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Influence of particle size and reactive oxygen species on cobalt chrome nanoparticle-mediated genotoxicity

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ABSTRACT

Patients with cobalt chrome (CoCr) metal-on-metal (MOM) implants may be exposed to a wide size range of metallic nanoparticles as a result of wear. In this study we have characterised the biological responses of human fibroblasts to two types of synthetically derived CoCr particles [(a) from a tribometer (30 nm) and (b) thermal plasma technology (20, 35, and 80 nm)] *in vitro*, testing their dependence on nanoparticle size or the generation of oxygen free radicals, or both. Metal ions were released from the surface of nanoparticles, particularly from larger (80 nm) particles generated by thermal plasma technology. Exposure of fibroblasts to these nanoparticles triggered rapid (2 h) generation of reactive oxygen species (ROS) that could be eliminated by inhibition of NADPH oxidase, suggesting that it was mediated by phagocytosis of the particles. The exposure also caused a more prolonged, MitoQ sensitive production of ROS (24 h), suggesting involvement of mitochondria. Consequently, we recorded elevated levels of aneuploidy, chromosome clumping, fragmentation of mitochondria and damage to the cytoskeleton particularly to the microtubule network. Exposure to the nanoparticles resulted in misshapen nuclei, disruption of mature lamin B1 and increased nucleoplasmic bridges, which could be prevented by MitoQ. In addition, increased numbers of micronuclei were observed and these were only partly prevented by MitoQ, and the incidence of micronuclei and ion release from the nanoparticles were positively correlated with nanoparticle size, although the cytogenetic changes, modifications in nuclear shape and the amount of ROS were not. These results suggest that cells exhibit diverse mitochondrial ROS-dependent and independent responses to CoCr particles, and that nanoparticle size and the amount of metal ion released are influential.

1. Introduction

Orthopaedic patients with metal-on-metal (MoM) hip replacements are exposed to CoCr nanoparticles as a result of wear of the implant [1–4]. Approximately 6.7×10^{12}–2.5×10^{14} particles (generally <50 nm) are generated by articulating CoCr surfaces in each patient every year [5,6]. Calls have been made for the establishment and validation of material characterisation protocols and biological testing methodologies to understand the potential toxicity of these nanoparticles. There is also an increase of circulating metal ions in the blood of these patients [5,7–17], who may be exposed to metal for up to 60 years after surgery. This internal surgical exposure to nanoparticles is different from the external exposure (such as environmental pollution/inhalation) as it bypasses many of the body's natural defences, for example by macrophage uptake or contact with fluids in the airways before entering the body.

While it is not known whether the release of Co and Cr ions is essentially from the whole implant, or by corrosion of wear debris and/or both; the mechanisms of Co^{2+} [18–21], and Cr ions

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(trivalent and hexavalent) [22–27] toxicity *in vitro* is well understood with oxidative stress mediated cyto-and genotoxicity playing a major role. We have previously observed increases in aneuploidy in peripheral blood lymphocytes of patients with CoCr-on-CoCr wear debris [28]. We also demonstrated that CoCr nanoparticles (30 nm) which were generated by a pin-on-plate tribometer were significantly more toxic than commercially available micron sized (2.9 μm) CoCr particles *in vitro* [29], demonstrating that CoCr particle size may be a key factor governing toxicity. Importantly, the CoCr wear debris generated *in situ* from MoM implants is often a heterogeneous mix of nanoscale particles.

Size dependent but concentration independent toxicity of nanoparticles has been demonstrated previously for other metal oxides such as TiO_2, CuO, ZnO [30,31]. There is very little known about the mechanisms of toxicity of differentially sized CoCr particles within the nano scale range. The toxicity of metal oxides may also be dependent on the extent of oxidative stress [32–34]. In this study, we have investigated whether there is a relationship between the size of the CoCr nanoparticle and the amount of genotoxicity. We have used a variety of assays to explore this. We have also explored the role of oxidative stress in the genotoxic response.

2. Materials and methods

2.1. Preparation and sterilisation of nanoparticles

2.1.1. Preparation of thermal plasma (ThP) particles

A water-cooled tungsten tip and copper crucible were used as cathode and anode electrodes, respectively. A piece of CoCr disc of 33 mm diameter and 10 mm thick was placed in the water-cooled copper crucible. A dynamic flow of Ar gas at 5L/min was applied between the tungsten tip and the CoCr disc maintained with a gap of 2 mm. A plasma was then generated between the tungsten tip and the CoCr disc using current and voltage listed in Table 1A. The plasma caused the CoCr disc to evaporate and the vaporised gas was quenched onto a water-cooled column, where the nanoparticles were collected for analysis. The composition of the alloy from which the thermal plasma particles were made, in terms of both weight% and atomic %, is shown in Table 1B.

2.1.2. Preparation of pin-on-plate (PoP) particles

Nanoparticles (29.5 ± 6.3 nm) of CoCr alloy were generated using a flat pin-on-plate tribometer. The composition of CoCr alloy (ASTM F1537) used to generate particles is listed in Table 1C. Particles were generated with a bi-directional motion of 5.6×10^{-2} ms^{-1} and a contact stress of 11 MPa over a 24 h period in water. The particles were recovered by filtration onto 100 nm pore sized 25 mm polycarbonate filter membranes and the mass of particles on the filters determined gravimetrically. The filters were placed in sterile pyrogen free-water and sonicated in a sonic bath for 1 h in order to resuspend and pool the particles.

2.1.3. Sterilisation, sonication and exposures

The metal nanoparticles were transferred into glass universals, weighed, washed in 100% ethanol and heated at 180 °C for 3 h as a mode of sterilisation. Latex nanoparticles were sterilised by filtering through a 0.2 μm filter. After sterilisation, the nanoparticles were resuspended in serum free growth medium by sonication (pulsed mode: 7 pulses each of 1–2 s long) using a titanium probe and Sonics VibraCELL VC130PB sonicator (maximum output power: 130W). The fibroblasts were exposed to the nanoparticles at doses of 0.0005 μm^3/cell to 500 μm^3/cell over a range of time points (2 h–5 days).

2.2. Dissolution of metal ions into the growth medium

All CoCr particles were incubated in growth medium for 24 h, 37 °C. At the end of the incubation period, the particle laden medium was centrifuged at 14,000 rpm for 20 min. The supernatant was analysed for trace metal by inductively coupled plasma mass spectrometry (ICP-MS) [Analytica Ltd, Sweden].

Table 1A
Conditions used to generate thermal plasma nanoparticles.

Sample	Current (A)	Voltage (V)
20 nm	350	40
34.8 nm	410	40
80 nm	500	40

Table 1B
Composition of alloy from which thermal plasma particles were prepared.

Element	C	Co	Cr	Fe	Mn	Mo	N	Ni	Si
Wt%	0.06	64.3	28.0	0.25	0.50	6.00	0.20	0.20	0.50
Atomic%	0.29	62.5	30.8	0.26	0.52	3.58	0.82	0.20	1.02

2.3. Characterisation of particles by transmission electron microscopy (TEM) and photon correlation spectroscopy (PCS)

The sizes of the particles were determined by TEM and PCS. Samples for TEM analysis were prepared by applying a drop of the appropriate dispersion onto a 3 mm diameter, carbon covered copper grid. Specimens were examined on a Jeol 1200EXmkII TEM operated at 120 keV. Digital images were recorded using an SIS Megaview 2 camera, and processed (particle sizing) using AnalySIS software. Energy dispersive X-ray analysis (EDX) was performed using an Oxford Instruments ISIS 300 system. For estimation of the size of particles by PCS, the particles were dispersed in growth medium filtered through a 0.22 μm millipore filter. The viscosity of the growth medium was determined using capillary viscometry. The refractive index and absorption of the particles were calculated at a wavelength of 488 nm taking into account the proportion of the metals in the Co/Cr alloy from which the particles were prepared. The particles were dispersed by ultrasonication through 7 pulses of 1–2 s, using a 25 μm amplitude, 11W probe. The vials were thermostatted at 37 °C and the dynamic light scattering measurements were carried out at an angle of 90° using a Brookhaven instrument, comprising a Coherent I70-2, 2W Ar ion laser tuned to 488 nm, a BI-200 goniometer and a BI-9000 digital correlator. Cumulants analysis yielded the quadratic diameter and polydispersity of the particles.

2.4. Determination of the electrophoretic mobility of CoCr nanoparticles

Particle electrophoretic mobilities were determined using a Malvern Instruments Zetasizer Nano Z and a disposable folded capillary cell. The particles were dispersed in growth medium by ultrasonication and equilibrated at 25 °C before the measurement was made. A standard latex sample with a zeta potential of −68 mV±7 mV was also run at the same time as a check. The data were analysed using Monomodal Analysis, as recommended by Malvern Instruments for samples with a conductivity >10 mS cm^{-1}, giving a mean value for the electrophoretic mobility but no distribution. Measurements were repeated at least twice. In addition, the electrophoretic mobility of 80 nm ThP particles was measured at 37 °C. Using the calculated ionic strength of the growth medium to find the Debye length, the zeta potentials were calculated using Henry's equation.

The isoelectric point of the 80 nm ThP CoCr particles was determined using a 'batchwise' titration method. The particles were dispersed using ultrasonication in 10^{-3} mol dm^{-3} KNO_3 which had been adjusted to cover a range of pH values from 4 to 10 using HNO_3 or KOH. Samples were equilibrated overnight before the equilibrium pH was recorded and the electrophoretic mobilities of the particles measured.

2.5. Cell culture

Primary human BJ fibroblasts were routinely cultured in growth medium comprised of minimal essential medium (MEM; Sigma) supplemented with 10% (v/v) foetal calf serum (FCS), L-glutamine (2 mM; Gibco), penicillin (10 U/ml), streptomycin (0.1 mg/ml) and amphotericin B (0.25 mg/ml) (Sigma, UK). When cells had reached 80% confluence, they were passaged (split ratio 1:10) by trypsinisation (Trypsin-EDTA;Gibco, UK). Cells were seeded at 3000 cells per well for experiments carried out in 96-well plates (ROS assays), and 50,000 cells per well on 13 mm Ø glass coverslips for those conducted in 12-well plates (Immunocytochemistry, and mFISH).

Table 1C
Composition of alloy used to make pin-on-plate particles.

Constituent element	Minimum composition (%)	Maximum composition (%)
Chromium	26	30
Molybdenum	5	7
Silicon	–	1
Manganese	–	1
Iron	–	0.75
Nickel	–	1
Carbon	0.15	0.35
Nitrogen	–	0.25
Cobalt	Balance	Balance