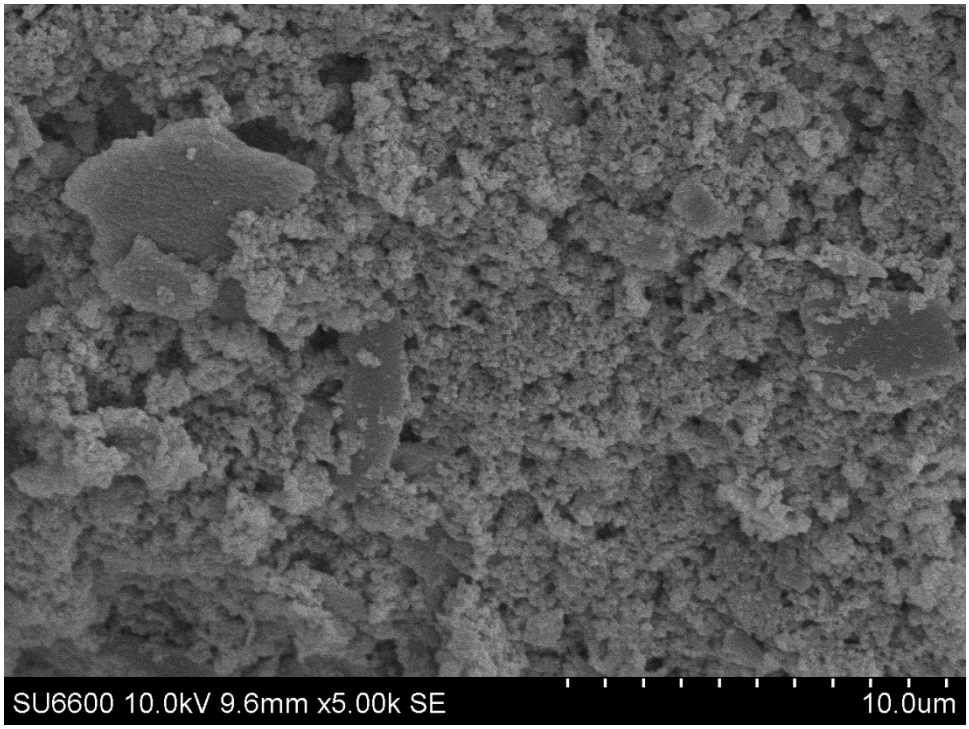

Image 1

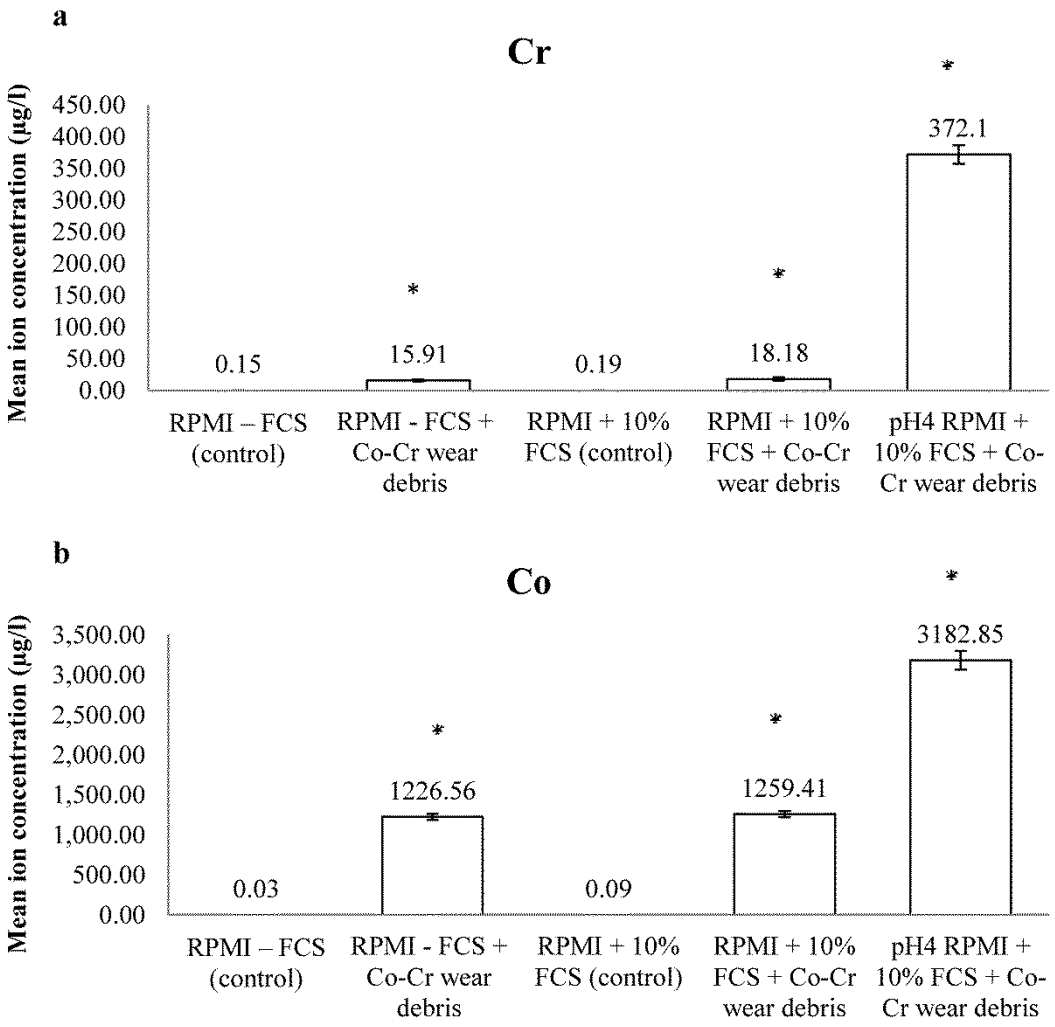
a
Cr
Mean ion concentration (µg/l)
450.00
400.00
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
0.15
15.91
0.19
18.18
372.1
*
*
*
RPMI – FCS (control)
RPMI - FCS + Co-Cr wear debris
RPMI + 10% FCS (control)
RPMI + 10% FCS + Co-Cr wear debris
pH4 RPMI + 10% FCS + Co-Cr wear debris
b
Co
Mean ion concentration (µg/l)
3,500.00
3,000.00
2,500.00
2,000.00
1,500.00
1,000.00
500.00
0.00
0.03
1226.56
0.09
1259.41
3182.85
*
*
*
RPMI – FCS (control)
RPMI - FCS + Co-Cr wear debris
RPMI + 10% FCS (control)
RPMI + 10% FCS + Co-Cr wear debris
pH4 RPMI + 10% FCS + Co-Cr wear debris

Figure 1

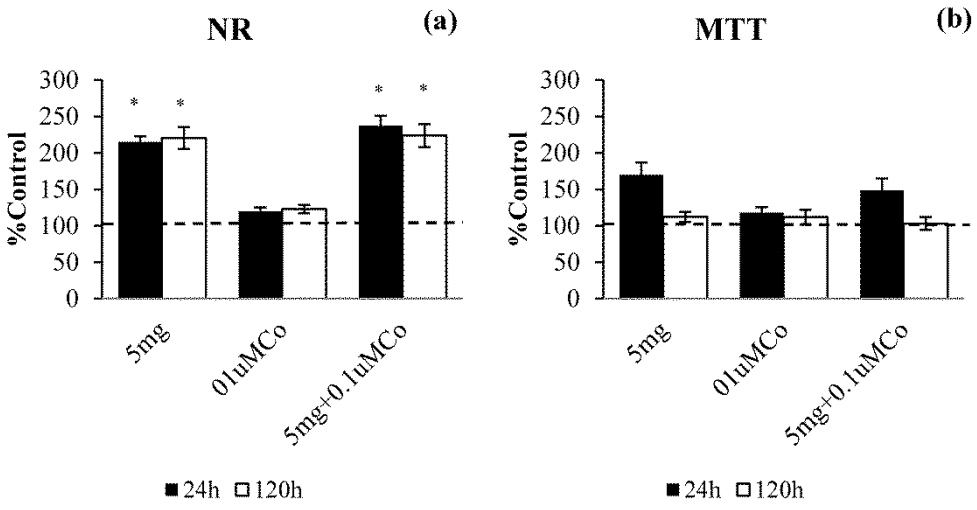
NR
(a)
MTT
(b)
% Control
300
250
200
150
100
50
0
5mg
01uMCo
5mg+0.1uMCo
24h
120h

Figure 2

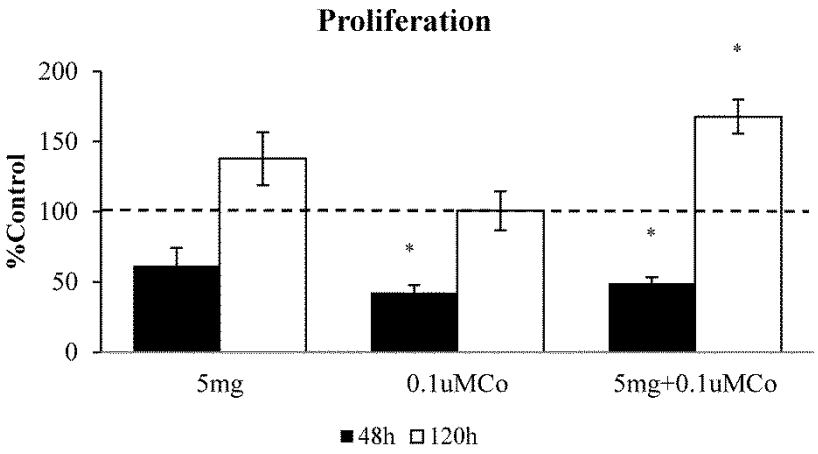
Proliferation
%Control
200
150
100
50
0
*
*
*
5mg
0.1uMCo
5mg+0.1uMCo
48h
120h

Figure 3

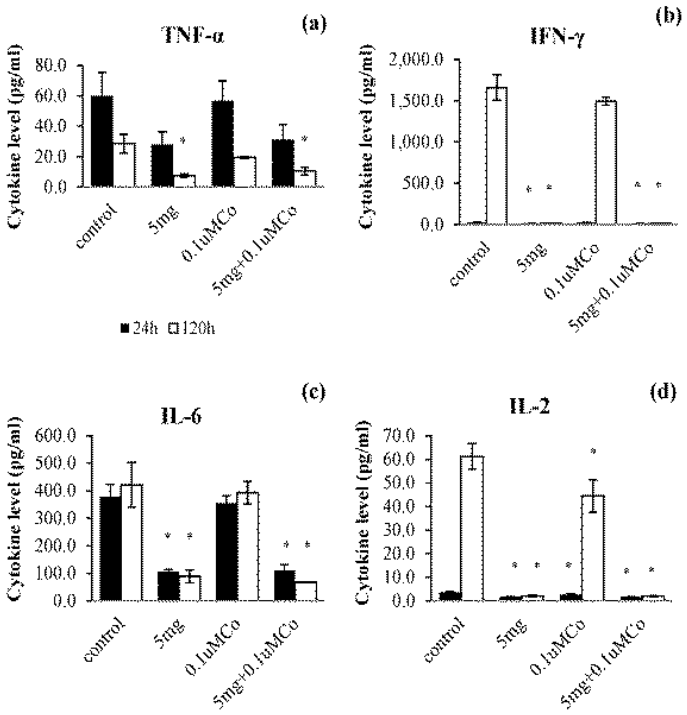
(a)
TNF-α
Cytokine level (pg/ml)
80.0
60.0
40.0
20.0
0.0
control
5mg
0.1uMCo
5mg+0.1uMCo
24h
120h
(b)
IFN-γ
Cytokine level (pg/ml)
2,000.0
1,500.0
1,000.0
500.0
0.0
control
5mg
0.1uMCo
5mg+0.1uMCo
(c)
IL-6
Cytokine level (pg/ml)
600.0
500.0
400.0
300.0
200.0
100.0
0.0
control
5mg
0.1uMCo
5mg+0.1uMCo
(d)
IL-2
Cytokine level (pg/ml)
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
control
5mg
0.1uMCo
5mg+0.1uMCo

Figure 4

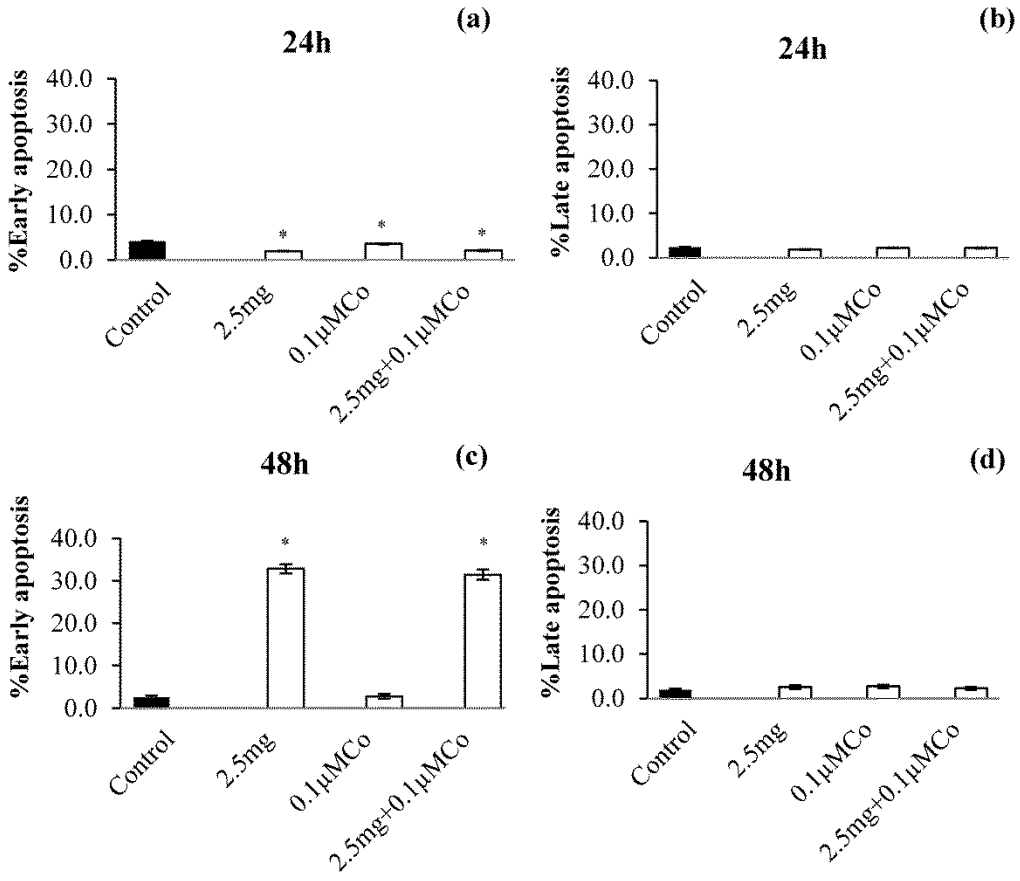
(a)
24h
%Early apoptosis
40.0
30.0
20.0
10.0
0.0
*
*
*
Control
2.5mg
0.1µMCo
2.5mg+0.1µMCo
(b)
24h
%Late apoptosis
40.0
30.0
20.0
10.0
0.0
Control
2.5mg
0.1µMCo
2.5mg+0.1µMCo
(c)
48h
%Early apoptosis
40.0
30.0
20.0
10.0
0.0
*
*
Control
2.5mg
0.1µMCo
2.5mg+0.1µMCo
(d)
48h
%Late apoptosis
40.0
30.0
20.0
10.0
0.0
Control
2.5mg
0.1µMCo
2.5mg+0.1µMCo

Figure 5