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Role of hypoxia inducible factor 1α in cobalt nanoparticle induced cytotoxicity of human THP-1 macrophages

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Key Words:
cobalt nanoparticle; cytotoxicity; hypoxia inducible factor; macrophages; TNFα

From the Contents

ABSTRACT

Cobalt is one of the main components of metal hip prostheses and cobalt nanoparticles (CoNPs) produced from wear cause inflammation, bone lyses and cytotoxicity at high concentrations. Cobalt ions mimic hypoxia in the presence of normal oxygen levels, and activate hypoxic signalling by stabilising hypoxia inducible transcription factor 1α (HIF1α). This study aimed to assess in vitro the functional role of HIF1α in CoNP induced cellular cytotoxicity. HIF1α, lysosomal pH, tumour necrosis factor α and interleukin 1β expression were analysed in THP-1 macrophages treated with CoNP (0, 10 and 100 μg/mL). HIF1α knock out assays were performed using small interfering RNA to assess the role of HIF1α in CoNP-induced cytotoxicity. Increasing CoNP concentration increased lysosomal activity and acidity in THP-1 macrophages. Higher doses of CoNP significantly reduced cell viability, stimulated caspase 3 activity and apoptosis. Reducing HIF1α activity increased the pro-inflammatory activity of tumour necrosis factor α and interleukin 1β, but had no significant impact on cellular cytotoxicity. This suggests that whilst CoNP promotes cytotoxicity and cellular inflammation, the apoptotic mechanism is not dependent on HIF1α.

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Introduction

Total hip replacements have revolutionised the management of elderly patients crippled with arthritis, with very successful long-term results[1] and significantly improved quality of life.[2] It is expected that hip prostheses should have a lifetime of at least 10 to 15 years in 90% of cases. However, some metal-on-metal (MoM) designs are resulting in much earlier failure rates, which may be attributed to the excessive exposure to metal debris released during wear.[3]

After implantation, all materials are subject to mechanical stress produced by the body, leading to wear, tear, loosening and even cracks within the components.[4] Cellular reactions at the cell-material interface can lead to corrosion and degradation of those materials resulting in clinical complications such as inflammation, bone lyses and cytotoxicity. In MoM hip replacements the body's innate immune system can react to metal debris at the bearing surfaces, which can cause an adverse reaction to metal debris.[5-9]

Cobalt (Co) is the main component of metal prostheses.[5, 10] It is an important trace element of the body and in normal situations plays a critical role in the synthesis of vitamin B12. However, excessive exposure to Co can be toxic to the cells[11] and is associated with several conditions including asthma, pneumonia and haematological abnormalities.[12, 13] The release of Co ions from hip implants during wear and tear results in macrophage infiltration, phagocytosis, necrosis and apoptosis with consequent inflammation and tissue mass formation.[5, 14-16] The underlying mechanism is not fully understood but it is known that Co acts as a hypoxia mimic in cell culture and stimulates the production of hypoxia inducible transcription factor 1α (HIF1α).[17]

Hypoxia is defined as a state when oxygen tension drops below normal limits, but Co activates HIF1α when the oxygen levels in the environment are normal. HIF1α is stabilised, translocated to the nucleus and dimerised with the constitutively expressed HIF1α to elicit the transcription of target genes necessary for increased oxygen demand (red cell production and angiogenesis), cell survival (growth factors) and paradoxically apoptosis (proteins within cell death pathway). HIF1 activity largely depends on the cell type and environmental milieu.[3, 17-19]

Recently Nyga et al.[20] reported that the HIF pathway plays an important role in Co nanoparticles (CoNPs)-induced cytotoxicity, and they expected that to study CoNPs and the HIF pathway was required to further elucidate the role of this molecular pathway in CoNPs and other metal nanoparticle cytotoxicity.

It was hypothesised that HIF1α plays an important role in CoNP-induced cellular cytotoxicity observed in THP-1 macrophages *in vitro*, therefor to knock out HIF1α gene expression was expected to reduce CoNP induced cytotoxicity. The expression of HIF1α and caspase 3 (**Additional file 1**) proteins were investigated *in vitro* following metal exposure and the role of HIF1α in CoNP-induced cellular cytotoxicity established using a HIF1α knock out model.

Methods

Cell culture

THP-1 macrophage cell line (American Type Culture Collection, Manassas, VA, USA; ATCC® TIB-202™) was recovered from liquid nitrogen stock and cultured in RPMI1640 with glutamax (Gibco, ThermoFisher Scientific, Paisley, UK) supplemented with 10% foetal bovine serum (Labtech International, Heathfield, West Sussex, UK) and penicillin/streptomycin (Gibco, ThermoFisher Scientific) at a seeding density of 3×10^4 cells/mL in a 10 cm diameter tissue culture dish. Cells were sub-cultured 1:10 dilution twice weekly to maintain stocks. THP-1 cells were activated to macrophages using 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, owned by Merck, Gillingham, UK) for 72 hours.

Cobalt nanoparticles

CoNPs, obtained from American Elements, Merelex Corp., Los Angeles, CA, USA (purity 99.8%, a mean size 30 nm), were suspended in 100% ethanol at 100 mg/mL, and kept at –20°C as stock solution to avoid corrosion or large agglomeration formation. The size and zeta potential of the particles were characterised using a Malvern Zetasizer Nanoseries (Malvern, Worcestershire, UK). For cell culture treatments, CoNP stock solution was diluted in RPMI1640 with glutamax to obtain a concentration of 0.1 mg/mL. A dose range 0.1–100 μg/mL was used for the study, which is equivalent to clinical exposure. It is suggested that the annual nanoparticles produced from MoM resurfacing is around 1 mg/mL in number; however, the nanoparticles in the tissue at any given time is unclear as it is vary case by case and also the distribution of the particle at local tissue is still unknown.

Cryo-scanning electron microscopy and back scatter observation

THP-1 cells were seeded onto clean and autoclave-sterilised 5 mm × 5 mm silicon wafers (Agar Scientific Ltd., Stansted, UK), which were cultured in 96 well plates (Greiner Bio One Ltd., Stonehouse, Gloucestershire, UK) at 1×10^5 cells/cm^2 (3×10^4 cells/well). THP-1 cells were activated by 100 nM PMA and incubated with CoNP (100 μg/mL) for 24 hours. Untreated activated THP-1 cells were used as a control. Cells were washed in phosphate buffered saline (PBS to remove floating CoNP and the silicon wafers were mounted on a metal stage for scanning electron microscope (SEM) and rapidly frozen in liquid nitrogen. The frozen sample was loaded onto the cryo-SEM chamber for evaporation and observed by SEM (Hitachi S4800; Hitachi Europe Ltd., Maidenhead, UK).

Detection of pH in lysosomes of THP-1 cells

THP-1 cells were seeded into 96 well tissue culture plates (black with transparent bottom, Greiner Bio One Ltd.) (1×10^5 cells/cm^2) and activated by 100 nM PMA for 72 hours before treatment with CoNP (1–10 μg/mL) for 24 hours. Untreated cells were used as a control. After 24 hours of incubation, the culture medium was replaced by fresh medium containing LysoSensor Yellow/Blue DND 160 probes (Molecular Probes, Life Technonolgies, Paisly, UK) according to manufacturer's instructions. These probes are fluorescent and label acidic organelles in live cells. In brief, the cells were treated with LysoSensor Yellow/Blue DND 160, diluted in culture medium at 1:1000. After 5 minutes of incubation, the probe containing medium was replaced by fresh culture medium and the cells were visualised using a Carl-Ziess LMS710 confocal microscope (Carl-Ziess UK Ltd., Cambridge, UK).

Hypoxia inducible transcription factor 1α silencing

Undifferentiated THP-1 cells were reverse transfected with human HIF1α-targeted or mismatched negative small interfering RNA (siRNA; Silencer® select siRNA from Applied Biosystems, Ambion, Huntington, UK) to a concentration of 16 nM using Lipofectamine RNAiMax transfection reagent (Life Technologies Ltd., Paisley, UK) in OptiMEM (no antibiotics). Knock down was confirmed following 72 hours transfection using the Total HIF1α Duoset® IC enzyme-linked immunosorbent assay (ELISA; R&D Systems, Abingdon, UK) and Western blot.

Detection of hypoxia inducible transcription factor 1α by enzyme-linked immunosorbent assay

Healthy and transfected THP-1 cells were seeded into 6 well tissue culture plates (1×10^6 cells/well) and activated by 100 nM PMA for 48 hours before treatment with CoNP (0, 0.1, 1, 10, 100 μg/mL) for 24 hours. Total HIF1α was measured in cell lysates using the Human/Mouse Total HIF1α Duoset® IC ELISA as per manufacturer's instructions. Briefly, cells were washed in PBS and solubilised on ice in the recommended lysis buffer and according to the manufacturer's instructions.

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Protein concentration was quantitated using the Bicinchoninic acid kit (Sigma-Aldrich) using bovine serum albumin as the protein standard, and 40 μg protein was used for the assay. The absorbance was read at 450 nm using the POLARstar Omega plate reader (Labtech International Ltd.) set to 450 nm.

Detection of hypoxia inducible transcription factor 1α by western blot

Healthy and transfected THP-1 cells were seeded into 6 well tissue culture plates (1×10^5 cells/well) and activated by 100 nM PMA for 48 hours before treatment with CoNP for 24 hours. Cells were solubilised using ice-cold RNeasy lysis buffer (with β-mercaptoethanol, 1:10) from RNeasy® Plus Mini Kit (Qiagen Ltd., Crawley, UK). The lysates were homogenised using a 25G needle and syringe and centrifuged at 150 × *g* at 4°C for 10 minutes. The protein concentration was quantitated using the Bicinchoninic acid kit (Thermo Fisher Scientific, London, UK) using bovine serum albumin as the protein standard. Proteins were separated on a 7.5% sodium dodecyl sulfate-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane. Blots were blocked with 5% non-fat milk powder in Tris-buffered saline-Tween 20 for 1 hour at room temperature before being stained with anti-human HIF1α antibody (1:500; BD Transduction Laboratories™, Oxford, UK) overnight at 4°C, or anti-human α-tubulin antibody (1:10,000; BD Transduction Laboratories™) for 1 hour at room temperature. After washing with Tris-buffered saline/Tween 20, the membranes were incubated with a horseradish peroxidise-conjugated anti-mouse IgG (1:2500; Sigma-Aldrich) for 1 hour at room temperature. Proteins were detected using an enhanced chemiluminescence detection system (Thermo Fisher Scientific) and visualised using the ChemiDoc (BioRad, Watford, UK).

Enzyme-linked immunosorbent assay detection of tumour necrosis factor α and interleukin 1β

Control and reverse transfected THP-1 cells were cultured in 24 well plates (1×10^5 cells/well) and activated by 100 nM PMA for 48 hours before treating with CoNP (0, 10, 100 μg/mL) for a further 24 hours. The conditioned media was collected, floating cells were removed by centrifugation and the media was stored at −20°C . Tumour necrosis factor α (TNFα) and interleukin 1β (IL-1β) were quantitated using the human TNFα or IL-1β Duoset (R&D systems) respectively.

Cytotoxicity studies

CoNP-induced cellular cytotoxicity was assessed in THP-1 macrophages using neutral red solution (Sigma-Aldrich), which is actively transported into the lysosomes of viable cells. Healthy and transfected THP-1 cells were seeded into 96 well plates (1×10^4 cells/well) and activated to macrophages using 100 nM PMA. After 24 hours the healthy and transfected THP-1 cells were treated with CoNP (0, 10, 100 μg/mL). After 24 hours CoNP treatment, Neutral red solution was added to the media (10%) and the cultures were incubated at 37°C for 2 hours. The plate was centrifuged for 5 minutes at 150 × *g* and the media was replaced with PBS. The plate was centrifuged again before removing the PBS and solubilising the neutral red in neutral red solubilisation solution (Sigma-Aldrich). After 10 minutes on a gyratory shaker the absorbance was read at 540 nm using the POLARstar Omega plate reader (Labtech International Ltd.). The cell viability changes were calculated as a percentage of non-treated cell viability.

Statistical analysis

Statistical comparisons were made using non-parametric statistics (Wilcoxon matched pairs) using Statistica 6.0 (Statsoft, Tulsa, OK, USA). Differences between groups were considered significant at $P < 0.05$. Data are expressed as mean ± standard error (SE).

Results

Characterisation of cobalt nanoparticle

Commercial CoNP are black coloured particles with an average size of 28 nm. They frequently form agglomerates due to their magnetic property. Once CoNPs are suspended in culture media they sedimented to form agglomeration, therefore, require vigorous mixing before dispersing to analyse or when seeding to cell culture. Ultrasonication in PBS for 5 minutes reduced the size of the agglomerations from 1.145 μm to 586.1 nm. However, ultrasonication for 10 minutes increased the size of agglomerations. By using Nano-sizer (Zetasizer Nano ZSP, Malvern, UK), the zeta potential of CoNP was measured as −14.6 mV, the conductivity 14.3 ms/cm and the mobility was around −1.144 μm·cm/V/s.

Microstructure and backscatter morphology of cobalt nanoparticle within the macrophages

Cryo-SEM can observe rapidly frozen cells without the process of fixation and dehydration. Under SEM the control THP-1 cells were around 10 μm diameter with relatively smooth surface, and they firmly attached to the culture substrates (**Figure 1A**). After CoNPs were seeded into culture medium, they formed cloudy sediment surrounding the THP-1 macrophages (**Figure 1B**). Higher magnification images showed the presence of CoNP within the macrophages (**Figure 1C–F**).

Detection of pH in lysosomes of THP-1 cells with cobalt nanoparticle treatment

LyoSensor Yellow/Blue DND 160 is a ratiometric probe that produces blue fluorescence in neutral environments and yellow fluorescence in more acidic environments.[21] Using this staining, normal cell cytoplasm is blue fluorescent, and lysosomes light yellow (**Figure 2A** and **B**). Whilst the THP-1 macrophages remained a neutral blue (**Figure 2C** and **E**), the lysosomes, having engulfed the CoNP, stained an intensely acidic yellow (**Figure 2D** and **F**). The CoNP could be clearly observed within the cells at higher magnification (**Figure 2G** and **H**). As the concentration of CoNP increased, the number of lysosomes within the cell and the staining intensity of the lysosomes increased indicating greater uptake and potentially a greater risk of cytotoxicity.

Detection of hypoxia inducible transcription factor 1α in total cell lysates of THP-1 cells with cobalt nanoparticle treatment

The presence of HIF1α was assessed within the total cell lysates of healthy and transfected THP-1 macrophages following

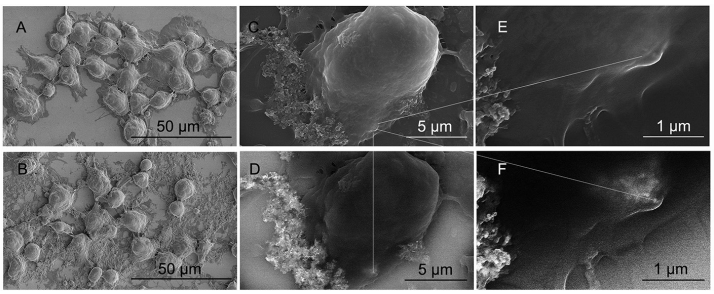

Figure 1. Micrographs of cryo-SEM illustrates THP-1 macrophages and their phagocytosis of CoNP. (A) Cell morphology of control THP-1 cells. (B) Cell morphology of THP-1 cells at 24 hours after CoNP (100 µg/mL). (C) High magnification of normal SEM showing a macrophage in contact with CoNPs. (D) Backscatter SEM of the same cells in C, revealing high electron density particles, in particular one cluster of CoNPs not showing in C. (E) Highlighted area demonstrating the cluster of CoNP not showing by normal SEM. (F) Highlighted area demonstrating phagocytosis of the cluster of CoNPs that were engulfed and submerged within the macrophage cell membrane. Scale bars: 50 µm in A, B; 5 µm in C, D; 1 µm in E, F. CoNP: cobalt nanoparticle; SEM: scanning electron microscope.

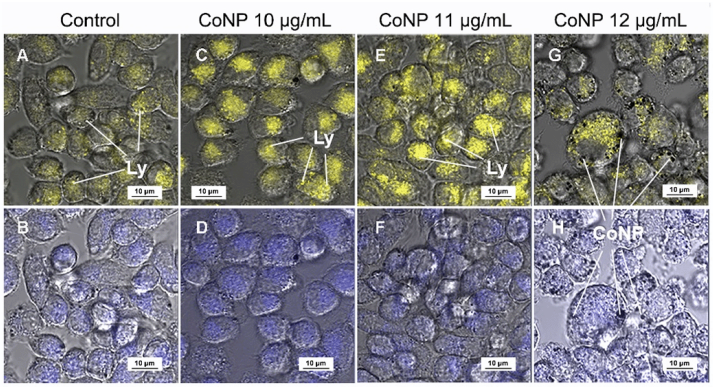

Figure 2. The pH within THP-1 macrophages at 24 hours after CoNP incubation was measured using LysoSensor Yellow/Blue DND 160 probes. (A–H) The blue fluorescence indicates neutral pH within the cell (A, C, E, G), whilst the yellow fluorescence shows the acidity of the lysosomes following the engulfment of CoNP (B, D, F, H). With increasing CoNP dose, the numbers and acidity of lysosomes within the cell increased whilst the pH within the cell remained neutral. CoNP: cobalt nanoparticle; Ly: lysosomes.

treatment with CoNP by ELISA (**Figure 3A**) and Western blot (**Figure 3B1** and **B2**). The higher doses of CoNP (10 and 100 µg/mL) resulted in a significant increase in HIF1α production in comparison to the control ($P < 0.01$). Concentrations above 100 µg/mL resulted in cell death (data not shown). CoNP-induced HIF1α accumulation was significantly reduced following transfection with HIF1α siRNA (**Figure 3A**).

Detection of tumour necrosis factor α and interleukin 1β concentration in THP-1 cells with cobalt nanoparticle treatment

TNFα (**Figure 3C**) and IL-1β (**Figure 3D**) secretion significantly increased at the higher CoNP doses (10 & 100 µg/mL) compared to the control (no CoNP) cultures at 24 hours ($P < 0.001$ or $P < 0.01$). Transfecting THP-1 macrophages resulted in a significant increase in TNFα and IL-1β regardless of CoNP concentration, but silencing HIF1α activity led to a further increase in both TNFβ ($P < 0.001$) and IL-1β ($P < 0.01$) secretion in comparison to the mismatched transfected control cultures.

Cobalt nanoparticle-induced cytotoxicity in THP-1 cells

Viability was assessed using solubilisation of Neutral red staining, which was directly proportional to the number of viable cells. Viability was significantly reduced following treatment with the higher concentrations of CoNP (10 & 100 µg/mL, $P < 0.05$; **Figure 3E**). The process of transfection, in the absence of CoNP, resulted in minor cytotoxicity to the cells, but no significant differences were observed between the mismatched control and the HIF1α transfection with HIF1α siRNA cultures.

Discussion

CoNP concentrations above 10 µg/mL induced significant cytotoxicity in THP-1 cells. This aligned with a significant increase in HIF1α, TNFα, and IL-1β. Significantly reducing HIF1α activity through transfection with siRNA, resulted in a greater increase in the pro-inflammatory mediators TNFα and IL-1β, but had no additional impact on CoNP-induced cytotoxicity. This suggests that whilst the higher doses of CoNP induce cytotoxicity, the mechanism is not directly or solely through the actions of HIF1α.

It is well known that macrophages phagocytose nanoparticles and some nanoparticles are toxic to macrophages. CoNPs are significantly more toxic to macrophages than same sized chromium nanoparticles,[10, 22] which emphasizes that it's not only particle size, but also chemical composition that plays an

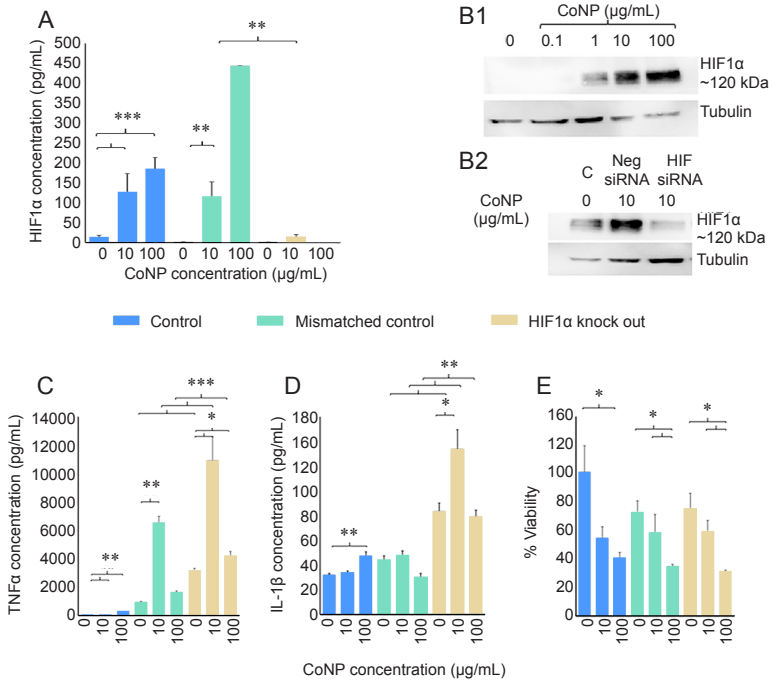

Figure 3. Analysis of HIF1α expression and the effect of HIF1α knockout on cytotoxicity and inflammatory factors (TNFα and IL-1β). (A) THP-1 cells were lysed for protein extraction 24 hours following treatment with CoNP (10 and 100 μg/mL). HIF1α concentration was measured by ELISA Duoset®. (B) Western blot of HIF1α expression. (B1) HIF1α concentration was increased with CoNP dose. (B2) Knockdown of HIF1α using siRNA showed a significantly reduced production of HIF1α protein in response to CoNP. (C, D) TNFα (C) and IL-1β (D) concentration in THP-1 cells with CoNP treatment were measured in the culture supernatants using enzyme-linked immunosorbent assay Duoset® 24 hours following CoNP treatment (0, 10, 100 μg/mL). Both TNFα (C) and IL-1β (D) secretion increased with CoNP dose in the healthy and transfected macrophages. Both TNFα and IL 1β increased significantly following transfection in comparison to the control. Cultures transfected with HIF1α siRNA showed significant up-regulation of TNFα and IL-1β in comparison to the mismatched control. (E) Percentage viability of healthy and transfected THP-1 cultures treated with CoNP (0, 10, 100 μg/mL) as measured by neutral red staining. Viability significantly reduced with increasing CoNP dose within the control, mismatched control and HIF1α knock out. There were no significant differences between the mismatched control and cultures transfected with HIF1α small interfering RNA. Data are expressed as mean ± SE. All experiments were repeated twice or three times with consistent results. $*P < 0.05$, $*P < 0.01$ (Wilcoxon matched pairs test). CoNP: cobalt nanoparticle; ELISA: enzyme-linked immunosorbent assay; HIF1α: hypoxia inducible transcription factor 1α; IL-1β: interleukin 1β; TNFα: tumour necrosis factor α.

important role in nanotoxicity. CoNP-induced cytotoxicity, demonstrated within this study, is consistent with previous reports[10, 20, 22] but the mechanism of nanotoxicity is not fully understood.

After phagocytosis nanoparticles are normally stored in phagosomes or lysosomes.[5] These are membrane-bound cytoplasmic vesicles where the engulfed materials are digested by enzymes in acidic conditions.[23] Inorganic metal particles, such as Co, are not digested by enzymes, but will be corroded by the acidic environment with the subsequent release of Co ions.[14, 24] This study demonstrated a build-up of lysosomal acidity with increasing CoNP dose, which is likely to result in intracellular corrosion and subsequent release of high levels of Co ions following cell death.

Metal implants are normally protected by an oxidant layer to avoid corrosion, however, in acidic conditions, protons may directly react to the metal oxidant and facilitate further corrosion *in vivo* and conversion to soluble Co ions.[25] Despite this, consistent reports show that the cytotoxic effects of CoNP are stronger than those of Co ions,[14, 20] and, *in vivo*, the concentration of Co ions are unlikely to be sufficient to cause cytotoxicity.[5, 10]

CoNP and soluble Co ions lead to allergic or inflammatory responses from the body[26, 27] and failing implants are usually associated with significant localised inflammation.[3, 16, 28] The key mediators are macrophages,[28] which have been shown to actively respond to many different biomaterials, including Co.[24] Couple this with reduced circulation, increased metabolic demand, hypoxia and release of pro-inflammatory mediators,[29] an escalating inflammatory response ensues, promoting further tissue damage and ultimately implant failure.

Co plays a key role in the HIF1α mediated pathway by directly inhibiting the oxygen-dependent prolyl hydroxylases that facilitate HIF1α degradation in normoxic conditions.[11, 30, 31] This enables the accumulation of HIF1α at the protein level, as demonstrated within this study. Stabilisation and dimerization of HIF1α with HIF1α in the nucleus elicits gene transcription controlling inflammation, angiogenesis and apoptosis *in vivo*.[11]

HIF1α has been shown to be a vital player in the manifestation of inflammation.[32] Co induces reactive oxygen species, which can induce inflammation and cytotoxicity in macrophages,[33-35] however, this is most likely due to the oxidative stress of Co ingestion rather than HIF1α upregulation,[20] and it is difficult to explain the complex clinical symptoms of MoM hip implant failure simply by reactive oxygen species reaction alone.

Increasing concentrations of Co led to a significant release of pro-inflammatory cytokines (TNFα and IL-1β) within 24 hours of treatment.[20, 36, 37] The decrease in TNFα and IL-1β at 100 μg/mL in comparison to 10 μg/mL may be explained by the high rates of cell death by 24 hours at 100 μg/mL and that these cytokines are only released from viable cells.[36] Both TNFα and IL-1β are central to the development and regulation of acute inflammation and have been found in high concentrations in tissue macrophages local to implant failure.[28] TNFα has also been reported to have a direct role in osteoclast recruitment and activation, which may contribute to implant loosening.[37] Hypoxia or Co exert much of their effects through the stabilisation of HIF1α, which either leads directly to the up-regulation of TNFα and IL-1β through modification of gene expression, or indirectly though up-regulation of other immune regulatory molecules such as IκB kinases and nuclear factor (NF)-κB. These, along with HIF1α, are also regulated by prolyl hydroxylases.[38,39] *In vivo*, knocking out HIF1α significantly reduced the ability of mouse myeloid cells to initiate an innate inflammatory response,[40] and challenging lung epithelium to Co in HIF1α-deficient mice resulted in them losing their pro-inflammatory phenotype.[32] This provides strong evidence for HIF1α in mounting an innate immune response *in vivo*.

In this *in vitro* study we showed a clear increase in pro-inflammatory mediator release in the absence of HIF1α, suggesting a mechanism for driving inflammation independent of HIF1α. In contrast, Nyga et al.[20] showed no change in IL-1β or TNFα protein release in the absence of HIF1α, but they used ascorbic acid to prevent HIF1α accumulation by maintaining the activity of prolyl hydroxylases, which also regulate other proteins such as NF-κB. In the absence of HIF1α, specifically knocked out using siRNA technology, it may be that NF-κB signalling is responsible for the increased production of TNFα and IL-1β. Indeed, HIF1α has been reported to restrict the transcription of NF-κB during inflammation,[41] so removing HIF1α may allow for enhanced NF-κB activity following hypoxia or Co treatment. This is certainly something to explore further.

CoNP-induced cytotoxicity correlated with an increase in HIF1α so we aimed to determine whether the increase in HIF1α was causal to apoptosis and cell death of THP-1 macrophages. HIF1α plays a complex but important role in apoptosis by regulating the expression of genes involved in either activating or inhibiting the cell death cascade.[18] Caspase 3 is part of the cell death cascade and has been previously shown to be involved in CoNP-induced macrophage cytotoxicity.[42] This study also showed an increase in caspase 3 with CoNP dose (**Additional Figure 1**), which correlated with cytotoxicity, but preventing HIF1α accumulation had no significant impact on macrophage cell death in comparison to the siRNA control.

Future work needs to investigate caspase 3 activation in the absence of HIF1α and equate this with macrophage cytotoxicity. Shi et al.[43] showed an upregulation of the activated fragment of caspase 3 following HIF1α siRNA knock out in human breast cancer cell lines, which suggests a direct role for HIF1α in preventing apoptosis. In fact, HIF1α is often found overexpressed in solid tumours rendering tumour cells resistant to apoptosis.[44] *In vivo*, macrophages are able to undergo rapid metabolic adaptation to changes in environmental milieu, such as hypoxia, in order to fight infection,[45] so the primary function of the hypoxia pathway and HIF1α must be survival. The role of HIF1α, however, appears to be very cell-type specific and the direct role for HIF1α in macrophage apoptosis to metal challenge is still unclear. Following MoM hip implants, Co could initiate a range of different responses depending on the patient's lifestyle or susceptibility to metal challenge. This may be the reason why failure rates vary so widely between individuals.

The role of HIF1α in Co-induced inflammation or cytotoxicity is clearly very complex. The potential cellular and molecular process involved in this study is shown in **Figure 4**.

There are a number of limitations of this study: (1) the reactive oxygen species was not detected in this study; (2) the direct causal factor of HIF1α independent apoptosis of this study is

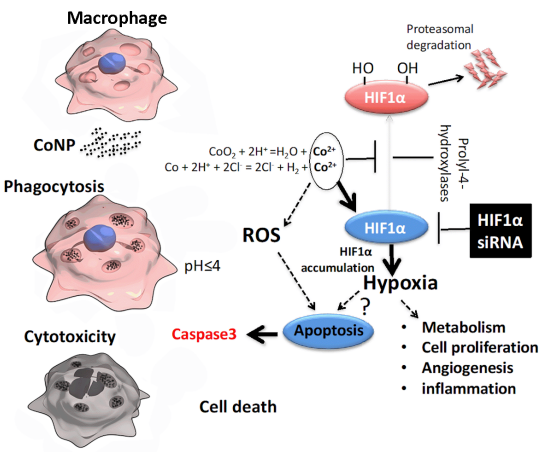

Figure 4. Schematic diagram. CoNP: cobalt nanoparticle; HIF1α: hypoxia inducible transcription factor 1α; ROS: reactive oxygen species; siRNA: small interfering RNA.

still unclear; (3) the downstream gene expression as a resultant of CoNP induced HIF1α increase needs further study.

In conclusion, inhibiting HIF1α protein accumulation alone is insufficient to prevent CoNP induced cytotoxicity, despite Co treatment leading to an increase in HIF1α stabilisation in a dose dependent manner. This suggests that whilst CoNP promotes cytotoxicity and cellular inflammation, the apoptotic mechanism is not dependent on HIF1α.

Author contributions

Original idea, day-to-day management, consumable, manuscript preparation: ZX; research design: WRF, ZX; cellular and molecular experiments, manuscript writing: WRF; cytotoxicity assay: ZL, WRF, XW; cell culture, HIF1α knockout: WRF; western blot and analysis: SEO; ELISA: XW; immunohistochemistry and confocal microscopy: HX; SEM image: AL; experimental support: VK; figure and graph preparation: ZL; data analysis: WRF, ZL, AL; manuscript revision: WRF, ZL, VK, ZX. All authors approved the final version of this manuscript.

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Conflicts of interest statement

The authors report no conflict of interest. No financial interest or benefit has arisen from the direct applications of this research.

Editor note: Zhidao Xia is an Editorial Board member of *Biomaterials Translational*.

The article was subject to the journal's standard procedures, with peer review handled independently of this Editorial Board Member and their research groups.

Additional files

Additional file 1: Immunocytochemistry of hypoxia inducible transcription factor 1α and caspase 3 of THP-1 cells after phagocytosis of cobalt nanoparticles.

Additional Figure 1: Bright field and confocal images illustrate the phagocytosis and the immunocytochemical staining of hypoxia inducible transcription factor 1α (HIF1α) and caspase 3 (Casp3) of THP-1 macrophages in response to different dose of cobalt nanoparticles (CoNPs).

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