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Title: Osteocyte physiology and response to fluid shear stress are impaired following exposure to cobalt and chromium: implications for bone health following joint replacement.

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Abstract

The effects of metal ion exposure on osteocytes, the most abundant cell type in bone and responsible for coordinating bone remodeling, remain unclear. However, several studies have previously shown that exposure to cobalt (Co^{2+}) and chromium (Cr^{3+}), at concentrations equivalent to those found clinically, affect osteoblast and osteoclast survival and function. In this study, we tested the hypothesis that metal ions would similarly impair the normal physiology of osteocytes. The survival, dendritic morphology and response to fluid shear stress (FSS) of the mature osteocyte-like cell-line MLO-Y4 following exposure to clinically relevant concentrations and combinations of Co and Cr ions was measured in 2D-culture. Exposure of MLO-Y4 cells to metal ions reduced cell number, increased dendrites per cell and increased dendrite length. We found that combinations of metal ions had a greater effect than the individual ions alone, and that Co^{2+} had a predominate effect on changes to cell numbers and dendrites. Combined metal ion exposure blunted the responses of the MLO-Y4 cells to FSS, including reducing the intracellular calcium responses and modulation of genes for the osteocyte markers Cx43 and Gp38, and the signaling molecules RANKL and Dkk-1. Finally, we demonstrated that in the late osteoblasts/early osteocytes cell line MLO-A5 that Co^{2+} exposure had no effect on mineralization, but Cr^{3+} treatment inhibited mineralization in a dose dependent manner, without affecting cell viability. Taken together these data indicate that metal exposure can directly affect osteocyte physiology, with potential implications for bone health including osseointegration of cementless components, and periprosthetic bone remodeling.

Keywords

Osteocytes; mechanical stimuli, bone remodeling, osseointegration, metal ions

Introduction

Osteocytes account for approximately 95% of the cell population of bone, and arise as terminally differentiated osteoblasts that have become embedded in the mineralizing osteoid matrix. Osteocytes connect to other osteocytes and cells on the bone surface to form an extensive dendritic network that senses mechanical strain and renders the osteocyte as the primary mechanosensory cell within bone[1].

The osteocyte network plays a master role in maintaining bone homeostasis through signaling pathways that orchestrate osteoblast and osteoclast activity necessary for normal bone health[2]. Osteocyte cell death and alterations in dendritic morphology and connectivity play a role in osteonecrosis of the femoral head, especially following chronic steroid treatment[3]. Decreased osteocyte dendritic connectivity is also found in osteoporosis and osteoarthritis, whilst osteomalacic bone exhibits an increased, but chaotic connectivity[4; 5], highlighting the importance of dendricity in pathophysiological conditions.

Successful osseointegration and survival of prosthetic joints also requires normal bone health. Data from several large national joint replacement registries has shown that hip replacement prostheses that use a large diameter metal-on-metal bearing made of a cobalt-chromium (Co^{2+}-Cr^{3+}) alloy have a survivorship that is profoundly poorer than prostheses that use non-metal-on-metal bearings[6-9]. This poor survivorship is due to complications including failed prosthesis osseointegration, osteolysis, femoral neck fracture, and hypersensitivity responses, and has resulted in a requirement for the regular clinical monitoring of indwelling prostheses[10], the recall of several prosthesis brands[11], and litigation against their manufacturers. It has also recently become more widely recognized that wear-accelerated corrosion at taper junctions of all modular prostheses generates metallic wear debris and impairs prosthesis survival[12-14].

Normal physiological concentrations of Co and chromium Cr in unexposed individuals are <0.3µg/L[15; 16], metal-on-metal bearings are known to cause an elevation in Co and Cr in both local tissues of the hip joint and systemically. In well-functioning prostheses at 10 year follow-up, median Co and Cr concentrations in the circulation have been reported to be 0.75µg/L (range: 0.3-50.1µg/L) and 0.95µg/L (range: 0.3-58.6µg/L) respectively[17]. Periprosthetic concentrations are several fold higher (median Co - 113µg/L, median Cr - 54 µg/L)[18], whilst patients with failing prosthesis have reported Co and Cr concentrations as high as 528µg/L (range: 0-13000µg/L) and 1844µg/L (range: 0-38600µg/L) respectively[19]. We recently demonstrated that exposure to cobalt (Co^{2+}) and chromium (Cr^{3+}) adversely affects both osteoclast and osteoblast survival and function, including the mineralization of prosthesis surfaces in vitro, at concentrations equivalent to those found clinically[20-22].

Previous studies have suggested that exposure to Co^{2+} in vitro has a cytotoxic and pro-inflammatory effect on osteocytes[23-26]. Here we develop understanding in this area by examining the effects of exposure to both Co^{2+} and Cr^{3+} at clinically relevant concentrations on osteocyte physiology – in particular their survival, dendritic morphology, response to fluid shear stress and mineralization.

Materials and Methods

Metal ion preparation and treatments

Cobalt (II) hexahydrate ($CoCl_2.6H_2O$) and chromium (III) chloride hexahydrate ($CrCl_3.6H_2O$) (Fluka, Gillingham, UK) served as salts for Co^{2+} and Cr^{3+} respectively. Working concentrations of each metal ion were prepared, as previously described[20]. The stability of these metal ions in culture media has been confirmed previously using flame-

atomic absorption spectroscopy[20]. Control treatment contained equivalent volume of sterile distilled water to maintain conditions, and referred to as 0µg/L treatments.

Cell culture

MLO-A5 cells are thought to represent the post-osteoblast, pre-osteocyte cells responsible for triggering mineralization of osteoid[27]. MLO-A5 cells were maintained at 37˚C in a humidified atmosphere of 95% air and 5% CO_2 with α-MEM GlutaMAX™ (ThermoFisher Scientific, Paisley, UK) containing 100U/mL penicillin, 100µg/mL streptomycin and 10% FBS (Life Technologies, Paisley, UK) (referred to as complete α-MEM).

MLO-Y4 cells were derived from the same transgenic mice as the MLO-A5 cells, but exhibit a more mature osteocyte phenotype, with characteristic dendritic morphology complemented with expression of osteocytic genes such as osteocalcin and connexin-43[28]. The cells were grown in rat-tail type 1 collagen (ThermoFisher Scientific, Paisley, UK) coated T75 flasks (0.15mg/mL in 0.02M acetic acid) and maintained at 37˚C in a humidified atmosphere of 95% air and 5% CO_2, with α-MEM+ containing 100U/mL penicillin, 100µg/mL streptomycin, 2.5% FBS and 2.5% BCS (complete α-MEM+).

MLO-Y4 survival and dendricity

To assess survival and dendricity, MLO-Y4 cells were cultured overnight in collagen coated 96-well plates at 2500 cells per well in complete α-MEM+ at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. Subsequently, the cells were treated with vehicle (0.5% FCS supplemented α-MEM+) ± metal ion treatments for 24 hours after which they were fixed and stained with 0.1% crystal violet[29] (Sigma-Aldrich, Gillingham, UK).

To quantify MLO-Y4 cell survival, cell numbers were counted from 5 non-overlapping random fields of view per treatment with 4 wells quantified for each treatment. Dendricity was assessed by counting the number of dendrites per cell and the average length of

dendrites, for dendrites greater than 5µm in length. Quantification was done using CellD imaging software (Olympus, Southend-on-Sea, UK) on 200X magnified images with Leica DMI4000B microscope.

Intracellular Calcium ($[Ca^{2+}]_i$) response to fluid flow

MLO-Y4 cells were cultured in complete α-MEM+ on collagen coated slides at a density of $15x10^3$ and $25x10^3$ cells per slide. Following a 24 hour incubation, the cells were treated with vehicle ± 50µg/L or 500µg/L of Co^{2+} and Cr^{3+} (1:1) for 30 minutes or 24 hours, respectively. Following the treatments, cells were loaded with a 5µM of Fluo-4AM (ThermoFisher Scientific, Paisley, UK), a fluorescent Ca^{2+} indicator for 45 minutes at 37°C to observe the cellular response to fluid shear-stress. The cells were then subjected to 20 seconds of mechanical stimuli using a laminar fluid-flow chamber to create a physiologically relevant fluid shear stress of 16 dynes/cm^2, previously demonstrated to induce increases in $[Ca^{2+}]_i$ in vitro[30-32]. Changes in $[Ca^{2+}]_i$ were monitored in real-time using Leica AF6000 time-lapse fluorescent microscope. Cellular fluorescence of individual cells was measured using ImageJ (http://imagej.nih.gov/ij/) and the average change in fluorescence for each cell relative to baseline was calculated and plotted. The area under the curve (AUC), a measure of cellular response, and peak intensity were calculated using GraphPad Prism® (GraphPad Software, La Jolla, CA) and expressed relative to control from the same experimental time-point. All experiments were repeated 3 times with 40 cells analyzed for each experiment.

RT-PCR

Immediately following mechanical stimuli, RNA was extracted from MLO-Y4 cells using ReliaPrep™ RNA Cell Miniprep System (Promega, Southampton, UK) and reverse transcribed to cDNA using the High Capacity RNA-to-cDNA Kit (ThermoFisher Scientific,

Paisley, UK). Quantitative RT-PCR was performed with 7900HT Fast Real-time PCR system using TaqMan® Gene expression assays (ThermoFisher Scientific, Paisley, UK) for RANKL, Gp38, Dkk-1 and Cx43 and gene expression was expressed relative to Gapdh and controls with no exposure to metal ions and fluid shear-stress using the ΔΔCT method. The expression of Gapdh did not vary across samples with respect to fluid shear stress and metal ion treatments (data not shown).

Mineralization assay

MLO-A5 cells were seeded in 48-well plates at a density of $2x10^4$ cells per well in 0.5mL complete α-MEM till they reached confluence (usually day 3). The media was then replaced with vehicle ± metal ion treatments supplemented with 10nM dexamethasone and 50μg/mL L-ascorbic acid (Sigma-Aldrich, Gillingham, UK) (referred to as osteogenic media) to promote osteoblast differentiation[20; 22; 33]. Vehicle ± metal ion treatments in osteogenic media was replenished every 2-3 days until two days prior to the end of experiment, when 5mM inorganic phosphate was added to the osteogenic media to promote mineralization. On day 7 the cells were fixed overnight in 100% ethanol and stained with 40mM Alizarin Red S (pH 4.2, Sigma-Aldrich, Gillingham, UK). The plates were washed extensively with 95% ethanol and air-dried prior to scanning on a flatbed scanner. The percentage area of mineralization per well was quantified using ImageJ (http://imagej.nih.gov/ij/) and expressed as percentage response to vehicle.

Statistical Analysis

All experiments were conducted on 3 separate occasions with 3 minimum repeats per occasion. The average of the individual repeat experiments were then used for statistical analysis. All values are reported as the mean ± standard deviation (SD) or mean ± 95% confidence interval (CI) and were analyzed using GraphPad Prism® (GraphPad Software, La

Jolla, CA). Data was tested for normality using the D'Agostino and Pearson omnibus normality test and analyzed by one-way analysis of variance (ANOVA) with post-test for linear trend or t-test to address specific questions. If a significant effect was found, specific interactions were examined by performing two-way analysis of variance with Sidak's multiple comparisons test. All tests were performed 2-tailed using a critical p-value of 0.05.

Results

Metal ions reduce MLO-Y4 osteocyte cell number in vitro

Co^{2+} ion treatment significantly decreased the number of MLO-Y4 cells over the full concentration range, whilst Cr^{3+} treatment had no effect on cell number (Figure 1A). Combined metal ion treatment showed a significant interaction (P=0.0396), reducing cell viability over the full concentration range (Figure 1A and Figure 1B), with 500µg/L Co^{2+}:Cr^{+3} combined treatment having a greater effect over Co^{2+} treatment alone.

Metal ions increase the dendricity of MLO-Y4 cells in vitro

Co^{2+} ion treatment increased the number of dendrites per cell over the full concentration range (Figure 2 A), whilst Cr^{3+} treatment had no significant effect. Combined metal ion treatment showed a significant interaction (P=0.0427), with concentrations of 5000µg/L Co^{2+}:Cr^{+3} reducing the increase in dendrite number observed with Co^{2+} treatment alone. An increase in average length of MLO-Y4 dendrites was also observed with metal ion treatments. Co^{2+} treatment increased the number of dendrites over the full concentration range (Figure 2B), whilst Cr^{3+} treatment had no significant effect. Co^{2+}:Cr^{3+} combined treatment similarly showed an increase in the number of dendrites over the full the concentration range (Figure 2B) but no significant interaction was observed (P=0.6861). The combined Co^{2+}:Cr^{3+} treatment did not have any further effect over that of Co^{2+} alone.

Metal ions reduce the intracellular Ca^{2+} response in MLO-Y4 following mechanical stimuli

The response of MLO-Y4 osteocyte cells to mechanical stimuli in the form of fluid shear-stress was assessed by measuring intracellular Ca^{2+} changes in real time. Following 30 minute and 24 hour exposure to Co^{2+}:Cr^{3+}, a reduction in cellular response (AUC) to mechanical stimuli was observed for 50µg/L and 500µg/L compared to the vehicle (Figure 3A and Figure 3B). A reduction in the peak response was also observed for both 50µg/L and 500µg/L Co^{2+}:Cr^{3+}compared to the vehicle, for both time-points. The effects were dose-dependent with 500µg/L Co^{2+}:Cr^{3+} showing reduced cellular and peak responses compared to 50µg/L following 30 minutes and 24 hour exposures.

MLO-Y4 gene expression in response to fluid shear-stress is altered with metal ion exposure.

The MLO-Y4 cells robustly expressed the osteocyte markers Cx43and Gp38, and the signaling molecules RANKL and Dkk-1, with the rank order of gene expression Gp38≥Cx43> RANKL > Dkk-1 (data not shown). Fluid shear stress (FSS) resulted in increased expression of RANKL (P=0.0272), Gp38 (P=0.0017), and Cx43 (P=0.01148), and a reduction in Dkk-1 expression (P=0.0245) compared to unstimulated cells (Figure 4A). These changes in gene expression following FSS were also observed for the 24 hour experimental group (increased RANKL (P=0.0415), Gp38 (P=0.0128), and Cx43 (P=0.0218), and reduced Dkk-1 (P=0.0245) (Figure 4B). Cells exposed to Co^{2+}:Cr^{3+} for 30 minutes and 24 hours prior to FSS had blunted responses, with the osteocyte markers Gp38 and Cx43 showing reduced expression in significant linear trend over the concentration range (Figure 4B, p=0.0338 and p=0.0107 respectively).

Metal ions reduce mineralization by late osteoblast/early osteocyte MLO-A5 cells

Using MLO-A5 cells we found no effect of individual Co^{2+} ion treatment on mineralization. However, Cr^{3+} reduced the mineralization of the MLO-A5 cells over the concentration range

equivalent to those observed in serum of patients with accelerated MOM bearing wear (Figure 5), without affecting cell viability (data not shown). Combined metal ion treatment similarly showed a significant reduction in mineralization over the same concentration range, however no interaction was seen ($p=0.8257$) or any further effect of Co^{2+}:Cr^{+3} combined treatment over the effect of Cr^{3+} treatment alone.

Discussion

The osteocyte is the most abundant type of bone cell and plays a critical role in mechanosensing and in the orchestration of bone remodeling. However, little is known about the effects of clinically-relevant metal exposure on this bone cell population. Here, we show that exposure to increased Co^{2+} and Cr^{3+} concentrations is detrimental to osteocyte physiology. We find that osteocyte cell survival and dendritic morphology are sensitive to metal ions at concentrations seen clinically in patients with prostheses. We also show for the first time that the typical osteocytic response to fluid shear stress, including intracellular calcium signaling and gene expression of osteocyte markers and bone remodeling cytokines, is impaired in the presence of these metal ions.

Reduction in MLO-Y4 osteocyte cell viability after 24 hour exposure to Co^{2+} occurred in a dose-dependent manner, consistent with and expanding on previous reports demonstrating a cytotoxic effect at high concentrations of Co^{2+} (0.05mM; ~3000μg/L) following 48 hours exposure[25]. The lack of any effect of Cr^{3+} alone is consistent with the observations of Kanaji and colleagues and the previously held dogma of the relative inability of Cr^{3+} to cross cell-membranes[34]. In contrast to this dogma, we have previously shown the intracellular localization of Cr^{3+} in osteoblasts[21], as well as an additive effect of Co^{2+} and Cr^{3+} treatment[22]. Thus the increased effect of combined ions at 500μg/L over the effect of Co^{2+} alone observed in this study suggests for the first time potential facilitation of Cr^{3+} entry into osteocytes, as

well as common downstream signaling. The fact that we don't observe any increased effect with combined metal ion treatment for concentrations at the higher end equivalent to those observed in the patient hip aspirates suggests that Co^{2+} is having the dominate effect.

As osteoblasts transition to osteocytes and mature, they form dendritic processes and establish highly oriented intercellular communication between the vascular space and the bone surface[1]. Reduced osteocyte dendricity has a negative impact on osteocyte function and on the mechanical properties of bone[35]. Several diseased states have been associated with alterations in osteocyte dendricity. A decrease in interconnectivity has been observed in osteoporotic and osteoarthritic bone, whilst osteomalacic bone exhibits an increased interconnectivity which is chaotic[4; 5]. We detected an increase in the number and length of dendrites in MLO-Y4 osteocytes cells treated with increasing concentrations of metal ions. One possible explanation for this observation is that it represents an attempt by the residual cell population to maintain or re-establish inter-cellular communication.

Osteocyte cell death plays a key role in the recruitment of osteoclasts and in bone resorption[36], whilst dendrites are found increased in and orientated to regions of potential bone remodeling[35]. The reduction in osteocyte survival and increase in dendricity that we have observed in vitro at clinically relevant concentrations of metal ions may alter periprosthetic bone remodeling and may compound the previously reported direct effects observed on osteoblasts and osteoclasts[20].

A change in intracellular calcium concentration is the immediate cellular response to mechanical stimuli and influences various downstream signaling events[37]. Consistent with this, fluid flow-induced increases in calcium signaling in MLO-Y4 cells were significantly reduced by combined metal ion treatment in a dose dependent manner, thus confirming a detrimental effect on osteocyte function. Furthermore, the mechano-responsive genes that are known to be upregulated in response to mechanical loading are increased in our system with

fluid flow [35; 38-41]. Exposure to metal ions suppressed the increased expression of osteocyte markers Gp38 and Cx43 after 24hours, but had no effect on the fluid flow-induced effects on RANKL or DKK1 expression. Whilst Gp38 is an early response gene responsible for dendrite elongation[35], Cx43 is involved cell-cell communication by forming gap-junctions and 'hemi-channels'[42]. It has been shown that prostaglandin E2 (PGE2) signalling from osteocytes occurs via Cx43 following mechanical strain[42] and mediates its osteogenic effect. The lack of upregulation of Cx43 with fluid shear stress following metal exposure may impede this autocrine/paracrine regulation of periprosthetic adaptive bone remodeling in patients with elevated metal levels. We appreciate that the selection of candidate genes in this study does not provide a comprehensive overview of the effects osteocytes might have on periprosthetic bone remodeling, but it does suggest an aberrance in regulation following fluid shear stress.

Finally, using the late osteoblasts/early osteocytes cell line MLO-A5 we found that Co^{2+} exposure had no effect on mineralization, but Cr^{3+} treatment inhibited mineralization in a dose dependent manner, whilst the MLO-A5 cells remained viable and produced a matrix that was left unmineralized. It is known that mineralization by osteoid-osteocyte cells occurs via the release of mineralized spheres that nucleate collagen fibrils and propagate calcification[43], and there is evidence that Cr^{3+} can form complexes with amino acids in collagen distorting its helical backbone[44]. Therefore Cr^{3+}-related distortion of the collagen structure may perturb matrix nucleation and eventual mineralization by MLO-A5 cells, as seen in this study.

We acknowledge that our study has limitation due to use of the osteocyte-like cell lines. Although recent studies have described methods for extraction of primary osteocytes from mouse long bones[45], the yields are generally low and would not have been sufficient for our experimental purposes. Therefore we have used the MLO-Y4 cell line which serve as a good osteocyte model as they respond to mechanical stimulation by releasing prostaglandin-E_2[46], ATP[47], and nitric oxide[48], integral to osteocytes' orchestration of adaptive bone remodelling.

We also used MLO-A5 cells with their higher expression of ALP and osteocalcin represent cells undergoing osteoblast-osteocyte transition which have the ability to mineralize the osteoid matrix they are embedded in[27]. Used together we believe that these models are a good representation of osteocyte behaviour with which to test our hypothesis, although we appreciate that some of the key regulators of bone homeostasis such as SOST/sclerostin and FGF23 are not expressed by these cell lines[49; 50].

In conclusion, our current data suggests that metal ions directly affect osteocyte cell number and dendricity. In addition, we have shown that the functional responses and gene expression following fluid shear stress is blunted in osteocytes exposed to metal ions with implications for adaptive bone remodeling in patients with prosthesis tribocorrosion.

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Figure 1. Effect of metal ions on MLO-Y4 cell number. A) Graph depicts the changes in MLO-Y4 cell numbers following 24hr treatment with clinically relevant concentrations of Co^{2+}, Cr^{3+} or Co^{2+}:Cr^{3+}. Cell numbers were counted from 5 non-overlapping random fields of view per well with 4 wells quantified for each treatment. All data are expressed as mean ± SD, relative to vehicle. Vertical dotted line separates the upper limit of previously reported ion concentrations equivalent to those in the patient serum and the hip aspirate. Significant linear trends across the concentration range analysed using One-way ANOVA are represented by arrows in the corresponding direction of effect and P values alongside the respective treatment condition. Asterisks are used to denote differences between Co^{2+} and combined Co^{2+}:Cr^{3+} treatments (* = P<0.05). B) MLO-Y4 cells stained with crystal violet - typical fields of view for vehicle (0µg/L), Co^{2+} (5000µg/L), Cr^{3+} (5000µg/L) and Co^{2+}:Cr^{3+} (5000µg/L each), scale bar = 50µm.

Figure 2. Metal ions increase the dendricity of MLO-Y4 cells in vitro

Graphs depict the changes in A) dendrite number and B) dendrite length per cell following 24hr treatment with clinically relevant concentrations of Co^{2+}, Cr^{3+} or Co^{2+}:Cr^{3+}. Dendrite (>5µm) numbers were counted and lengths measured for cells in 5 non-overlapping random fields of view per well with 4 wells quantified for each treatment. All data are expressed as mean ± SD, relative to vehicle. Vertical dotted line separates the upper limit of previously reported ion concentrations equivalent to those in the patient serum and the hip aspirate. Significant linear trends across the concentration range analysed using One-way ANOVA are represented by arrows in the corresponding direction of effect and P values alongside the respective treatment condition. Asterisks are used to denote differences between Co^{2+} and combined Co^{2+}:Cr^{3+} treatments (*** = P<0.0001).

Figure 3. Metal ions reduce the intracellular Ca2+ response in MLO-Y4 following mechanical stimuli. The graphs illustrate changes in intracellular Ca^{2+} mediated fluorescence due to fluid shear-stress (16 dynes/cm^2) following exposure to 50µg/L and 500µg/L of Co^{2+} and Cr^{3+} for A) 30 minutes and B) 24 hours. Data are from 3 separate experiments with 40 cells analysed for each experiment and are represented as mean ± 95%CI. The inset graphs illustrate the changes in area under curve (AUC) and peak intensity at both time points (*** = P<0.0001).

Figure 4. MLO-Y4 gene expression in response to fluid shear-stress is altered with metal ion exposure. The effect of fluid shear stress on osteocyte gene expression was assessed in the absence or presence of 50µg/L and 500µg/L of Co^{2+} and Cr^{3+} exposure for A) 30 minutes and B) 24 hours. Changes in MLO-Y4 gene expression are depicted as $2^{-\Delta\Delta Ct}$ relative to Gapdh and untreated cells without application of fluid shear stress. All data are mean ± 95%CI from 3 separate experiments with 3 technical repeats per experiment. Comparisons between untreated cells and cells following fluid shear stress are represented by asterisks (* = P<0.05; ** = P<0.01). Significant linear trends across the concentration range analysed using One-way ANOVA with P<0.05.are denoted as ‘f’.

Figure 5. Metal ions reduce mineralization by late osteoblast/early osteocyte MLO-A5 cells. Percentage area mineralised by MLO-A5 cells following exposure to clinically relevant concentrations of Co^{2+} and Cr^{3+}. All data are expressed as mean ± SD, relative to vehicle. Vertical dotted line separates the upper limit of previously reported ion concentrations equivalent to those in the patient serum and the hip aspirate. Significant linear trends across the concentration range analysed using One-way ANOVA are represented by arrows in the corresponding direction of effect and P values alongside the respective treatment condition.