

***In vitro* macrophage response to
nanometer-size particles
from materials used in hip implants**

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Abstract

Wear particle-induced inflammation leading to periprosthetic osteolysis remains a major cause of hip implant failure. As polyethylene particles from conventional metal-on-polyethylene implants have been associated with these failures, an interest in lower wear metal-on-metal (MM) bearings has emerged. However, the biological effects of nanometer-size chromium oxide particles, predominant type of wear particles produced by MM implants, remain mostly unknown. Therefore, this study aimed to determine the cytotoxicity of nanometer-size Cr_2O_3 particles on macrophages *in vitro*, by analyzing their effects on cell mortality and cytokine release and comparing them with those of similarly-sized alumina (Al_2O_3) particles (known to be relatively bioinert). Results showed that at high concentrations, nanometer-size Cr_2O_3 particles can be cytotoxic to macrophages, inducing significant decreases in total cell numbers and increases in necrosis. Results also showed that, at high concentrations, the cytotoxicity of Cr_2O_3 particles was overall higher than that of Al_2O_3 particles, even though Cr_2O_3 and Al_2O_3 are both stable forms of ceramic materials. However, it appeared to be lower than that of previously reported conventional polyethylene and CoCrMo particles. Therefore, chromium oxide particles may not be the main culprit in initiating the inflammatory reaction in MM periprosthetic tissues.

List of Acronyms

Al ₂ O ₃	-	Alumina
ANOVA	-	Analysis of variance
CC	-	Ceramic-on-ceramic
CoCrMo	-	Cobalt chromium molybdenum
CPE	-	Ceramic-polyethylene
Cr ₂ O ₃	-	Chromium oxide
DMEM	-	Dulbecco's Modified Eagle Medium
ELISA	-	Enzyme-linked immunosorbent assay
FBS	-	Fetal bovine serum
FS	-	Forward scatter
FITC	-	Fluorescein isothiocyanate
HXPE	-	Highly crosslinked polyethylene
IL	-	Interleukin
LAL	-	Limulus Amebocyte Lysate
LPS	-	Lipopolysaccharide
MC	-	Million cycles
MCP-1	-	Monocyte chemotactic protein-1
MIP-1 α	-	Macrophage inflammatory protein-1 alpha
MM	-	Metal-on-metal
MPE	-	Metal-on-polyethylene
PARP	-	poly(ADP-ribose) polymerase
PBS	-	Phosphate buffered saline
PE	-	Polyethylene
PI	-	Propidium iodide
PS	-	Phosphatidylserine
SEM	-	Scanning electron microscopy

SS	-	Side scatter
TEM	-	Transmission electron microscopy
Ti6Al4V	-	Titanium alloy
THA	-	Total hip arthroplasty
THR	-	Total hip replacement
TNF- α	-	Tumour necrosis factor alpha
UHMWPE	-	Ultra high molecular weight polyethylene

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1 Background

1.1 Total Hip Arthroplasty

Total hip arthroplasty (THA) is one of the most common and most successful orthopaedic procedures performed [1]. Primary THAs are necessary in case, for example, of degenerative osteoarthritis, osteonecrosis and acute fractures [2], and a successful surgery will relieve pain and improve joint function [1]. In 2006, over 24,000 hip replacement surgeries were performed in Canada [2], a figure which had increased by nearly 60% since 1997 [2]. The demand for hip replacements is expected to continue rising [3] as our baby boomer population (i.e., those born between 1946-1964) ages and as increasingly younger patients are demanding implants [1].

A hip replacement may be a resurfacing implant (a bone-conserving method where the femoral head is resurfaced and the acetabulum is replaced) (Figure 1), or a total hip replacement (THR) using a femoral stem device (where the femoral head and acetabulum are replaced) [2] (Figure 1). Stem-type devices are by far the more practiced option. Indeed, in Canada, hip resurfacings accounted for fewer than 2% of implants between 2003-2007 [2].



Figure 1: A hip resurfacing implant (left) and a THR stem-type device (right) [4].

For successful implementation, the implant must be well fixed in the host bone. The fixation method for the implant may be cemented, cementless (sometimes with screws for the acetabular cup) or hybrid (i.e., different for the stem and the acetabular cup). The majority of implants from 2003-2007 in Canada were cementless [2].

The prosthetic head is made of a metal or ceramic and articulates with a metal, polymer or ceramic acetabulum. The most common pairing of bearing surfaces is metal-on-polyethylene (MPE), followed by ceramic-on-ceramic (CC) and metal-on-metal (MM) articulations (78%, 11% and 9% of hip replacements in 2006, respectively [2]). Ceramic-on-polyethylene (CPE) and ceramic-on-metal implants are also available but are not widely used in Canada [2]. The polyethylene (PE) used in the MPE or CPE bearing surfaces is either conventional ultra high

molecular weight PE (UHMWPE) or, more recently, highly cross-linked PE (HXPE). CC implants are typically made of alumina (Al_2O_3) and MM implants are made of cobalt chromium molybdenum (CrCoMo) alloy.

First generation MM implants were developed the 1960s and gained popularity in response to poor wear performance from some Charnley MPE implants that were available at the time [5, 6]. These MM implants included the McKee, Farrar, Ring and Sivash designs and offered lower wear compared to the MPE bearings [5, 6]. Seizing, imprecise tolerances between components and high frictional torque causing some early failures of the MM articulations brought about a popularization of MPE bearings [5-7]. However, many MM implants remained successfully implanted for long periods with no evidence of osteolysis [8-10]. Similarly, in the 1970s, CC implants were introduced as a low wear alternative to MPE bearings, but reports of high fracture rates in some cases [11, 12] caused the CC implants to lose favour to MPE bearings. In the 1990s, a high incidence of osteolysis causing aseptic loosening associated with MPE implants was recognized [13], which motivated a return to alternative bearing surfaces and the development of current MM and CC articulations [14]. Newer MM implants with larger diameter heads as well as lower surface roughness and out-of-roundness were developed to improve stability and wear over previous versions [8, 15]. Today's CC implants are medical grade and comprise purer compositions with finer grain sizes and more homogeneous densification [12] making them much less prone to fracture [16].

Of all the THAs in 2006 in Canada, approximately 13% were revisions [2]. The most common

reason for required revisions was implant failure due to aseptic loosening, itself caused by periprosthetic osteolysis [2]. As each revision surgery (along with osteolysis induced by previous implant wear particles and stress shielding) severely reduces proximal femoral bone stock [17], multiple revision surgeries should be avoided, if at all possible.

1.2 Periprosthetic Osteolysis

A major cause of implant failure is aseptic loosening, to which wear particles largely contribute by inducing periprosthetic osteolysis [2, 18, 19]. Articulating implants produce wear particles which disseminate into periprosthetic tissues [20]. Macrophages, whose role is to ingest and degrade foreign bodies [21], engulf the particles [21-23] by the mechanisms of phagocytosis (particles sized from about 0.15 μm to 10 μm) and pinocytosis (for smaller particles) [24]. Since the macrophages cannot fully degrade the particles, they become activated [25] and initiate an inflammatory response [18], undergo apoptosis [26] or necrosis [27], and release pro-inflammatory mediators (cytokines and chemokines) [18, 19, 23, 28]. The cytokines and chemokines act to influence the behaviour of other cells causing increased differentiation of osteoclasts and decreased osteoblast function, resulting in osteolysis (i.e., bone loss) around the implant [18, 25, 29]. Additionally, the bone-forming ability of osteoblasts is limited after exposure to wear debris [30, 31], and lysosomal enzymes and metalloproteinases released by the cells in the periprosthetic environment act directly on bone to cause further bone loss [31, 32]. Osteolysis can be identified radiographically as osteolytic regions are characterized by

radiolucencies of the bone [33]. Macrophages have been shown to be the primary cell type in inflammatory periprosthetic tissues [33].

Monocytes (precursors of macrophages) are produced in the bone marrow and are mobilized to the vascular compartment for intra-corporeal circulation. To enter the tissues where they mature into macrophages, monocytes adhere to the blood vessel endothelium and migrate across the blood vessel wall [23]. Under normal conditions, monocytes enter the tissues as resident macrophages which play an important role in keeping the tissues clear of antigen and debris. Cells from the monocyte-macrophage lineage are known for their heterogeneity and plasticity [34]. Exposure of the monocytes to different cytokines and microbial products can cause the cells to differentiate, become activated and hold specialized functions [34]. Such macrophages may be referred to as being polarized and are classified as M1 or M2 types. M1 macrophages have been reported to be activated by cytokine interferon gamma ($\text{IFN}\gamma$), selected cytokines (i.e., $\text{TNF-}\alpha$) as well as microbial products, while M2 macrophages comprise activated macrophages with the exclusion of the M1 type and include cells exposed to IL-4 or IL-13, immune complexes, IL-10, and glucocorticoid hormones [34]. In general, M1 cells produce inflammatory cytokines efficiently, while M2 cells vary in their production capacity of these proteins [34].

Cytokines and chemokines released from macrophages are the main players in the inflammatory response [21, 25, 35]. As previously mentioned, once activated, macrophages may produce a variety of cytokines and chemokines (cell signaling molecules) to influence the

behavior of other cells [23, 36, 37]. During inflammation, cytokines attract inflammatory cells (neutrophils, monocytes, lymphocytes and macrophages) to the site of inflammation [23, 36, 37]. Macrophages and the released cytokines (such as tumor necrosis factor alpha (TNF- α), Interleukin-1 (IL-1), and Interleukin-6 (IL-6)) as well as chemokines (such as monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1 alpha (MIP-1 α)) are usually found in large quantities in periprosthetic tissues of failed implants [18, 25, 35]. For *In vitro* macrophages have been shown to release TNF- α after being stimulated with bone cement, high density polyethylene (HDP), Al₂O₃, Ti6Al4V, CoCrMo and Ti particles [22, 38-42], MCP-1 after incubation with Ti6Al4V and Al₂O₃ particles [40, 43], and MIP-1 α after being stimulated by Ti6Al4V and bone cement particles [44].

Inflammatory mediator signaling by cytokines and chemokines can induce differentiation of macrophage lineage cells and osteoclast precursors into osteoclasts and promote recruitment of osteoclasts to the periprosthetic region [23, 37]. Many cytokines and chemokines indirectly modulate the activity of osteoclasts by inducing the expression of receptor activator of NF κ B ligand (RANKL), an important regulator of osteoclast differentiation and maturation [37, 45]. Although the RANK/RANKL pathway has been recognized as playing a key role in the onset and perpetuation of osteoclastogenesis associated to periprosthetic osteolysis [45], other pathways may also play a role. For example, the inflammasome danger signalling pathway has been reported to mediate macrophage reactivity in human macrophages in response to metal ions and CoCrMo particles [46].

Cell apoptosis and necrosis are two distinct modes of cell death that have been observed in periprosthetic tissues [26, 27]. Apoptosis is an active form of programmed cell death, sometimes referred to as “cell suicide”, that is initiated in response to stress or because a cell is not needed [26, 47]. Cell morphology changes include shrinkage followed by chromatin condensation (pyknosis), cell surface blebbing, organized nuclear and DNA fragmentation, and finally the formation of apoptotic bodies (having intact membranes) which are removed by phagocytes (such as macrophages) [26, 47]. As the cell membrane maintains its integrity throughout the process, the release of mediators (cytokines) into the extracellular environment is limited [48]. Apoptosis can be beneficial when controlled, for example during embryonal development [48]. In contrast to apoptosis, necrosis is a passive, uncontrolled form of cell death that occurs due to tissue injury and in acute inflammatory response [26, 48]. Cells undergo swelling, complete disintegration of organelles, uncontrolled DNA fragmentation, and cell membrane deconstruction and rupture [26, 48, 49]. Intracellular contents leak from the cells, releasing potentially inflammatory mediators (cytokines and chemokines) which elicit and perpetuate inflammatory reactions [26, 47, 48]. A notable difference between apoptosis and necrosis is in the association with inflammation – while necrosis is known to cause and participate in inflammatory response, a lesser defined association exists between apoptosis and inflammation [26, 48].

1.3 Implant Wear

Implant wear plays an important role in the incidence and intensity of cytokine release, cell death, and osteolysis around the implant [14, 19, 21, 29, 50-52]. Higher levels of activity post-surgery have been reported to cause greater volumes of implant wear [1, 29]. Implant design [18], implantation time [53], lubrication at the joint [54, 55], surgical technique [29, 55], and patient-related factors (such as age, height, weight and gender) [29] also influence implant wear rates and patterns.

1.3.1 Wear Particles

Wear particles are continually generated during articulation of the implant [29]. Each type of implant bearing surface produces particles with different characteristics (such as size and shape) at different wear rates [5, 29, 50, 53-74].

1.3.1.1 UHMWPE and HXPE wear particles

UHMWPE is used in conventional MPE hip replacements. These bearings have been implanted for over forty years and were developed for their relative wear resistance at that time as well as ease of alignment [75]. Some studies illustrating the characteristics of UHMWPE wear particles are listed in Table 1.

Table 1: UHMWPE particle size and shape, and wear rate of metal-UHMWPE bearings (MC = million cycles).

Particle size	Particle shape	Wear Rate
<u><i>In vitro</i></u> Averages of 0.1 μm to 1.96 μm [76, 77] <u><i>In vivo</i></u> Averages of 0.07 μm to 6.3 μm (for the round) and up to 200 μm in length (for the needle incl. fibrils and shreds) [50, 78-80]	Round [50, 76, 79, 80] and needle-shaped [50, 79, 80]	<u><i>In vitro</i></u> Linear wear at 0.056 mm/million cycles (MC) [81]; volumetric wear between 13 mm^3/MC and approximately 30 mm^3/MC [5, 81] <u><i>In vivo</i></u> Linear wear between 0.10 mm/year and 0.26 mm/year [66, 72, 81, 82]; volumetric wear between 55.5 mm^3/year and 74 mm^3/year [5, 81].

Due to an association between UHMWPE particles and osteolysis [20, 81, 83-85], HXPE has been developed for hip implants because of its higher wear resistance, and has been gaining popularity in recent years [2]. HXPE has been reported to exhibit lower volumetric wear and generate smaller wear particles compared to UHMWPE [56, 65, 66, 76, 83, 86]. However, it is also more brittle. Some studies illustrating the characteristics of HXPE wear particles are listed in Table 2.

Table 2: HXPE particle size and shape, and wear rate of metal-HXPE bearings (MC = million cycles).

Particle size	Particle shape	Wear Rate
<u><i>In vitro</i></u> Sizes in the range of around 0.01 μm to 1 μm [76, 77], mostly <0.55 μm [76] and even <0.1 μm [77] <u><i>In vivo</i></u> Average of 0.66 μm [65]	Mostly round (granular) [65, 76, 77], with only a small proportion of fibrils and flakes [76]	<u><i>In vitro</i></u> Linear wear at 0.013 mm/MC [81]; volumetric wear at 8.0 mm^3/MC [81]. <u><i>In vivo</i></u> Linear wear between 0.01 mm/year and 0.06 mm/year [56, 66, 83, 86]; volumetric wear at 15.4 mm^3/year [81]; and 5.33×10^7 particles per gram in tissues [65].

1.3.1.2 *Al₂O₃ wear particles*

CC implants are typically made of a dense Al₂O₃. This material was chosen for desirable qualities such as high hardness, non-corrosive properties, and a relatively bioinert composition [87]. However, although rather rare, brittle fracture of ceramic implant bearing surfaces can occur [88, 89].

These implants produce Al₂O₃ wear particles at very low wear rates [14, 67, 69, 71, 90]. Such particles were reported to be in the micrometer-size range when characterized using scanning electron microscopy (SEM) after being isolated from periprosthetic tissues [91-93]. However, they have also been reported to exhibit a bimodal size distribution in the nanometer and micrometer ranges when characterized using transmission electron microscopy (TEM) after being isolated from a hip simulator under microseparation conditions [71] or from periprosthetic tissues [63]. Some studies illustrating the characteristics of Al₂O₃ wear particles are listed in Table 3.

Table 3: Al₂O₃ particle size and shape, and wear rate of CC bearings (MC = million cycles).

Composition	Size	Shape	Wear Rate
Al ₂ O ₃	<u><i>In vitro</i></u> Bimodal distribution of 1-35 nm and 0.02-10 µm (under microseparation, using TEM) [71] <u><i>In vivo</i></u> Averages of 0.44 µm to 0.71 µm (using SEM) [91-93]; bimodal distribution of 5-90 nm and 0.05-3.2 µm (using TEM) [63].	Mainly round [70]	<u><i>In vitro</i></u> Linear wear at 16.1 ± 7.5 µm/MC run-in & 0.4 ± 10.1 µm/MC steady state [54]; volumetric wear at 0.5 mm³/MC run-in and 0.05 mm³/MC to 0.1 mm³/MC steady-state [60, 69], or 0.004 mm³/MC to 1.74 mm³/MC [5, 70, 71] <u><i>In vivo</i></u> Linear wear undetectable [72, 90]; volumetric wear at 1-5 mm³/year [67]

1.3.1.3 Chromium oxide and CoCrMo particles

As previously mentioned, MM implants are made of CoCrMo alloy. CoCrMo alloy was chosen for its high hardness, high toughness and wear resistance [53, 58]. MM implants have shown relatively low wear, smaller wear particles and lower levels of osteolysis compared to conventional MPE implants [73, 74, 94-96]. CoCrMo alloy offers the advantage of being self-polishing *in vivo* [54, 57, 68, 97], as well as fluid film lubrication and low wear when performing well [54, 55].

This bearing has been shown to produce mainly chromium oxide particles with some CoCrMo particles [53, 58, 59, 61, 64]. Oxidation states of chromium include +6, +3 and +2, where +3 is the most stable. It is not yet clear what forms of chromium oxides are produced by the MM implant wear, hence why they are referred to as “chromium oxide” particles in the present

study. However, Cr₂O₃ particles were chosen for the analysis of the biological effects as they are stable and commercially available and Cr₂O₃ is one of the principle oxides of chromium. Some studies illustrating the characteristics of MM wear particles are listed in Table 4.

Table 4: MM particle composition, size and shape, and wear rate of MM bearings (MC = million cycles).

Composition	Particle size	Particle shape	Wear Rate
Chromium oxide (predominant particle type) [53, 58, 59, 61, 64]	<u><i>In vitro</i></u> Averages mainly around 40 nm to 50 nm [58, 59, 98] (vary with cycle numbers, plus small differences due to alloy [58,59]) <u><i>In vivo</i></u> Averages mainly around 30 nm to 60 nm [53, 59, 61, 64] (vary with implantation time [53, 59])	Mainly round to oval [53, 58, 59, 61, 64, 98]	<u><i>In vitro</i></u> Linear wear at $23.4 \pm 5.7 \mu\text{m}/\text{MC}$ run-in & $5.6 \pm 7.3 \mu\text{m}/\text{MC}$ steady-state [54]; volumetric wear between $0.119 \text{ mm}^3/\text{MC}$ and $1.23 \pm 0.5 \text{ mm}^3/\text{MC}$ [5, 70] <u><i>In vivo</i></u> Between $4.2 \mu\text{m}/\text{year}$ and $6 \mu\text{m}/\text{year}$ [97, 99]; volumetric wear at $6 \text{ mm}^3/\text{year}$ or less [68, 97, 100].
CoCrMo [53, 58, 59, 61, 64]	<u><i>In vitro</i></u> Averages around 70 nm to 80 nm [58, 59] (vary with cycle numbers, plus small differences due to alloy [58, 59]) <u><i>In vivo</i></u> Averages around 50 nm to 120 nm [53, 59, 61, 64] (vary with implantation time [53, 59])	Mainly needle-shaped [53, 58, 59, 61, 64]	

1.3.2 Biological Effects of Wear Particles

The biological response to wear particles has been shown to vary primarily with particle composition, size, shape and number [14, 21, 22, 29, 50-52, 79].

Size: Multiple studies have shown that particles in the phagocytosable range (i.e., up to about 10 μm) [22, 101] can be inflammatory [33, 41, 51, 101-103], the most pro-inflammatory range being between 0.1 μm and 1.0 μm [33, 52].

Shape: Elongated (needle-shaped) particles have been reported to be more pro-inflammatory than round particles [102].

Number: In general, the inflammatory response tends to be particle number-dependent; a larger number of particles induces a more pronounced inflammatory response than a lesser number of equivalent particles [22, 41, 51].

1.3.2.1 *Particles from MPE implants*

Conventional MPE implants (made of UHMWPE) have been associated with a high incidence of periprosthetic osteolysis [81, 83-85]. As depicted in Table 1, UHMWPE wear particles are relatively large [50, 76] and are produced in great numbers due to relatively high volumetric wear rates of conventional MPE bearings [82] compared to wear particles from other types of implants such as CC and MM. UHMWPE particles have been shown to induce cell mortality, primarily necrosis both *in vitro* [104] and *in vivo* [26, 105]. They have been shown to induce the release of several inflammatory cytokines, including TNF- α *in vitro* [41, 76, 104] and *in vivo* [106, 107], IL-1 β and IL-6 *in vitro* [41], in a murine calvaria model [108] and *in vivo* [107], as well as IL-1 α *in vitro* [76].

Short term *in vivo* studies have shown a decreased incidence of periprosthetic osteolysis with implants made of HXPE over UHMWPE [81, 83-85, 109], likely due to the significant decrease in wear particle size [65, 76] as well as volumetric and linear wear [66, 81, 83] (see Table 2). In comparable doses, however, HXPE particles have been shown to induce similar to more TNF- α compared to UHMWPE particles *in vitro* [21, 76, 110]. Overall, the HXPE bearing surface shows promise compared to conventional PE bearing surface; however long term studies have yet to be conducted [14, 66].

1.3.2.2 *Particles from CC implants*

CC implants made of alumina have been associated with low levels of periprosthetic osteolysis [87, 111-113] and aseptic loosening [72]. These low wear implants produce small Al₂O₃ particles (see Table 3 above) which have been shown to be rather bioinert *in vitro* [112] and when implanted into murine calvaria [108], causing relatively low levels of *in vitro* cell mortality [22, 39, 62, 114] and low levels of cytokine release from human monocytes [115, 116], human macrophages [40] and mouse macrophages [104]. These particles have been shown to induce primarily cell apoptosis *in vitro* [104, 117].

1.3.2.3 *Particles and soluble ions from MM implants*

Although the inflammatory response in periprosthetic tissues from failed MM implants has been reported to be lower than that in tissues surrounding conventional MPE implants, cytokines associated with bone resorption and implant loosening can still be found in MM periprosthetic tissues [94, 107]. In addition, MM wear products may elicit a hypersensitivity

reaction [118-120] which may play a role in some early MM implant failures that have been increasingly reported recently [121-123].

Most *in vitro* studies on the biological effects of MM wear particles have been conducted with CoCrMo particles. Some studies have shown limited cytokine release with both nanometer- and micrometer-size CoCrMo particles (low levels of TNF- α [124], IL-6 [124, 125], IL-1 β [40] and MCP-1 [40]). However, other studies have reported cytotoxic effects of such particles, including cell mortality [62, 114]. Some studies have also shown that micrometer-size CoCrMo particles can induce inflammatory cytokine release including TNF- α *in vitro* [40] and in a murine calvaria model [126], as well as IL-6 and IL-8 *in vitro* [40]. Micrometer-size CoCrMo particles have also been reported to induce osteolysis and increase the content and activity of osteoclasts in an animal model [126], as well as decrease the proliferation of mesenchymal stem cells *in vitro* [127].

Sources of corrosion products from the MM implants may include chemically reactive wear particles, head-neck interface fretting and passive oxide layer disruption [128, 129]. Cobalt and chromium ions can form at these sites [62], bind to proteins [119, 130], and disseminate throughout the body [131-133]. Significantly higher ion levels in the serum, synovial fluid, blood and urine have been reported post-implantation [131-133]. Cr⁶⁺ ions have been found to be highly cytotoxic [128]. Co²⁺ ions are known to be more cytotoxic than Cr³⁺ ions [134, 135], but both have been shown to induce cell mortality (including apoptosis and necrosis) [134-137] and cytokine/chemokine release *in vitro* [128, 136, 138, 139].

While some studies have reported on the cytotoxicity of CoCrMo particles and soluble ions, the biological effects of clinically relevant chromium oxide particles (which have been shown to be the predominant particle type produced by MM implants [53, 58, 59, 61, 64]) have not been examined specifically. One study measuring the effects of nanometer-size chromium particles (most likely chromium oxide particles due to the propensity of nanometer-size chromium particles to instantaneously oxidize) reported no significant decrease in macrophage number *in vitro*, despite a substantial decrease in cell number due to these particles depicted in the results of that study [135]. Without further research into the biological effects of these particles, the effects of MM wear products cannot be fully understood.

2 Hypotheses and Objectives of the Study

It is critical to optimize the life-span of hip replacements in order to reduce or eliminate the need for revision surgeries. Studying and comparing the macrophage response to clinically relevant wear particles is an important step in isolating contributing factors in the inflammatory response leading to wear particle-induced periprosthetic osteolysis. Certain indicators of inflammation, such as macrophage mortality and cytokine release, can provide clues in determining relative osteolytic capacities of specific types of implant wear particles.

2.1 Hypotheses

Wear particle composition, size, shape, and concentration have been shown to influence the macrophage response and the overall inflammatory response in periprosthetic tissues ([14, 21, 22, 29, 50-52]). Round to oval chromium oxide particles (predominant type of particles produced by MM implants [53, 58, 59, 61, 64]) may play a role in the inflammatory response around MM implants. The cytotoxicity of these particles has not been established and may depend on their size and concentration. Therefore, the hypotheses of the study were as follows:

- 1) macrophage response to Cr_2O_3 particles is dependent on particle size and concentration of particles per cell; and
- 2) because both Cr_2O_3 and Al_2O_3 are two stable forms of ceramics, the cytotoxic effects of nanometer-size Cr_2O_3 on macrophages are similar to those of Al_2O_3 particles.

2.2 Objectives

The overall goal of this study was to establish the cytotoxicity of clinically-relevant chromium oxide particles on macrophages *in vitro*. The specific objectives were:

- 1) to determine the effects of 60 nm vs. 700 nm Cr_2O_3 particles at different concentrations; and
- 2) to compare the effects of 60 nm Cr_2O_3 particles with those of similarly sized (50 nm) Al_2O_3 particles.

The biological effects of the particles were determined by measuring indicators of inflammatory response, including cell mortality (total cell number, apoptosis and necrosis) and cytokine/chemokine release. The 60 nm Cr₂O₃ and 50 nm Al₂O₃ particles were chosen specifically for their clinical relevancy (as shown in Table 3 and Table 4). The 700 nm Cr₂O₃ were chosen to isolate the effects of particle size. All particles were commercially available.

3 Materials and Methods

3.1 Particles

The following particles were commercially obtained:

Table 5: Particle characteristics and sources.

Particle composition	Mean particle diameter	Particle shape	Source
Cr ₂ O ₃	60 nm	Mostly round	Sigma, St. Louis, MO
Cr ₂ O ₃	700 nm	Mostly round	Acros, Geel, Belgium
Al ₂ O ₃	50 nm	Mostly round	American Elements, Los Angeles, CA

Particles were sterilized for 1-3 hours in 70% ethanol followed by 10 minutes in an ultrasonic bath to break up clumps and allow particle resuspension. The particles were then washed three times in phosphate buffer saline (PBS) (Wisent, St. Bruno, QC) to remove traces of the

ethanol prior to incubation with the cells. The particles were resuspended in Dulbecco's Modified Eagle Medium (DMEM) (Wisent, St. Bruno, QC) with 5% fetal bovine serum (FBS) (Wisent, St. Bruno, QC) and then serially diluted to achieve the following concentrations:

- 0.5 million, 1.5 million, 2.5 million and 3.5 million particles per macrophage (60 nm Cr₂O₃);
- 500, 1000, 1500 and 2000 particles per macrophage (700 nm Cr₂O₃); and
- 0.5 million, 1.5 million, 2.5 million and 3.5 million particles per macrophage (50 nm Al₂O₃).

As the biological effect of particle volume per cell was of interest, the volume of particles per cell was calculated using the following formula, assuming spherical particles:

$$V = \frac{4}{3}\pi r^3 \times n$$

(1)

Where,

V = volume of particles per cell (μm³)

r = radius of spherical particle (μm)

n = number of particles per cell

Particles were tested for endotoxins using the ToxinSensor™ kit (Genscript, Piscataway, NJ).

Briefly, particles were sterilized, washed and serially diluted (as described above) in PBS.

Particles were then collected and resuspended in 1 ml of kit-provided Limulus Amebocyte Lysate (LAL) reagent water and incubated for 1 hour in a 37°C oven, followed by sonication for 1 hour to release the endotoxins, as previously described [38, 41]. Samples were centrifuged for collection and analysis of supernatants, and kit-provided standards and reagents were prepared as per the manufacturer protocols. Color changes (due to endotoxins catalyzing the activation of enzymes) were read by absorbance using a Biotek Synergy 4 Microplate reader (Biotek, Winooski, VA) at 545 nm with subtraction of blank. Absorbance values were compared against a linear standard curve to calculate endotoxin concentrations (EU/ml). Sensitivity of the ToxinSensor™ kit was 0.25 EU/ml. Particles tested negatively for endotoxins, all particles having levels <0.005 EU/ml.

3.2 Cells

J774 mouse macrophages (ATCC, Burlington, ON) were used in this study due to their morphological similarities with macrophages found at the bone-cement interface [140, 141]. Macrophages were sub-cultured in DMEM supplemented with 5% FBS, in standard cell culture conditions, i.e., at 37°C and 5% CO₂, in a humidified environment.

Cells were exposed to particles at the following concentrations: 0.5 million, 1.5 million, 2.5 million and 3.5 million particles per cell (60 nm Cr₂O₃); 500, 1000, 1500, 2000 particles per cell (700 nm Cr₂O₃); and 0.5 million, 1.5 million, 2.5 million and 3.5 million particles per cell (50 nm Al₂O₃), as previously mentioned. Half a million macrophages were used for each condition,

resuspended in 1 ml of medium containing the particles. Macrophages incubated without particles served as negative controls, and macrophages incubated with lipopolysaccharide (LPS) (Sigma Aldrich, St. Louis, MO) were used as positive controls for cytokine quantification, as previously reported [42]. Incubations were conducted in 5 ml tubes for up to 24 hours in standard cell culture conditions as described above. Since the J774 macrophages are adherent cells, a rotator was used to ensure constant resuspension of the particles and cells in the medium, similarly to previous studies [22, 39].

3.3 Flow cytometry principle

Flow cytometry is a powerful technique for compiling characteristics of a large cell population by allowing analysis of individual cells at very high flow rates (up to >1000 cells/second) [142]. A flow cytometer (Figure 2) comprises a laser, a flow cell with a flow channel, a lens, filters for isolating different wavelengths of light, and photomultiplier tubes for sensing and measuring the outputs [142, 143]. Suspended cells are hydrodynamically focused into a single file stream of sheath fluid (mainly a saline solution) in the flow channel as illustrated in Figure 3 and are moved through a laser beam in a quartz flow chamber. The photomultipliers (light and fluorescence detectors) measure scattering and fluorescence of the light simultaneously. The signals reaching the photomultipliers are amplified and presented by the software in histograms (showing intensity distribution for any one parameter) and dot-plots (intensity distribution for two parameters) [143]. Forward scatter (FS) measures scatter of light near the forward direction (giving some indication of cell size), and side scatter (SC) measures light

scatter near ninety degrees from the angle of incidence (reflecting the complexity within the cell, such as granularity of the membrane, nucleus and cytoplasm) [144]. The excitation of fluorophores (such as fluorescein isothiocyanate FITC) produces fluorescent light when excited by the laser [143].



Figure 2: Picture of the flow cytometer used for the present study (Beckman Coulter FC 500).

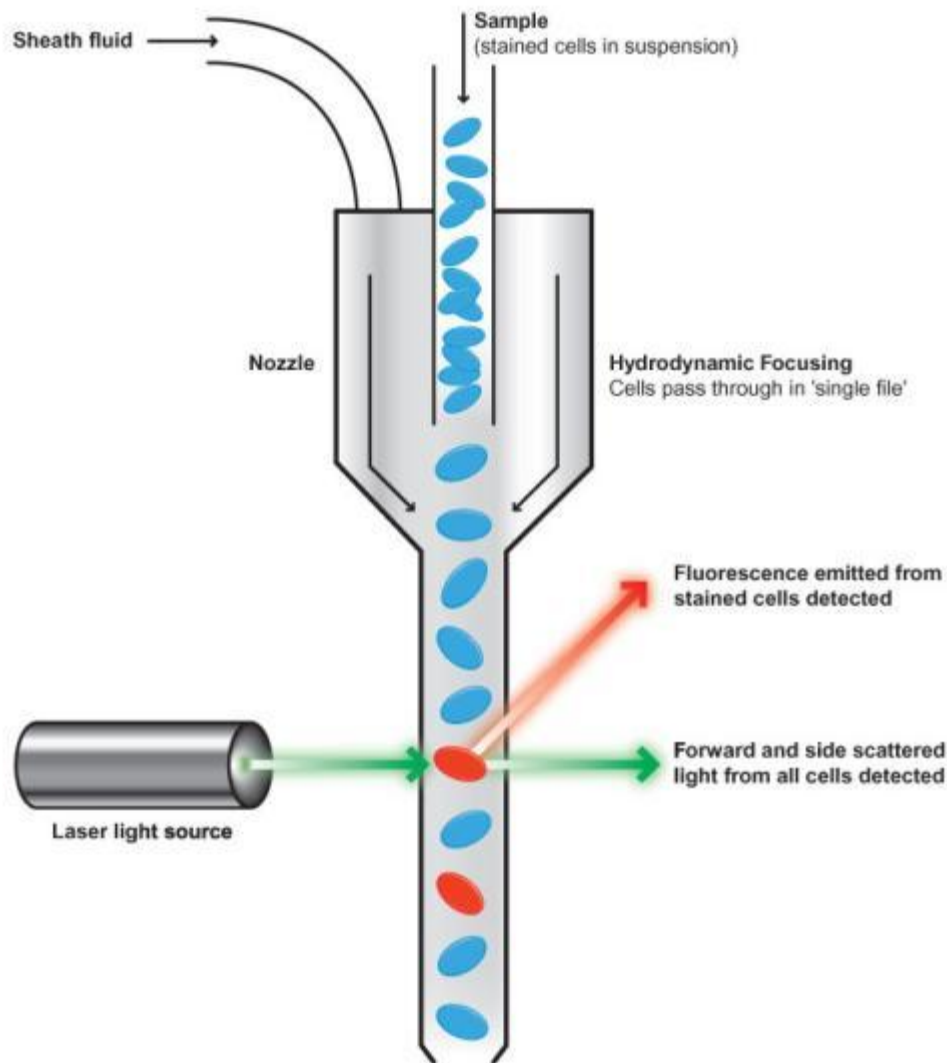


Figure 3: A schematic representation of a flow cell using hydrodynamic focusing to align the cells to pass in a single file through the laser beam [145].

3.4 Phagocytosis

Changes in cell size, granularity and color, indicating particle phagocytosis, were observed under light microscopy after up to 24 hours incubation. Flow cytometry was also used to observe changes in cell complexity (measured by SS), as previously described [22].

3.5 Cell Mortality

Cell mortality was analyzed by counting total cell number (viable and dead) using light microscopy, and by quantifying apoptosis and necrosis using Annexin V-Propidium Iodide (PI) assay (Trevigen, Gaithersburg, MD) as well as cell death enzyme-linked immunosorbent assay (ELISA), after 20-24 hours incubation with the particles, as described below. Cell mortality was further measured in kinetic experiments by counting total cell number at 6-, 12- and 24-hour time points.

3.5.1 Total Cell Number

After incubation with the particles, viable and dead cells were counted using the trypan blue exclusion assay, which is a simple and well-accepted method for measuring cell mortality [146]. The molecular weight of the trypan blue dye permits diffusion through damaged membranes only, and provides a visual indication of dead vs. viable cells [146]. For each count, a 10 μ l aliquot was pipetted onto a haemocytometer from a 110 μ l sample (itself prepared with 100 μ l of cell suspension and 10 μ l of trypan blue (Sigma Aldrich, St. Louis, MO)). Cells in the four corner squares of the nine-square counting grid were counted, where dead cells appeared blue. As each square had a surface area of 1 mm² and a depth of 0.1 mm (with coverslip in place), each square represented 0.1 mm³, or 10⁻⁴ ml [146]. Therefore the following equation was used to calculate viable, dead, and total number of cells per ml (when four squares were counted), accounting for Trypan Blue dilution by multiplying cells/ml by a factor of 1.1:

$$\frac{\text{Cells}}{\text{ml}} = \frac{\text{Cells counted}}{4} * 10^4 \text{ml}^{-1} * 1.1$$

(2)

3.5.2 Early Apoptosis & Late Apoptosis-Necrosis (Flow Cytometry)

Early apoptotic vs. late apoptotic-necrotic cells were quantified by flow cytometry using an Annexin-V/PI assay (Trevigen, Gaithersburg, MD). The assay allowed quantification of early apoptotic cells (which stained Annexin-V FITC only), as explained below. Late apoptosis and necrosis were differentiated using Cell Death Detection ELISA Plus kit (Roche Diagnostics, Indianapolis, IN), as previously described [137].

Annexin-V is a protein that binds to the negatively charged phospholipid, phosphatidylserine (PS), which becomes exposed on the cell surface in the early stages of apoptosis [147, 148]. Conjugating Annexin-V with FITC fluorochrome (excitation 488 nm / emission wavelength 518 nm) allows fluorescent detection of apoptotic cells under the green wavelength of the flow cytometer. PI is a fluorescent molecule (excitation 488 nm / emission 620 nm) that, due to changes in integrity of the cell membrane, enters and stains the DNA of dead cells and becomes visible in necrotic and late apoptotic cells [49]. PI-positive cells fluoresce under the red wavelength of the flow cytometer.

After incubation with the particles for up to 24h, cells were centrifuged for 6 minutes at 150Xg in preparation for the flow cytometry data collection and analysis. The sample supernatants were frozen at -80°C for subsequent cytokine quantification by ELISA. Cells were then washed with 500 µl PBS and stained with Annexin-V and PI according to the manufacturer protocol. Samples were incubated for 15 minutes in the dark followed by quenching with 400 µl of ice-cold binding buffer. Collection of data was done using a FC 500 flow cytometer (Beckman Coulter Inc., Brea, CA). Percentages of positively and negatively stained cells were calculated by the flow cytometer software (CXP Analysis 2.2) in the analysis mode. Each sample was run until 10,000 events were acquired or for a maximum of 300 seconds.

Cells that were in the early stages of apoptosis stained positive for Annexin-V FITC only (PI-negative), as PS translocation occurred and the cell membrane remained intact (a characteristic of early apoptosis). Cells that were PI-positive but Annexin-V negative were likely necrotic. Double-stained cells (positive for both Annexin-V and PI) were likely late apoptotic cells. However, a clear distinction between late apoptosis and necrosis is not possible with the Annexin-V/PI assay because apoptotic cells can evolve to become necrotic and create an overlap between apoptotic and necrotic cell death [137]. A cell death ELISA (specifically Cell Death Detection ELISA Plus, Roche Diagnostics, Indianapolis, IN) was therefore used to differentiate these types of cell death.

3.5.3 Late Apoptosis & Necrosis (Cell Death ELISA)

An ELISA is a common tool used to confirm and measure the presence of a protein in a sample [144, 149]. The Cell Death Detection ELISA Plus (Roche Diagnostics, Indianapolis, IN) measures levels of mono- and oligo-nucleosomes within the cell cytoplasm after cell lysis (late apoptosis) and in the supernatants (necrosis). A nucleosome is a unit comprising DNA wound around a protein core composed of histones [150]. Mono- and oligo-nucleosomes are DNA fragments that are found in the cell cytoplasm of apoptotic cells and are released from damaged membranes of necrotic cells [49, 151]. Thus, cell necrosis and late apoptosis were differentiated using the Cell Death Detection ELISA Plus, as previously described [137]. Samples (i.e., cell lysates or supernatants) and a mixture of antibodies (anti-histone-biotin-labeled and anti-DNA-peroxidase-conjugated) were added to microplate wells pre-coated with streptavidin. Histones in the mono/oligo-nucleosomes in the sample (in the cell lysate for late apoptosis, and in the supernatant for necrosis) bound to the first antibody (the anti-histone biotin-labeled), which also bound to the streptavidin coated well, while the DNA components of the nucleosomes bound to the second antibody (the anti-DNA-peroxidase-conjugated) [152]. A photometrically detectable peroxidase substrate (ABTS) was then added, which also bound to the second antibody (the anti-DNA-peroxidase-conjugated). The substrate was converted to a detectable and quantifiable signal by colorimetry [149], and the intensity of the color in the well was interpreted as relative amounts of mono/oligo-nucleosomes in the sample [152]. The test principle of the cell death ELISA is depicted in Figure 4.

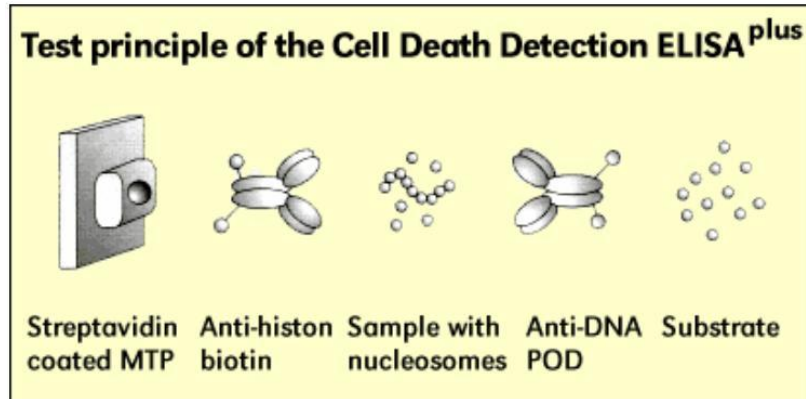


Figure 4: Test principle for the Cell Death Detection ELISA Plus (Roche Diagnostics, Indianapolis, IN) [152].

Briefly, samples were centrifuged at 200Xg for 8 minutes. Supernatants were collected. Cell pellets were lysed using the kit-provided lysis buffer for 30 minutes followed by centrifugation at 200Xg for 10 minutes, and lysate supernatants were collected. Reagents were prepared as per the manufacturer instructions, and samples were analyzed in kit-provided pre-treated wells as per the protocol. Substrates linked to bound nucleosomes in the samples provided proportional color changes in the wells. Apoptosis and necrosis were quantified by measuring the light absorbance in the wells with a Synergy 4 plate reader (Biotek, Winooski, VA). For relative quantities of nucleosomes (ratio over control, i.e. cells with no particles), an enrichment factor was calculated for each sample, using the following formula:

$$EnrichmentFactor = \frac{Sampleabsorbance}{Controlabsorbance}$$

(3)

Absorbance levels were measured at 405 nm using 490 nm as reference wavelength and with subtraction of background (incubation buffer only), as per the manufacturer instructions. The

minimum detectable dose was nucleosomes from 150 cells per well, according to the manufacturer instructions.

3.6 Cytokine Release

The levels of TNF- α , MCP-1 and MIP-1 α into sample supernatants were measured by ELISA with the following kits: Mouse TNF- α ELISA (R&D Systems, Minneapolis, MN), Mouse CCL2/JE/MCP-1 ELISA (R&D Systems, Minneapolis, MN), and Mouse CCL3/MIP-1 α ELISA (R&D Systems, Minneapolis, MN). Culture supernatants were thawed and gently mixed prior to processing as per manufacturer instructions. Cytokine and chemokine levels were calculated as mean pg/ml by interpolation from the standard curves. The minimum detectable dose was 5.1 pg/ml, 2 pg/ml, and 1.5 pg/ml for the TNF- α , MCP-1 and MIP-1 α assays, respectively. Kits were specific enough to avoid cross-reactivity of other recombinant cytokines.

3.7 Statistics

Each experiment was repeated at least three (and up to six) times and samples in each experiment were run in triplicates.

Mean values and pooled standard deviations were calculated with the equations below, and are presented in the Results (Section 4).

$$\text{Mean Value} = \frac{1}{k} \sum_{i=1}^k \left[\frac{1}{n} \sum_{i=1}^n x_i \right]$$

$$\text{Pooled Standard Deviation} = \left(\sum_{i=1}^k \frac{(n_i - 1) \cdot s_i^2}{(n_i - 1)} \right)^{1/2}$$

(4)

Where,

n = number of replicates
k = number of repeated experiments
x = measured value
s = standard deviation

Statistical analysis was performed using the two-way analysis of variance (ANOVA) with replicates test and 5% as the level of significance (SPSS Statistics 17.0).

4 Results

4.1 Phagocytosis

Cells with and without particles were observed using a haemocytometer under light microscopy. Cells which had ingested particles appeared darkened and more granular (compared to control), as depicted in Figure 5. Cells appeared to ingest Cr₂O₃ and Al₂O₃ particles indiscriminately. Phagocytosis of the particles occurred prior to the first kinetic time-point of 6 hours.

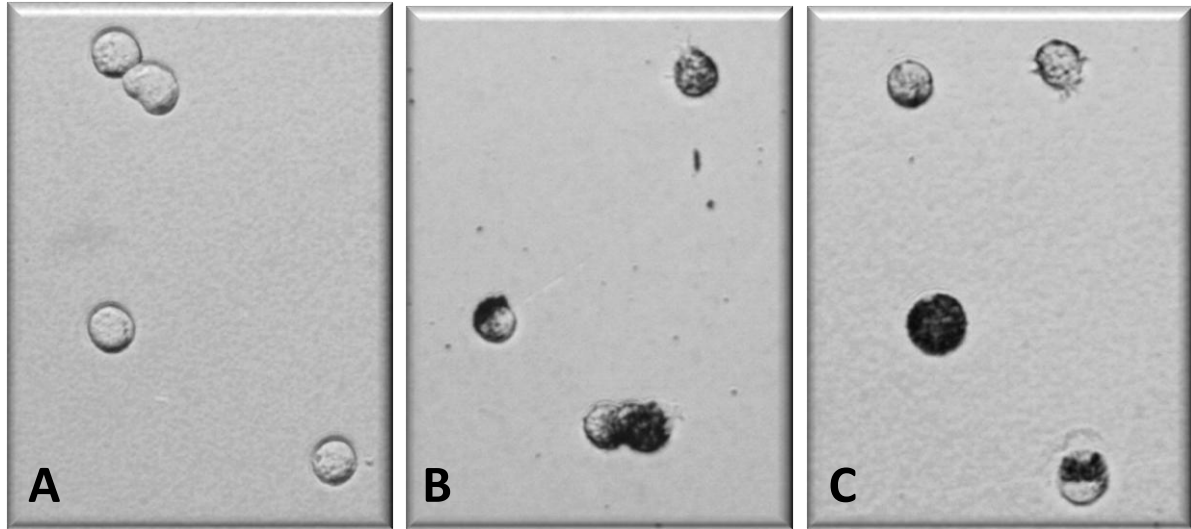


Figure 5: Light microscopy pictures of J774 macrophages after 20-24h incubation with: (A) no particles (control); (B) 0.5 million Cr_2O_3 particles per cell; and (C) 0.5 million Al_2O_3 particles per cell (200X magnification).

Phagocytosis was confirmed using flow cytometry, revealing an increase in SS for all particle types, at all concentrations analyzed.

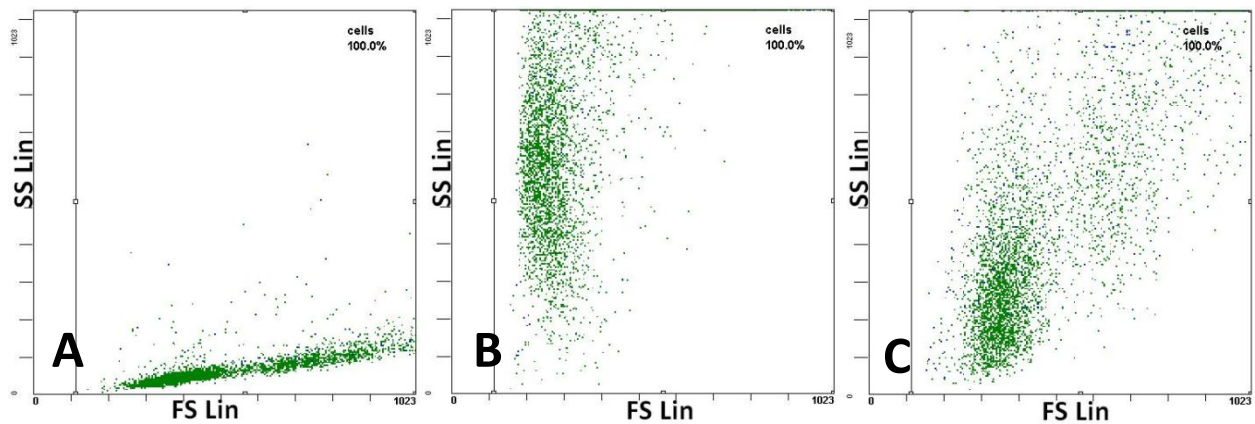


Figure 6: Examples of FS-SS dot plots acquired after 20-24h incubation of the macrophages with: (A) no particles (control); (B) 0.5 million Cr_2O_3 particles per cell; and (C) 0.5 million Al_2O_3 particles per cell.

4.2 Mortality and Cytokine Release

4.2.1 60 nm Cr₂O₃ Versus 700 nm Cr₂O₃ Particles

Overall, 60 nm and 700 nm Cr₂O₃ particles induced significant increases in macrophage mortality, as depicted by decreases in total cell number (Figure 7), and significant increases in late apoptosis-necrosis (PI-positive cells) (Figure 11) as well as necrosis (measured by cell death ELISA) (Figure 13) after incubation with the particles, at all concentrations analyzed. Levels of early apoptosis did not significantly increase (vs. control) (Figure 9), and levels of late apoptosis remained low overall (Figure 13). Comparing the effects of 60 nm versus 700 nm Cr₂O₃ particle volumes revealed a potential volume effect on the cells (Figure 8, Figure 10, Figure 12 & Figure 14).

4.2.1.1 Total Cell Number

Trypan blue cell counts revealed a particle concentration-dependent decrease in total cell numbers for macrophages cultured with either 60 nm or 700 nm Cr₂O₃ particles. A significant decrease was observed for all concentrations analyzed ($p < 0.005$ vs. control at all concentrations), up to 67% with 3.5 million particles per macrophage for the 60 nm particles (Figure 7A) and up to 69% with 2000 particles per macrophage for the 700 nm particles (Figure 7B) at 24h. Comparing the effects of 60 nm with 700 nm Cr₂O₃ particle volume revealed that the total cell numbers may have been dependent upon a combination of particle size and concentration, which can translate into a particle volume (heretofore referred to as a volume effect), as shown in Figure 8.

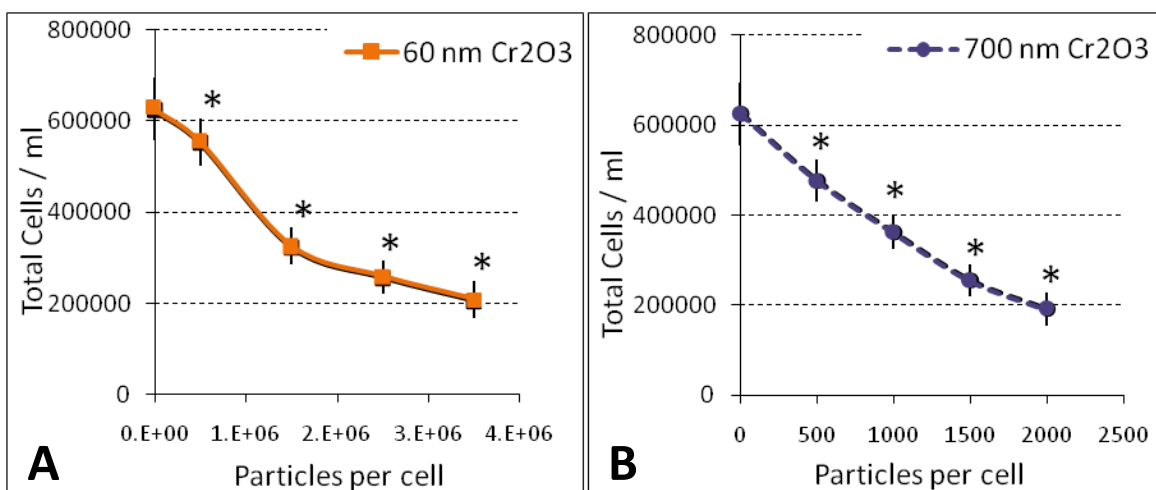


Figure 7: Effects of (A) 60 nm and (B) 700 nm Cr₂O₃ particles on the total cell number after 20-24h incubation (* indicates $p < 0.005$ vs. control). Data shows mean $\pm$ STD of 6 experiments performed in triplicates.

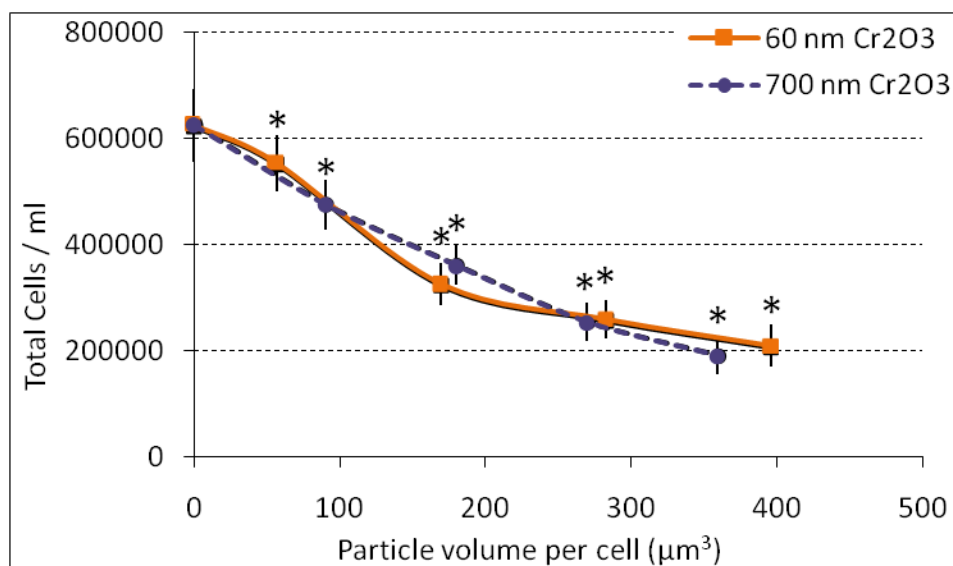


Figure 8: Effects of 60 nm and 700 nm Cr₂O₃ particle volume on total cell number after 20-24h incubation (* indicates $p < 0.005$ vs. control). Cells were incubated with different concentrations of 60 nm or 700 nm particles. Particle volumes were calculated based on the sizes and concentrations, considering spherical particles. Data shows mean $\pm$ STD of 6 experiments performed in triplicates.

4.2.1.2 Early Apoptosis & Late Apoptosis-Necrosis (Flow Cytometry)

Neither the 60 nm nor the 700 nm Cr₂O₃ particles induced a significant increase in early apoptosis (Annexin-V positive/PI negative cells) ($p > 0.05$ at all concentrations vs. control) (Figure

9A and B). Comparing the effects of 60 nm with 700 nm Cr_2O_3 particle volume revealed that the percentage of early apoptosis may have been dependent on particle per cell volume (i.e., potential volume effect) (Figure 10).

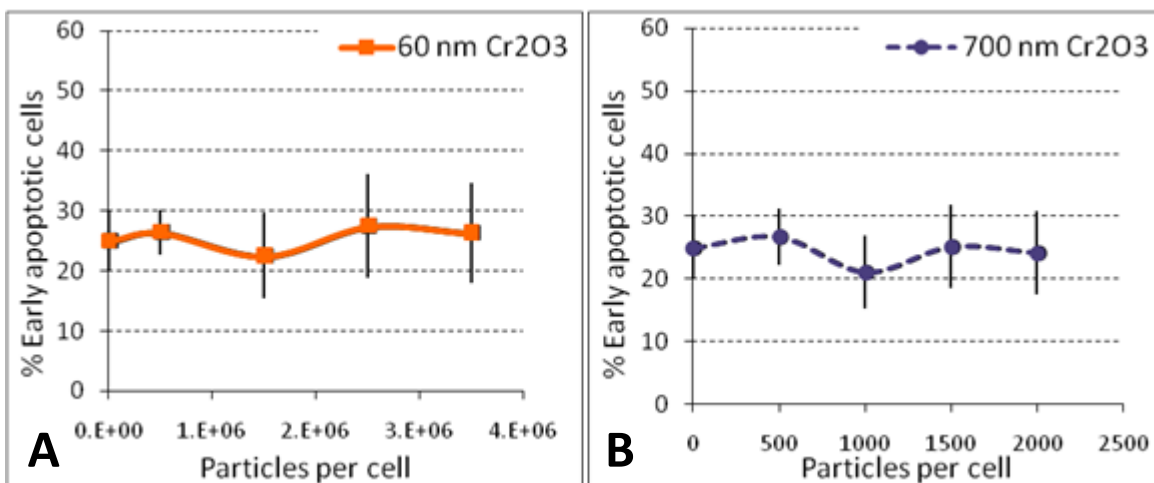


Figure 9: Effects of (A) 60 nm and (B) 700 nm Cr_2O_3 particles on early apoptosis of macrophages after 20-24h incubation. Percentages of early apoptosis were determined using flow cytometry. Data shows mean $\pm$ STD of 6 experiments performed in triplicates.

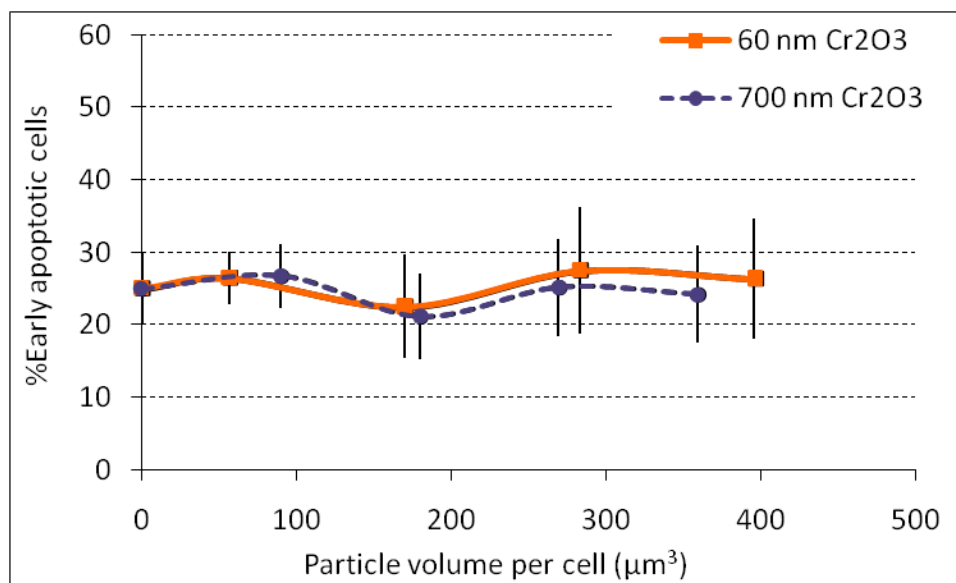


Figure 10: Effects of 60 nm and 700 nm Cr_2O_3 particle volume on early apoptosis of macrophages after 20-24h incubation. Cells were incubated with different concentrations of 60 nm and 700 nm particles. Particle volumes were calculated based on the sizes and concentrations, considering spherical particles. Percentages of early apoptosis were determined using flow cytometry. Data shows mean $\pm$ STD of 6 experiments performed in triplicates.

Percentages of PI-positive cells, indicating late apoptotic-necrotic cells, increased significantly with both 60 nm and 700 nm Cr_2O_3 particles at all concentrations ($p < 0.001$ vs. control, for all concentrations), reaching a maximum of 16% with 2.5 million particles per macrophage for the 60 nm particles and a maximum of nearly 19% with 1500 particles per macrophage for the 700 nm particles (Figure 11A and Figure 11B, respectively). Interestingly, percentages appeared to plateau at the highest concentrations of 60 nm particles, and even significantly decreased with 2000 particles per macrophage for the 700 nm particles ($p < 0.001$ for 1500 particles per cell vs. 2000 particles per cell).

As observed for the total cell numbers and the percentages of early apoptosis, comparing the effects of 60 nm and 700 nm Cr_2O_3 particle volumes on the percentage of PI-positive cells revealed that cell response may have been determined by particle per cell volume (volume effect) (Figure 12).

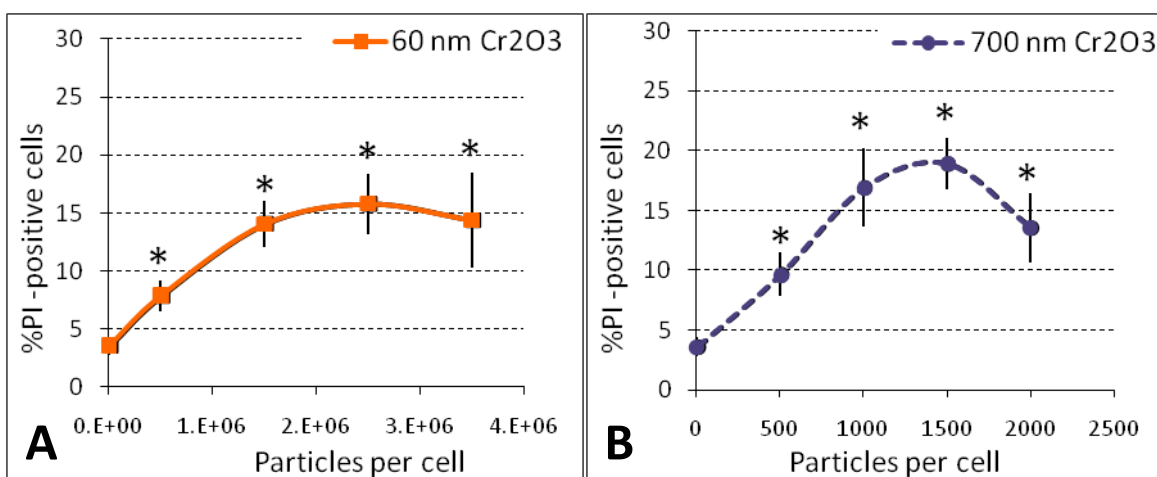


Figure 11: Effects of (A) 60 nm and (B) 700 nm Cr_2O_3 particles on late apoptosis-necrosis (PI-positive cells) of macrophages after 20-24h incubation (* indicates $p < 0.001$ vs. control). Percentages of PI-positive cells were determined using flow cytometry. Data shows mean $\pm$ STD of 4 experiments performed in triplicates.

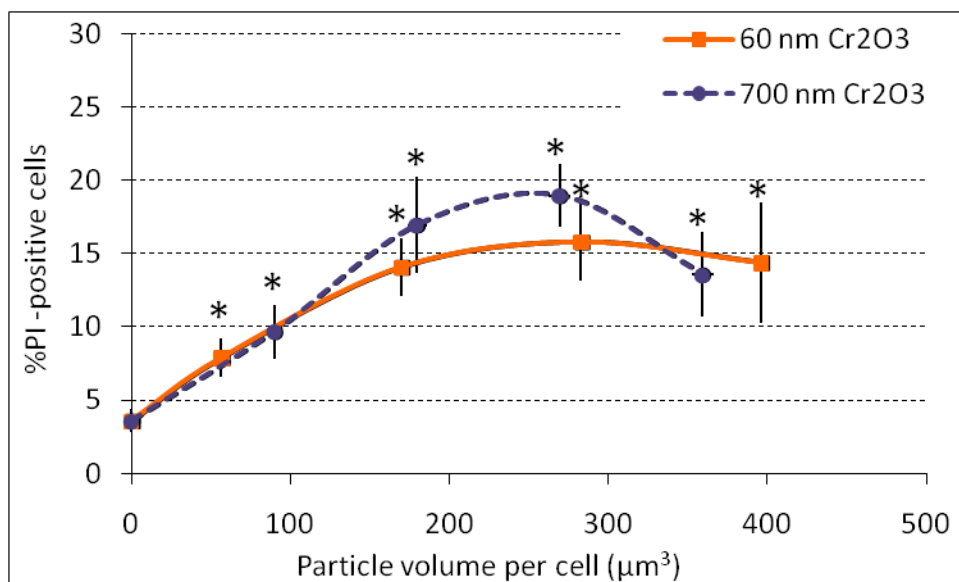


Figure 12: Effects of 60 nm and 700 nm Cr₂O₃ particle volume on late apoptosis-necrosis (PI-positive cells) of macrophages after 20-24h incubation (* indicates $p < 0.001$ vs. control). Cells were incubated with different concentrations of 60 nm or 700 nm particles. Particle volumes were calculated based on the sizes and concentrations, considering spherical particles. Percentages of PI-positive cells were determined using flow cytometry. Data shows mean $\pm$ STD of 4 experiments performed in triplicates.

4.2.1.3 Late Apoptosis & Necrosis (Cell Death ELISA)

Late apoptosis and necrosis induced by the particles were differentiated using a cell death ELISA (Cell Death Detection ELISA Plus). The ratios of mono/oligo-nucleosome levels in cell lysates over control (reflecting the levels of late apoptosis over control) and the ratios of mono/oligo-nucleosomes in culture supernatants over control (reflecting the levels of necrosis over control) are reported and presented as enrichment factors.

Levels of late apoptosis remained low for both sizes of Cr₂O₃ particles. The 60 nm Cr₂O₃ particles did not induce a significant increase in the level of late apoptosis except at the concentration of 3.5 million particles per macrophage, with a mono/oligo-nucleosome enrichment factor of 1.9 over control ($p = 0.006$) (Figure 13A). Similarly, the 700 nm Cr₂O₃

particles did not induce a significant increase in late apoptosis except at the concentration of 2000 particles per cell, with a mono/oligo-nucleosome enrichment factor of 1.7 over control ($p=0.002$) (Figure 13B).

Both the 60 nm and the 700 nm Cr_2O_3 particles induced significant increases in necrosis at all concentrations ($p<0.002$ for the mono/oligo-nucleosome enrichment factor at all concentrations vs. control). As shown in Figure 13A and Figure 13B, the enrichment factors reflecting levels of necrosis reached a maximum of 5.7 at the concentration of 3.5 million particles per macrophage for the 60 nm particles and 6.5 at the concentration of 2000 particles per macrophage for the 700 nm particles. Comparing the effects of 60 nm and 700 nm Cr_2O_3 particle volumes revealed a potential volume effect both for late apoptosis (Figure 14A) and for necrosis (Figure 14B). Overall, particles of both sizes induced primarily necrotic (over late apoptotic) cell death at all concentrations.

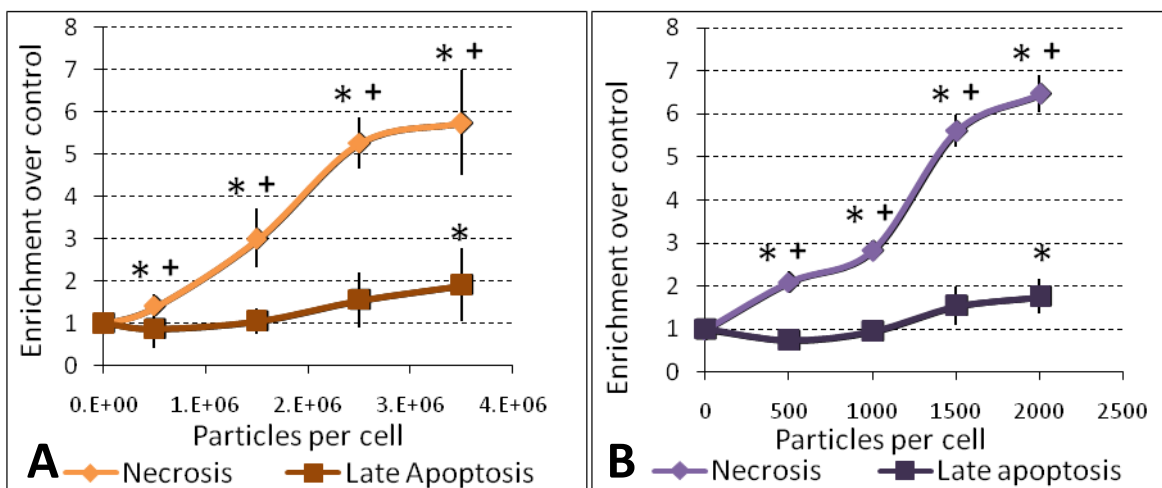


Figure 13: Effects of (A) 60 nm and (B) 700 nm Cr₂O₃ particles on macrophage late apoptosis and necrosis after 20-24h incubation, as determined using the Cell Death Detection ELISA Plus kit (Roche Diagnostics) (* indicates $p < 0.002$ vs. control; + indicates $p < 0.05$ comparing enrichment factors for necrosis versus late apoptosis, for a given particle concentration). Enrichment factor indicates the ratio of mono/oligo-nucleosomes in cell lysates (late apoptosis) or supernatants (necrosis) over respective negative controls. Data shows mean $\pm$ STD of 4 experiments performed in triplicates.

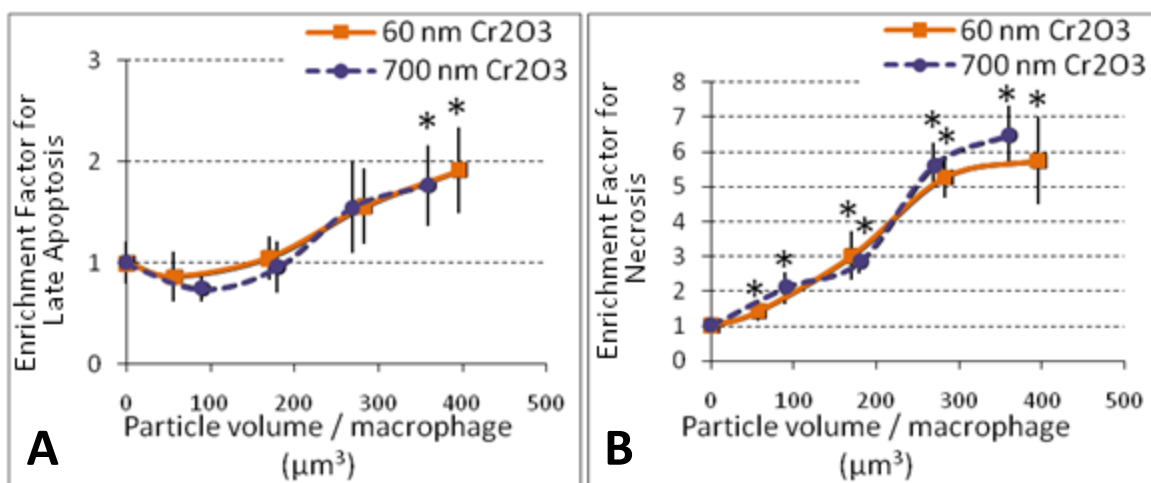


Figure 14: Effects of 60 nm and 700 nm Cr₂O₃ particle volume on (A) macrophage late apoptosis and (B) macrophage necrosis after 20-24h incubation, as determined using the Cell Death Detection ELISA Plus kit (Roche Diagnostics) (* indicates $p < 0.01$ vs. control). Macrophages were incubated with different concentrations of 60 nm or 700 nm size particles for 20-24h. Particle volumes were calculated based on particle sizes and concentrations, considering spherical particles. Enrichment factor indicates the ratio of mono/oligo-nucleosomes in cell lysates (late apoptosis) or supernatants (necrosis) over respective negative controls. Data shows mean $\pm$ STD of 4 experiments performed in triplicates.

4.2.1.4 Cytokines/Chemokines

Neither 60 nm nor 700 nm Cr_2O_3 particles induced a significant increase in TNF- α (Figure 15), MCP-1 (Figure 16) or MIP-1 α (Figure 17) ($p>0.05$ vs. control for all cytokines and for both particle sizes, at all concentrations analyzed). Positive controls with LPS exhibited an average increase of approximately 17 fold, 2 fold and 12 fold above the TNF- α , MCP-1 and MIP-1 α control levels (cells alone), respectively. For each cytokine, a potential volume effect was noted as depicted in Figure 15 (TNF- α), Figure 16 (MCP-1) and Figure 17 (MIP-1 α).

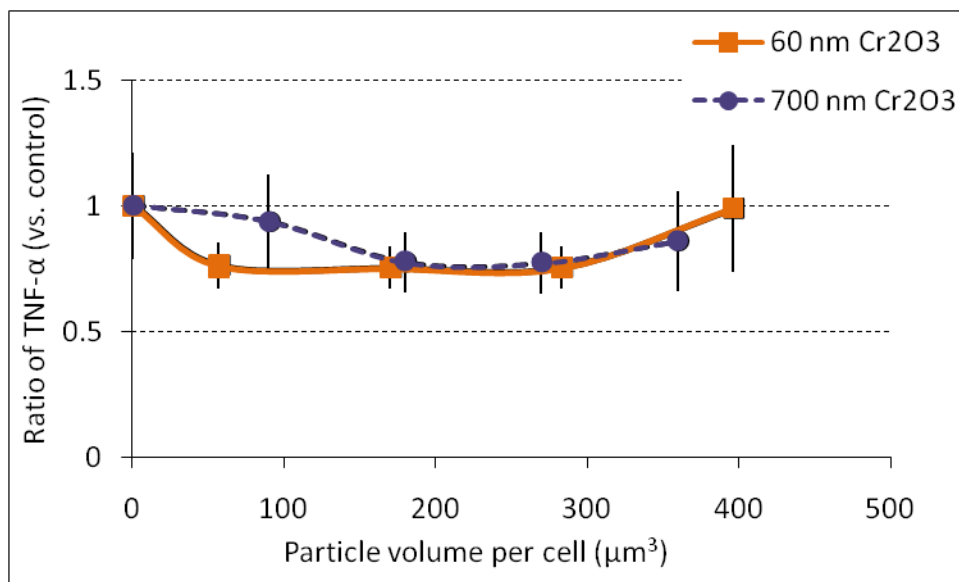


Figure 15: Effects of 60 nm and 700 nm Cr_2O_3 particle volume on TNF- α release after 20-24h incubation. Macrophages were incubated with different concentrations of 60 nm or 700 nm Cr_2O_3 particles. Particle volumes were calculated based on the sizes and concentrations, considering spherical particles. Results are expressed as ratios of the negative control level (cells with no particles) and are the mean $\pm$ STD of 3 experiments performed in triplicates.

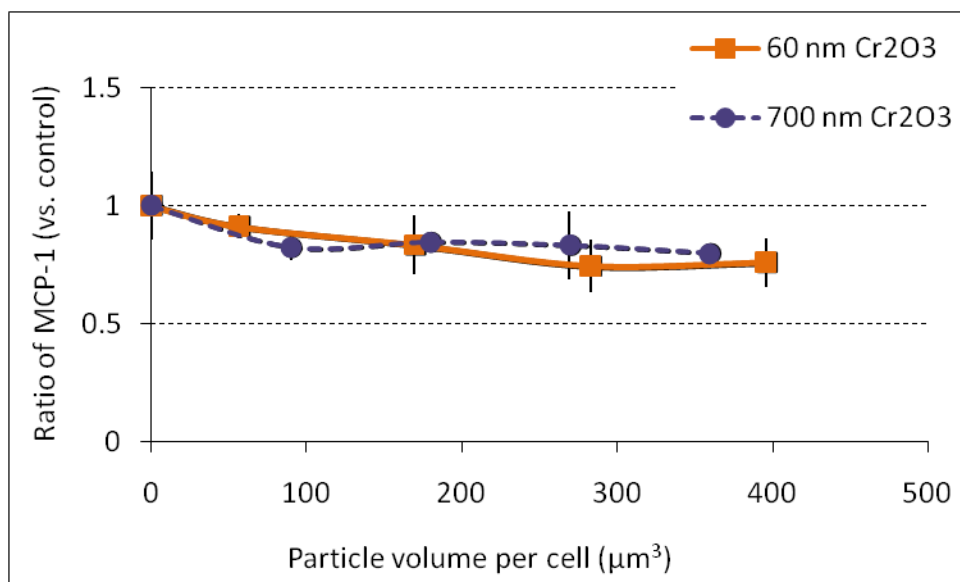


Figure 16: Effects of 60 nm and 700 nm Cr_2O_3 particle volume on MCP-1 release after 20-24h incubation. Macrophages were incubated with different concentrations of 60 nm or 700 nm Cr_2O_3 particles. Particle volumes were calculated based on the sizes and concentrations, considering spherical particles. Results are expressed as ratios of the negative control level (cells with no particles) and are the mean $\pm$ STD of 4 experiments performed in triplicates.

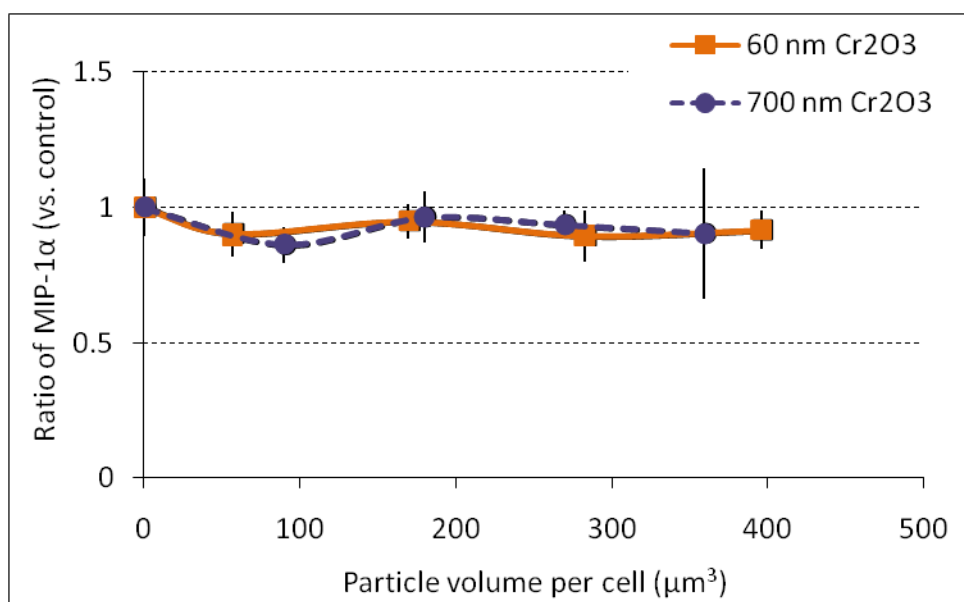


Figure 17: Effects of 60 nm and 700 nm Cr_2O_3 particle volume on MIP-1 α release after 20-24h incubation. Macrophages were incubated with different concentrations of 60 nm or 700 nm Cr_2O_3 particles. Particle volumes were calculated based on the sizes and concentrations, considering spherical particles. Results are expressed as ratios of the negative control level (cells with no particles) and are the mean $\pm$ STD of 3 experiments performed in triplicates.

4.2.2 60 nm Cr₂O₃ Versus 50 nm Al₂O₃ Particles

Generally, studies comparing the effects of 60 nm Cr₂O₃ and 50 nm Al₂O₃ particles demonstrated that Cr₂O₃ particles induced a significantly larger decrease in total cell number (Figure 18) and significantly more necrosis compared to Al₂O₃ particles at concentrations higher than 0.5 million particles per cell (Figure 24). Levels of early apoptosis induced by Cr₂O₃ particles remained low, and were significantly lower than those induced by Al₂O₃ particles at all concentrations except 3.5 million particles per cell (Figure 21). Levels of late apoptosis also remained low, but were significantly higher than those induced by Al₂O₃ for most particle concentrations (Figure 24). Finally, kinetic studies revealed that concentrations higher than 0.5 million particles per cell of either Cr₂O₃ or Al₂O₃ particles induced macrophage mortality within 6 hours of exposure (Figure 19 & Figure 20).

4.2.2.1 *Total Cell Number*

Results of total cell numbers obtained with the 60 nm Cr₂O₃ particles (while comparing with the 50 nm Al₂O₃ particles) were similar to those obtained when comparing these particles with the 700 nm Cr₂O₃ (Figure 7A in paragraph 4.2.1.1). Results showed that both 60 nm Cr₂O₃ and 50 nm Al₂O₃ particles caused a significant decrease in total cell number for all concentrations analyzed ($p < 0.001$ vs. control for Cr₂O₃ particles, and $p < 0.005$ vs. control for Al₂O₃ particles), reaching up to 72% and 42% decreases with 3.5 million Cr₂O₃ and Al₂O₃ particles per cell, respectively (Figure 18). Cr₂O₃ particles induced a greater decrease in total cell number compared to Al₂O₃ particles, which was significant at concentrations of 1.5 million, 2.5 million

and 3.5 million particles per macrophage ($p < 0.001$ for all of these concentrations), as depicted in Figure 18.

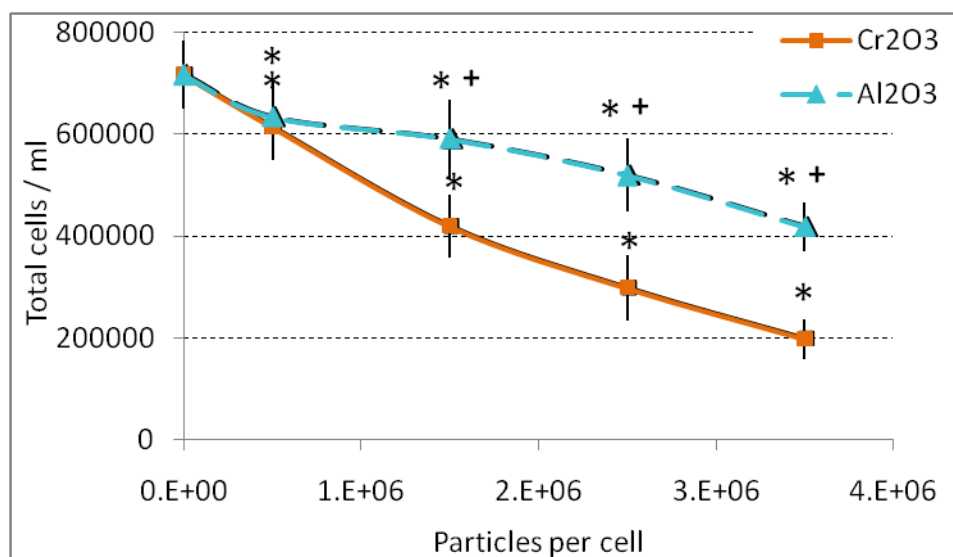


Figure 18: Effects of 60 nm Cr₂O₃ and 50 nm Al₂O₃ particles on total cell number after 20-24h incubation (* indicates $p < 0.005$ vs. control; + indicates $p < 0.001$ when comparing the effects of Cr₂O₃ vs. Al₂O₃ particles at the same concentration). Data shows mean $\pm$ STD of 5 experiments performed in triplicates.

Kinetic studies:

Kinetic experiments measuring differences in total cell number over a 24-hour period revealed that, after only 6h of incubation, 60 nm Cr₂O₃ particles induced a significant decrease in total cell number, up to 11%, 23%, 57% and 59% with 0.5 million, 1.5 million, 2.5 million and 3.5 million particles per cell, respectively ($p < 0.02$ vs. control), as shown in Figure 19. Total cell number further decreased significantly with 1.5 million, 2.5 million and 3.5 million Cr₂O₃ particles per cell, and increased significantly for 0.5 million particles per cell, between 6-12 hours ($p < 0.05$ for 12h vs. 6h). No significant change in total cell numbers occurred between 12-24 hours with concentrations of 1.5 million, 2.5 million and 3.5 million particles per

macrophage ($p>0.05$ for 24h vs. 12h). In contrast, total cell numbers in control samples (cells with no particles) and with 0.5 million Cr_2O_3 particles significantly increased between 12-24 hours ($p<0.001$).

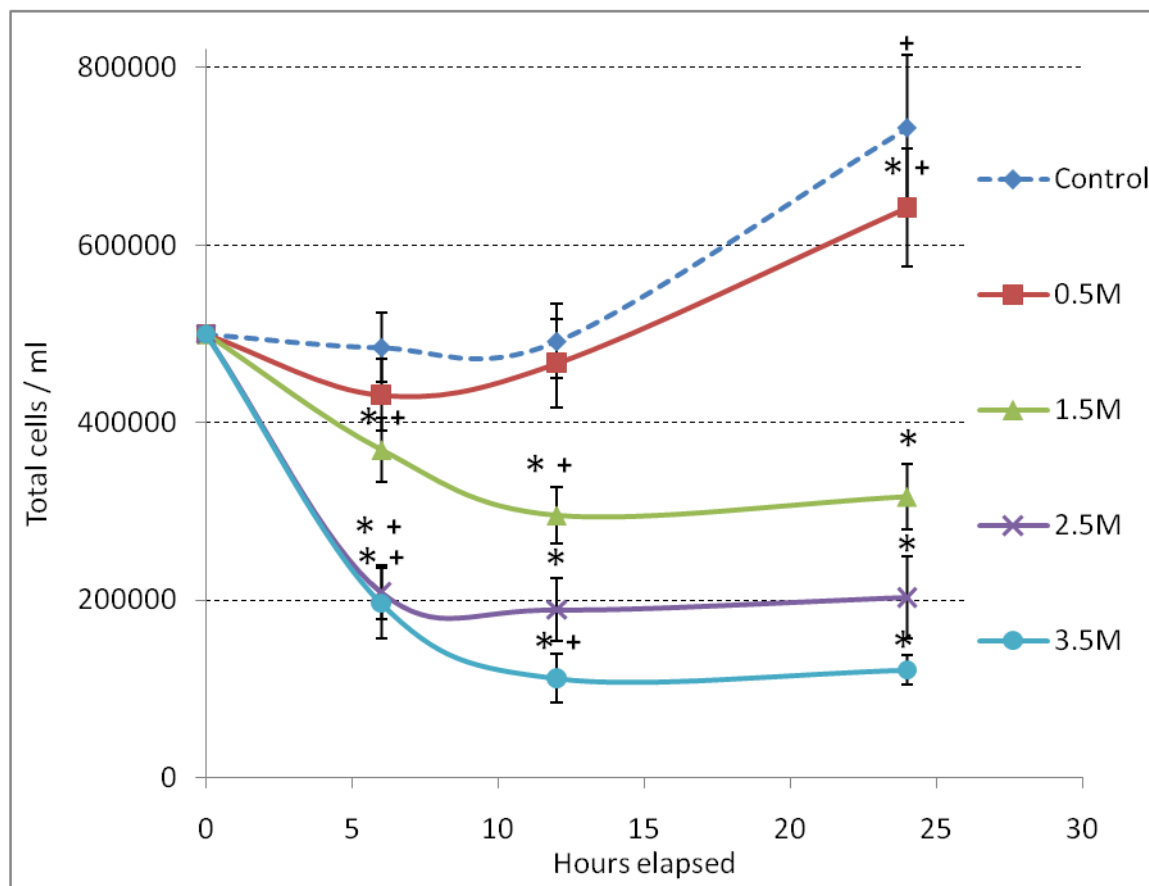


Figure 19: Effects of 60 nm Cr_2O_3 particles on total cell number after 6 hours, 12 hours and 24 hours incubation (* indicates $p<0.02$ vs. control; + indicates $p<0.05$ when comparing total cell numbers at two consecutive time points for a given particle concentration). Data shows mean $\pm$ STD of 3 experiments performed in triplicates.

Similar trends were noted for cells incubated with 50 nm Al_2O_3 particles, as shown in Figure 20. After 6 hours, total cell numbers had significantly decreased by 12%, 26% and 36% in the presence of 1.5 million, 2.5 million and 3.5 million Al_2O_3 particles per cell, respectively ($p<0.05$ vs. control). No significant change in total cell numbers occurred between 6-12 hours with any concentration of Al_2O_3 particles ($p>0.05$ for 12h vs. 6h). Total cell numbers did also not

significantly change between 12-24 hours with 2.5 million and 3.5 million particles per cell. On the other hand, they significantly increased in control samples (cells with no particles) and with 0.5 million and 1.5 million Al_2O_3 particles per cell ($p < 0.02$ for all). At all time points, total cell numbers with 0.5 million Al_2O_3 particles per cell were not significantly different than those in control samples ($p > 0.05$ for all time points).

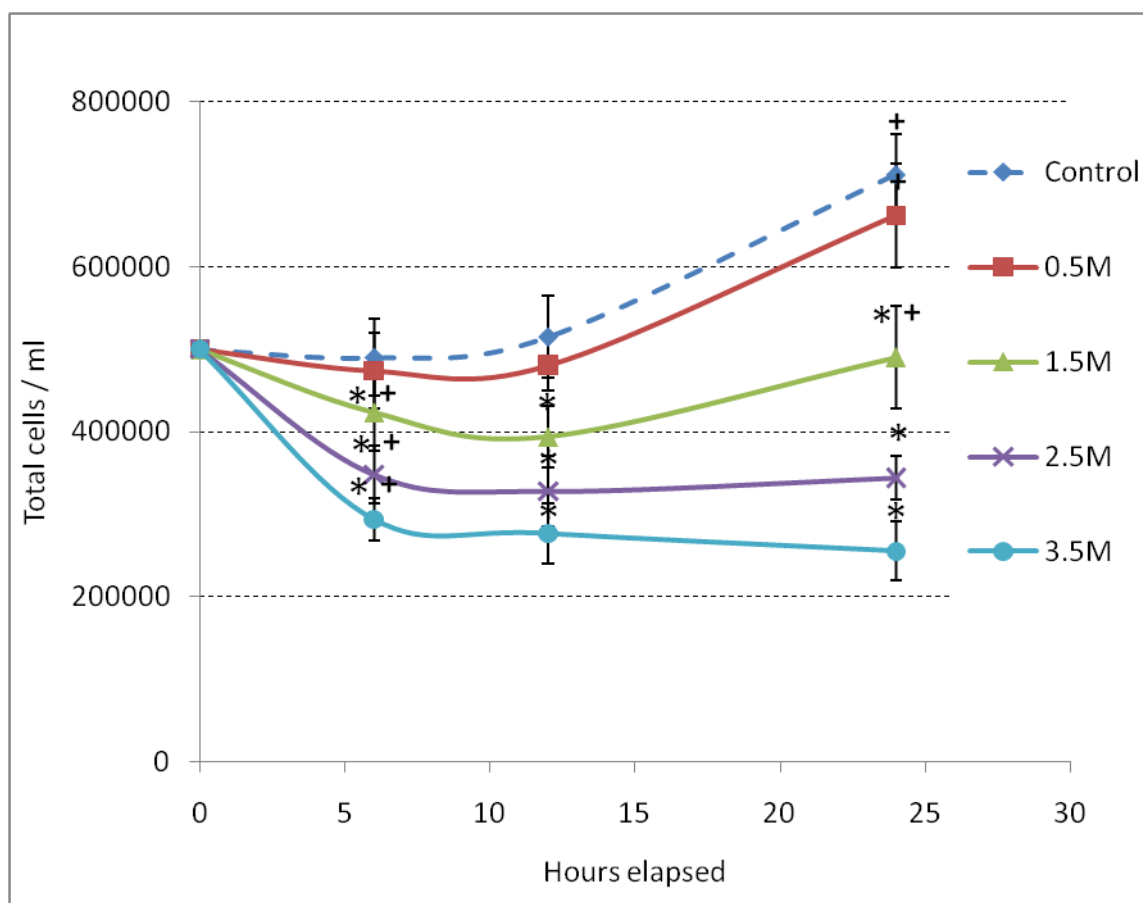


Figure 20: Effects of 50 nm Al_2O_3 particles on total cell number after 6 hours, 12 hours and 24 hours incubation (* indicates $p < 0.05$ vs. control; + indicates $p < 0.02$ when comparing total cell numbers at two consecutive time points for a given particle concentration). Data shows mean $\pm$ STD of 2 experiments performed in triplicates.

In summary, significant decreases in total cell numbers were observed during the first 6 hours of incubation with 60 nm Cr_2O_3 or 50 nm Al_2O_3 particles. Cr_2O_3 particles induced an additional

significant decrease between 6-12 hours at 1.5 million, 2.5 million and 3.5 million particles per cell, whereas no significant change was observed with Al_2O_3 particles during this time period. Total cell number did not significantly decrease between 12 and 24 hours of incubation with either particle type. However, a significant increase was observed during this period for control samples, samples having 0.5 million Cr_2O_3 particles per cell and samples having 0.5 million or 1.5 million Al_2O_3 particles per cell.

4.2.2.2 *Early Apoptosis & Late Apoptosis-Necrosis (Flow Cytometry)*

While the 60 nm Cr_2O_3 particles did not induce a significant increase in early apoptosis except at 3.5 million particles per macrophage (where it reached 6% over control ($p < 0.02$), i.e. still remained low), the 50 nm Al_2O_3 particles induced a significant increase in early apoptosis at all concentrations analyzed ($p < 0.002$ for all concentrations, vs. control), with a maximum of 22% over control at 1.5 million particles per macrophage (Figure 21). The level of early apoptosis with Al_2O_3 particles gradually decreased at 2.5 and 3.5 million particles per macrophage, although it remained significantly higher than that with Cr_2O_3 particles at 2.5 million particles per cell ($p < 0.001$). Overall, comparing the effects of Cr_2O_3 versus Al_2O_3 particles revealed that the Al_2O_3 particles induced a significantly higher percentage of early apoptotic cells at all concentrations except at 3.5 million particles per cell ($p < 0.005$ for Cr_2O_3 vs. Al_2O_3 at 0.5 million, 1.5 million and 2.5 million particles per macrophage), as shown in Figure 21.

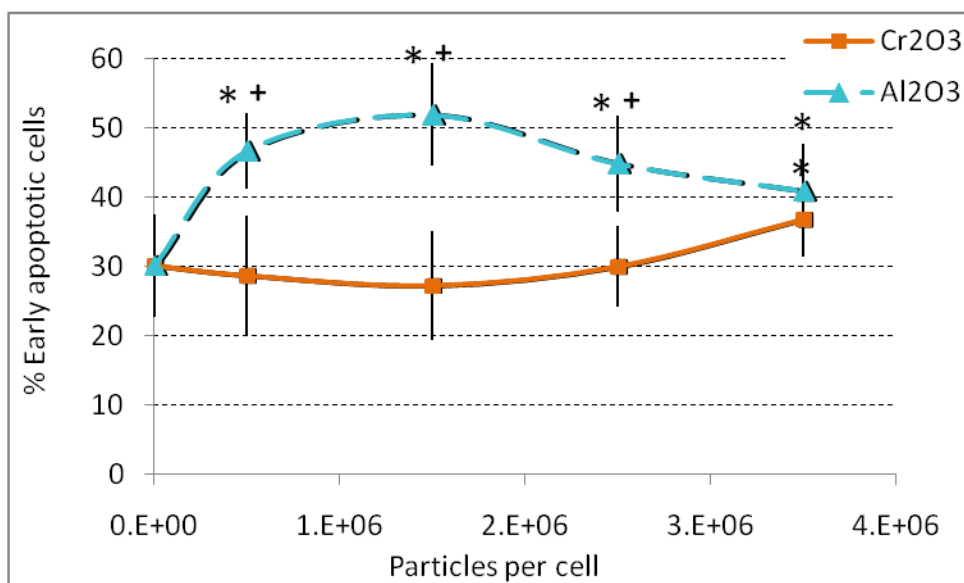


Figure 21: Effects of 60 nm Cr₂O₃ and 50 nm Al₂O₃ particles on early apoptosis of macrophages after 20-24h incubation (* indicates $p < 0.002$ vs. control; + indicates $p < 0.005$ when comparing Cr₂O₃ vs. Al₂O₃ particles at the same concentration). Percentages of early apoptosis were determined using flow cytometry. Data shows mean $\pm$ STD of 5 experiments performed in triplicates.

Both Cr₂O₃ and Al₂O₃ particles induced a significant increase in late apoptosis-necrosis (PI-positive cells) at all concentrations analyzed ($p < 0.001$ for each concentration, vs. control) (Figure 22). The percentages of late apoptosis-necrosis (PI-positive cells) were significantly higher for cells incubated with Al₂O₃ particles compared to Cr₂O₃ particles, at all concentrations ($p < 0.005$ when comparing Cr₂O₃ vs. Al₂O₃ particles at any given concentration). Indeed, Cr₂O₃ particles induced a maximum increase of 14% over control at 2.5 million particles per macrophage (similar to results when comparing 60 nm versus 700 nm Cr₂O₃ particles - see Figure 11 in paragraph 4.2.1.2), while Al₂O₃ particles induced a maximum increase of 22% over control at 3.5 million particles per macrophage. However, although percentages were statistically significantly higher for cells incubated with Al₂O₃ particles, differences remained within 4% for all concentrations but 3.5 million particles per cell (where an 11% difference was noted).

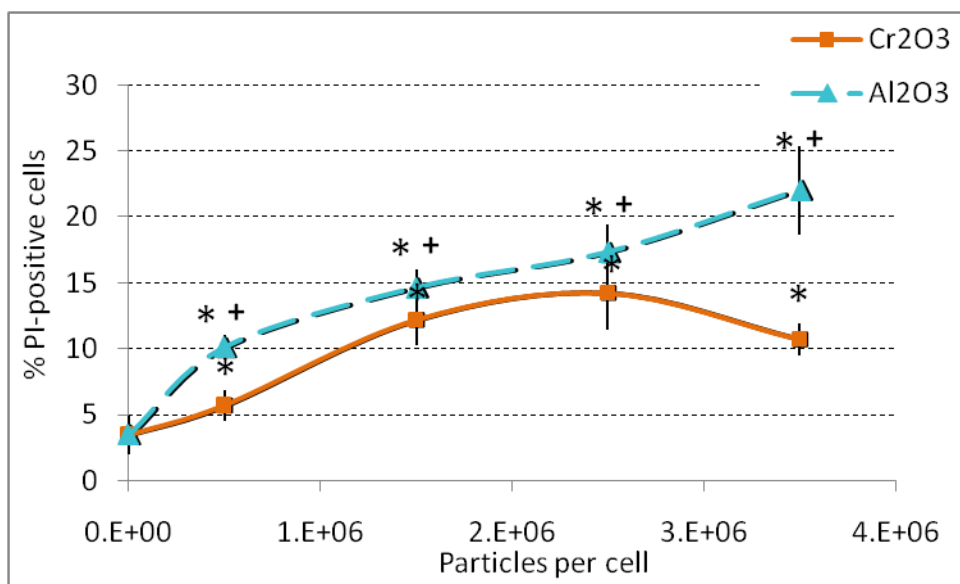


Figure 22: Effects of 60 nm Cr₂O₃ and 50 nm Al₂O₃ particles on late apoptosis-necrosis (PI-positive cells) of macrophages after 20-24h incubation (* indicates $p < 0.001$ vs. control; + indicates $p < 0.005$ when comparing Cr₂O₃ vs. Al₂O₃ at a given concentration). Percentages of PI-positive cells were determined using flow cytometry. Data shows mean $\pm$ STD of 5 experiments performed in triplicates.

4.2.2.3 Late Apoptosis & Necrosis (Cell Death ELISA)

As previously mentioned, the ratios of mono/oligo-nucleosome levels in cell lysates over control (reflecting the levels of late apoptosis over control) and the ratios of mono/oligo-nucleosomes in supernatants over control (reflecting the levels of necrosis over control) are reported and presented as enrichment factors.

Levels of late apoptosis remained low for cells incubated with either Cr₂O₃ or Al₂O₃ particles. Although Cr₂O₃ particles induced a significant increase in late apoptosis at 1.5 million, 2.5 million and 3.5 million particles per macrophage, the maximum enrichment factor (2.2 over control at 3.5 million particles per cell ($p < 0.001$) - similar to that reported in the 60 nm vs. 700 nm Cr₂O₃ study (Figure 13A, paragraph 4.2.1.3)) remained low compared to the maximum

enrichment factor reflecting the level of necrosis (Figure 23A). The Al_2O_3 particles did not cause a significant increase in late apoptosis except at 3.5 million particles per cell ($p < 0.001$ vs. control), where the levels of mono/oligo-nucleosomes in the cell lysates reached an enrichment factor of 1.7 over control, as shown in Figure 23B. Comparing the effects of Cr_2O_3 and Al_2O_3 particles revealed that the Cr_2O_3 particles induced significantly more late apoptosis than Al_2O_3 particles at all concentrations analyzed ($p < 0.05$ when comparing Cr_2O_3 vs. Al_2O_3 for all concentrations), although the enrichment factors remained close (within 0.8 (ratio over control) of each other) at all concentrations, as illustrated in Figure 24A.

Both Cr_2O_3 and Al_2O_3 particles induced a significant increase in necrosis (depicted by an increase in the ratios of mono/oligo-nucleosomes in cell supernatants over negative controls) at all concentrations analyzed, except at 0.5 million Al_2O_3 particles per macrophage ($p < 0.05$ vs. control for 1.5 million, 2.5 million and 3.5 million Al_2O_3 particles per cell). However, Cr_2O_3 particles induced significantly more necrosis than Al_2O_3 particles at all concentrations analyzed ($p < 0.005$), with a maximum enrichment factor of 9.6 with 2.5 million particles per macrophage (versus a maximum enrichment factor of 4.4 with 3.5 million Al_2O_3 particles per macrophage) (Figure 24B). Overall, both Cr_2O_3 and Al_2O_3 particles induced predominantly necrosis (over late apoptosis), as shown in Figure 23 with significantly higher levels observed with Cr_2O_3 particles.

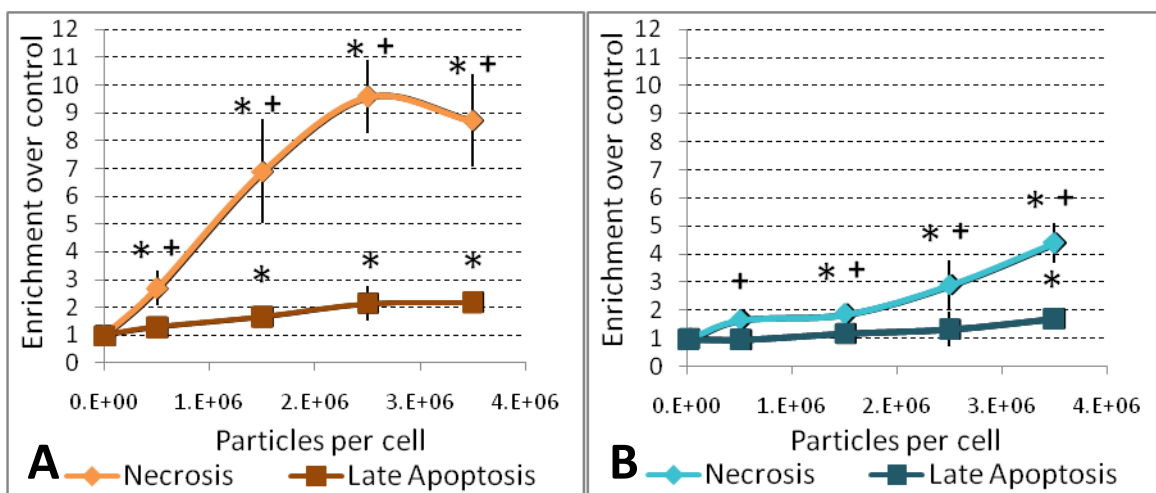


Figure 23: Effects of (A) 60 nm Cr_2O_3 and (B) 50 nm Al_2O_3 particles on macrophage late apoptosis and necrosis after 20-24h incubation, as determined using the Cell Death Detection ELISA Plus kit (Roche Diagnostics) (* indicates $p < 0.05$ vs. control; + indicates $p < 0.05$ comparing enrichment factors for necrosis versus late apoptosis, for a given particle concentration). Enrichment factor indicates the ratio of mono/oligo-nucleosomes in cell lysates (late apoptosis) or supernatants (necrosis) over respective negative controls. Data shows mean $\pm$ STD of 4 experiments performed in triplicates.

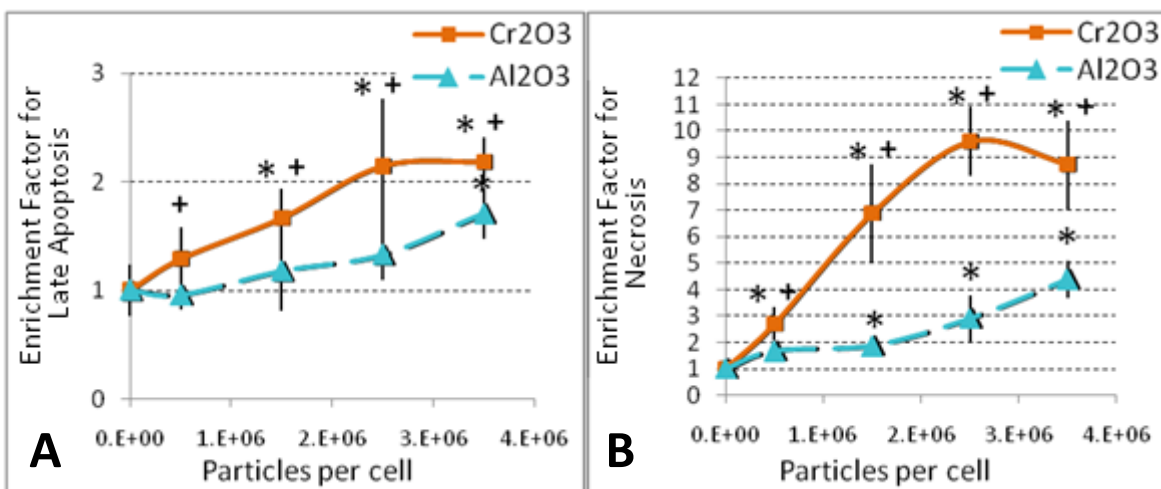


Figure 24: Effects of 60 nm Cr_2O_3 and 50 nm Al_2O_3 particles on (A) macrophage late apoptosis and (B) macrophage necrosis after 20-24h incubation, as determined using the Cell Death Detection ELISA Plus kit (Roche Diagnostics) (* indicates $p < 0.001$ vs. control; + indicates $p < 0.005$ when comparing Cr_2O_3 vs. Al_2O_3 at a given concentration). Enrichment factor indicates ratio of mono/oligo-nucleosomes in cell lysates (late apoptosis) or supernatants (necrosis) over respective negative controls. Data shows mean $\pm$ STD of 4 experiments performed in triplicates.

4.2.2.4 Cytokines/Chemokines

Neither Cr_2O_3 nor Al_2O_3 particles induced a significant increase in $\text{TNF-}\alpha$ release (Figure 25). No significant increase in MCP-1 was observed, except with 3.5 million Al_2O_3 particles, reaching 1.5 fold above the control level ($p=0.02$) (Figure 26). No significant increase in MIP-1 α release was observed, except with 0.5 million Cr_2O_3 particles per cell, reaching 1.2 fold above the control level ($p<0.001$) (Figure 27). Positive controls with LPS exhibited an average increase of approximately 46 fold, 4 fold and 6 fold above the $\text{TNF-}\alpha$, MCP-1 and MIP-1 α control levels (cells alone), respectively.

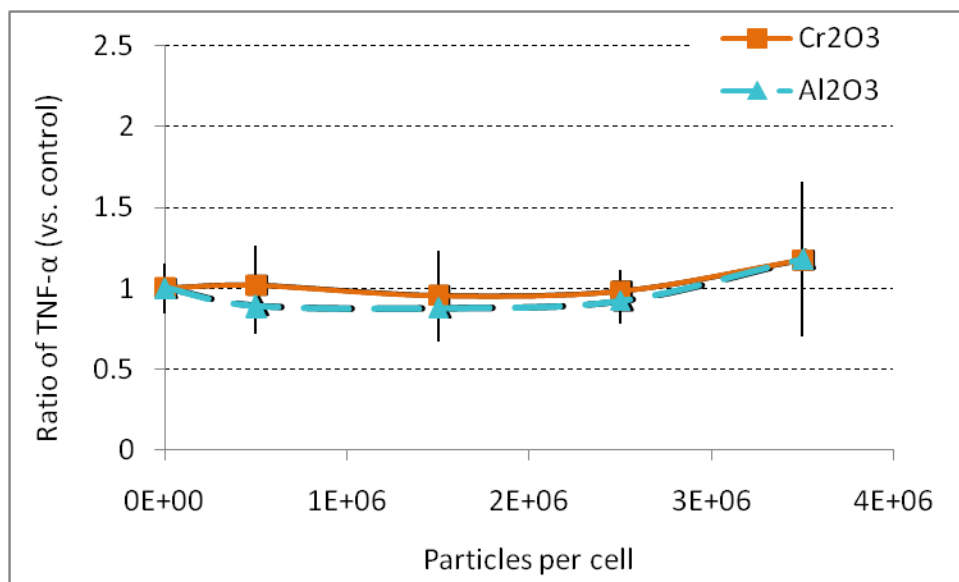


Figure 25: Effects of 60 nm Cr_2O_3 and 50 nm Al_2O_3 particles on $\text{TNF-}\alpha$ release after 20-24h incubation. Macrophages were incubated with different concentrations of Cr_2O_3 or Al_2O_3 particles. Results are expressed as ratios of the negative control level (cells with no particles) and are the mean $\pm$ STD of 3 experiments performed in triplicates.

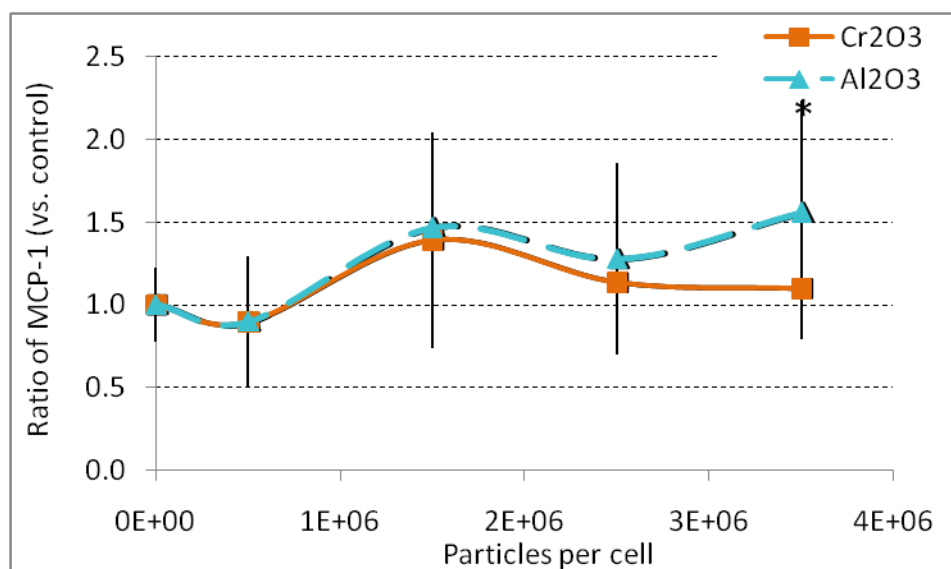


Figure 26: Effects of 60 nm Cr_2O_3 and 50 nm Al_2O_3 particles on MCP-1 release after 20-24h incubation (* indicates $p=0.02$). Macrophages were incubated with different concentrations of Cr_2O_3 or Al_2O_3 particles. Results are expressed as ratios of the negative control level (cells with no particles) and are the mean $\pm$ STD of 4 experiments performed in triplicates.

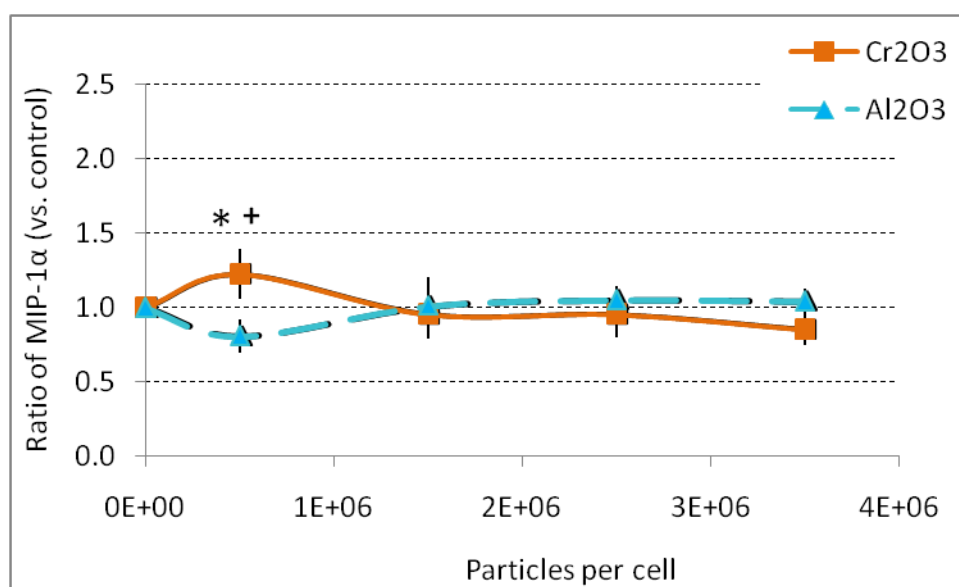


Figure 27: Effect of 60 nm Cr_2O_3 and 50 nm Al_2O_3 particles on MIP-1 α release after 20-24h incubation (* indicates $p=0.001$ vs. control; + indicates $p<0.001$ when comparing Cr_2O_3 with Al_2O_3 particles at a given concentration). Macrophages were incubated with different concentrations of Cr_2O_3 or Al_2O_3 particles. Results are expressed as ratios of the negative control level (cells with no particles) and are the mean $\pm$ STD of 4 experiments performed in triplicates.

5 Discussion

Despite the fact that nanometer-size chromium oxide particles are the predominant type of particles generated by MM implants [53, 58, 59, 61, 64], the biological response to these particles has not been described specifically. Therefore, the present study aimed to determine the cytotoxic effects of clinically relevant Cr_2O_3 particles, firstly by analyzing the effect of particle size, and secondly by comparing the effects with those of another type of similarly-sized ceramic particle (Al_2O_3), known to elicit a relatively low level inflammatory response [22, 39, 40, 62, 104, 108, 112, 114-116]. The effects of the particles on cultured macrophages were evaluated by measuring cell mortality (i.e., overall cell number, apoptosis and necrosis) as well as inflammatory cytokine (TNF- α) and chemokine (MCP-1 and MIP-1 α) release after incubation with the particles.

5.1 Particles

Commercially available 60 nm Cr_2O_3 particles were used in this study. As per the manufacturer specifications, they were mainly round. These characteristics are consistent with size, composition and shape of particles isolated from MM periprosthetic tissues *in vivo* [53, 59, 61, 64] and particles generated by hip simulators *in vitro* [58, 59]. Thus, these commercial particles were considered as clinically relevant. Larger Cr_2O_3 particles (700 nm) were also chosen as they offered equivalent characteristics to the 60 nm Cr_2O_3 particles with the exception of particle size, allowing for analysis of the effect of particle size and volume on macrophage response.

Finally, Al_2O_3 particles were selected for comparison with the Cr_2O_3 particles because both types of particles are forms of stable oxide ceramics produced by low-wear hip implant bearing surfaces [63, 70], and furthermore because the Al_2O_3 particles have been reported as being relatively bioinert [22, 39, 40, 62, 104, 108, 112, 114-116] and could therefore serve as a reference material.

Particle concentrations were selected to cover a large range of cytotoxic effects on macrophages. The lowest concentrations (0.5 million particles per macrophage for 60 nm Cr_2O_3 and 50 nm Al_2O_3 particles, and 500 particles per macrophage for 700 nm Cr_2O_3 particles) produced results similar to control conditions for many parameters measured. The highest concentrations of particles (3.5 million particles per macrophage for 60 nm Cr_2O_3 and 50 nm Al_2O_3 particles, and 2000 particles per macrophage for 700 nm Cr_2O_3 particles) induced such a significant decrease in cell number (particularly for cells incubated with Cr_2O_3 particles) that higher concentrations would have resulted in insufficient cells for analysis by flow cytometry. The 700 nm Cr_2O_3 particle concentrations were also further chosen for comparison of particle volume per cell versus the 60 nm Cr_2O_3 particles.

With regard to clinical relevance of the particle concentrations used, *in vivo* wear rates of MM implants have been estimated at 6 mm^3 or less per year [68, 97, 100]. This annual volumetric wear rate is equivalent to 5.3×10^{13} spherical 60 nm particles generated per year (which is in the range of previously reported particle numbers in MM tissues [61]), or 1×10^{15} particles generated over a twenty year period (a typical life expectancy for a well functioning MM

implant [99, 153]). While investigating the concentration of UHMWPE particles from conventional MPE implants in periprosthetic tissues, Elfick et al. found a median volume of 0.106 mm^3 of UHMWPE particles per gram of wet tissue [154]. Knowing that the volumetric wear of MM implants is approximately one-tenth that of conventional MPE implants (producing UHMWPE particles) [81, 97] (shown in Table 1 and Table 4), a volume of approximately 0.011 mm^3 particles per gram of wet tissue can be roughly estimated for MM implants. Approximating that all particles have similar properties as the Cr_2O_3 particles used in the present study (i.e., 60 nm diameter, spherical and with a density of $5.21 \times 10^6 \text{ g/m}^3$, resulting in a particle volume of $1.13 \times 10^{-13} \text{ mm}^3$ each), 0.011 mm^3 particles per gram of wet tissue would correspond to 9.73×10^{10} particles (this is without considering that some wear particles from MM implants are CoCrMo particles, with some needle-shaped). The number of macrophages in periprosthetic tissues is difficult to estimate because monocytes (which mature into macrophages) can be produced on an as-needed basis, and are recruited in large numbers to sites of debris or inflammation. It is estimated that the average human body contains about 0.2 trillion macrophages and other mononuclear phagocytic cells [155], or 0.43 million monocytes per cm^3 of healthy tissue (which would be equal to 0.43 million monocytes per gram if approximating the density of tissue to the density of water) [156]. Therefore, if the numbers of particles and macrophages per gram of periprosthetic tissues are approximated to about 9.73×10^{10} and 0.43 million, respectively, the particle concentration in periprosthetic tissues may be estimated to 0.23 million particles per macrophage. Interestingly, although the lowest Cr_2O_3 particle concentration of 0.5 million particles per cell in the present study is about two times higher than the particle concentration estimated in periprosthetic tissues, results with this

concentration were often comparable with controls (cells with no particles). This suggests that although high concentrations of nanometer-size Cr_2O_3 particles can induce cell mortality *in vitro* (including necrosis as discussed below), the particle loads required likely exceed the amounts of particles expected to be generated *in vivo*. Of note is that the present study was based on an *in vitro* (controlled and isolated) model with a single cell type. It could therefore not provide the exogenous stimulation that would continually be occurring *in vivo* (e.g., physical and chemical influence from other cell types). For this reason, higher concentrations of particles per cell are usually required *in vitro* to elicit a cell response and one could expect a stronger response *in vivo* with lower doses of particles.

Of course, in order to devise this simplistic approximation of particles per cell *in vivo*, many assumptions were necessary. As previously mentioned, MM implants do not generate exclusively 60 nm spherical Cr_2O_3 particles, and the particles are produced over the course of many years and not introduced all at once (affording time for macrophage ingestion, particle transport and cell mortality). The approximation could therefore be an overestimation since particle migration due to diffusion and cellular transport has been shown to be inversely proportional to particle size [154] and nanometer-size particles would disseminate more readily than micrometer-size particles, resulting in fewer particles in periprosthetic tissues. Also, macrophage infiltration is expected at sites of debris or inflammation, increasing the number of the cells and thereby decreasing the concentration of particles per cell. On the other hand, wear particles, which are not fully digestable by the macrophages, accumulate in the tissues over the years, increasing the concentration of particles/macrophage. In addition, the density

of tissue is most likely higher than that of water, and the conversion of the number of macrophages per cubic centimeter to number per gram of tissue would lead to a lower number and thereby inflate the concentration of particles per macrophage. Hence, although the above estimation of particle per cell is an interesting point of comparison for the present study, it is difficult to ascertain how closely it actually reflects the *in vivo* scenario.

A limitation of the present study is that the particles were obtained commercially. Hence, particle characteristics (e.g., surface roughness, etc.) were not controlled and may differ from particles generated *in vivo*. Additionally, although the particles tested negatively for endotoxins, a positive control sample other than the kit standards was not used and should be considered in future studies. Finally, there was a slight difference in the Cr_2O_3 and Al_2O_3 particle size (60 nm Cr_2O_3 particles and 50 nm Al_2O_3 particles) as 60 nm alumina particles were not commercially available. It is, however, unlikely that such a small difference in size would cause a significant difference in the cell response.

5.2 Phagocytosis

Phagocytosis was observed using light microscopy and flow cytometry as previously reported [22]. Results revealed that macrophages indiscriminately ingested Cr_2O_3 and Al_2O_3 particles, starting within the first 6 hours of exposure.

A limitation of light microscopy is that this technique did not allow the visualization of the particles inside the cells and therefore, some particles that appeared to be phagocytosed may have actually been adhering to the outer surface of the cell membrane. As particle adhesion to macrophage membrane can cause cell activation without phagocytosis [23], confirmation of particle internalization should be further considered. Transmission electron microscopy (TEM) would allow visualization of the intracellular compartment to confirm particle ingestion.

Flow cytometry FS-SS dot plots revealed that all particles induced an increase in SS compared to controls. A limitation was encountered with concentrations equal or higher than 1.5 million particles per cell for the 60 nm Cr_2O_3 particles and 50 nm Al_2O_3 particles, and concentrations equal or higher than 1000 particles per cell for the 700 nm Cr_2O_3 particles, as such concentrations induced increases in SS beyond the upper detection limit. This prevented a quantitative analysis of SS increase. Using the log scale during the acquisition could be considered for further analysis of SS changes in the future.

5.3 Mortality and Cytokine Release

Well-established techniques including trypan blue exclusion assay [146], flow cytometry [22], cell death ELISA [137] and cytokine/chemokine ELISA [38, 44] were used to measure total cell number, cell mortality (including the distinction of apoptosis and necrosis) and cytokine/chemokine release. Overall, Cr_2O_3 particles at both sizes and 50 nm Al_2O_3 particles increased cell mortality, as exemplified by decreases in total cell numbers and increases in

apoptosis (either early or late) and necrosis with increasing particle concentrations. Levels of TNF- α cytokine as well as MCP-1 and MIP-1 α chemokines did not increase significantly over controls, with the exception of two isolated cases in the Cr₂O₃ vs. Al₂O₃ study only, where MCP-1 reached 1.5 fold above the control level (with 3.5 million Al₂O₃ particles per cell), and where MIP-1 α reached 1.2 fold above the control level (with 0.5 million Cr₂O₃ particles per cell).

Although less pronounced than necrosis, an association has been drawn between apoptotic cell death and inflammation [26]. None of the two analyzed Cr₂O₃ particle sizes induced a significant increase in early apoptosis over controls, except for 3.5 million particles per cell (in the Cr₂O₃ vs. Al₂O₃ study). On the other hand, Al₂O₃ particles up to 2.5 million particles per macrophage induced a significant increase in early apoptosis (up to about 20% over control), which is consistent with previous reports of apoptosis with micrometer-size Al₂O₃ particles *in vitro* [104, 117]. These results show that a different cellular reaction was initiated in response to similarly-sized Cr₂O₃ and Al₂O₃ particles, despite the fact that they are both ceramics. At concentrations higher than 1.5 million particles per macrophage, the level of early apoptosis with Al₂O₃ particles gradually decreased. This could be because at these higher concentrations, more cells were undergoing late apoptosis and/or necrosis rather than early apoptosis. This explanation is supported by the cell death ELISA results, which show a significant increase in necrosis at concentrations higher than 1.5 million particles per macrophage (Figure 24B, paragraph 4.2.2.2). Levels of early apoptosis in the control samples were consistently high (25-30%). This may be attributed to the use of a rotator during incubation which kept the cells and particles in constant suspension, but may have induced macrophage activation and early

apoptosis [24, 29, 35]. In addition, because the culture tubes were being rotated, they were tightly capped and thus gas exchange (CO_2) was restricted, which could have further affected the level of early apoptosis.

Results with the cell death ELISA revealed that late apoptosis remained low but significantly increased with 60 nm Cr_2O_3 particle concentrations, as depicted by mono/oligo-nucleosome enrichment factors reaching 1.9 and 2.2 (ratios over control level) with 3.5 million particles per cell (for studies comparing 60 nm Cr_2O_3 with 700 nm Cr_2O_3 and with Al_2O_3 particles, respectively). Late apoptosis induced by Al_2O_3 particles also remained low, and only significantly increased with 3.5 million particles per cell, with mono/oligo-nucleosome enrichment factor reaching 1.7 (ratio of control level). At the lowest concentration for each particle type (0.5 million particles per cell – more similar to particle concentrations estimated *in vivo*), levels of late apoptosis were not significantly higher than controls. These results suggest that while high concentrations of clinically relevant Cr_2O_3 and Al_2O_3 particles are capable of inducing low levels of late apoptosis in macrophages, lower concentrations (approaching what may be expected *in vivo*) did not appear to do so. The presence of late apoptosis (albeit at low levels) due to Cr_2O_3 particles despite the non-significant level of early apoptosis is surprising. It is possible that the cells underwent apoptosis faster when exposed to Cr_2O_3 compared to Al_2O_3 particles, and therefore reached the late apoptosis stage earlier. A kinetic study of the effects of these particles on macrophage apoptosis could be valuable in deciphering this trend. However, a limitation of the present study lies in the interpretation and analysis of apoptosis using the Annexin-V FITC/PI assay with flow cytometry. This assay is based on the translocation of PS to

the outer leaflet [157]. The continuum between FITC Annexin-V histogram positive to negative peaks observed in the present study (when measuring early apoptosis) suggests that the translocation of PS does not occur extensively in J774 macrophages, as previously noted in another study using the same cells [137]. Additionally, it has been reported that some cells (including macrophages) constitutively expose PS on their membrane outer leaflets [157, 158], which could further explain the lack of a clear demarcation between the Annexin-V FITC positive and negative histogram peaks. Additional techniques to analyze apoptosis (such as DNA laddering and poly(ADP-ribose) polymerase (PARP) cleavage) could be useful in supporting the conclusions of the present study.

Despite the presence of apoptosis, especially with Al_2O_3 particles, the primary mode of cell death of macrophages incubated with both particle types was necrosis, as demonstrated by cell death ELISA. Indeed, the enrichment factors of mono/oligo-nucleosomes in cell culture supernatants (reflecting necrosis) after exposure to 60 nm Cr_2O_3 particles reached levels as high as 5.8 and 9.6 (ratios over controls) (from studies comparing 60 nm with 700 nm Cr_2O_3 and 50 nm Al_2O_3 particles, respectively), and up to 4.4 after exposure to 50 nm Al_2O_3 particles. Overall, Cr_2O_3 particles appeared to induce a significantly higher level of necrosis than Al_2O_3 particles ($p < 0.001$ when comparing the mono/oligo-nucleosome enrichment factor levels after incubation with 60 nm Cr_2O_3 vs. 50 nm Al_2O_3 particles at all concentrations). However, it is noted again that the numbers of particles per cell used to induce these levels of necrosis were quite high compared with what is expected *in vivo*. The lowest concentration of Cr_2O_3 particles (still approximately two fold the estimation of an *in vivo* concentration, as discussed above)

induced necrosis with mono/oligo-nucleosome enrichment factors of 1.4 and 2.7 over control (for studies comparing 60 nm Cr₂O₃ with 700 nm Cr₂O₃ and 50 nm Al₂O₃ particles, respectively).

Although the same treatment was imparted on the cells with 60 nm Cr₂O₃ particles across the two studies (comparing 60 nm Cr₂O₃ particles with 700 nm Cr₂O₃ and 50 nm Al₂O₃ particles), a considerable discrepancy in the magnitude of mono/oligo-nucleosome enrichment factors in the supernatants (reflecting necrosis) was noted (maximum enrichment factors over controls of 5.8 and 9.6, respectively). Because the treatments were the same, similar levels were expected. This discrepancy may have been caused by the use of different cell line lot numbers that could have responded slightly differently to particles in the different experiments. Trends, however, remained consistent through both studies.

Levels of late apoptosis-necrosis (PI-positive cells) increased significantly for all particle types, up to 16% and 14% for the 60 nm Cr₂O₃ particles (while comparing with 700 nm Cr₂O₃ and 50 nm Al₂O₃ particles, respectively) and up to 22% for the Al₂O₃ particles. The percentages of these cells were significantly higher for Al₂O₃ particles than Cr₂O₃ at all concentrations, although they remained close (within 4% of each other) for all but the highest concentration. This confirms that Cr₂O₃ and Al₂O₃ particles can cause late apoptosis and/or necrosis in macrophages, and suggests that Al₂O₃ particles may have a slightly greater effect than the Cr₂O₃. The fact that the cell death ELISA demonstrated significantly higher levels of more mono/oligo-nucleosomes in both the lysates and supernatants of cells exposed to Cr₂O₃ compared to Al₂O₃ particles (translating into higher levels of late apoptosis and necrosis,

respectively) may appear contradictory. However, while the cell death ELISA reflected the level of necrosis in the supernatants (measuring DNA fragmentation of previously ruptured cells), PI stained the late apoptotic and necrotic cells that yet remained in solution. Therefore, of the remaining cells in solution, the percentages of late apoptotic and/or necrotic cells in the Al_2O_3 particle samples were higher (vs. Cr_2O_3 particles), while the supernatants of the Cr_2O_3 particle samples contained more DNA fragmentation indicating more previously ruptured necrotic cells.

Necrosis is a mode of cell death that has been associated with an inflammatory response since cell membranes become damaged and inflammatory cytokines are released into the extracellular environment [26, 47, 48]. Despite the presence of necrotic cell death with both Cr_2O_3 and Al_2O_3 particles, TNF- α cytokine did not significantly increase, and MCP-1 and MIP-1 α chemokines remained low and increased significantly with only two conditions (1.5 and 1.2 fold control values, respectively). Kinetic studies of the effects of particles on total cell number revealed that the cells incubated with either Cr_2O_3 or Al_2O_3 particles were rapidly dying after exposure to the particles. Indeed, significant decreases in total cell number, sometimes resulting in numbers similar to those observed at 24 hours, were seen after only 6 hours of incubation. Considering that macrophages require several hours to produce and release cytokines and chemokines in response to a stimulus, it appears that they may have died before many of these inflammatory mediators could have been produced. Future kinetic studies analyzing gene expression of these mediators by the macrophages may be useful in confirming this theory.

Overall comparison of the effects of 60 nm vs. 700 nm Cr₂O₃ particles, and 60 nm Cr₂O₃ vs. 50 nm Al₂O₃

When combining particle size and concentration into an overall particle volume (calculated assuming spherical particles), macrophages appeared to respond similarly to equivalent volumes of 60 nm and 700 nm Cr₂O₃ particles per cell, suggesting that the volume of Cr₂O₃ particles rather than the absolute particle size or number dominated the macrophage response. Thus, future *in vitro* studies using Cr₂O₃ particles may benefit from considering and comparing the effects of particle volumes per cell rather than focusing on absolute particle size or number.

Results also revealed that, at high concentrations, 60 nm Cr₂O₃ particles induced a significantly greater decrease in total cell number, significantly less early apoptosis, and significantly more late apoptosis and necrosis, compared to Al₂O₃ particles. These results suggest that although both Cr₂O₃ and Al₂O₃ are forms of stable oxide ceramics, the macrophages responded differently to these particles. At high concentrations (1.5 million particles per macrophage or higher – i.e., about 6 fold the estimated *in vivo* concentration or higher, as described above in paragraph 5.1), Cr₂O₃ particles appear to be more cytotoxic than Al₂O₃ particles, inducing a larger decrease in the overall cell number and more necrosis *in vitro*.

Effects of Cr₂O₃ particles compared to other types of wear particles

Although decreased cell numbers and higher levels of apoptosis and necrosis were observed in cells incubated with high concentrations of Cr₂O₃ particles, levels remained low compared to those previously reported in cells incubated with lower numbers of micrometer-size UHMWPE

particles [102, 104, 159, 160]. Indeed, previous *in vitro* studies showed that fewer micrometer-size UHMWPE particles induced significant cell mortality [102, 104, 159, 160] and pro-inflammatory cytokine release [41, 76, 104, 159]. This suggests a lower cytotoxicity of nanometer-size Cr₂O₃ particles compared to micrometer-size UHMWPE particles.

Studies measuring the biological response to particles produced by MM implants have mainly been focused on the effect of CoCrMo particles, and in particular those of micrometer-size particles which are not the most clinically relevant and thus may be of limited value and applicability [58]. Some of these studies have reported significant decreases in cell viability [114] and significant increases in TNF- α [40, 126]. Few studies examining the biological effects of nanometer-size CoCrMo particles (i.e., more clinically relevant) suggest that these particles have cytotoxic effects which, in light of the current study, appear to be high compared to the Cr₂O₃ particles. For example, Germain et al. found that, in volumes similar to those obtained with the lowest concentration used in the present study (50 μm^3 per cell), nanometer-size CoCrMo particles (mean size: 29.5 nm) induced a 43% decrease in macrophage viability after one day of incubation [62]. Likewise, Brown et al. reported a significant decrease of approximately 45% in fibroblast viability after one day, as well as a significant increase in TNF- α release by human peripheral blood mononuclear cells *in vitro* due to nanometer-size CoCrMo particles (mean size: 40 nm), in volumes similar to those obtained with the lowest concentration used in the present study (50 μm^3 per cell) [20].

The study of Kwon et al. reporting the effects of nanometer-size chromium particles on macrophage viability is perhaps the most comparable one to the present study [135]. It is likely that the particles used by these authors were in fact a form of chromium oxide considering the propensity for nanometer-scale metal particles to instantaneously oxidize [161]. Although the authors reported no significant effect of these particles on cell number, results depict a substantial decrease of up to about 30% fewer cells after one day, which corroborate with the results of the present study. Nevertheless, differences exist between Kwon et al. and the present studies. These differences include the use of the RAW 264.7 murine macrophage cell line supplemented with 10% FBS by Kwon et al. (vs. J774 murine macrophages with 5% FBS in the present study). Additionally, to determine cell viability, Kwon et al. used the Alamar Blue proliferation assay and a Live/Dead assay for determining the proportion of live to dead cells while the trypan blue exclusion assay and cell counting with light microscopy was applied in the present study. Furthermore, Kwon et al. did not disclose the number of particles placed in culture with the cells. Therefore, the direct comparison of the results remains difficult.

6 Conclusion & Future Studies

This study showed that:

- 1) at high concentrations, Cr_2O_3 particles may be cytotoxic to macrophages, inducing significant decreases in total cell numbers and increases in necrosis *in vitro*;

- 2) the macrophage response to Cr_2O_3 may be dependent on the volume of particles rather than the absolute Cr_2O_3 particle size or particle number;
 - 3) similarly-sized Cr_2O_3 and Al_2O_3 particles appear to elicit a different response from macrophages *in vitro* even though they are both stable forms of ceramic materials;
 - 4) both 60 nm Cr_2O_3 and 50 nm Al_2O_3 particles in high concentrations induce macrophage mortality within 6 hours, which may explain why levels of TNF- α , MCP-1 and MIP-1 α remained low (leaving insufficient time for cytokine/chemokine production); and
 - 5) while the cytotoxicity of nanometer-size Cr_2O_3 particles may be higher than that of Al_2O_3 particles, it appears lower than that of previously reported UHMWPE and CoCrMo particles.
- Therefore, chromium oxide particles may not be the main culprit in initiating the inflammatory reaction in MM periprosthetic tissues.

As the biological effects of nanometer-size chromium oxide particles (predominant wear particle-type produced by MM implants [53, 59, 61, 64, 136]) were not previously reported, the findings of this study are both novel and significant. Furthermore, the findings that large numbers of nanometer-size Cr_2O_3 particles are more cytotoxic than similar numbers of nanometer-size Al_2O_3 particles, but apparently less cytotoxic than UHMWPE and CoCrMo particles (as previously reported in the literature) introduce a relative measure of the osteolytic potential for this particle type that was previously unknown. Thus, this study provides an important contribution to the field of MM implant wear products and their biological effects.

Future studies may include a kinetic analysis of the induction of macrophage apoptosis by Cr_2O_3 and Al_2O_3 particles, a kinetic analysis of cytokine/chemokine gene expression induced by Cr_2O_3 particles, as well as a comparison of the biological effects of nanometer-size Cr_2O_3 versus CoCrMo and HXPE particles. Additionally, it would be interesting to investigate the effects of lower concentrations of Cr_2O_3 particles over a longer incubation period. Finally, longer term future studies should include the analysis of the effects of clinically relevant particles from MM implants in animal models.

7 References

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