Effects of Cobalt and Chromium Ions at Clinically Equivalent Concentrations after Metal-on-Metal Hip Replacement on Human Osteoblasts and Osteoclasts: Implications for Skeletal Health

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ABSTRACT

Metal-on-metal hip replacement (MOMHR) using large diameter bearings has become a popular alternative to conventional total hip arthroplasty, but is associated with elevated local tissue and circulating levels of chromium (Cr) and cobalt (Co) ions that may affect bone health. We examined the effects of acute and chronic exposure to these metals on human osteoblast and osteoclast formation and function over a clinically relevant concentration range previously reported in serum and within hip synovial fluid in patients after MOMHR. SaOS-2 cells were cultured with Co^{2+}, Cr^{3+} and Cr^{6+} for 3 days after which an MTS assay was used to assess cell viability, for 13 days after which alkaline phosphatase and cell viability were assessed and 21 days after which nodule formation was assessed. Monocytes were isolated from human peripheral blood and settled onto dentine disks then cultured with M-CSF and RANKL plus either Co^{2+}, Cr^{3+} or Cr^{6+} ions for 21 days from day 0 or between days 14 and 21. Cells were fixed and stained for TRAP and osteoclast number and amount of resorption per dentine disk determined. Co^{2+} and Cr^{3+} did not affect osteoblast survival or function over the clinically equivalent concentration range, whilst Cr^{6+} reduced osteoblast survival and function at concentrations within the clinically equivalent serum range after MOMHR (IC_{50} =2.2μM). In contrast, osteoclasts were more sensitive to metal ions exposure. At serum levels a mild stimulatory effect on resorption in forming osteoclasts was found for Co^{2+} and Cr^{3+}, whist at higher serum and synovial equivalent concentrations, and with Cr^{6+}, a reduction in cell number and resorption was observed. Co^{2+} and Cr^{6+} within the clinical range reduced cell number and resorption in mature osteoclasts. Our data suggests that metal ions at equivalent concentrations to those found in MOMHR affect bone cell health and may contribute to the observed bone-related complications of these prostheses.

Key words: metal-on-metal hip replacement, hip resurfacing, cobalt, chromium, human osteoblast, human osteoclast.

1 INTRODUCTION

In recent years total hip replacement using large diameter metal-on-metal bearings (MOMHR), either as a hip resurfacing procedure or using a stemmed femoral prosthesis, has become a common alternative to conventional total hip arthroplasty (THA) for the treatment of young and active arthritis patients because of advantages of lower volumetric wear and dislocation risk [1]. However, the clinical outcomes of hip replacement using these prostheses have been mixed. Data from the National Joint Register for England and Wales (2008) demonstrated a 3-year revision rate for hip resurfacing of 4.4% (95%CI 4.0 to 5.0) compared with 1.3% (1.2 to 1.4) for cemented THA (www.njrcentre.org.uk). The Australian arthroplasty register (1997 to 2005) also reported a higher 3-year revision rate for hip resurfacing versus THA (3.1% (2.7 to 3.6) versus 2.1% (1.9 to 2.5%) www.dmac.adelaide.edu.au/aoanjrr). The most common adverse events necessitating revision surgery after MOMHR include early periprosthetic fracture, osteolysis, failure of prosthesis osseo-integration resulting in aseptic loosening, unexplained pain, and inflammatory masses [2-7].

Circulating physiological levels of cobalt and chromium are normally <0.25µg/L (0.005µM). Elevated levels of cobalt and chromium occur in both the hip synovial fluid and in peripheral blood after MOMHR. Whole blood concentrations of cobalt and chromium after MOMHR of up to 4.6µM and 2.3µM, respectively [8], and local hip synovial fluid levels of up to 30µM and 25µM, respectively, have been measured in-vivo [9]. Whilst circulating metal levels are usually highest over the first few months after implantation, persistent elevation occurs as late as 10 years after surgery [10].

Previous studies have shown that short-term exposure to these metal species may affect human osteoclast and osteoblast survival and function. High concentrations of cobalt^{2+} (Co^{2+}), chromium^{3+} (Cr^{3+}), and chromium^{6+} (Cr^{6+}) ions is toxic to osteoblasts and reduces cell activity *in-vitro* [11-13]. Few data are available on the effect of cobalt and

chromium exposure on primary human osteoclasts, although Nichols and Puleo showed short-term exposure to Co and Cr ions at sub-lethal doses resulted in decreased resorptive activity in rat osteoclasts [14]. In contrast, Rousselle et al. found exposure of rabbit osteoclasts to Cr^{3+} had no effect on rabbit osteoclast function [15]. Sankaramanivel et al. have shown that rats treated intraperitoneally with potassium dichromate (Cr^{6+}) over 5 days led to accumulation of chromium in the femur, and was associated with reduced systemic assays of alkaline phosphatase and tartrate-resistant acid phosphatase, suggesting an impact on both bone formation and resorption [16]. However, the longer-term effect of chronic exposure of both human osteoblasts and osteoclasts to these ions at clinically relevant concentrations, more akin to clinical exposure both systemically and at the level of the hip joint, is unknown.

We hypothesise that chronic exposure of local bone cells to metal ions may contribute to the clinical bone-related complications after MOMHR. The aims of this study were to investigate the effect of both short-term and chronic Co^{2+}, Cr^{3+}, and Cr^{6+} ion exposure at clinically relevant concentrations after MOMHR on human osteoblast and osteoclast proliferation and function, and on mature primary human osteoclasts. A dose-ranging methodology was used including metal ion levels covering the normal physiological range, through systemic levels found after MOMHR, to the high concentrations reported in hip joint synovial fluid aspirates after MOMHR.

2 MATERIALS AND METHODS

2.1 Metal ion preparation and validation of concentrations

Co^{2+} and Cr^{3+} were purchased as cobalt (II) chloride hexahydrate and Chromium (III) chloride hexahydrate from Sigma-Aldrich Company Ltd, Gillingham, UK. Cr^{6+} was purchased as chromium (VI) oxide from BDH, Lutterworth, UK. Stock solution for each metal ion at 0.2M was prepared in 50ml of sterile water and stored at 4°C prior to use. The

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0.2M stock solutions were serially diluted in sterile distilled water to give aliquots of 100X the working concentration range for the treatment of cells. These were then diluted in Dulbecco's modified Eagle's medium (DMEM© GLUTAMAX™) supplemented with 0.5% FCS and 1% penicillin – streptomycin (10000units penicillin, 10000ug/ml streptomycin), which from here on will be referred to as vehicle. Control treatments were prepared to contain 1% of distilled sterile water in vehicle to maintain conditions, referred to as 0μM treatments.

The final metal ion concentrations in the test solutions were confirmed using flame-atomic absorbance spectroscopy. Co^{2+}, Cr^{3+} and Cr^{6+} predicted versus measured concentration showed close agreement (linear regression, r^2 = 1.00, 0.85 and 0.98 for Co^{2+}, Cr^{3+} and Cr^{6+}, respectively).

2.2 Osteoblast Cell Culture

Human SaOS-2 cells (a human osteosarcoma-derived osteoblast cell line) were cultured in T75 flasks containing Dulbecco's modified Eagle's medium (DMEM© Glutamax™, Gibco® Invitrogen, Paisley, UK) supplemented with 10% FCS, 100 IU/mL of penicillin and 100μg/mL of streptomycin (Sigma, Poole, UK), hereafter termed complete DMEM. The cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2 until required for assay.

2.3 Osteoblast Viability and Proliferation

SaOS-2 cells were seeded at into 96-well plates at $5x10^3$ cells per well in 0.1mL of complete DMEM and left to adhere overnight. The medium was then replaced with DMEM supplemented with 0.5% FCS and 100 IU/mL of penicillin and 100μg/mL of streptomycin (referred hereon in as vehicle) ± metal ion treatments and incubated for 3 or 13 days at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Vehicle ± treatments were

replenished every 3rd and 4th day consecutively for cells cultured for 13 days. At the end of the culture period a CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay was performed according to the manufacturer's instructions (Promega, Southampton, UK). The assay utilises dehydrogenase enzymes found in metabolically active cells to convert 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) into an aqueous soluble formazan product. The absorbance of the formazan product produced by the cells was read at 490nm using a SpectraMax M5e Microplate Reader (Molecular Devices, Sunnyvale, CA). Values were expressed as percentage response relative to vehicle.

2.4 Osteoblast synthetic function

2.4.1 Alkaline Phosphatase (ALP) Activity

SaOS-2 cells were seeded into 24-well plates at $15x10^3$ cells per well in 1mL of complete DMEM, left to adhere overnight and then the medium was replaced with vehicle ± metal ion treatments and incubated for 13 days 37°C in a humidified atmosphere of 95% air and 5% CO_2. The vehicle ± treatments were replenished every 3rd or 4th day. Cells were washed with PBS, lysed in nuclease-free water and frozen at -80°C following completion of culture. Cell lysates were obtained after three freeze and thaw cycles. ALP activity was measured using p-nitrophenyl phosphate (pNPP) (Sigma) as the chromogenic ALP substrate in the presence of Mg^{2+} ions in a buffered solution. The absorbance was read at 405nm using a SpectraMax M5e Microplate Reader. Values were expressed as percentage response relative to vehicle. DNA content was quantified using Quant-iT™ PicoGreen dsDNA Assay Kit (Invitrogen, Paisley, UK) according to the manufacturer's instructions. ALP activity was normalized to DNA content and ALP/DNA was then expressed as percentage response relative to vehicle.

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2.4.2 Osteoblast mineralization

Once the SaOS-2 cells had reached confluency the cells were treated with vehicle supplemented with 10^{-8}M Dexamethasone and 50μg/ml ascorbic acid (referred to as osteogenic medium) ± metal ion treatment. Metal ion treatments in osteogenic medium were changed every 3rd or 4th day. Two days prior to experiment end, 10μl of 5mM inorganic phosphate 4.2pH was added to the existing treatments within each well. On day 21 cells were then washed once in PBS, fixed in 100% ethanol, rinsed in PBS and incubated in 40mM alizarin red (pH 4.2; Sigma) for 1 hour at room temperature. The cells were then washed extensively in 95% ethanol and air-dried. The plates were scanned on a high-resolution flat-bed scanner. The percentage of each well stained with alizarin red was quantified using Image J software (NIH: http://rsb.info.nih.gov/ij/) and expressed as percentage response relative to vehicle.

2.5 Human Osteoclast Culture

Osteoclasts were generated from human peripheral blood monocytes taken from healthy volunteers as previously described and with research ethics committee approval [17]. Sterilisation of 6mm diameter coverslips (Richardson's of Leicester, Leicester, UK) was performed by baking at 180° for 2 hours. Dentine disks (http://www.dentinedisks.com/) were sonicated and sterilised by washing in 70% ethanol overnight. Venous blood was obtained from healthy volunteers and separated using Histopaque®-1077 (Sigma). The monocyte fraction was collected, washed and then re-suspended in α -MEM Glutamax (Gibco® Invitrogen, Paisley, UK). An appropriate volume of cell suspension containing $5x10^5$ cells was then added to pre-wetted coverslips or dentine disks in a 96 well plate. Cells were incubated for a minimum of 1 hour to allow adherence to the dentine or glass surface. Non-adherent cells were subsequently washed away with α -MEM. Adherent cells were incubated in 100μl α-MEM Glutamax containing 10% FCS, 100U/mL penicillin,

100μg/mL streptomycin (Sigma) (referred to as complete α -MEM) and supplemented with 25ng/mL M-CSF, 30ng/mL recombinant RANKL (Insight Biotechnology, Wembley, UK) at 37°C in a humidified atmosphere of 93% air and 7% CO_2 for three weeks. To determine the effect of osteoclastogenesis metal ion treatments were added from day 3, whilst for effects on mature osteoclast activity metal ion treatments were added after the onset of resorption (typically 14 days). Complete α -MEM containing 25ng/mL M-CSF, 30ng/mL RANKL and treatments was replaced every 2-3 days.

2.6 TRAP staining

Dentine disks were TRAP stained as previously described [18, 19]. Briefly, the disks were fixed in 10% buffered formalin. Disks were then incubated in pre-warmed Acetate-tartrate buffer (0.1M Sodium tartrate (Sigma) in 0.2M Acetate buffer (Sigma), pH5.2) at 37°C for 5 mins, followed by 30 mins incubation at 37°C in 20mg/mL Naphthol AS-BI phosphate (Sigma) /Dimethylformamide (Fisher Scientific, Loughborough, UK) in acetate-tartrate buffer. The disks were then incubated in acetate-tartrate buffer hexazotised pararosaniline solution. The disks were rinsed in water and counterstained in Gill's haematoxylin.

2.7 Quantification of Osteoclast Number and Resorption

Resorbing osteoclasts were identified on dentine disks as a TRAP positive cell in or in close proximity to resorption pits and quantified from 8 random fields of view per disk. Resorption lacunae were identified in the same 8 random fields of view per disk and the plan area of resorption was determined by point counting as previously described[20]. All values were expressed as percentage response relative to vehicle.

2.8 Statistical Analysis

All treatment comparisons were made versus vehicle (0μM). Logistic curve fitting was performed using the least squares (ordinary fit) method and a standard Hill slope to calculate IC_{50} values where effects on bone cell proliferation were found. Data sets were analysed using ANOVA with Dunnett's multiple comparison post-test or the Kruskal-Wallis test with Dunn's multiple comparison post-test, depending on the normality of the data distribution. All analyses were conducted 2-tailed using a critical p-value of 0.05 using GraphPad Prism® (GraphPad Software, La Jolla, CA).

3 RESULTS

3.1 Effect of short–term exposure to metal ions on osteoblast viability

Treatment up to 2000μM of all ions caused a reduction in the number of viable, metabolically active osteoblasts compared to vehicle after 3 days (Figure 1, ANOVA $p<0.0001$). Cr^{6+} had the greatest effect on cell viability, and Cr^{3+} the least. The concentration above which there was a reduction in osteoblast viability was 100μM ($p<0.001$) for Co^{2+}, 10μM for Cr^{6+} ($p<0.0001$), and 450μM for Cr^{3+} ($p<0.0001$). The ion concentration at which osteoblast viability was reduced by 50% (IC_{50} value), calculated by logistic curve fitting, was 135μM for Co^{2+}, and 2.2μM for Cr^{6+}. No IC_{50} was calculated for Cr^{3+} as 50% inhibition was not achieved over the clinical concentration range examined.

3.2 Effect of chronic exposure to metal ions on osteoblast viability and function

Osteoblast proliferation over 13 days was not affected by metal ion concentrations of Co^{2+} or Cr^{3+} up to 5μM (Figure 2A). However, Cr^{6+} at 1 and 5μM reduced osteoblast proliferation over 13-days exposure ($p<0.05$ and $p<0.0001$ respectively). These concentrations of Cr^{6+} had had no effect on short-term osteoblast proliferation.

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ALP activity was reduced over 13 days exposure to all metal ions at 100μM ($p<0.001$ for Co^{2+}; $p<0.0001$ for 135 and 175μM *measured* Cr^{3+} and Cr^{6+}, respectively: Figure 2B). In addition, Cr^{6+} exposure also reduced ALP activity to undetectable levels at concentrations of 10μM and 1μM ($p<0.05$ and $p<0.0001$, respectively). When ALP activity was corrected for cell number using DNA content, only Cr^{6+} reduced ALP activity at the cellular level (10μM =$p<0.05$ and 100μM =$p<0.001$). Thus, the suppressed osteoblast activity was largely a function of reduced cell number rather than reduced activity per cell. Mineralisation activity, measured by Alizarin red staining after 21-days culture in osteogenic medium, was reduced with all metal ion treatments at 100μM ($p<0.0001$, Figure 2C). Cr^{6+} at 10μM also reduced mineralisation activity ($p<0.0001$).

3.3 Effect of Co^{2+}, Cr^{3+}, and Cr^{6+} on osteoclastogenesis and resorption

Treatment with Co^{2+} ions had no effect on osteoclast number from 0.01μM up to approximately 1μM (Figure 3A). The IC_{50} for $C0^{2+}$ was 12μM, and 200μM reduced the number of TRAP positive osteoclasts to near zero ($p<0.0001$). Total resorption followed a slightly different pattern, with a transient rise in resorption in the sub-micromolar range (EC_{50}=0.4μM), followed by complete suppression of resorption at 200μM (Figure 3B and 4A-C, $P<0.001$). Treatment with Cr^{3+} resulted in a biphasic response pattern for both osteoclast number and resorption (Figure 3A-B and 4D-F), with concentrations of up to approximately 0.1μM resulting on increased number (EC_{50} = 0.14μM) and resorption (EC_{50} = 0.27μM). Above this concentration osteoclast number returned to physiological baseline and resorption declined to zero at [Cr^{3+} =200μM, $P<0.001$] (IC_{50} 50μM). Cr^{6+} ions had the greatest inhibitory effect on the formation of functional osteoclasts. Increasing Cr^{6+} resulted in a reduction in the number of osteoclasts (IC_{50} = 0.37μM) and total resorption (IC_{50} = 0.30μM), (Figure 3A-B and 4G-I, $p<0.0001$ for 1μM to 100μM).

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3.4 Effect of Co^{2+}, Cr^{3+}, and Cr^{6+} on mature, active osteoclasts

To determine the effects of metal ions on mature, fully functional and active human osteoclasts, human monocytes were isolated, settled onto dentine disks and cultured as above but in the absence of metal ions to allow the fusion cells and formation of osteoclasts. The onset of resorption (an indicator of fully functional and active osteoclasts) was monitored daily from day 10 and once resorption had been detected (typically after 14 days), the osteoclast culture medium was then replaced to include 0.01μM to 200μM Co^{2+} and Cr^{3+} and 0.01μM to 100μM Cr^{6+} ions for the last 7days of culture. The pattern of response for Co^{2+} and Cr^{3+} was different to that seen for newly forming osteoclasts in that no transient increase in cell number or activity was found, and that the inhibitory effects of all ions were seen at a lower ion concentration. Seven days treatment with Co^{2+} ions ≥10μM reduced mature osteoclast number (IC_{50} = 5.4μM ($p<0.001$, Figure 3C). Total amount of resorption per disk was only reduced at the high (200μM) concentration ($p<0.0001$ and $p<0.001$, Figure 3D and 4J-L). Treatment with Cr^{3+} ions reduced mature osteoclast number and resorption per disk, but only at the 200μM dose ($p<0.05$, Figure 3C-D and 4M-O, IC_{50} for osteoclast number = 221μM and IC_{50} for resorption per disk = 77μM). No trend towards increased osteoclast number or resorption was seen for mature osteoclasts at the lower Cr^{3+} concentration range. Cr^{6+} ions had the greatest effect on osteoclast number and resorption. Cr^{6+} at concentration ≥10μM caused a reduction in osteoclast number and resorption per disk ($P<0.01$ all analyses, Figure 3C-D and 4P-R, IC_{50} for osteoclast number = 1.8μM and IC_{50} for resorption per disk = 3.9μM).

4 DISCUSSION

In this study we examined the effect of chronic exposure of human osteoblast and primary human osteoclast cells to Co and Cr ions at concentrations including the clinically

equivalent range defined by previous reports of measured metal levels in the serum and hip synovial fluid taken from patients after MOMHR. We found that ions of both metals affected osteoblast and osteoclast cell proliferation and function. These effects were greatest for Cr^{6+}, then Co^{2+}, with Cr^{3+} showing the least effect. The observed responses also varied with metal ion concentration, cell type and cell maturity.

Our findings are consistent with in-vitro studies using animal cells that supra-physiological concentrations of cobalt and chromium ions induce apoptosis in human osteoblast-like cells *in-vitro* in a dose-dependent manner [12], and suppress osteoblast synthetic function [11, 21, 22]. Our dose-ranging data suggest that chronic exposure to Co^{2+} or Cr^{3+} do not have a major effect on cell viability or synthetic function at concentrations equivalent to those metal levels found in serum or synovial fluid in patients after MOMHR, whilst exposure to Cr^{6+} has a profound effect on cell viability at concentrations equivalent to the serum chromium range after MOMHR.

In contrast, our data suggest that an effect of Co and Cr on human primary osteoclasts occurs within the clinically observed concentration range and varies with cell maturity. At systemic levels these ions may have a mild stimulatory effect on developing osteoclasts, but at higher concentrations and in mature osteoclasts their effect is inhibitory. The reason for this difference might be explained by the substrate resorbing activity of the exposed cell, as mature resorbing osteoclasts may accumulate more intracellular metal ions through phagocytic activity versus developing osteoclasts, and thus demonstrate a greater toxic effect due to greater internalisation of the metal. In support of the increased resorption transient seen in the serum range, Patntirapong et al have shown that cobalt ions in solution or incorporated into calcium phosphate coated plastic at clinically-relevant concentrations increase murine osteoclast differentiation and resorption *in-vitro* [23]. Whilst cobalt ions do not localise to bone, chromium salts do have an affinity for bone [24], being trapped in the bone matrix, and thus levels in the bone microenvironment may exceed

those found in serum. Albrecht et al have also suggested a possible indirect route for osteoclast activation in response to metal ions, showing that exposure of human peripheral blood mononuclear cells to Co^{2+} ions *in-vitro* results in upregulation of IL-1α, IL-1β, and IL-6 expression, that may in turn increase osteoclast birth rate and resorption [25].

Differences in the cellular responses to Co^{2+}, Cr^{3+}, and Cr^{6+} are likely complex, with several mechanisms operating. Co^{2+} and Cr^{6+} ion complexes are highly soluble and readily cross cell membranes via the anion transporter, whilst Cr^{3+} complexes are less soluble at physiological pH and cell membrane permeability to Cr^{3+} is low [26]. These physico-chemical characteristics may explain, in part, the lower toxicity of Cr^{3+} relative to the other ions to both osteoblasts and osteoclasts. The high toxicity of Cr^{6+} may be explained by its rapid transport across cell membranes and subsequent reduction to Cr^{3+} within the cell by glutathione resulting in an increase in oxidative stress leading to cell death.[27]

It is currently unclear which chromium species are released from prosthesis surfaces after MOMHR. Metal ion release as a result of corrosion, distinct to that arising from the process of wear, has been identified as a significant contributor to systemic metal release after MOMHR [7, 28]. Merritt and Brown have shown that Cr^{6+} is released during the corrosion of orthopaedic implants and is present systemically in-vivo [29]. However, De Flora et al have observed that circulating whole blood has a capacity to sequester and reduce approximately 200mg of Cr^{6+}/day [30], which is in excess of that released from MOMHR bearings. Thus, bone cells in the prosthesis microenvironment may be subject to released Cr^{6+}, and our data show that at clinically relevant levels this would be highly toxic to local osteoblasts and osteoclasts. A recent speciation study of chromium complexes by microfocus x-ray spectroscopy using a synchrotron beam in retrieved tissues around failed MOMHR prostheses showed chromium is present mainly as chromium (III) phosphate.[31] However, as Cr^{3+} has poor cell membrane permeability, its presence may arguably be

accounted for by its entering the cell as Cr^{6+} then being reduced to Cr^{3+}, and giving rise to the necrotic lesions for which the biopsies were taken.

Our observation of the toxicity of Co^{2+} to osteoclast cells at synovial fluid levels and to osteoblasts at concentrations 3-5 times that found in local tissues after MOMHR may occur through a similar mechanism to that observed in previous studies of lung toxicology. High concentrations of Co^{2+} are thought to induce cell damage by stabilising hypoxia inducible factors (HIF) that bind to DNA and initiate hypoxia-related gene expression and are normally degraded under normal oxygen tensions, resulting in HIF pathway activation and cellular apoptosis [32, 33].

Our observations that Co and Cr ions at clinically identified levels after MOMHR has several clinical implications for local bone health. Supressed osteoblast activity may explain early aseptic loosening as a failure of primary osseo-integration. In support of this concept, Long et al have reported a 15% failure rate for the Durom acetabular prosthesis in 207 hips within 2 years following implantation [34]. In all cases but 1 aseptic loosening of the prosthesis was the mode of failure, and in 13 prostheses examined in detail at retrieval, all showed failure of osseo-integration of bone onto the fixation surface. Femoral neck narrowing has commonly been reported after MOMHR and may contribute to fracture risk [35]. It has been suggested that narrowing occurs as a result of elevated hydrostatic fluid pressures in these patients, however, and alternative mechanism may be through osteoclast activation at the bone surface due to elevated metal levels. In support of this increased osteoclast numbers have been identified histologically on periosteal surfaces in fracture cases with femoral neck narrowing after MOMHR (Pat Campbell, personal communication). At a systemic bone health level, our data suggest that metal ions release may be sufficient to impact on osteoclast cell activity and number that in turn may affect bone mass and remodelling. The long term implication of systemic metal release after MOMHR for systemic bone health remains to be elucidated.

5 Acknowledgements

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6 Legend to Figures

Figure 1 Effect of metal ions on osteoblast viability. SaOS-2 cells were cultured with clinically relevant levels of Co^{2+}, Cr^{3+} and Cr^{6+} for 3 days after which time a MTS assay was used to assess cell viability. All values expressed relative to vehicle (100%). n= 6 wells per experiment, 3 replicate experiments. Values are mean ± SEM. Arrows represent upper limit of previously reported ion concentrations in-vivo.

Figure 2 Effect of metal ions on osteoblast function. SaOS-2 cells were cultured for 13 days after which time MTS assay was performed to assess proliferation (A) and ALP activity measured (B). Mineralisation was assessed in cells cultured in osteogenic medium in the presence of metal ions for 3-weeks (C). n= 3-6 wells per experiment, 3 replicate experiments. $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$. (*spectroscopy measured concentrations were 100μM for Co^{2+}, 125μM for Cr^{3+}, and 175μM for Cr^{6+}). Line within boxes indicates median, boxes represent interquartile range, and whiskers are range.

Figure 3 Effect of metal ions on human osteoclasts. A-B) Osteoclast formation. Monocytes were isolated from human peripheral blood, settled onto dentine disks then cultured with M-CSF and RANKL plus either Co^{2+}, Cr^{3+} or Cr^{6+} ions for 21 days. **C-D) Mature osteoclasts.** Cells were cultured with M-CSF and RANKL for 14 days prior to the addition of metal ions (C-D). Cells were fixed and stained for TRAP with the number of osteoclasts and amount of resorption per dentine disk determined. n=5 wells per experiment, 3 replicate experiments. *p<0.05, **p<0.01, ***p<0.001. Logistic curve fitting was used to calculate the approximate EC_{50} and IC_{50} values.

Figure 4. Metal ions and osteoclast number and dentine resorption. Peripheral blood mononuclear cells from healthy donors were cultured on dentine slices in recombinant M-

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CSF and RANKL-supplemented medium to generate multinucleated-osteoclasts. At day 21, dentine slices were fixed in 10% buffered formalin, stained for tartrate-resistant acid phosphatase (TRAP) and counterstained with Gill's haematoxylin. In 'A', the stars correspond to TRAP positive osteoclasts and the arrows point to the resorption pits. Metal ions were introduced in the culture at day 3 (for A-I) at the indicated concentrations. For J-R, metal ions were introduced once resorption was observed, generally day 14, indicating mature osteoclasts.. *Typical fields of view of cells following treatment;* A-C and J-L treated with Co^{2+}, D-F and M-O with Cr^{3+}, G-I and P-R with Cr^{6+}. Scale bar 200µm.

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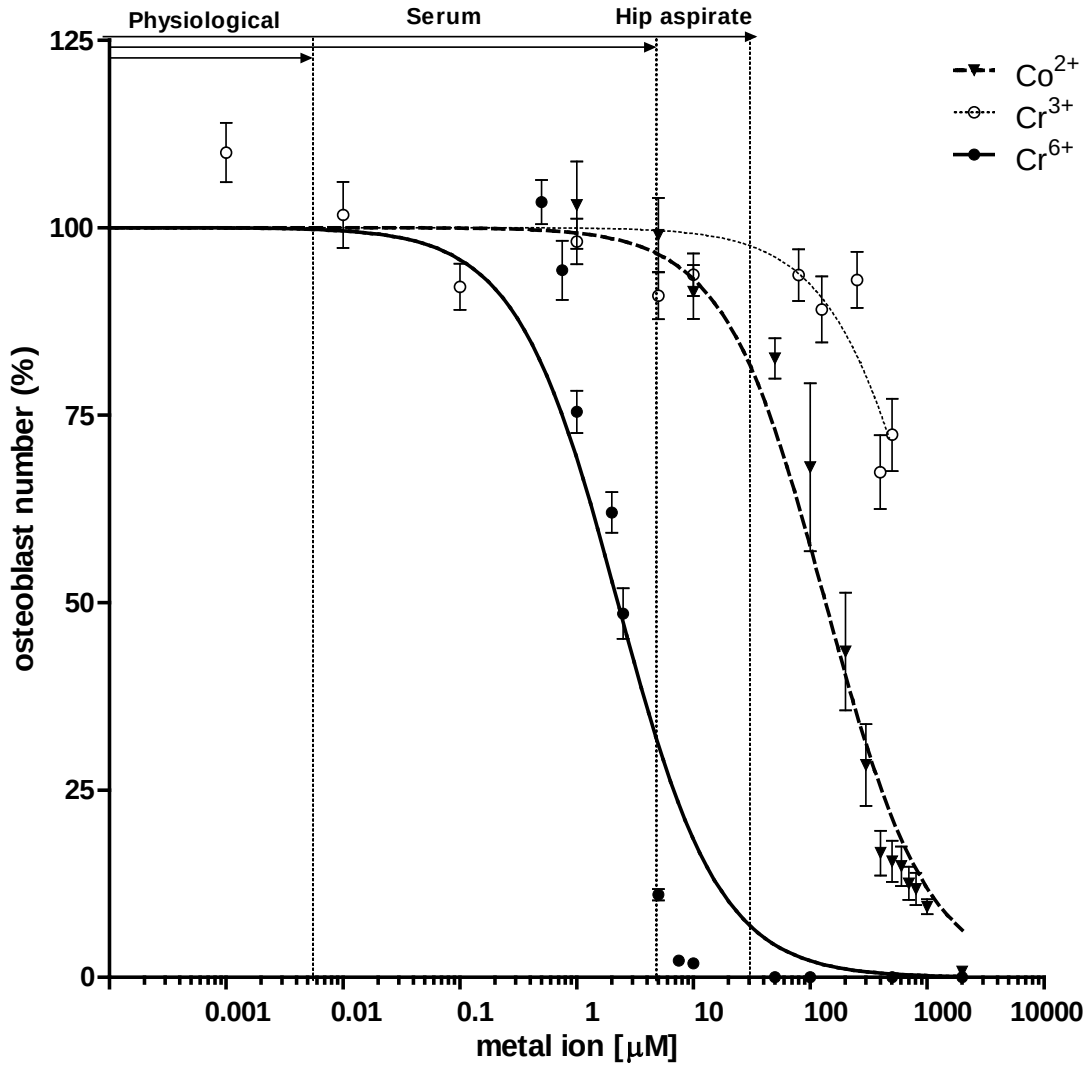
Figure 1



Effect of metal ions on osteoblasts and osteoclasts

Figure 2

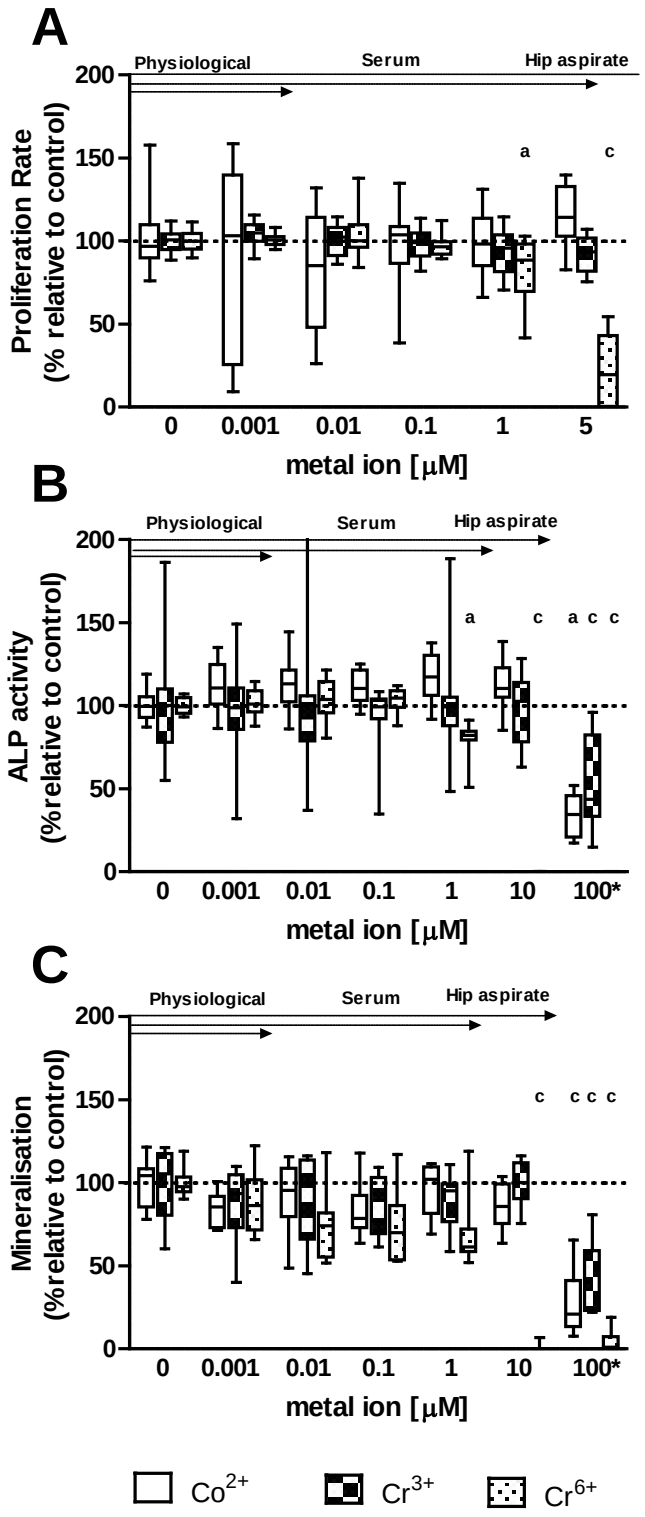


Figure 3

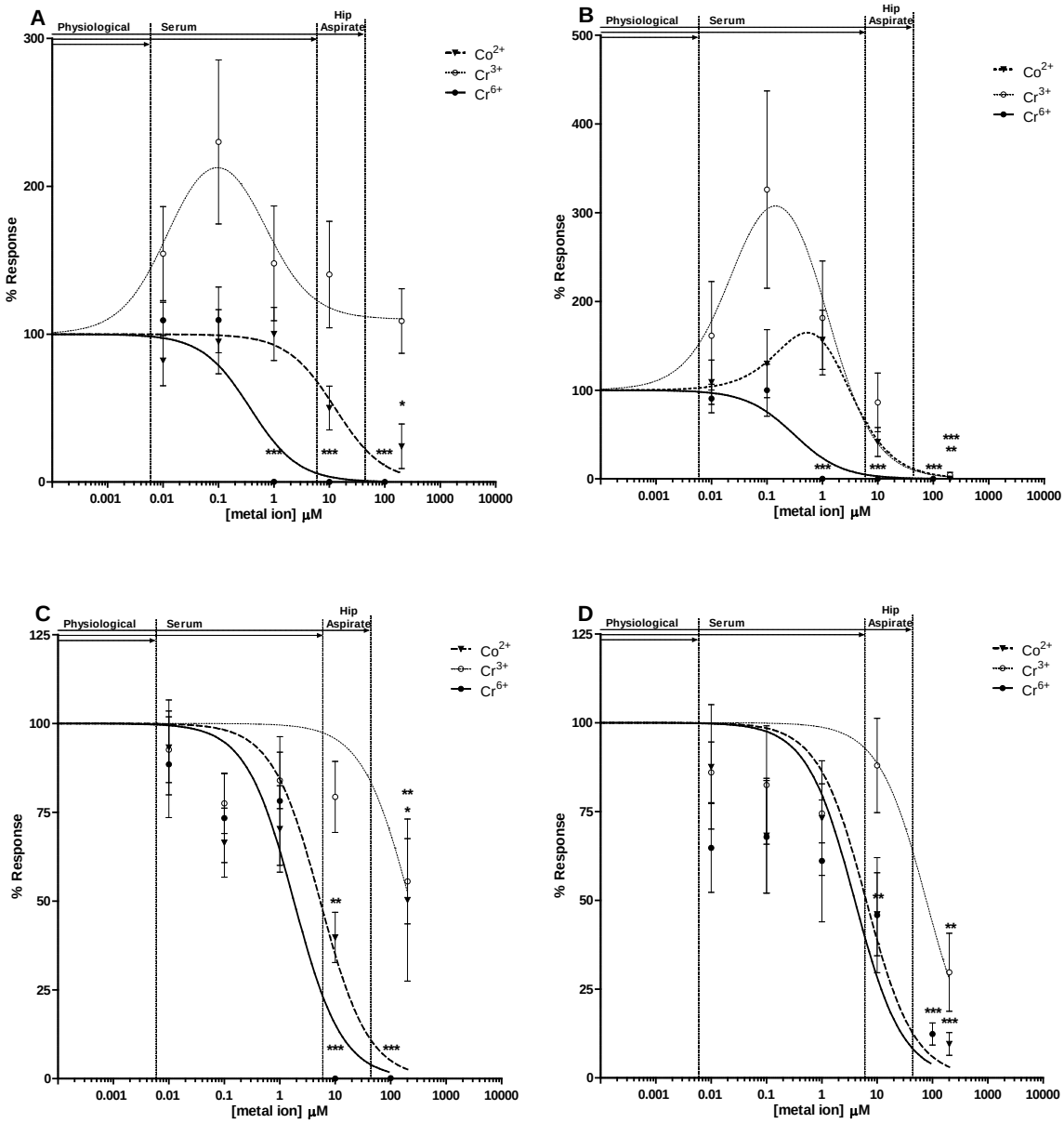

A
B
C
D
Physiological
Serum
Hip Aspirate
Co2+
Cr3+
Cr6+
% Response
[metal ion] μM
0.001
0.01
0.1
1
10
100
1000
10000

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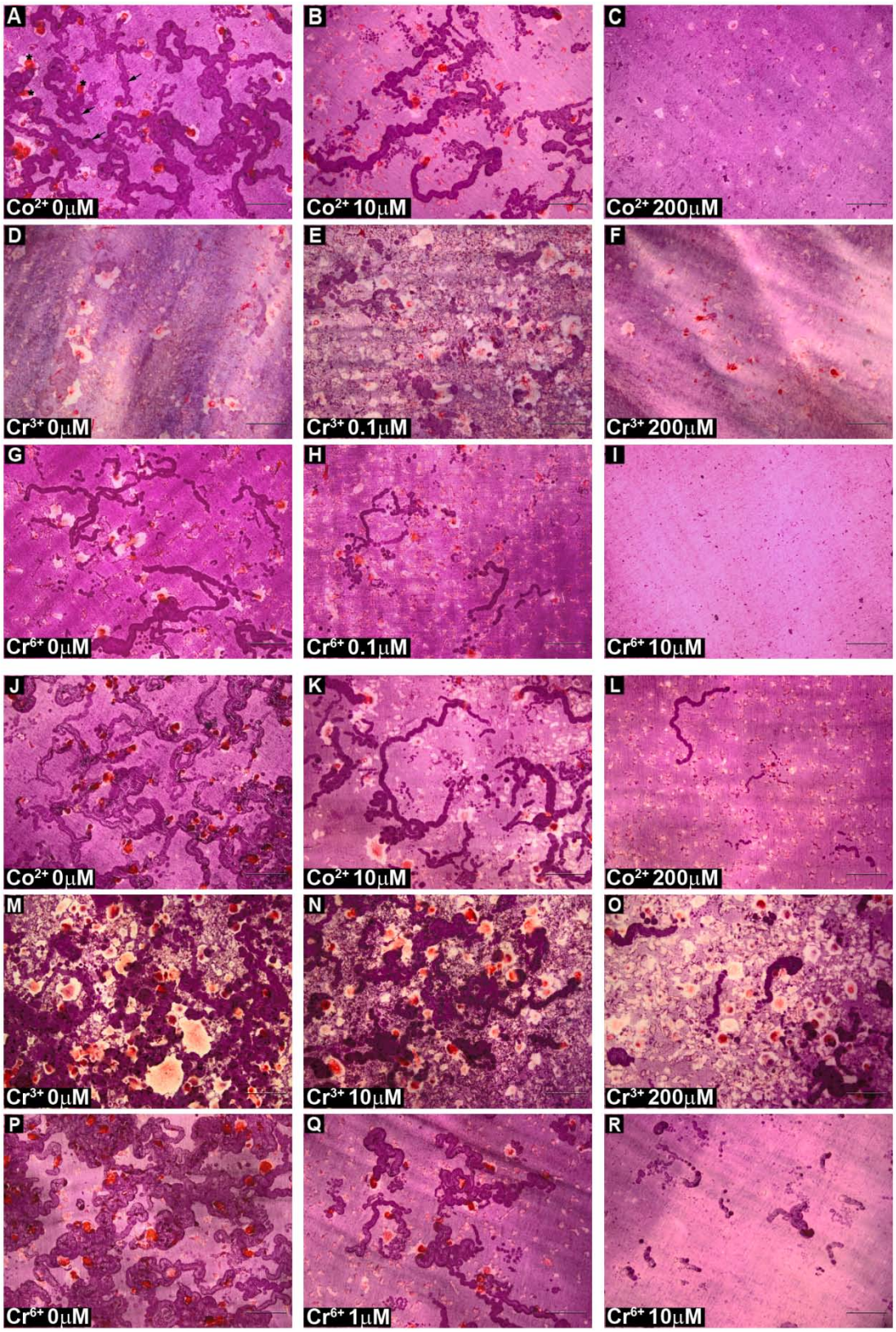
Figure 4



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