Cytotoxic and apoptotic effects of cobalt and chromium ions on J774 macrophages – Implication of caspase-3 in the apoptotic pathway

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The aim of this study was to evaluate the cytotoxic and apoptotic effects of cobalt and chromium ions on macrophages in vitro, and analyze the implication of caspase-3 in the apoptotic pathway. J774 mouse macrophages (5 x 10^5 cells/ml) were exposed for up to 24 h to 0–10 ppm Co^{2+} and 0–500 ppm Cr^{3+}. The cytotoxic effect of ions was measured by Trypan blue exclusion. DNA analysis on agarose gel was used as a specific test for detection of DNA fragmentation into oligonucleosomes that occurs in apoptotic cells. The proteolytic cleavage of poly(ADP-ribose)polymerase (PARP), closely associated with the induction of apoptosis, was also analyzed along with the appearance of the active fragment of caspase-3, implicated in several apoptosis pathways. Results demonstrated that both Co^{2+} and Cr^{3+} ions induce macrophage mortality in a dose-dependent manner. However, Co^{2+} is more toxic inducing a cell mortality up to 28% with only 10 ppm vs. 37% with 500 ppm of Cr^{3+}. DNA analysis demonstrated that both Co^{2+} and Cr^{3+} ions induce DNA fragmentation, between 6-10 ppm Co^{2+} and 250-500 ppm Cr^{3+} after 24 h incubation. PARP cleavage and the appearance of caspase-3 active fragment were observed after 6 h with both Co^{2+} and Cr^{3+} ions, with a stronger signal after 24 h and 10 ppm of Co^{2+} or 500 ppm of Cr^{3+}. In conclusion, this study demonstrates that after 24 h incubation, both Co^{2+} and Cr^{3+} ions can induce macrophage mortality, and more specifically apoptosis. The results also suggest that apoptosis occurs via a caspase-3 pathway. However, the relative importance of necrosis and apoptosis and the effects of longer exposure times on the induction of macrophage death by these metal ions remain to be investigated.

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Introduction

Wear particles leading to periprosthetic osteolysis and subsequent loosening is a critical process that limits the longevity of total hip arthroplasty (THA) [1–2]. Polyethylene particles have been the main culprit in initiating osteolysis. Because of their potential for improved wear performance [3–7], there has been a revived interest in metal–metal (MM) bearings and they have been considered as an alternative to the use of metal–polyethylene bearings. However, metal ion toxicity remains a major cause for concern. Indeed, there are multiple sources of metallic corrosion products in the MM periprosthetic environment, including wear particles [8], the corrosion and the fretting of the head–neck interface [9, 10], and the disruption of the passive oxide layer during dynamic loading conditions [11]. These metallic corrosion products can circulate in both local and systemic manner, penetrate cell plasma membrane, bind cellular proteins or enzymes, or modulate cytokine expression.

Although there have been a few studies reported on the toxicity and the ability of metal ions and more specifically cobalt and chromium ions to induce cytokine release in cultured cell systems [12, 13], little is known about the mechanism of cell death induced by these ions. Previous studies conducted in our laboratory demonstrated the presence of macrophage apoptosis in both in vivo pseudomembranes from failed metal-polyethylene THAs [14] and in vitro particle-stimulated macrophages [15]. Granchi et al. also demonstrated the induction of apoptosis by cobalt and chromium ions from metal extracts on human peripheral blood mononuclear cells [16]. Apoptosis is an active form of cell death that requires the participation of active cellular processes [17]. The explosion of interest in apoptosis lies in the fact that it is under positive and negative regulation through

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evolutionary conserved biochemical pathways. One of the highlights of apoptotic cell death, as it applies to periprosthetic osteolysis, is that the whole process terminates in the elimination of dead macrophages without the induction of a high inflammatory reaction [18]. The identification of an apoptosis-related pathway in the macrophage response to wear particles and ions may then be a desired therapeutic endpoint and could provide crucial data for a rational approach in the treatment and/or prevention of periprosthetic osteolysis.

The aim of this study was to evaluate the cytotoxic and apoptotic effects of cobalt and chromium ions on J774 macrophages, and analyze the implication of caspase-3 in the apoptotic pathway.

Materials and methods
Cell culture
J774 mouse macrophages (ATCC, Rockville, MD, USA) were cultured and maintained in RPMI 1640 medium (Bio Media Canada, Drummondville, Quebec, Canada) supplemented with 5% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. J774 cells were exposed to Co^{2+} (CoCl₂, Fisher Scientific, Ville St-Laurent, Quebec, Canada) and Cr^{3+} (CrCl₃, Sigma Chemicals, Oakville, Ontario, Canada) at 5 × 10⁵ cells/ml of culture media with 0–10 ppm Co^{2+} and 0–500 ppm Cr^{3+} in tubes for cell mortality and in Petri dishes (35 × 10 mm) for Western blot analyses. Cells in 25 cm² culture flasks (5 × 10⁶ cells) were used for DNA laddering. Macrophages without ions served as negative control. Incubations were conducted at 37 °C in a 5% CO₂ environment.

Measure of cell mortality
After 24 h incubation with Co^{2+} or Cr^{3+}, the cytotoxic effect of ion stimulation on macrophages, evaluated by cell mortality, was obtained by measuring the percentage of dead macrophages by Trypan blue exclusion.

DNA laddering
DNA fragmentation into oligonucleosomes was used as a marker of apoptosis. DNA was isolated as recently described by Petit et al. [19]. DNA extracts were loaded onto 1.5% agarose gel containing 50 μg/ml ethidium bromide, run at 50 volts for about 2 h on Mupid-2 Minigel (Helixx Technologies, Scarborough, Ontario, Canada). DNA was then visualized directly upon illumination with UV light and photographed using Polaroid 667 film (ASA 3000).

Protein extraction and Western blot analysis
The appearance of poly(ADP-ribose)/polymerase (PARP) proteolysis, a marker of apoptosis, and the active fragment of caspase-3, implicated in several apoptotic pathways, were analyzed by Western blot as previously described [19]. Briefly, cells (5 × 10⁵) were homogenized in 50 μl of lysis buffer, cell extracts were spun at 5000 × g for 15 min, and the supernatants were collected as the source of total proteins. Protein samples were denatured at 100 °C, loaded on an 8% or 14% gel (PARP and caspase-3, respectively) and separated by SDS-PAGE. Blotting was performed using anti-PARP (1:1500) or anti-caspase-3 (3 μg/ml) as primary antibodies and peroxidase-conjugated anti-mouse IgG (1:10000) for PARP and anti-rabbit IgG (1:10000) for caspase-3 as secondary antibodies (Zymed Laboratories Inc., South San Francisco, CA). NEN Renaissance chemiluminescence substrates were used for detection. Autoradiographies were performed using Kodak X-Omat LS X-ray film.

Results
Cell mortality
Trypan blue analysis of cytotoxicity at 24 h demonstrated that both Co^{2+} and Cr^{3+} ions induce macrophage death in a dose-dependent manner. However, Co^{2+} is more toxic, with a significant mortality observed with 6 ppm Co^{2+} (12%) compared to 14% with 250 ppm Cr^{3+}. The

![Figure 1](https://example.com/figure1.png)

**Figure 1** Effects of Co^{2+} and Cr^{3+} ions on macrophage mortality. J774 macrophages were incubated for 24 h with Co^{2+} and Cr^{3+} ions and cell mortality was determined by Trypan blue exclusion. Results are the mean ± STD of nine experiments.