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Biological effects of cobalt-chromium nanoparticles and ions on dural fibroblasts and dural epithelial cells

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ABSTRACT

The introduction of metal-on-metal total disc replacements motivated studies to evaluate the effects of cobalt-chromium (CoCr) nanoparticles on cells of the dura mater. Porcine fibroblasts and epithelial cells isolated from the dura mater were cultured with clinically-relevant CoCr nanoparticles and the ions, generated by the particles over 24 h, at doses up to 121 μm^3per cell. Cell viability and production of proinflammatory cytokines was assessed over 4 days. The capacity of the particles to induce oxidative stress in the cells was evaluated at 24 h. The CoCr particles and their ions significantly reduced the viability of the dural epithelial cells in a dose-dependent manner but not the fibroblasts. Both cell types secreted IL-8 in response to particle exposure at doses of 60.5 μm^3 (epithelial cells) and 121 μm^3 (fibroblasts, epithelial cells) per cell. No significant release of IL-6 was observed in both cell types at any dose. Reactive oxygen species were induced in both cell types at 50 μm^3 per cell after 24 h exposure. The data suggested novel differences in the resistance of the dural epithelial cells and fibroblasts to CoCr nanoparticle/ion toxicity and demonstrated the inflammatory potential of the particles. The data contributes to a greater understanding of the potential biological consequences of the use of metal-on-metal total disc prostheses.

1. Introduction

Degeneration of the intervertebral discs may lead in some individuals to a painful and disabling condition [1]. First line treatment generally includes non-invasive approaches such as rest, physiotherapy and analgesics. Patients are subjected to operative procedures when they do not respond to conservative treatment. A surgical procedure such as arthrodesis (spinal fusion) [2] is employed, however, its limitations include increased mechanical load at the adjacent and non-adjacent vertebrae and decrease in spine mobility that can lead to adjacent vertebrae degeneration [3]. For these reasons European surgical specialists on total hip and knee joint replacements started to experiment with total replacement of lumbar discs two decades ago, but the early operations led to some serious complications such as slippage of the artificial disc forward causing compression and damage to the aorta and the vena cava. It took nearly a decade for new designs to be developed and approved by the U.S. Food and Drug Administration following which more than 20,000 procedures were performed worldwide with more than 3000 in the United States alone [4]. This improved procedure of total disc replacement (TDR) has been developed with the objective of providing relief from pain and restoring motion between degenerated vertebrae [5]. New generation of artificial discs developed for TDR have gained increased popularity and preliminary studies have shown promising results [6,7]. Such prosthetic discs have the advantage of motion preservation and alleviation of adjacent segment degeneration [8]. The materials used in TDR have been adopted from experience in total hip arthroplasty. The two major types of TDR are the metal-on-polyethylene and metal-on-metal (MoM) implants [9]. The most extensively clinically utilised, studied and tested metal-on-polyethylene artificial discs are the Prodisc and the SB Charité total discs. The Flexicore and the Maverick total discs are two types of MoM artificial discs that can be used in TDR surgery [10].

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However, there is currently very limited data (only case reports) available on the effectiveness and complications associated with spinal arthroplasty with virtually no long-term studies on the evaluation of the durability and sequelae of TDR surgeries. Case reports such as Berry *et al.* 2010 [11], Guyer *et al.* 2011 [12] and Cabraja *et al.* [13] revealed that MoM TDR can lead to the formation of granulomatous tissue with significant level of metalosis that can occlude or dislocate the surrounding arteries and veins and also cause spinal stenosis through infiltration of the spinal canal. This is of particular concern for MoM TDR, given the potential for toxicity of metal wear particles and the proximity to the spinal cord.

It is well established that prosthetic metal wear particles are in the nanometre size range [14,15]. The wear particles from simulators are of mean size smaller than 50 nm with round and irregular morphology [15] and similarly the mean size of the wear particles retrieved from periprosthetic tissues is also less than 50 nm (range: 6–834 nm) with round to oval shape and irregular boundaries [14]. These nanometre sized particles may disseminate to systemic regions away from the implant site such as local or distant lymph nodes, bone marrow, liver and spleen [16]. Significantly higher levels of cobalt and chromium ions have been reported in the serum of patients with MoM hips than those without [17] and this is a cause for concern as this may present a potential risk of carcinogenicity in humans and is directly associated with soft tissue changes [18]. Metal wear debris also has the capacity to induce host hypersensitivity [19,20]. Papageorgiou *et al.* [21], described the cytotoxic and genotoxic effects of CoCr nanoparticles on human fibroblasts *in vitro*. It has also been shown recently that CoCr nanoparticles and ions can cause DNA damage in cultured human fibroblasts even across cellular barriers [22] but this study had received considerable criticism [23].

There is an increasing concern regarding the development of periprosthetic soft tissue reactions in patients with some designs of MoM surface hip replacements [24]. These reports are beginning to raise questions about the long-term clinical implications of such pathologic observations associated with MoM prostheses, which are yet unknown. Similar cases have been presented in association with MoM TDR. Zeh *et al.* [25] examined 10 patients with Maverick lumbar disc replacements for an average of 5.6 years and reported the presence of CoCr ions in the serum. Cavanaugh *et al.* [26] published a case study in which a patient developed a delayed immune reaction to a MoM cervical artificial disc 9 months after surgery. In a recent case study, Berry *et al.* [11] were the first to report the formation of a large granuloma in a patient in response to metallic wear particles generated by a lumbar Maverick disc implantation. Moreover, Guyer *et al.* [12] presented a case study wherein four patients receiving MoM TDR developed periprosthetic necrosis and lymphocytic reactions resulting in failure of the TDR surgery. In one case report by Cabraja *et al.* [13], growth of this granulomatous tissue continued even after stabilization and reduction of the total disc arthroplasty device motion and so the clinicians were forced to remov the implant altogether.

It is possible that the wear particles produced in MoM TDRs can potentially interact with the dura mater (meninges). It is therefore imperative to determine the biological effects of these particles on the cells of the dura mater. The aim of this study was to investigate the biological effects of clinically relevant nanometre-sized CoCr wear particles on porcine dural cells extracted from the meninges that surround the spinal cord and are present in the immediate vicinity of artificial intervertebral discs implanted during TDR surgery. Porcine dural fibroblasts and epithelial cells were isolated and cultured with CoCr nanoparticles and the ions generated by the CoCr nanoparticles over 24 h at varying volumetric concentrations. Cellular responses were assessed using assays of cell viability, cytokine release and oxidative stress.

2. Materials and methods

2.1. Cobalt-chromium nanoparticle preparation and characterisation of size and sedimentation potential

Particles were generated in a 6 station pin-on-plate tribometer with water as the lubricant under a force of 80 N for 40 h. The pins and plates were manufactured from medical grade wrought CoCr alloy ASTM F1537 with smooth counterfaces (Ra: 0.01–0.02 μm). Wear particles were sterilised at 180 °C for 4 h and their mass determined by gravimetric analysis. The particles were then suspended in sterile water to yield a stock concentration of 1 $mg \cdot ml^{-1}$. A sample of the particle suspension was filtered through 0.015 μm polycarbonate filter membranes (Millipore Limited, UK). The isolated particles were characterized using field emission gun scanning electron microscopy (FEGSEM; FEI, The Netherlands) and Image Pro-Plus® imaging software. A total of 100 particles per image were analysed for 3 images.

In order to assess the particle sedimentation potential, CoCr-particle suspensions were prepared in dH_2O, DMEM (10% foetal bovine serum) and M199 medium (20% foetal bovine serum) (dural fibroablast and epithelial cell culture media). The particles were sonicated in a sonicating water bath (Grant Instruments Limited, UK) for 10 min and then the absorbance was measured at 204 nm over 2 s intervals for a period of 6 h using a M359 spectrophotometer (CamSpec, UK). The A/A_0 (A: Absorbance at time point; A_0: Absorbance at time = 0) was calculated for each time point and then plotted on a graph.

2.2. Ion release from the cobalt-chromium alloy nanoparticles

In order to measure the cobalt (Co), chromium (Cr) and molybdenum (Mo) ion concentrations that were released from the CoCr alloy nanoparticles over a period of time, two sets of clinically relevant particle suspensions were prepared at doses equivalent to 62, 6.2, 0.62, 0.062 $\mu g \cdot ml^{-1}$ in culture medium. One set of the particle doses was incubated at 37 °C in 5% (v/v) CO_2 in air for 1 day and the other set was incubated under the same conditions for 5 days. After incubation, the particle suspensions were centrifuged for 20 min at 3000 g and the supernatants containing the ions released from the particles were analysed. The Co and Cr ion levels in the supernatants were measured by graphite furnace atomic absorption spectroscopy whereas the Mo ion levels were measured by inductively coupled plasma mass spectroscopy.

2.3. Extraction of the dura mater

A UK Home Office protocol was employed to humanely sacrifice a female pig (65 kg) at the University of Leeds farm, which was then immediately transferred to the dissection table within few minutes of death. The skin covering the dorsal region was disinfected by swabbing with iodine solution and approximately 7–8 vertebrae from the thorax (T2–T10) were aseptically extracted. Following this, the intact extracted region of the vertebral column was transferred to the tissue culture laboratory under sterile conditions for further aseptic dissection. The dissected spinal cord with the attached meninges was then completely immersed in an antimicrobial solution (200 ml) containing Nystatin (100 $U.ml^{-1}$; Sigma–Aldrich, UK) and Gentamicin (50 $ng \cdot ml^{-1}$; Sigma–Aldrich, UK) in phosphate buffered saline (PBS; Sigma–Aldrich, UK) for 1 h at room temperature.

2.4. Cell isolation and culture

Samples of the fresh porcine dural membrane were dissected into ~1 cm^2 sections and then seeded in a 6-well culture plate (Fisher Scientific, UK). The tissue sections were cultured in M-199 medium (Lonza Biopharmaceuticals, UK) supplemented with 20% (v/v) foetal bovine serum (FBS; Lonza Biopharmaceuticals, UK), penicillin-streptomycin (50 $U.ml^{-1}$; Lonza Biopharmaceuticals, UK), L-glutamine (2 mM; Lonza Biopharmaceuticals, UK), sodium pyruvate (0.1 $mg \cdot ml^{-1}$; Sigma–Aldrich, UK), heparin (1 $U.ml^{-1}$; LEO Laboratories Limited, UK) and endothelial growth factor (0.015 $mg \cdot ml^{-1}$; Sigma–Aldrich, UK). The cells were allowed to outgrow for 7 days and culture expanded in supplemented M-199 medium by harvesting and transfers to 75 cm^2 culture flasks (Fisher Scientific, UK).

2.5. Separation of dural fibroblasts and epithelial cells

The cells were harvested after 7 days of growth using trypsin/EDTA (Lonza Biopharmaceuticals, UK) and centrifuged (200 g) and re-suspended in PBS supplemented with 0.1% (w/v) bovine serum albumin (BSA; Sigma–Aldrich, UK). The resulting cell suspension was centrifuged and re-suspended in PBS/BSA at a density of 2×10^6 $cells \cdot ml^{-1}$. The cells were separated using CD31 labelled magnetic Dynabeads® (endothelial cell-specific antibody, Invitrogen, UK). The Dynabeads (25 $\mu l \cdot ml^{-1}$) were incubated with the cell suspension at 2–8 °C with moderate rotation for 1 h, placed on a magnet (Invitrogen, UK) for 3 min. The flow-through was collected and the cells bound to the column were washed in PBS/BSA. This process was repeated twice. The unbound cells were also subjected to 3 steps of magnetic separation. The bound epithelial cells were collected by centrifugation (200 g) and re-suspended in supplemented M-199 medium while the unbound