Neural Cell Responses to Wear Debris from Metal-on-Metal Total Disc Replacements

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1.0 Abstract

Purpose: Total disc replacements (TDRs), comprising all-metal articulations, are compromised by wear and particle production. Metallic wear debris and ions trigger a range of biological responses including inflammation, genotoxicity, cytotoxicity, hypersensitivity and pseudotumour formation, therefore we hypothesise that, due to proximity to the spinal cord, glial cells may be adversely affected.

Methods: Clinically relevant cobalt chrome (CoCr) and stainless steel (SS) wear particles were generated using a six-station pin-on-plate wear simulator. The effects of metallic particles (0.5 μm^3 – 50 μm^3 debris per cell) and metal ions on glial cell viability, cellular activity (glial fibrillary acidic protein expression) and DNA integrity were investigated in 2D and 3D culture using live/dead, immunocytochemistry and a Comet assay, respectively.

Results: CoCr wear particles and ions caused significant reductions in glial cell viability in both 2D and 3D culture systems. Stainless steel particles did not affect glial cell viability or astrocyte activation. In contrast, ions released from SS caused significant reductions in glial cell viability, an effect that was especially noticeable when astrocytes were cultured in isolation without microglia. DNA damage was observed in both cell types and with both biomaterials tested. CoCr wear particles had a dose-dependent effect on astrocyte activation, measured through expression of GFAP.

Conclusions: The results from this study suggest that microglia influence the effects that metal particles have on astrocytes, that SS ions and particles play a role in the adverse effects observed and that SS is a less toxic biomaterial than CoCr alloy for use in spinal devices.

Key Words

Total Disc Replacements (TDR); Metal wear particles; Cobalt Chromium; Stainless steel; glial cells

2.0 Introduction

Significant economic and patient issues associated with early failure of metal-on-metal (MOM) total hip and resurfacing replacements have been the focus of the public and scientific media recently [1]. These events have led to concerns over other MOM implant systems, including those used in spinal surgery, where a number of articulating total disc replacements (TDRs) are still in use. TDRs comprise structures that have a large number of interfaces, e.g. endplate/bone interface, endplate screws and bone as well as the articulating surfaces. All of these interacting surfaces may produce debris and/or ions with the potential to invoke adverse biological

responses, which may lead to failure and ultimately device revision. Metallic TDRs generate wear, debris and/or corrosion products [2-4] therefore cytotoxic and immunological responses may become a significant problem in the future. Of particular concern is a recent case report describing metallosis and the formation of pseudotumours around a metal-on-metal TDR, similar to failing hip resurfacing arthroplasties [5]. There is evidence documenting the release of metallic particulates from other spinal devices and the inflammatory responses that ensue, including cellular infiltrates and cytokine release (predominantly TNF-α) [6-12]. Clinically there are reports that describe neurological symptoms, including radicular pain and paraparesis, in patients with metallosis associated with spinal devices [7, 13]. Inflammation would appear to be a key factor in the development of such symptoms, however, other reports also describe late operative site pain [8,9].

The effects of ions and metal debris remain largely unknown for the neural structures in the spine. Cells of the central nervous system (CNS) are protected by the meninges. Disruption of these anatomical features makes the spinal cord and adjacent neural structures particularly vulnerable to exposure to tribo-corrosive products. Recent research has shown that porcine dural cells and dural organ cultures exposed to cobalt chromium (CoCr) nanoparticles produced significantly elevated levels of the pro-inflammatory chemokine IL-8 [14,15], which adversely affected the barrier function of the endothelial cell layer [16]. In addition, significant alterations to the structural integrity of the dura mater were observed together with elevated expression of matrix metalloproteinases (MMP-1, -3, -9, -13) and tissue inhibitor of metalloproteinase-1 indicating that tissue remodelling and collagen fibre loosening were prevalent in the presence of CoCr particles. In combination these effects may enhance nanoparticle penetration into the dura mater and enable access to the cord tissue. Indeed, there is evidence that particles introduced epidurally have the ability to cross both the dural barrier in vivo [11] and the blood spinal cord barrier, the latter has been exploited for drug delivery [17]. In addition, it has been shown that rapid CSF fluid flow occurs from the subarachnoid space into the spinal cord canal via the peri-vascular spaces and these may allow nanoparticle access to these tissues [18, 19]. This has led to the hypothesis that the barrier functions protecting the spinal cord will be compromised by exposure to metal wear particles and/or ions and that subsequent adverse responses to these species by neural cell populations will result in neurotoxicity, leading to complications and subsequent failure of spinal implants. This is likely to be determined by material type, with some materials (e.g. titanium, PEEK) being less neurotoxic and some materials causing adverse effects. It should be noted that both titanium and PEEK are not or are no longer used as bearing materials due to poor tribologial properties and inadequate wear resistance of titanium and a lack of evidence regarding the tribologial performance of PEEK as a bearing surface compared to the more commonly used polymer, ultra high molecular weight polyethylene (UHMWPE). The present study investigated the response of primary rat glial cells to CoCr and stainless steel particles and ions from implant biomaterials in 2D and 3D cell culture systems over time. Particulate and ion effects on neural cell viability, DNA integrity and glial fibrillary acidic protein (GFAP) expression in astrocytes were the focus of the study.

3.0 Materials and methods

3.1 Wear particle generation and characterisation

Clinically relevant cobalt–chromium and stainless steel wear particles were generated using an in house manufactured pin-on-plate tribometer and distilled water as the lubricant as described by Germain et al. [20]. Both the pin and plate were made from either medical grade wrought cobalt–chromium alloy (according to ASTM F1537) with a high carbon content or medical grade stainless steel (316L). Debris was generated using smooth (R_a 0.01-0.02 μm) counterfaces with a multidirectional motion (rotation ±30°, stroke length 28 mm, frequency 1 Hz) and a contact stress of 11 MPa over 80h. For particle characterisation, a 1 ml aliquot of each lubricant was sequentially filtered through 5, 1, 0.1 and 0.015 μm sized polycarbonate filters (Whatman) and filters examined using high resolution scanning electron microscopy (SEM), at magnifications up to x150K, combined with energy dispersive x-ray (EDX) analysis to determine particle composition. Particle size and area distributions were generated using Image Pro Plus 6.0 (Media Cybernetics, USA). Stock solutions (1 $mg.ml^{-1}$) of each debris type were generated from sterile, endotoxin-free particles [20]. Particles were dispersed by sonication prior to use.

3.2 Neural cell isolation and culture

All experiments were performed according to the UK Animals (Scientific Procedures) Act (1986) and approved by the appropriate institutional ethical committee. Primary astrocyte cultures were prepared from the cortices of postnatal 2 day-old Wistar rat pups as described by East et al [21]. The tissue was chopped and cells isolated by incubation with 250 $\mu g.ml^{-1}$ trypsin. Digests were repeatedly triturated using a 1 ml pipette to break down any larger clumps of tissue. The resultant cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin/streptomycin (100 $U.ml^{-1}$ and 100 $\mu g.ml^{-1}$, respectively) and with 10% (v/v) foetal calf serum (FCS). This suspension was dispensed into tissue culture flasks that had been coated with poly-D-lysine (50 $\mu g.ml^{-1}$) and placed in a humidified incubator at 37 °C with 5% CO_2:95% air. Cells were expanded in culture for 10-14 days to reach confluence. After this time cell types were separated by shaking at 150 rpm for 4h to remove microglia and less adherent cell types from the resulting astrocytes. Both cell types were passaged into fresh media before being exposed to wear particles and/or ions.

3.3 Culture of cells with particles and ions

Primary rat astrocytes and microglia in isolation and in co-culture were cultured with CoCr or stainless steel wear particles or ions (0.05 μm^3 – 50 μm^3 per cell) for up to 5 days in both 2D and 3D culture systems. In the 2D system, cells were seeded at 1 x 10^4 cells per well (n=6) in supplemented DMEM medium in poly-D lysine coated 96-well plates. Particles were added and cell viability was assessed after 1, 3 and 5 days using the ATP-Lite assay (Perkin Elmer, Windsor, UK). DNA damage was also assessed using the alkaline Comet assay (Trevigen, Maryland, USA) at 24h, 48h and 5 day time points [22]. After staining with SYBR Gold (ThermoFisher Scientific, UK) slides were imaged using a Zeiss fluorescent microscope and the Olive tail moment was used to calculate DNA damage over time using Comet IV Lite software (Perceptive Instruments, UK). Data was expressed as mean comet tail moment ± 95% confidence limits (n= 6).

For 3D systems, cells were seeded at 1 x 10^6 ml^{-1} into collagen gels (First Link, UK; 2 $mg.ml^{-1}$) along with the appropriate volume of particles suspended in DMEM. A volume of 100 µl of collagen gel containing cells and particles was seeded into 96-well plates. Particles were dosed at 0.5, 5, 50 μm^3 per cell (n=6) and cell viability assessed using a live/dead cytotoxicity assay for mammalian cells (ThermoFisher Scientific, UK) after 48h and 5 days in culture. The expression of glial fibrillary acidic protein (GFAP), a marker of astrocyte activation and reactive gliosis, was assessed at the same time points using a polyclonal primary antibody (Agilent Technologies UK Ltd) and an anti-rabbit DyLight® 549 (Vector Laboratories, UK) secondary antibody. Hoescht 33258 was used to stain cell nuclei. Cells were imaged at 6 locations within each gel (n=6) using a LSM510 confocal laser scanning microscope (Carl Zeis Ltd) leading to 36 images per test. The number of live and dead cells were calculated and immunopositive cells were scored (images were negative (GFAP expression not detectable) or positive (GFAP upregulated to detectable level)) using Image J software (NIH, USA) compared to the negative control images.

For the culture of primary glial cells with CoCr or SS ions, the appropriate mass of particles (equivalent to 0.5, 5, 50 μm^3 particles per cell) was incubated with supplemented DMEM for 24h at 37°C in 5% (v/v) CO_2 in air with agitation. The particle suspensions were centrifuged at 160,000 x g and the supernatants containing ions were added to wells containing cells suspended in collagen gel. The same assays as described above were performed to assess the effect of metal ions on cell viability and the expression of GFAP.

3.4 Statistical Analysis

Data was presented as mean ± 95% confidence limits of the mean and statistical analysis performed using a two-way ANOVA. Significant differences between means were determined using Tukeys post hoc analysis. A p value of $p<0.05$ was indicative of statistical significance

4.0 Results

4.1 Characterisation of Cobalt Chromium and Stainless Steel Particles

Particles were isolated through sequential filtration through 5, 1, 0.1 and 0.015 µm filters, which were coated with platinum and imaged using high resolution FEGSEM. Cobalt chromium particles were viewed as agglomerates on all filters (Fig. 1A-D). At higher magnifications (> x30K) on the 0.1 and 0.015 µm filters, particles were round to oval and in the nanoscale. The majority of particles produced were between 10 and 120 nm and the mode was 30-39 nm (Fig. 2). Particles were identified as comprising cobalt, chromium, molybdenum and oxygen as the major elements (Online Resources 1). Stainless steel particles were observed as irregular granular particles on the larger pore size filters and round nanoscale particles on the smaller pore size filters (Fig. 3A-D). The particles ranged in size from 10 nm to larger than 1 mm and the mode was between 30 and 39 nm (Fig. 4) similar to CoCr. The elemental composition of the stainless steel particles was comprised largely of iron and nickel plus carbon and oxygen (Online Resource 2).

4.2 The effect of CoCr and stainless steel particles on primary astrocytes and microglia in 2D culture

The effect of increasing volumes of cobalt chrome particles on the viability of primary astrocytes and microglia in 2D co-culture was assessed using the ATP Lite™ assay over time. After 24h there were no significant effects on cell viability, however, after 3 and 5 days in culture a statistically significant reduction in viability was observed for all particle doses (Fig. 5A; $p<0.05$, ANOVA). With stainless steel particles there was an initial adverse effect on viability observed after 24h at concentrations of between 0.5 and 50 μm^3 per cell and at day 5 at the highest particle dose only (Fig. 5B; $p<0.05$, ANOVA).

Only the highest concentration of CoCr particles (50 μm^3 per cell) had a significant effect on DNA integrity in the first 24h of culture (Fig. 6A; $p<0.05$ ANOVA plus Tukey). After 48h all concentrations of CoCr particles adversely affected DNA integrity, however, after 5 days only the 50 μm^3 particle concentration caused a significant increase in DNA damage (Fig. 6A). When astrocytes were cultured in isolation in 2D monolayer culture with CoCr particles, all particle concentrations caused significant DNA damage from 24h onwards (Fig. 6B; $p<0.05$ ANOVA and Tukey). Stainless steel particles compromised DNA integrity from 24h an effect that was dose dependent (Fig 6C). When primary astrocytes were cultured in isolation with stainless steel particles, significant effects on DNA integrity were observed at 2 and 5 days with the 5 and 50 μm^3 particle per cell concentrations (Fig, 6D; $p<0.05$ ANOVA and Tukey).

4.3 The effect of CoCr and stainless steel particles on primary astrocytes and microglia in 3D culture

A live dead assay was used to determine the effect of wear particles on the viability of primary astrocytes and microglia in co-culture and primary astrocytes in isolation in a 3D collagen hydrogel cell culture system. Gels were imaged using fluorescence microscopy and the percentage of living cells calculated. After 2 days in culture 50 μm^3 of CoCr particles adversely affected cell viability (Fig 7A; $p<0.05$ ANOVA plus Tukey). After 5 days in culture both the 5 and 50 μm^3 per cell of CoCr particles significantly reduced cell viability (Fig. 7A; $p<0.05$, ANOVA and Tukey post hoc test). Significant effects on viability were observed when the primary astrocytes were cultured with only the 50 μm^3 CoCr particle concentration after two and five days in culture (Fig. 7B; $p<0.05$ ANOVA and Tukey post hoc test).

No adverse effects on cell viability were observed when primary astrocytes and microglia were cultured together or in isolation with any concentration of stainless steel debris at any time point (Fig 7C and 7D).

4.4 The effect of ions released from CoCr and stainless steel wear debris on the viability of primary astrocytes and microglia in 3D culture

There were no adverse effects on cell viability of CoCr ions at any concentration at the 48h time point (Fig. 8A). However, a significant reduction in viability at the 5 μm^3 per cell concentration of cobalt chrome ions was observed after five days in culture (Fig 8A; $p<0.05$ ANOVA and Tukey post hoc test). When primary astrocytes were cultured in isolation with CoCr ions, all doses affected cell viability significantly (Fig. 8B; $p<0.05$, ANOVA and Tukey post hoc test) after 2 days. After 5 days the ions released from 5 μm^3 and 50 μm^3 CoCr

particles significantly affected cell viability (Fig. 8B). No adverse effects were observed when primary astrocytes and microglia were cultured in 3D co-culture with ions released from stainless steel particles at concentrations of 0.5 and 5 μm^3 per cell (Fig. 8C). However, after 5 days in culture, ions from the highest concentration of stainless steel debris (50 μm^3 per cell) adversely affected cell viability (Fig. 8C; $p<0.05$ ANOVA and post hoc Tukey test). When astrocytes were cultured in isolation with ions from stainless steel particles, after 2 days only the 5 μm^3 per cell concentration had a significant effect on cell viability, however by day 5 all concentrations of ions adversely affected cell viability (Fig. 8D).

4.5 The effect of CoCr and stainless steel wear particles on the level of expression of glial fibrillary acidic protein (GFAP) in primary astrocytes in the presence and absence of microglia

When primary astrocytes were co-cultured with microglia in 3D culture with CoCr particles (0.5 – 50 μm^3 per cell), the highest concentration of particles (50 μm^3 per cell) resulted in increased expression of GFAP at both the two and five day time point (Table 1). However, lower concentrations of CoCr wear particles only caused increased expression of GFAP after 5 days. Stainless steel particles failed to increase GFAP expression to detectable levels in astrocytes at any particle concentration at any time point. When primary astrocytes were cultured in isolation in 3D culture with CoCr wear particles, the 5 and 0.5 μm^3 per cell concentrations of particles increased the expression of GFAP after 5 days. Again, stainless steel particles did not cause increased expression of GFAP at any concentration or time point (Table 2).

5.0 Discussion

5.1 Characterisation of Wear Particles

The particles of CoCr generated in this study were similar in terms of size and morphology to previous studies [20, 22], and comparable to those generated in metal-on-metal total disc replacement devices [2]. The stainless steel particles were similar in morphology to the CoCr particles, however, their size range was larger, which is in line with the different material properties of the two metals, particularly with respect to chemistry, hardness, and microstructure e.g. grain size [23].

5.2 Effects of wear particles on cell viability and DNA integrity in 2D culture

When CoCr wear particles were cultured with primary rat astrocytes and microglial cells in 2D culture cell viability was adversely affected by all doses of particles. This is in line with previous studies that exposed monocytes and fibroblasts to CoCr wear particles [20]. When cells were exposed to stainless steel particles in 2D culture, initially an adverse effect was observed, however, this effect was only consistent at the highest particle concentration at the 5 day time point. Other studies have observed variable cellular toxicity associated with stainless steel particles, with some cell types e.g. monocytes adversely affected [24] and others not affected e.g. osteoblasts [25]. The different effects are postulated to be due to different sensitivities of cell lines and

different compositions of the two metals, with CoCr having a higher chromium content than stainless steel (27-30% compared to 16-18%) and the absence of cobalt in stainless steel [26].

When glial cells were exposed to CoCr and SS particles and DNA integrity analysed, again there were differences between the two biomaterials. High doses of CoCr particles significantly affected DNA integrity of glial cells. In isolation, astrocytes were more sensitive to CoCr particles. These results suggested that when microglial cells were present, these cells influenced the effects that the particles had on the astrocyte cells, often with the microglial cells experiencing adverse effects in the form of DNA damage, whilst appearing to protect the astrocytes from these effects in the co-culture. After culture with SS particles, adverse effects on glial cell DNA integrity were observed after 24h with all concentrations of particles. In contrast astrocytes in isolation were not affected by stainless steel particles.

5.3 Effects of wear particles and ions on cell viability and cell reactivity in 3D culture

When primary astrocytes and microglia were cultured with increasing particle volumes of CoCr a dose dependent effect on cell viability was observed. When primary astrocytes were cultured in isolation with increasing particle volumes of CoCr, only the highest particle dose significantly reduced cell viability. Interestingly the removal of microglia from the culture environment appeared to reduce the sensitivity of primary astrocytes to CoCr wear particles, suggesting the resident macrophage cell type, the microglia, influence the effects that the particles have on astrocytes. When cells were exposed to CoCr ions, the results suggested that astrocytes in isolation were more sensitive than when glial cells were co-cultured. We postulate that this may be due to either active uptake (phagocytosis) of large volumes of particles into microglia, followed by the release of cytokines or signalling factors that trigger subsequent cell death in the astrocytes. When astrocytes were cultured in isolation with particles, uptake may occur via pinocytosis and hence be much slower, resulting in lower levels of toxicity [15,16].

In contrast, there were no adverse effects on cell viability when glial cells were cultured with increasing particle volumes of stainless steel at any particle dose, or at any time point. These results echo the results in the 2D culture system, indicating that differences in elemental composition between the two biomaterials may be responsible for the different effects on cell viability. In contrast, adverse effects on cell viability were observed when glial cells were exposed to ions from stainless steel particles, particularly when astrocytes were cultured in isolation, indicating that in the absence of microglia, astrocytes were more sensitive to ions from implant biomaterials.

Upregulation of the expression of glial fibrillary acidic protein (GFAP) to a variety of stimulants including injury [21] and biomaterial particles [27], has been widely reported in the literature. CoCr particles caused increased expression of GFAP at low particle concentrations by the five day time point, however, often the higher concentrations of particles did not. We postulate that these higher particle concentrations caused significant reductions in cell viability, and with a large proportion of the cells dead or dying, this masked the

detection of GFAP. Stainless steel particles had no effect on the GFAP expression in glial cells at any dose or any time point.

In summary, CoCr particles adversely affected glial cell viability and triggered increased GFAP expression, indicating astrocytes developed a reactive phenotype and were subjected to significant DNA damage. Cobalt Chromium ions also had adverse effects on cell viability. In contrast stainless steel particles rarely affected cell viability and did not elicit increased expression of GFAP. However, SS particles caused significant DNA damage and glial cells appeared to be more sensitive to stainless steel ions compared to CoCr ions, where the former were found to adversely affect cell viability at low concentrations and early time points. The results indicated that DNA damage was the result of interaction with ionic species rather than the particles themselves.

The particles used in the present study were generated using a simple configuration pin-on-plate wear simulator using water as a lubricant. In a previous study [2], the biocompatibility of CoCr wear particles generated from CoCr alloy on CCr alloy TDRs in serum and water were compared with particles generated in water using the same pin-on-plate set up used in the present study. In L929 fibroblast cells, Pasko [2] found that there was no difference in the viability of L929 cells exposed to the different types of particles, with higher volumes of particles (5 and 50 μm^3 of particles per cell) causing adverse effects on cell viability in a similar way that CoCr particles affected neural cells in the present study. The use of particles from joint simulators using serum lubricants causes issues with microbial contamination, endotoxin contamination and degraded proteins, which all affect the cellular responses, making it difficult to determine the effects of the particles alone. Simple configuration pin-on-plate simulators provide a quick, cost effective way to generate large volumes of debris for cell culture easily avoiding these issues.

This study has revealed for the first time that clinically-relevant wear particles from metallic biomaterials have numerous adverse effects on the cells of the spinal cord and caution should be exercised when using these materials in spinal devices and instrumentation.

6.0 References

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Fig. 1 High resolution FEGSEM images of cobalt chromium particles generated by pin-on-plate summation in water lubricant. A) Aggregate particles isolated on a 5 μm filter, x 120 magnification; B) Aggregates of cobalt chromium particles isolated on a 1 μm filter, x 400 magnification; C) Aggregates of cobalt chromium particles isolated on a 0.1 μm filter, x 30,000 magnification; D) high magnification image of cobalt chromium aggregates isolated on a 0.1 μm filter, x60,000 magnification. Arrows indicate large irregular micron sized particles. ** indicates agglomerate of submicron sized particles.

Fig. 2 Size distribution of cobalt chromium particles generated in a six-station pin-on-plate wear simulator using water as the lubricant

Fig. 3 High resolution FEGSEM images of stainless steel particles generated by pin-on-plate summation in water lubricant. A) Aggregate particles isolated on a 5 μm filter, x 120 magnification; B) Aggregates of stainless steel particles isolated on a 1 μm filter, x 120 magnification; C) Aggregates of stainless steel particles isolated on a 1 μm filter, x 400 magnification; D) high magnification image of stainless steel aggregates isolated on a 0.1 μm filter, x60,000 magnification

Fig. 4. Size distribution of stainless steel particles generated in a six-station pin-on-plate wear simulator using water as the lubricant

Fig. 5 The effect of cobalt chrome and stainless steel particles on the viability of primary astrocytes and microglia in co-culture in 2D monolayer culture over five days. Cell viability measured using ATP-Lite assay after exposure to increasing concentrations of A) CoCr and B) stainless steel particles; *significant difference between means (Two way ANOVA plus Tukey, $p<0.05$) when compared to the cell only negative control

Fig. 6 The effect of increasing cobalt chrome and stainless steel particle volumes on the integrity of primary glial cell DNA over five days co-cultured in 2D culture, mean tail moment ± 95% confidence intervals, hydrogen peroxide was used as a positive control. A) effect of CoCr particles on astrocyte and microglial DNA in co-culture, B) effect of CoCr particles on astrocyte DNA cultured in isolation, C) effect of stainless steel particles on astrocyte and microglial DNA in co-culture, D) effect of stainless steel particles on astrocyte DNA cultured in isolation. *Indicates a significant increase in level of DNA damage expressed as a value for tail moment ($p<0.05$) when compared with cell only negative control using Two-way ANOVA. • Indicates a significant decrease in level of DNA damage expressed as a value for tail moment ($p<0.05$) when compared with cell only negative control using Two-way ANOVA

Fig. 7 The effect of increasing volumes ($0.5\mu m^3$-$50\mu m^3$ per cell) of CoCr and stainless steel particles on the viability of primary glial cells cultured together and in isolation in 3D in culture over five days. Cell viability measured using a live/dead assay, mean percentage of living cells ± 95% confidence intervals. DMSO was used as a positive control. A) effect of CoCr particles on astrocyte and microglial viability in co-culture, B) effect of CoCr particles on astrocyte viability cultured in isolation, C) effect of stainless steel particles on astrocyte and microglial viability in co-culture, D) effect of stainless steel particles on astrocyte viability

cultured in isolation. *Indicates a significant reduction in viability ($p<0.05$) when compared with cell only negative control using Two-way ANOVA and Tukey post hoc test

Fig. 8. The effect of increasing concentrations of ions from CoCr and stainless steel wear particles ($0.5\mu m^3$-$50\mu m^3$) on the viability of primary glial cells in 3D co-culture and in isolation over five days. Cell viability measured using a live/dead assay, mean percentage of living cells ± 95% confidence intervals. DMSO was used as the positive control. A) effect of CoCr ions on astrocyte and microglial viability in co-culture, B) effect of CoCr ions on astrocyte viability cultured in isolation, C) effect of stainless steel ions on astrocyte and microglial viability in co-culture, D) effect of stainless steel ions on astrocyte viability cultured in isolation. *Indicates a significant reduction in viability ($p<0.05$) when compared with cell only negative control using Two-way ANOVA and post hoc Tukey test

Table 1. The effects of cobalt chrome and stainless steel particles on the expression of GFAP by primary astrocytes in the presence of microglia after two and five days in culture

Table 2. The effects of cobalt chrome and stainless steel particles on the expression of GFAP by primary astrocytes after two and five days in culture

Online Resources 1. High resolution scanning electron micrograph and corresponding trace produced by energy dispersive X-ray analysis (EDX) of cobalt chromium particles

Online Resources 2. High resolution scanning electron micrograph and corresponding trace produced by energy dispersive X-ray analysis (EDX) of stainless steel particles

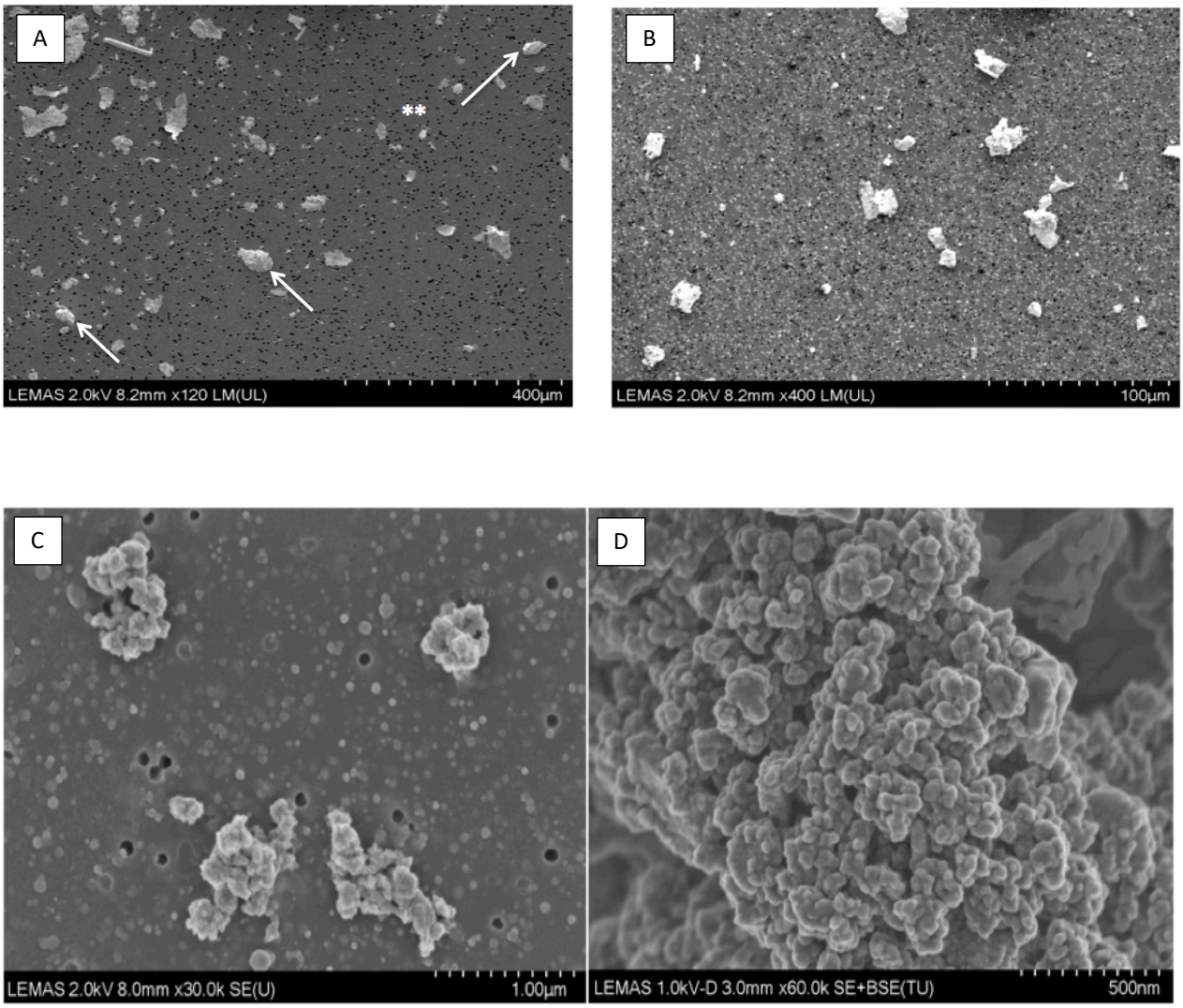


Fig. 1 High resolution FEGSEM images of cobalt chromium particles generated by pin-on-plate summation in water lubricant. A) Aggregate particles isolated on a 5 µm filter, x 120 magnification; B) Aggregates of cobalt chromium particles isolated on a 1 µm filter, x 400 magnification; C) Aggregates of cobalt chromium particles isolated on a 0.1 µm filter, x 30,000 magnification; D) high magnification image of cobalt chromium aggregates isolated on a 0.1 µm filter, x60,000 magnification.

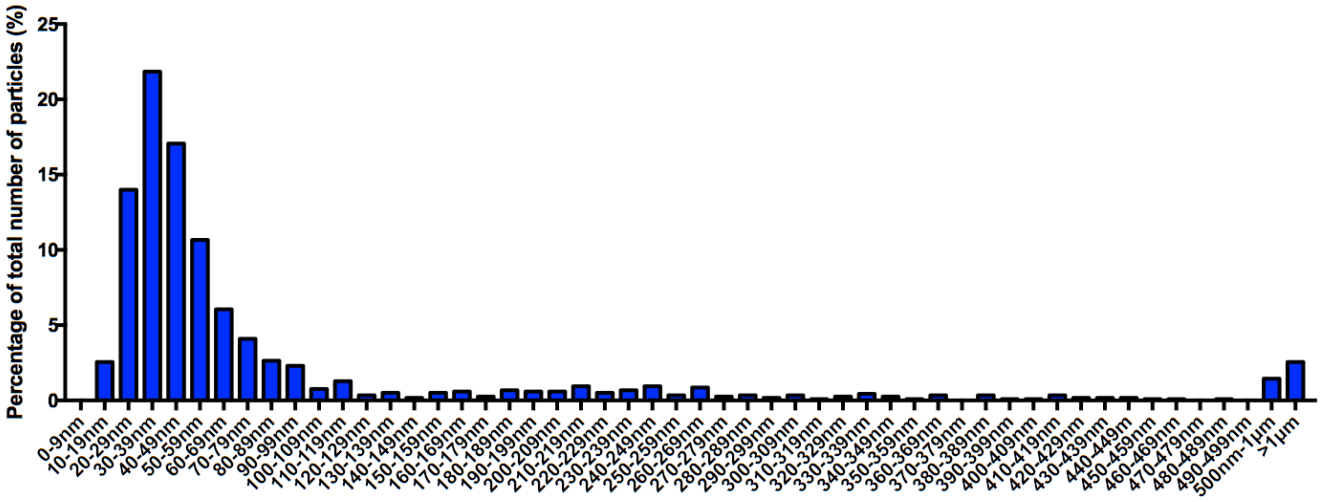


Fig. 2 Size distribution of cobalt chromium particles generated in a six-station pin-on-plate wear simulator using water as the lubricant.

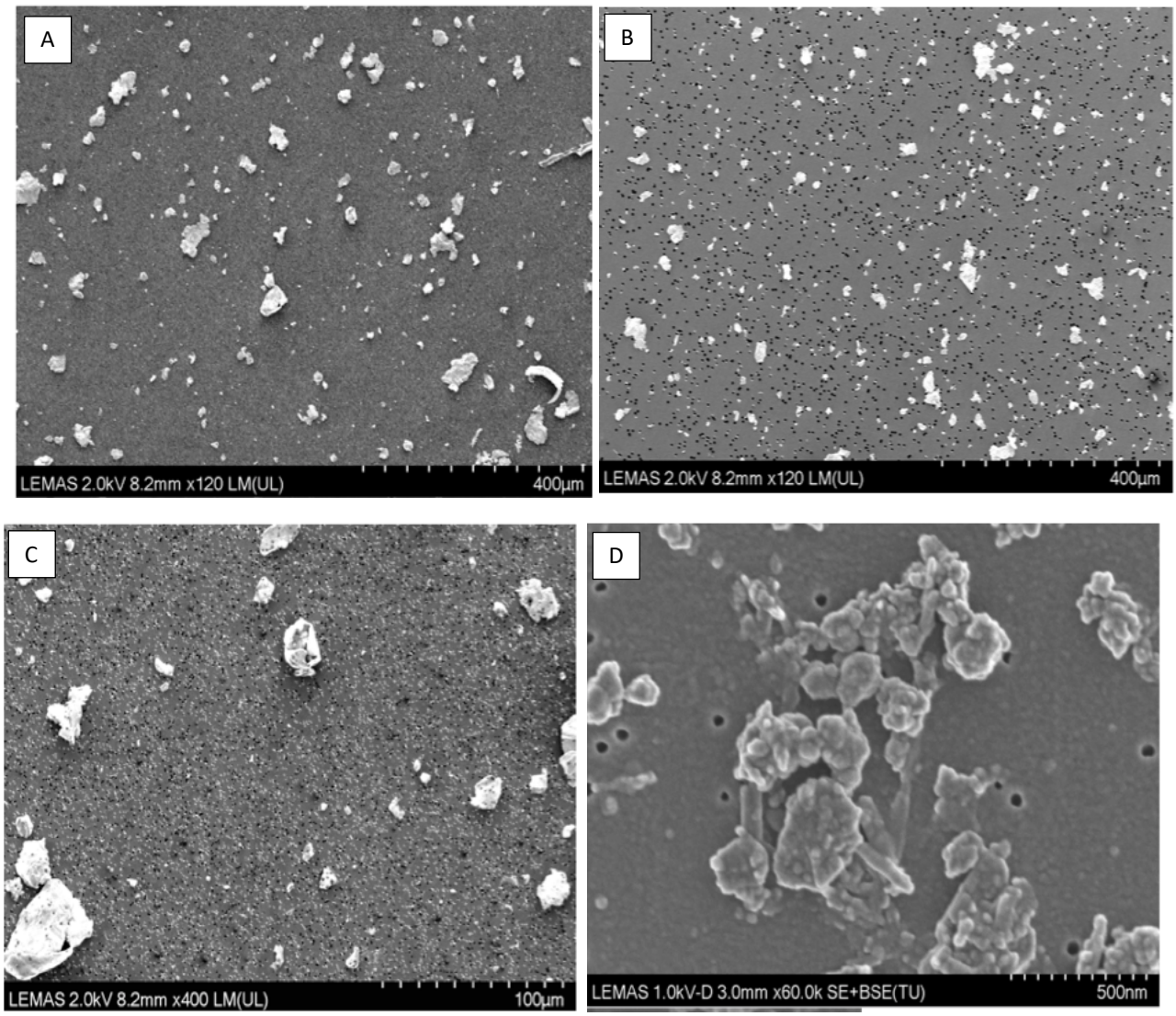


Fig. 3 High resolution FEGSEM images of stainless steel particles generated by pin-on-plate summation in water lubricant. A) Aggregate particles isolated on a 5 µm filter, x 120 magnification; B) Aggregates of stainless steel particles isolated on a 1 µm filter, x 120 magnification; C) Aggregates of stainless steel particles isolated on a 1 µm filter, x 400 magnification; D) high magnification image of stainless steel aggregates isolated on a 0.1 µm filter, x60,000 magnification.

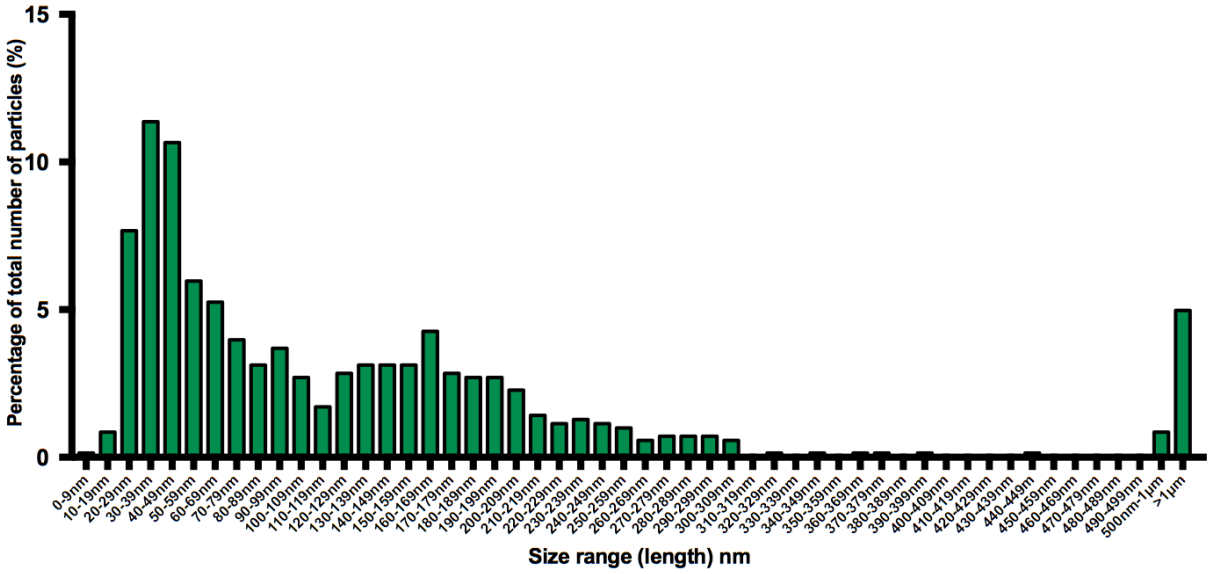


Fig. 4. Size distribution of stainless steel particles generated in a six-station pin-on-plate wear simulator using water as the lubricant.

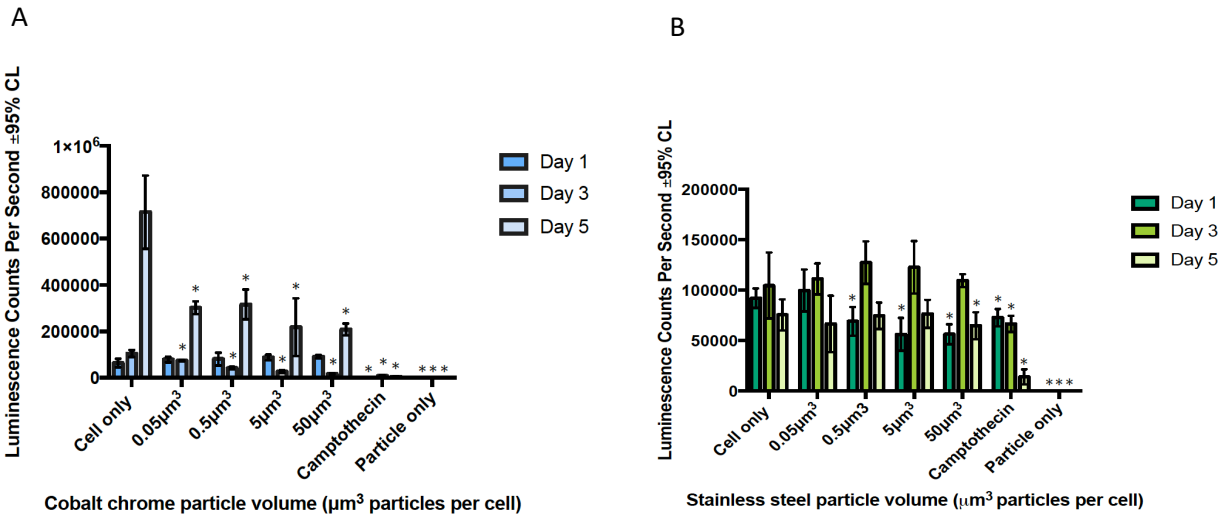


Fig. 5 The effect of cobalt chrome and stainless steel particles on the viability of primary astrocytes and microglia in co-culture in 2D monolayer culture over five days. Cell viability measured using ATP-Lite assay after exposure to increasing concentrations of A) CoCr and B) stainless steel particles; *significant difference between means (Two way ANOVA plus Tukey, $p<0.05$) when compared to the cell only negative control.

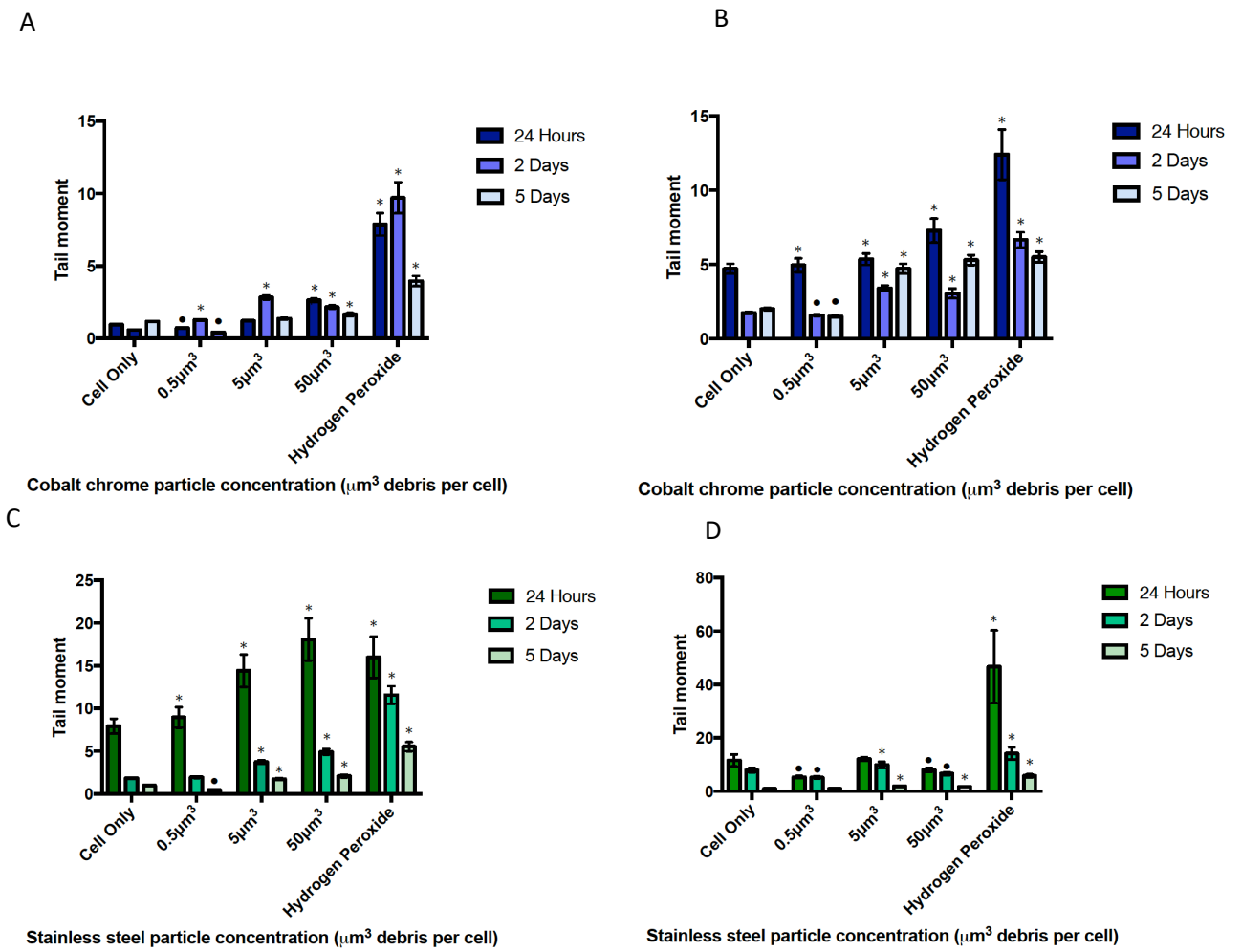


Fig. 6 The effect of increasing cobalt chrome and stainless steel particle volumes on the integrity of primary glial cell DNA over five days co-cultured in 2D culture, mean tail moment ± 95% confidence intervals, hydrogen peroxide was used as a positive control. A) effect of CoCr particles on astrocyte and microglial DNA in co-culture, B) effect of CoCr particles on astrocyte DNA cultured in isolation, C) effect of stainless steel particles on astrocyte and microglial DNA in co-culture, D) effect of stainless steel particles on astrocyte DNA cultured in isolation. *Indicates a significant increase in level of DNA damage expressed as a value for tail moment ($p<0.05$) when compared with cell only negative control using Two-way ANOVA

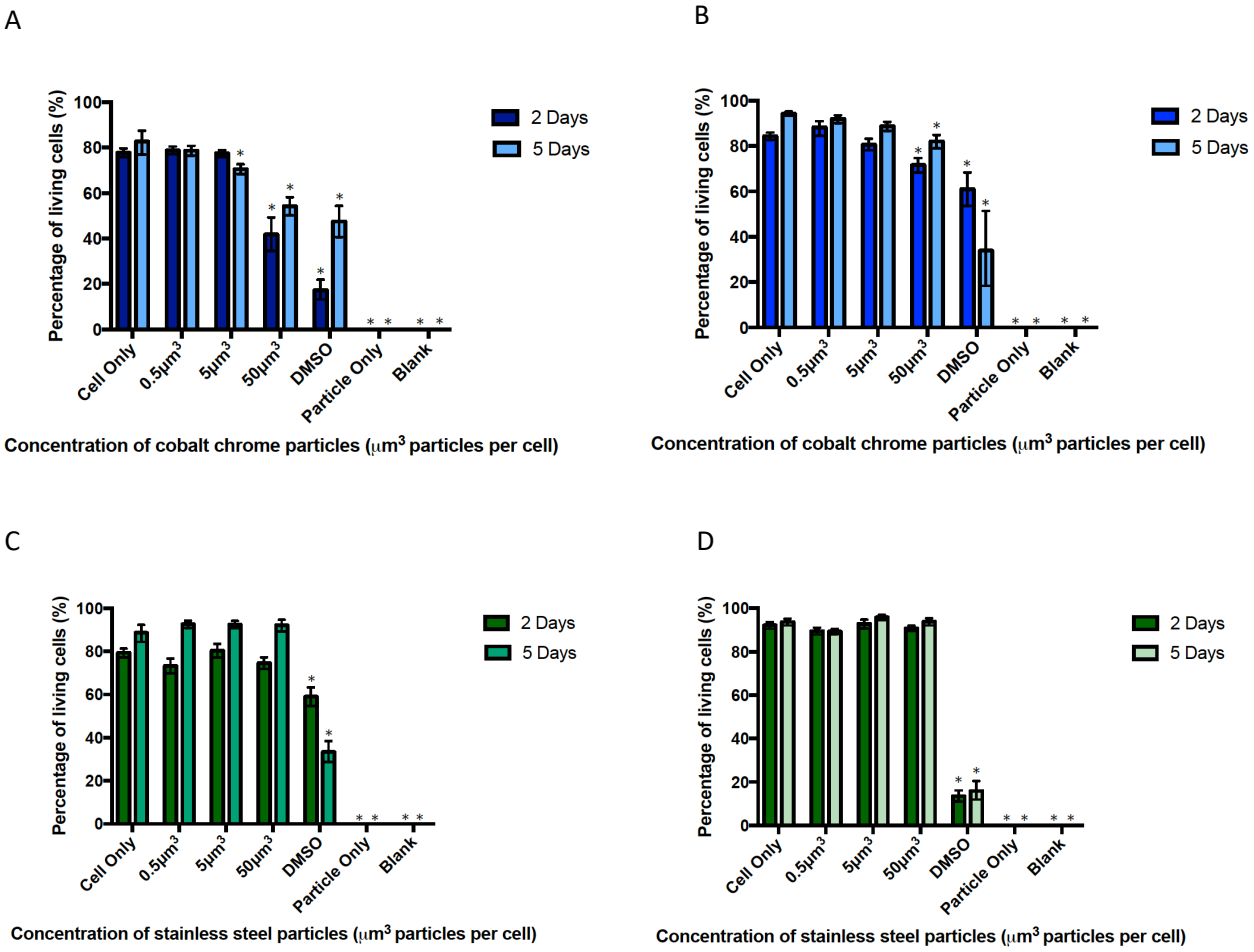


Fig. 7 The effect of increasing volumes ($0.5\mu m^3$-$50\mu m^3$ per cell) of CoCr and stainless steel particles on the viability of primary glial cells cultured together and in isolation in 3D in culture over five days. Cell viability measured using a live/dead assay, mean percentage of living cells ± 95% confidence intervals. DMSO was used as a positive control. A) effect of CoCr particles on astrocyte and microglial DNA in co-culture, B) effect of CoCr particles on astrocyte DNA cultured in isolation, C) effect of stainless steel particles on astrocyte and microglial DNA in co-culture, D) effect of stainless steel particles on astrocyte DNA cultured in isolation. *Indicates a significant reduction in viability ($p<0.05$) when compared with cell only negative control using Two-way ANOVA and Tukey post hoc test.

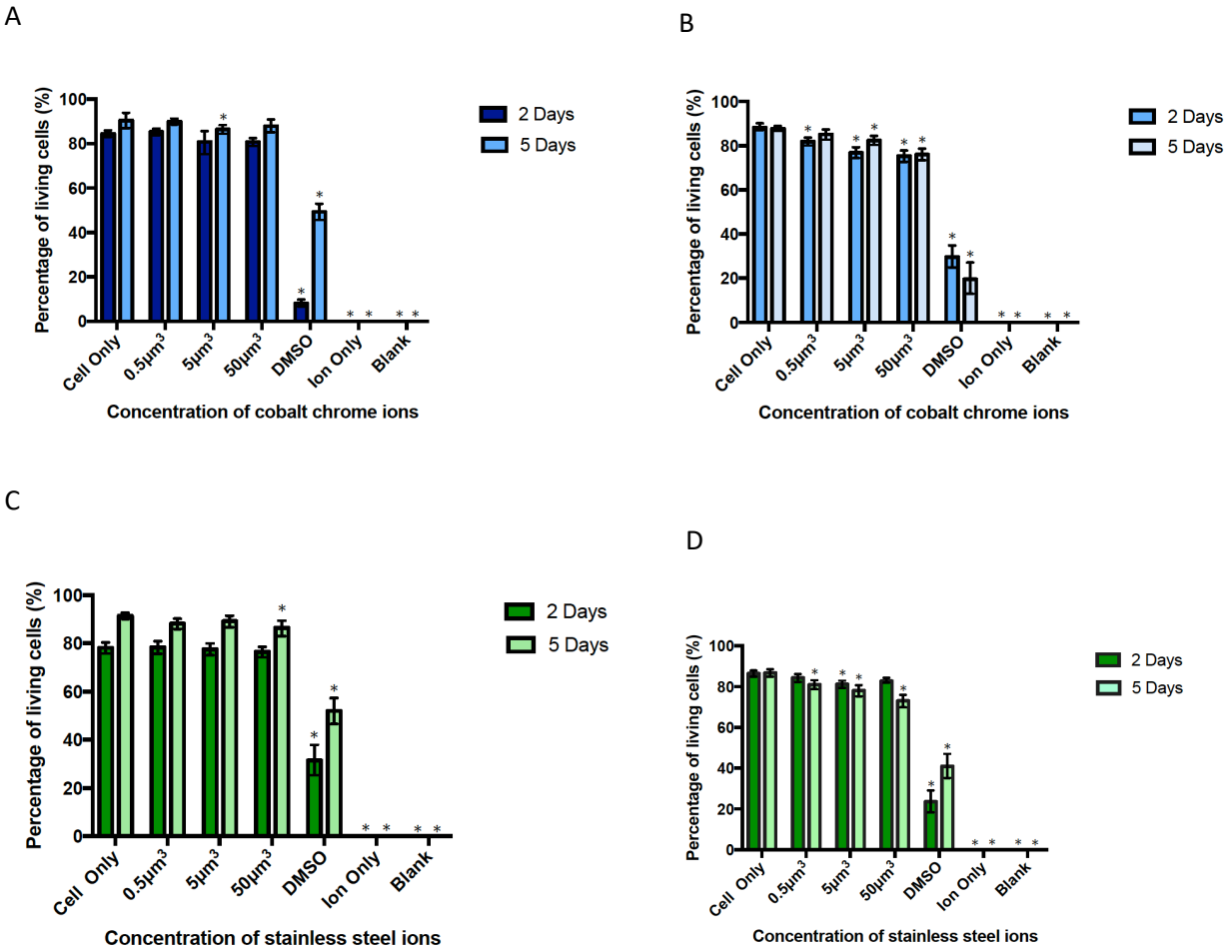


Fig. 8. The effect of increasing concentrations of ions from CoCr and stainless steel wear particles (0.5µm^3-50µm^3) on the viability of primary glial cells in 3D co-culture and in isolation over five days. Cell viability measured using a live/dead assay, mean percentage of living cells ± 95% confidence intervals. DMSO was used as the positive control. A) effect of CoCr ions on astrocyte and microglial DNA in co-culture, B) effect of CoCr ions on astrocyte DNA cultured in isolation, C) effect of stainless steel ions on astrocyte and microglial DNA in co-culture, D) effect of stainless steel ions on astrocyte DNA cultured in isolation. *Indicates a significant reduction in viability ($p<0.05$) when compared with cell only negative control using Two-way ANOVA and post hoc Tukey test.

Table 1. The effects of cobalt chrome and stainless steel particles on the expression of GFAP by primary astrocytes in the presence of microglia after two and five days in culture

	Cobalt chrome 2 days	Cobalt chrome 5 days	Stainless steel 2 days	Stainless steel 5 days
50μm^3 debris per cell	+	+	-	-
5μm^3 debris per cell	-	+	-	-
0.5μm^3 debris per cell	-	+	-	+/-
TGF-β1 (positive control)	+	+	+	+
Cell only	-	-	-	-
Particle only	-	-	-	-
Blank	-	-	-	-

+ indicates the presence of GFAP positive cells; - indicates the absence of GFAP positive cells;

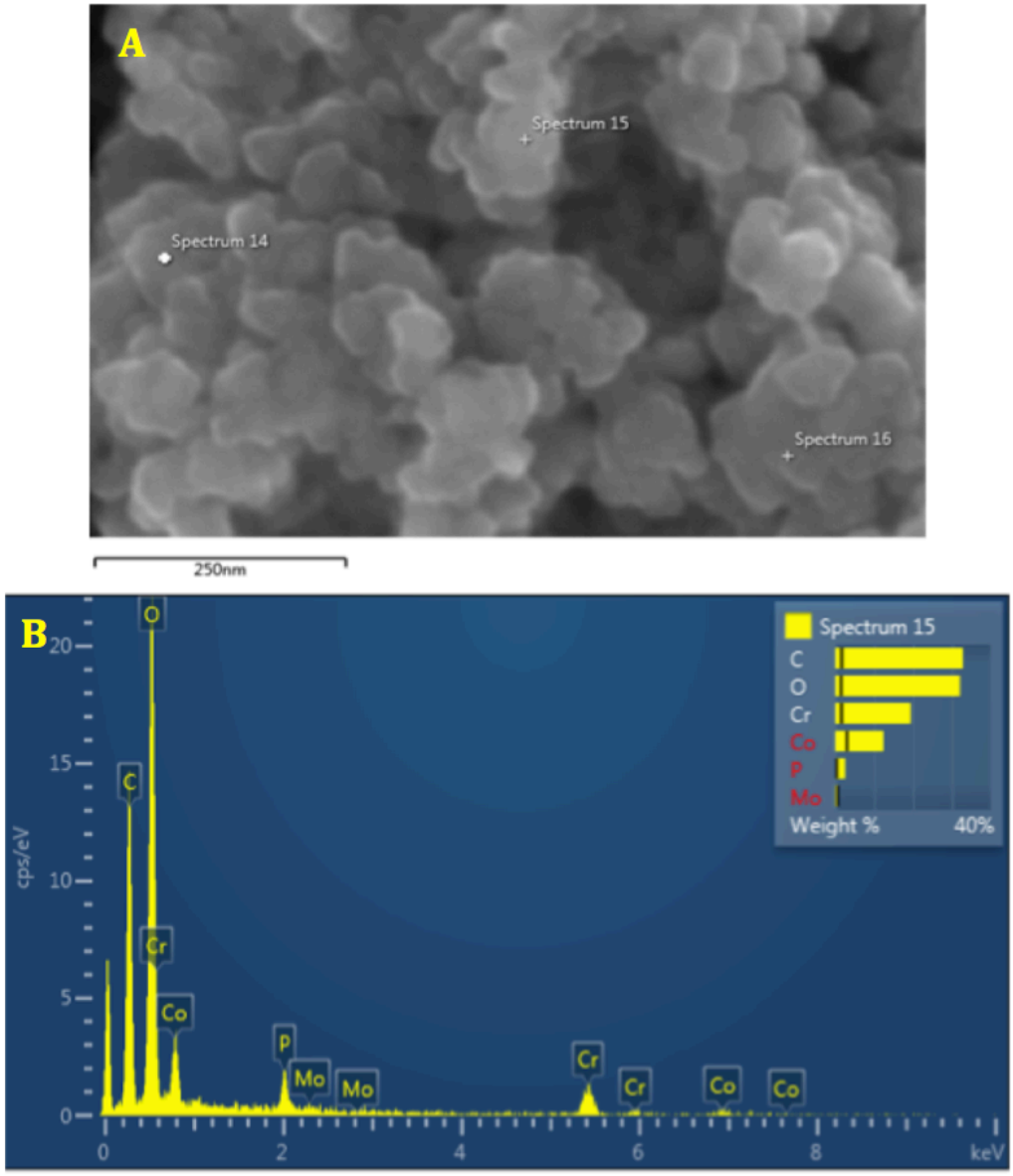
+/- indicates equal numbers of GFAP positive and negative cells

Table 2. The effects of cobalt chrome and stainless steel particles on the expression of GFAP by primary astrocytes after two and five days in culture

	Cobalt chrome 2 days	Cobalt chrome 5 days	Stainless steel 2 days	Stainless steel 5 days
50μm^3 debris per cell	-	-	-	-
5μm^3 debris per cell	-	+	-	-
0.5μm^3 debris per cell	-	+	-	-
TGF-1β (positive control)	+	+	+	+
Cell only	-	-	-	-
Particle only	-	-	-	-
Blank	-	-	-	-

+ indicates the presence of GFAP positive cells; - indicates the absence of GFAP positive cells;

+/- indicates equal numbers of GFAP positive and negative cells



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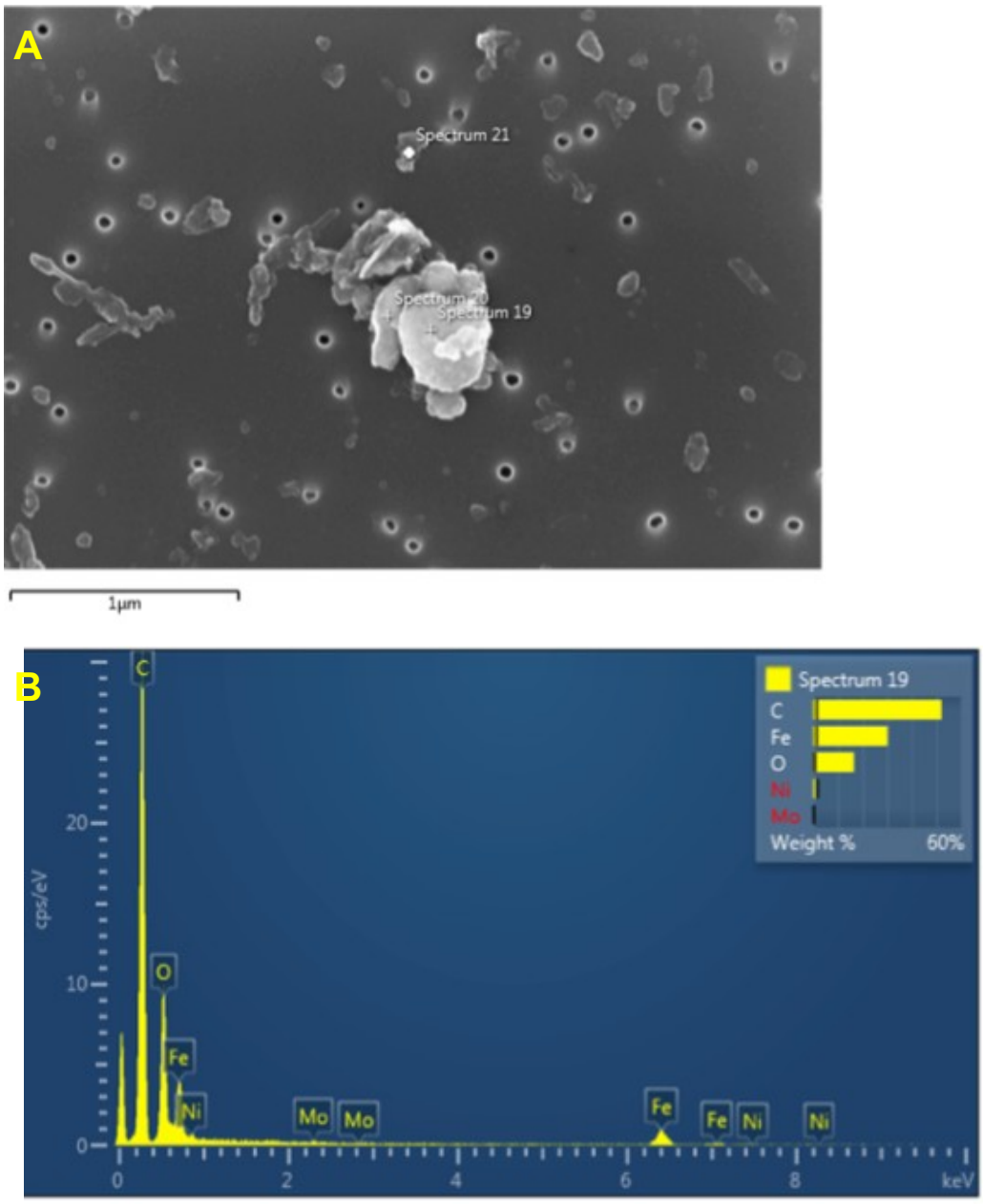

Online Resources 2. High resolution scanning electron micrograph and corresponding trace produced by energy dispersive X-ray analysis (EDX) of stainless steel particles