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 (5×10^5 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^+ 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+} (CoCl$_2$, Fisher Scientific, Ville St-Laurent, Quebec, Canada) and Cr^{3+} (CrCl$_3$, Sigma Chemicals, Oakville, Ontario, Canada) at 5×10^5 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 cm^2 culture flasks (5×10^6 cells) were used for DNA laddering. Macrophages without ions served as negative control. Incubations were conducted at 37 °C in a 5% CO_2 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 (5×10^5) were homogenized in 50 µl of lysis buffer, cell extracts were spun at $5000 \times g$ 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 : 10 000) for PARP and anti-rabbit IgG (1 : 10 000) 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
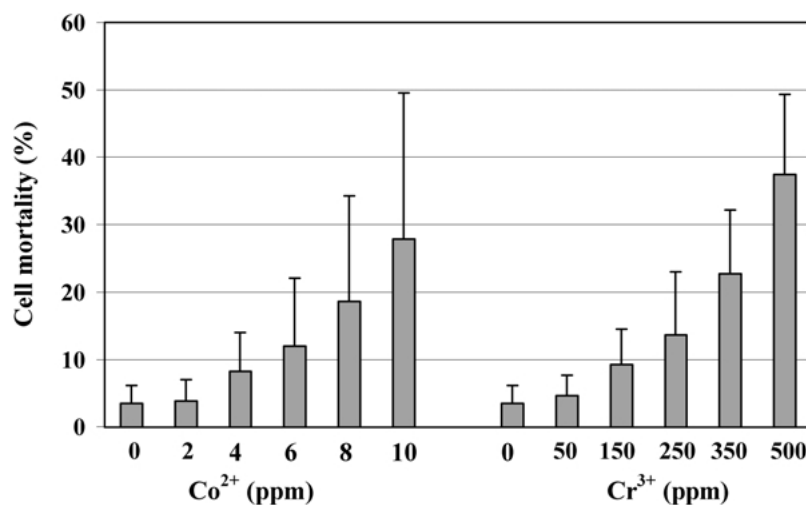

Figure 1 Effects of Co^{2+} and Cr^{3+} ions on macrophage mortality. J774 macrophages were incubated for 24 h with Co^{2+} and Cr^{3+} ions and cell mortality was determined by Trypan blue exclusion. Results are the mean $\pm$ STD of nine experiments.

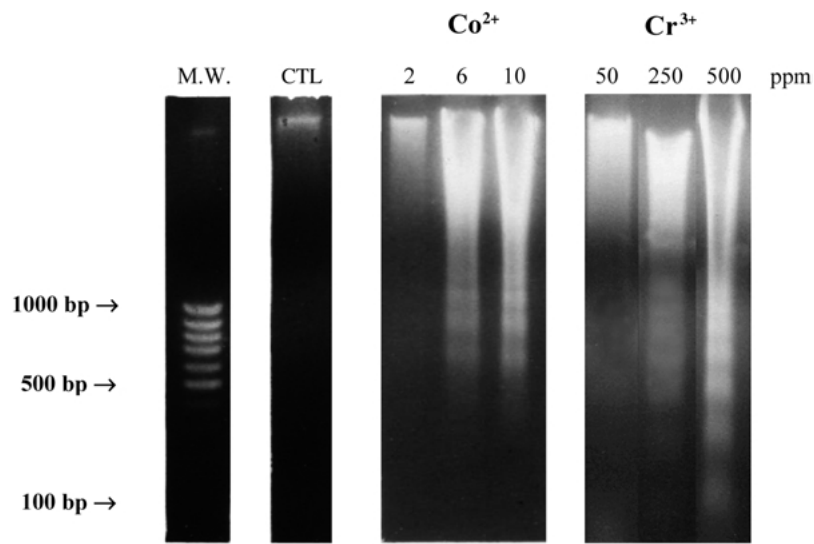*Figure 2* Effects of Co^{2+} and Cr^{3+} ions on macrophage DNA fragmentation. J774 macrophages were incubated for 24 h with Co^{2+} and Cr^{3+} ions and DNA fragmentation was analyzed on 1.5% agarose gel. Results are representative of three experiments.

mortality increased up to 28% with 10 ppm Co^{2+} vs. 37% with 500 ppm of Cr^{3+} (Fig. 1).

DNA laddering

DNA analysis demonstrated that both Co^{2+} and Cr^{3+} ions can induce macrophage apoptosis. After 24 h incubation, DNA fragmentation started to occur at 6 ppm Co^{2+} and 250 ppm Cr^{3+} with a stronger signal at 10 ppm Co^{2+} and 500 ppm Cr^{3+}. This fragmentation did not occur in control cells or with lower ion concentrations (Fig. 2).

PARP cleavage and caspase-3 activation

Western blot analysis revealed that both Co^{2+} and Cr^{3+} ions induce the appearance of the proteolytic fragment of PARP (85 kDa) starting after 6 h, and with a stronger signal after 24 h with 10 ppm Co^{2+} and 500 ppm Cr^{3+} (Fig. 3). The caspase-3 active fragment (17 kDa) was also observed after 6 h with Co^{2+} and Cr^{3+} with a stronger signal after 24 h incubation with 10 ppm Co^{2+} and 500 ppm Cr^{3+} (Fig. 4). The appearance of caspase-3 active fragment and the proteolysis of PARP preceded the induction of DNA fragmentation.

Discussion

In the present study, cytotoxic and apoptotic effects of cobalt and chromium ions were analyzed on J774 macrophages *in vitro*. Cytotoxicity can be reflected in many ways, i.e. cellular damage leading to cell death,
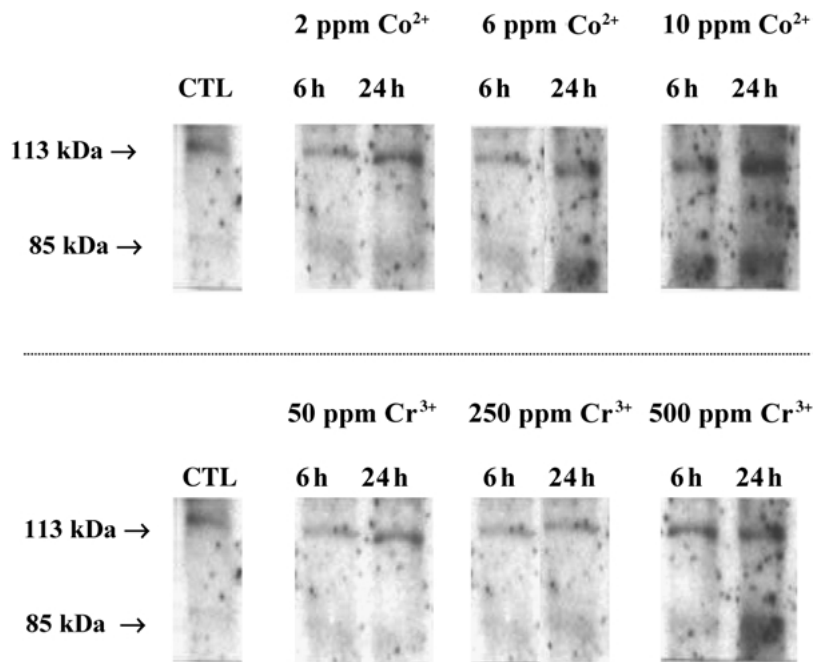

Figure 3 Effects of Co^{2+} and Cr^{3+} ions on macrophage PARP expression. J774 macrophages were incubated for 6 h and 24 h with Co^{2+} and Cr^{3+} ions and PARP expression was analyzed by Western blot. Results are representative of three experiments.
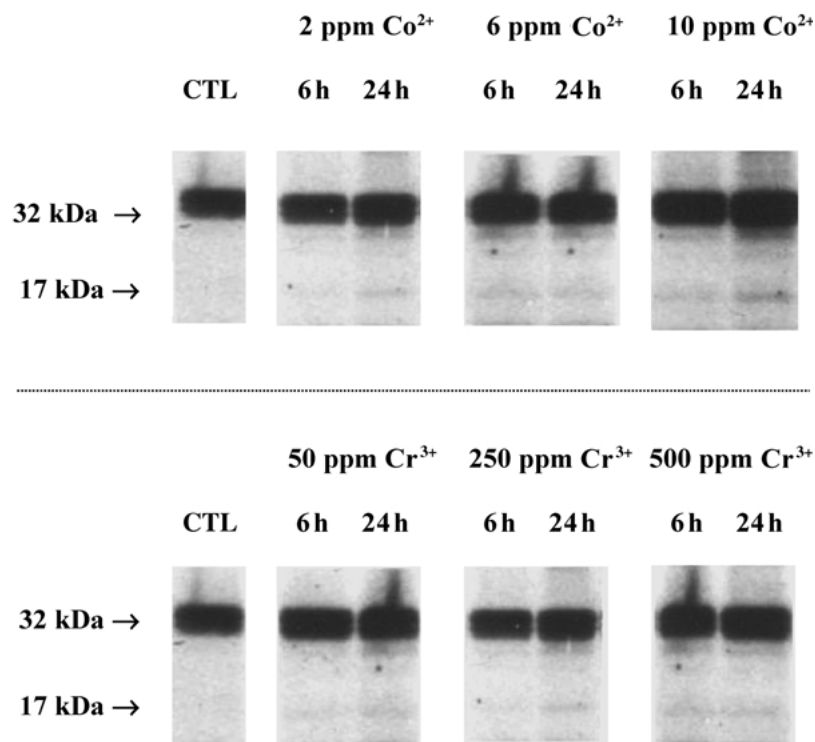
Figure 4 Effects of Co^{2+} and Cr^{3+} ions on macrophage caspase-3 expression. J774 macrophages were incubated for 6 h and 24 h with Co^{2+} and Cr^{3+} ions and caspase-3 expression was analyzed by Western blot. Results are representative of three experiments.

modified proliferation or altered metabolism. In the present study, cytotoxicity was analyzed in terms of cell mortality, and then apoptosis was analyzed as a specific type of cell death that may be a desired therapeutic endpoint in the treatment and/or prevention of periprosthetic osteolysis. Caspase-3 was finally analyzed as a potential pathway leading to apoptosis.

The J774 mouse macrophage cell line was used in this model, as in several other studies [15, 19–25], since it has been demonstrated to be morphologically similar to macrophages at the bone–cement interface and respond to cement in a similar way as macrophages at this interface [22]. Moreover, comparable results have been found using a human macrophage-like model [26, 27] or J774 macrophages [19] when looking at TNF-α release induced by ceramic and UHMWPE particles.

Results in the present study demonstrated that both Co^{2+} and Cr^{3+} ions can induce macrophage mortality. However, Co^{2+} is more toxic than Cr^{3+}, inducing a cell mortality up to 28% with only 10 ppm vs. 37% with 500 ppm of Cr^{3+}. This may be explained by a higher capacity of Co^{2+} to penetrate the cells. It is also worth noting that at the highest concentrations of Cr^{3+}, cell mortality may have been slightly misinterpreted due to a visual interference between the dark brown color of living cells caused by the Cr^{3+} ions adhering on cell membrane or in the cytoplasm and the dark blue color induced by the Trypan blue staining of dead cells. It may also be possible that Cr^{3+} ions binding to cell membrane partially prevented the Trypan blue to enter the cells, inducing an underestimation of cell mortality. Nevertheless, these results support previous studies reported in the literature on the toxicity of metal ions [12, 13, 16]. However, whereas Wang *et al.* demonstrated that cobalt and chromium ions could induce the release of cytokines, they did not find any cytotoxic effect [12]. This difference with our results can be attributed to differences in cell types, culture media, ion species and concentrations. Our choice of concentrations higher than those found in body fluids [28, 29] was based on the fact that the exact concentrations of ions in the immediate periprosthetic tissues are unknown and are most likely higher than those in body fluids where they get diluted and are eventually partially eliminated. Moreover, the present study was conducted using an *in vitro* model of cells that were not prestimulated. Therefore, these cells respond to higher concentrations than prestimulated cells [30, 31], or than cells *in vivo* that are stimulated and are interacting with many other environmental signals.

Although the toxicity of cobalt and chromium ions has been reported in a few cultured cell system studies, little is known about the mechanism of cell death induced by these ions. Cells can die by two principal pathways, necrosis or apoptosis. As previously mentioned, one major difference between apoptosis and necrosis is that the whole process of apoptosis terminates in the elimination of dead macrophages without the induction of a high inflammatory reaction whereas necrosis is characterized by a higher inflammatory reaction leading to severe tissue reaction and injury. The results of the present study demonstrated that, at 24 h exposure, both Co^{2+} and Cr^{3+} ions, at the concentrations analyzed, could induce macrophage apoptosis, characterized by the fragmentation of DNA into oligonucleosomes, considered a gold standard in the demonstration of apoptosis [32]. Moreover, results also demonstrated the appearance of the 85-kDa proteolytic fragment of PARP. This proteolytic cleavage of PARP, a nuclear protein implicated in DNA repair, is closely associated to the induction of apoptosis [33, 34]. These results support a

previous report on human peripheral blood mononuclear cells, demonstrating that, at 24 h, both Co and Cr ions induced apoptosis [16]. However, in the latter study, Co and Cr ions came from metal extracts. Therefore, the ions exposed to the cells were most likely a mixture of different valences, and the concentrations were not the same as the ones used in the present study. Our choice of using chloride solutions of $CoCl_2$ and $CrCl_3$ to expose macrophages to Co^{2+} and Cr^{3+} and not to higher valences such as Cr^{6+}, was based on the fact that Cr ions with a higher valence are much more unstable in solution and it becomes difficult to quantify the observations in a dose-dependent manner.

The interest in apoptosis lies in the fact that this form of cell death is under the control of multiple proteins, including the caspases. The present study demonstrated that up to 24 h, both Co^{2+} and Cr^{3+} ions can induce the appearance of the active fragment of caspase-3, a protease of the caspase family that is implicated as ''executioner'' in the majority of the apoptosis pathways, and more specifically in the cleavage of PARP [35]. Therefore, the demonstration of the early appearance (starting at 6 h) of both the proteolytic fragment of PARP and the active fragment of caspase-3 preceding irreversible DNA fragmentation at 24 h, suggests that both Co^{2+} and Cr^{3+} ions induce apoptosis via a caspase-3 pathway.

In conclusion, the identification of a caspase-3 apoptosis-related pathway in the macrophage response to cobalt and chromium ions could provide crucial data for a rational approach in a better understanding of the overall tissue response in MM THAs. 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.

Acknowledgments

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