Published in final edited form as:

Nanotoxicology. 2018 November ; 12(9): 941–956. doi:10.1080/17435390.2018.1498929.

Differential toxicity of processed and non-processed states of CoCrMo degradation products generated from a hip simulator on neural cells

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Abstract

Physico-chemical characteristics of the CoCrMo degradation products have played an important role in cytotoxicity and clinical complications on the orthopedic patients who have metal implants. Previous studies have limited reflection on the physicochemical characteristics of the degradation products generated *in vivo*, which are very different from individual metal particles and/or ions obtained from different commercial sources. In this study, we aimed to understand the differences in toxicity induced by the degradation products in as-synthesized form as well as those obtained after post-processing. The degradation products were generated using a hip-simulator by maintaining physiological conditions closer to *in vivo* and separated into two batches, one with processing by washing and drying called processed degradation products (PDP) and another batch as 'as-synthesized' degradation product (DP). We studied the dose-dependent toxicity response by neural cells derived from induced pluripotent stem cells. The results of the study show that as-synthesized DPs are more toxic to neural cells even at lower concentrations studied with evident low TC50 (1-5 µg/ml) concentrations compared to PDP (25 µg/ml). Flow cytometric analysis showed a significant ($p < 0.01$) increase in uptake of the particles after 24h and corresponding ROS production in DP treated cells. RT-PCR analysis of oxidative specific gene expression showed, elevated mRNA levels of NADPH oxidase 1, nuclear transcription factor, Superoxide dismutase-2 and Glutaredoxin-2 in DP treated cells after 6h. The results of the study provided a clear evidence of the differential response of neural cells on the degradation products as a function of concentrations and their chemical nature.

Keywords

CoCrMo; neurotoxicity; metal particles; flow cytometry; particle uptake; Total Hip Replacement

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⁶Conflicts of Interest: None

1.0 Introduction

Total Joint Replacement (TJR) surgeries are the standard treatment option for patients with end-stage clinical intervention for the joint related disabilities (Fitzgerald et al. 2004, Jones et al. 2005, Harding et al. 2014, Wanderling et al. 2017). The most commonly replaced joint is the knee, followed by the Total Hip Replacements (THR). Currently, around 300,000 THRs are annually performed in the US, and this is expected to increase to 572,000 by 2030. It has been reported that as 2014, approximately 4.5 million Americans have undergone THR procedure in the US (Maradit Kremers et al. 2015). Different combinations of materials have been employed at the articulation (ball and socket), such as the metal-on-metal (MoM), Metal-on-polyethylene (MoP), Metal-on Ceramic (MoC) and Ceramic on ceramic (CoC) (Kurtz et al. 2007, Bozic et al. 2009). Many MoM devices were recalled due to the concern arisen from FDA on the adverse tissue reaction and other side effects on the patients (Hip Replacement Recall - Stryker, Zimmer & DePuy Hip Recalls n.d., Health 2018a, 2018b). MoP or CoP implants are currently in use with uncertain limitations on their long-term survival. A long-standing clinical concern or potential risk is the morbidity of biological and immunological responses (Hallab and Jacobs 2009) caused by implant degradation products (DPs).

Irrespective of the type of implants (MoP or MoM), the DPs released from the implants, in the form of nano and micro-particles, metal ions, and polyethylene particles are disseminated into the surrounding tissue as well as to remote locations in the body. Such particles can also be developed as metallo-organic species by mixing with surrounding organic species such as proteins or blood or tissues. The adverse effects of this process are becoming apparent and were critical to the failure of many THR designs (Liao et al. 2013, Hallab 2009, Jacobs et al. 2003, Hallab and Jacobs 2009). Substantial scientific evidence has been reported regarding the toxicity of CoCrMo alloy particles, as well as Lymphocyte ions in fibroblasts, macrophages, lymphocytes, osteoblasts, and osteoclasts *in vitro* and *in vivo* studies (Catelas et al. 2005, Papageorgiou et al. 2007, Madathil et al. 2010, Akbar et al. 2011, Zijlstra et al. 2012, VanOs et al. 2014, Abudayyak et al. 2016, Samelko et al. 2016). Recent clinical studies have reported polyneuropathy with progressive sensory disturbance, hearing loss, and hypothyroidism with sural nerve biopsy indicating axonopathy and cardiomyopathy (Charette et al. n.d., Moniz et al. n.d., Oldenburg et al. 2009, Ikeda et al. 2010) in THR patients who had systemically elevated levels of chromium (Cr) and cobalt (Co). Neurotoxicity due to metals such as Al, Cu, V, As and Pb were reported decades ago and their consequences as Alzheimer's Disease, Parkinson's Disease, etc. were well established. Plenty of reports are available showing the toxicity of cobalt and chromium to the peri-prosthetic tissues (Hallab et al. 2004, Davies et al. 2005, Boardman et al. 2006, Caicedo et al. 2010) though very limited information with regard to neurotoxicity.

As mentioned earlier, it was well established that the degradation products (DPs) of the implants materials are not just metal particles alone. Instead, they may present as metal-protein complexes, free metallic ions, inorganic metal salts or oxides and as organic storage form such as hemosiderin (Jacobs et al. 2003). Also, previous studies reported that the physicochemical characteristics and the wear particles vary on the method of generation, and the bioactivity of these DPs varies significantly based on their physicochemical

characteristics (Bowsher et al. 2006, Pajarinen et al. 2009, Billi and Campbell 2010, Prokopovich 2014, Mirshafiee et al. 2016). The particles characterized from the peri-prosthetic tissues of the retrieval implant show a variety of geometrical shapes and dimensions such as round, oval, needle, spike, etc., with varying degrees of sizes (Campbell et al. 1995, Doorn et al. 1998, Billi and Campbell 2010, Madl, Kovochich, et al. 2015, Madl, Liong, et al. 2015). However, still, there are disparities in the analysis of physicochemical characteristics of the wear particles, or degradations products (in a broad meaning) from tissue samples due to the variation in isolation methodologies adopted by the researchers. Different isolation techniques have specific impact on the characteristics of the particles. Moreover, some of the reported studies had used such degradation products by purchasing from commercial agencies, which is very far from the chemical states of the *in vivo* DPs generated from the hip implants.

Inconclusive results may affect the overall outcome of the implant based toxicology studies. *In vitro* hip-simulator studies showed that the debris generated using a serum (simulated synovial fluid) has similar characteristics to that of patient tissue samples (Madl, Liong, et al. 2015). Both tissue sample and hip simulator generated particles are in nanometer size range and low cobalt content. Hence, toxicity studies using micron-sized particles may not provide clinically relevant toxicity results. Moreover, the majority of studies tested CoCl_2 or CrCl_3 , Cr_6O_2 , Cr_3O_4 , $\text{K}_2\text{Cr}_2\text{O}_7$ (potassium dichromate), as the source of Co and Cr ions (Ponti et al. 2009, Catalani et al. 2012). In addition, commercially available CoCr-based nano- and micro-particles were also used to evaluate the cytotoxicity of wear particles from orthopedic implants and prostheses. The above-mentioned particles from different sources have varying degrees of clinical relevance. It is important to consider that the toxicity of wear products *in vivo* can be an outcome of the cumulative effect of different forms of metal debris in the degradation product.

Hence, in this study, we focused to evaluate the cumulative effect of CoCrMo wear particles generated from hip simulator on the cytotoxic response. In addition, we compared the toxic effect of DPs obtained from the hip simulator to that of PDPs, to understand the differential response due to processing conditions. Here we have used human neural cells derived from induced Pluripotent Stem Cells (iPSCs) as a model system primarily for two reasons; 1) In order to avoid the use of immortalized cell lines and 2) To provide preliminary investigation on the neurotoxicity of CoCr wear particles.

2.0 Results:

2.1. Physico-chemical characterization of the wear particles:

The processed and unprocessed degradation products generated from a hip simulator were characterized by DLS, SEM, EDS, and TEM. SEM images revealed that the particles are agglomerated in the PDP (Figure 1 a) was comparatively high with regard to DP (Figure 1b). Elemental analysis of PDP and DP confirmed the presence of both Co and Cr species in the particles. Representative EDX is shown in Figure 1c. DLS analysis showed that the size (diameter) of CoCrMo as synthesized particle (DP) was 119 ± 138.4 nm with 65% particles are under 150nm (Figure 1f,g). Where after processing of the degradation products, the particle size increased to 387.6 ± 216.3 nm with 89.1% are above the size of 220nm. In

addition, TEM studies showed clear evidence of protein corona formation around the metal particles in the case of DP, confirming the different surface characteristics on DP particles (Figure 1g,h) compared to PDP (Figure 1d,e). The presence of protein corona around DP was further confirmed via SDS-PAGE and the intensity of the band were obtained using image J software (Figure 2a-b).

2.2. Characterization of iPSCs and iPSC derived neurons:

Human iPSC lines derived from normal fibroblast were characterized using pluripotency markers Nanog, Tra-1-60 and SSEA4 and published previously by Denton et al., 2014(Denton et al. 2014). In addition, the expression of pluripotency markers was confirmed by RT-PCR for SOX2, Nanog, and Oct4. Further, the iPSC lines (Figure 3a) were differentiated into neural lineage for the generation of telencephalic forebrain neurons. The neural cells lineage showed characteristic long axonal processes (Figure 3b). Immunocytochemistry studies were carried out to evaluate the expression of Tbr1 and β -tubulin III. Tbr1 is a transcription factor expressed by glutamatergic neurons during the neuronal development, and the neuronal activation upregulates the expression of Tbr1. β -tubulin III is a microtubule element exclusively expressed in neurons(Li et al. 2005, Chuang et al. 2014). Here, the differentiated neural cells after 1 week are Tbr1⁺ and β III-tubulin⁺, confirming the presence of mature forebrain neurons (Figure 3c).

2.3. Cytotoxicity of processed and unprocessed degradation products to iPSC derived neural cells:

Before conducting cytotoxicity studies, endotoxin levels of the metal particles prepared from the hip simulator was evaluated. The concentrations of endotoxin found in the test preparations were in the range of 0.05-0.07EU/ml, which are is far less level than the activation threshold(Li et al. 2016) for the cells to evoke toxic response (figure 2c). The percentage of viability of cells after 3, 6, 24 and 48 h of treatment with DP and PDPat varying concentrations were evaluated in terms metabolic activity by alamarBlue assay (Figure 4a-d). It was observed that after 3 h, the viability decreased to 30% at 100 μ g/ml of DP, whereas there is no significant reduction in cell viability after corresponding PDP treatment. However, after 6 h, only approximately 10-15% cells were viable at 100 μ g/ml in the case of DP and whereas, only 30% cell death was observed in PDP. The trend was continued to 24 h and 48 h where 60% cells died even at 3.125 μ g/ml concentration of DP. It was clear from the TC50 data that the toxic concentration of DP was significantly lower compared to PDP. Before 10 h of time intervals, the toxic concentration for DP was 10-14 μ g/ml, in contrast to 25-28 μ g/ml of PDP. After 20 h, the TC50 of DP decreased to 1-5 μ g/ml, while it remains same in the case of PDP (Figure 4d).

2.4. Cellular uptake of wear particles by Flow cytometry:

Uptake of wear particles after 24 h of incubation with DP and PDP was determined from side scattering in flow cytometry (figure 5a-d). A dose-dependent uptake of particles by neural cells was observed after DP and PDP treatment for a period of 24 h. There is no significant difference in side scattering were observed between different concentrations, up to 3 μ g/ml of PDP. Whereas, at higher concentrations, side scattering significantly higher to that of the control. The results were consistent with the percentage of cell viability observed

after 24 h of particle treatment. However, uptake of DP is significantly higher at all concentrations studied compared to PDP except 0.1 $\mu\text{g/ml}$, which is a strong correlation with cell viability data. Further, we have quantified the metal ions present in the DP and PDP treated cells (100 $\mu\text{g/ml}$) from the cell extract using ICP-MS. The results showed elevated levels of Co and Cr ions inside the DP treated cells compared to PDP treated cells after 24 hours. The results were in correlation with the increased toxicity of DP by the neural cells (Figure 2d).

2.5. ROS production Assay:

Reactive oxygen species generation was determined after 3 h of particle treatment (low concentrations 3, 1 and 0.1 $\mu\text{g/ml}$), where there is no significant change in the viability in both DP and PDP treated cells. Interestingly, irrespective of the dose, DP induce ROS production soon after 3 h of incubation. There is a significant increase in the production of ROS in DP treated cells compared respective concentrations of PDP as well as control (figure 6a). The production of ROS is generally due to the interaction of nanoparticles from within the cell or associated with the cell membrane during the internalization process. This indicates that nanoparticles and metal ions in the DP were involved in a quick internalization by the cells. Due to higher particle size (1 μm), the particles in PDP were less likely to be interacting with the cells leading to the lower level of ROS production. However, by increasing the incubation time, the ionic leakage from the particles can increase resulting in a gradual increase in the percentage of cell toxicity. The phase contrast image of cells after 24 h of 0.1 $\mu\text{g/ml}$ of DP and PDP treatment shows cell body detachment with axonal damage as indicated in the figure 6b. DP induces more damage to the cells even at lower concentration compared to PDP. Control cells revealed well-adhered cell body and healthy axons.

2.6. Apoptosis/Necrosis Assay:

To determine the cellular death mechanism of neural cells, while exposed to DP and PDP, we used flow cytometry analysis of apoptosis using Annexin V/PI staining. Concentrations ranging from 50 $\mu\text{g/ml}$ to 0.78 $\mu\text{g/ml}$ were used for the study. From the flow cytogram, it was evident that, even at 0.78 $\mu\text{g/ml}$ concentrations of DP, a higher population of cells undergo necrosis. Necrosis and apoptosis are significantly higher in DP at all concentrations studied than PDP which again confirming the increase in toxicity elicited by as-synthesized degradation products in comparison to processed particles (figure 7a-b).

2.7. Oxidative stress-specific gene expression by qRT-PCR:

In order to confirm the ROS mediated cell death, we have evaluated the oxidative stress specific gene expression by evaluating the mRNA levels of NADPH oxidase 1 (NOX), nuclear transcription factor ($\text{NF}\kappa\text{B}$), Superoxide dismutase-2 (SOD-2) and Glutaredoxin-2 (GrX2). In addition, to understand the role of caspase-mediated apoptosis in inducing cell death due to ROS, we have analyzed the expression of Caspase-3. Cell viability results showed that the DP is highly cytotoxic at 24 h of incubation higher than 3 $\mu\text{g/ml}$ concentration. Hence, in this experiment we incubated, 3, 1, and 0.1 $\mu\text{g/ml}$ of DP and PDP and treatment period of 6 h to get an adequate amount of RNA to perform qRT-PCR. The data were normalized with untreated cells as a control. After 6 h of incubation, NOX-1

mRNA levels were 16.3 ± 5.4 higher in 3 $\mu\text{g/ml}$ of DP treatment compared to control untreated cells (figure 8). There is 15.12 ± 2.5 , and 6.08 ± 1.33 fold increase observed after incubation with 1 and 0.1 $\mu\text{g/ml}$ of DP, respectively. However, there was no increase in expression of NOX observed in the case of PDP treatment. The expression of GrX2 was found to be 1.3 ± 0.08 , 4.3 ± 2.2 and 2.3 ± 0.26 fold higher in 3, 1 and 0.1 $\mu\text{g/ml}$ of DP treated cells in comparison to control as well as PDP. Similarly, SOD-2 mRNA level was elevated to 11.5 ± 0.7 , 15.6 ± 2.8 and 2.3 ± 0.07 fold than control. However, in the case PDP, SOD-2 expression found to be elevated to 2-3 fold higher in all the concentration studied. On the contrary, there is no significant increment in the level of caspase-3 observed in both PDP and DP treatment groups, suggesting the possibility of the involvement of caspase-3 independent pathway in cell death mechanism, which requires further research.

3. Discussion

In this study, we attempt to differentiate the response of difference in physicochemical characteristics of wear particles on the cellular response by changing only the processing condition of the wear particles generated from an *in vivo* analog; hip simulator. The current study is primarily focusing on the biological effects of different processing conditions of wear particles using neural cells as the model primary cells by providing basic material characterization necessary to prove the difference in physical and chemical characteristics.

3.1 Wear particle generation and clinical concern

Even though currently, the clinical outcome of MoP or CoP hip implants are satisfactory in clinical practices and superior compared to MoM contemporary implants (Amstutz et al. 1969, Pourzal et al. 2009), the degradation products are a clinical concern. In fact, the major limitation associated with MoP is the degradation of the polyethylene cup and generation of large micron-sized polymer wear particles (Pourzal et al. 2009). Irrespective of the type of implant combinations (MoM or MoP or CoP), the modular junction constitutes the metallic component, which is primarily of CoCrMo alloys. The fretting corrosion of metals at the modular junction is believed to be the predominant cause and source of wear particles into the periprosthetic region. A number of experimental studies have suggested that the CoCrMo wear debris can cause local and systemic side effects (Gill et al. 2012, Polyzois et al. 2012, Behl et al. 2013, 2013). In correlation with experimental research, several clinical complications were reported including neurological complications.

Considering CoCrMo implant, the toxicity issues exerted by metal ions such as Co and Cr to osteoblasts, macrophages, monocytes and their cellular mechanism of action is well established. Majority of the conditions evokes oxidative stress-mediated cell death as per the previous reports. However, the materials commonly considered for previous investigations include CoCl_2 or CrCl_3 , Cr_6O_2 , Cr_3O_4 , $\text{K}_2\text{Cr}_2\text{O}_7$ (potassium dichromate), as the source of Co and Cr ions. In addition, commercially available CoCr-based nano- and micro-particles were also used to evaluate the cytotoxicity of wear particles from orthopedic implants and prostheses. Since the biological response of any nano/micro-particles is known to be highly influenced by the physicochemical properties such as size, shape, density, concentration, and density, etc. it is very critical to maintain the characteristics as close to *in vivo* as possible to

understand the actual toxicity levels. Wear particles *in vivo* are exposed to protein-rich medium; synovial fluid, which can influence the colloidal stability, reduce the rate of agglomeration as well as sedimentation (Garrett et al. 1983, Behl et al. 2013), which in turn may have a significant effect on the toxicity. In this study we have used pin-on-ball tribocorrosion hip simulator for generating wear particle by maintaining the physiological conditions as close as possible. The test conditions are detailed in the methodology session. The loading conditions was 16N, which correspond to around the max pressure of 300-350 MPa (point contact-based on the Hertzian calculation). The walking frequency of 1Hz, the amplitude $\pm 30^\circ$ were used, to mimic the expected condition human functional requirements (Mathew et al 2011 & 2012) (Wimmer et al. 2010, Mathew et al. 2011, 2012). Previous studies provided evidences for the difference in physico-chemical characteristics (shape, size and ionic composition) of the wear particles generated under different conditions of the hip simulator (Billi and Campbell 2010, Gill et al. 2012, Bijukumar et al. 2018). In addition, the type of lubricant solution in the hip simulator also influence on the particle characteristics. Catelas et al (Catelas et al. 2004), compared the characteristics of wear particles generated from the hip simulator to that of periprosthetic tissue samples from the revision surgery. It was revealed that the particles generated in the simulator were similar in chemical composition, size, and shape to the particles isolated from patients (Catelas et al. 2004). Even though the tissue digestion involve enzymatic digestion to minimize the changes in physicochemical characteristics, the particles isolated have comparable chemical composition, size and shape to that of isolated from the patients. In our study, we have collected the particles as-synthesized, without further digestion processes. Previous studies have considered complex digestion processes which includes harsh digestion methods such as acidic/alkaline digestion and enzymatic digestion for isolating wear particles. Enzymatic digestion can be of different types 1) papain and proteinase K (2) papain and proteinase K with NaOH and (3) papain and proteinase K with yeast lytic enzyme an zymolase (Schmiedberg et al. 1994, Catelas et al. 2001, Brown et al. 2007). Each digestion technique will have specific impact on the characteristics of the isolated particles (Catelas et al. 2001). Commercially available CoCr particles are usually generated from plasma spray or ball milling process. Hence, in order to prepare processed particles (PDP), we have used high speed centrifugation to do multiple washing and further heat treatment to dry the particles which can partially mimic the commercial process. Many studies have proven that the *in vitro* simulator testing is an accurate method to predict the performance of the joint replacements (Barbour et al. 1999, Leslie et al. 2009, Hadley et al. 2013, Wang et al. 2017).

3.2 The difference between DPs and processed DPs

(a) Physico-chemical characterization: In our study, SEM and TEM of degradation products before and after processing clearly showed the difference in physicochemical characteristics. The particles size were significantly high (increased to 1 μ m and more) after processing. However, as synthesized wear particles were surrounded by a thin layer of protein (protein corona), which can cause a differential response in cellular toxicity. It was reported earlier that upon contact with body fluids, the nanomaterials will be covered with proteins, and which in turn define the physicochemical properties of the materials. Additionally, the type of proteins involved in corona formation determines the biological activity (uptake and toxicity) of the nanoparticles.

(b) Cellular response: It is interesting to note that, as synthesized DPs elicit early and higher toxicity in comparison to PDP. The TC50 of DP after 24 h was 3.125 µg/ml in comparison to 50-100 µg/ml of PDP. This variation in toxicity can be correlated with the smaller particle size of DP and the protein corona around it, which can help the entry of particle into the cells. It can also consider that the proteins, particular albumin could enhance ionic release as observed in the case of silver nanoparticles⁵⁴. Hence, an increment in ion release observed inside the cells in the case of DP can be justified (Figure 2e). In depth evaluation of the particle characterization is one of the limitations of the current study. The difference in metal ion composition in DP and PDP and the release kinetics will be required for the complete understanding of the differential toxicity response.

Further, cellular uptake results confirmed that there is a dose dependent side scatter of the DP treated cells after 24h treatment. Where as, cells up taken the PDP was significantly low mainly attributed to their large size compared to DP. In order to understand the difference in toxicity mechanism, we have evaluated the ROS generation induced by DP and PDP. It was found that after 3h of incubation with DP, the ROS generation was significantly elevated even at lowest concentration analyzed (0.1µg/ml). However, PDP failed to induce ROS activity at their lower concentration studied.

Further, we evaluated the differential response of DP and PDP on cell death mechanism. Similar to previous results, after 24h, DP induces necrosis at higher concentrations (100-10µg/ml) and apoptosis at lower concentrations such as 0.1-3 µg/ml . The results were in correlation with the previous study by Catelas *et al*(Catelas et al. 2005) that Co and Cr ions will cause cellular death via necrosis and apoptosis. In addition, the results were consistent with the recent report by Posada *et al.* where they observed apoptosis only after 24h by the lymphocyte cells exposed with commercially available CoCr wear debris obtained from Depuy International(Posada et al. 2015). However, as-synthesized DP induces phosphatidyl serine expression on the cell surface even at lower concentrations (3-0.1µg/ml). However, interestingly, the gene expression studies did not show any caspase-3 upregulation compared to control, suggesting the possibility of caspase independent cell death mechanism. Further investigations are required for detailed understanding on the difference in molecular mechanism of cell death evoked by as-synthesized DPs. Gene expression analysis revealed upregulation of oxidative stress specific genes such as-NOX-1, GrX2, and NFκB which were significantly upregulated due to DP treatment compared to PDP indicating their difference in toxicity response, these results can be correlated with ROS expression studies.

3.3 Differential cellular response to Degradation products

As reported previously by Catelas *et al* (Catelas et al. 2004) and Pourzal *et al* (Pourzal et al. 2014), the wear particles generated from hip simulator using the serum as lubricant will have a characteristic particle size below 100 nm. Most of the particles generated in a protein-rich medium will have a tendency to form protein corona around the particles, which may stabilize the particles in solution. However, isolation of particles from protein solution, by various processing conditions, will remove the protein coat and are susceptible to agglomeration compared to those particles in lubricant (serum) solution. In addition, the

temperature treatment during synthetic post processing can change the particles characteristics, such as particle size, shape and surface chemistry(Liu et al. 2012, Varga et al. 2016). As previously shown by Yan *et al*, (Yan et al. 2013) even in the unfolded form, protein-coated particles can activate phagocytosis. However, there are still disparities in the cellular uptake mechanisms of protein-coated nanoparticles due to the variation in the plasma protein adsorbed and other physical and chemical aspects of the protein corona. According to Monopoli et al. (Monopoli et al. 2011), a hard corona can form on the surface of nanoparticles after exposing them into a protein concentration similar to *in vivo*. Hence, the type of proteins and the conformation on NP surfaces, as well as the type of cells are crucial to dictate their cellular uptake mechanism. Recently, Ribeiro et al provided evidence for this by showing albumin adsorption on TiO₂ particles followed by calcium and phosphate ions; all together masking the NP and further enhancing its internalization by the bone cells (Ribeiro et al. 2016).

Based on the results obtained in our study, an overall summary of this work is schematically represented in Figure 9. It is worth to note that the internalization mechanisms of DP and PDP by the neural cells are not under consideration of this study. However, one possible mechanism by which small sized DP enter cells can be via pinocytosis as reported by other nanoparticle related research (McGee et al. 2000, Shang et al. 2014). Large macromolecular agglomerates, as well as NPs, are selectively transported into the cells via endocytosis mainly pinocytosis through clathrin dependent and clathrin independent endocytosis(Shang et al. 2014). In addition, receptor mediated uptake mechanism also observed in some nanoparticles. Very small sized particles can enter through direct diffusion through the cell membrane(Shang et al. 2014). The higher ionic concentrations of metals (Co, Cr and Mo) present inside the cells (Figure 2e) can be attributed to the entry of higher amount of nanoparticles/ions from DP in to the neural cells compared to PDP, which in turn leads to the higher ROS production and can cause subsequent cytotoxicity. Such difference in toxicity response by neurons to the ionic as well as nanoparticle form of metals was previously reported by Strickland et al. 2016 and Sun et al. 2016 in the case of silver nitrate and Silver NP(Strickland et al. 2016, Sun et al. 2016).

3.4 Limitations and future directions

The authors acknowledge the limitation of the current study that, the report meant only to provide the difference in cellular response due to processing condition of the DP since the DPs generated from hip simulator are closer to *in vivo* conditions. In-depth evaluation of the molecular mechanism, role of each constituent in wear particles in evoking toxicity at their complex environment, their cellular internalization and toxicity are underway. In addition, the concentrations of the degradation products used in the study are not comparable to that of the patient levels. The study utilized a range of concentrations to gain a better understanding of differential response by the cells. A tissue specific response associated with a clinically relevant dose of CoCr degradation product is planned for future prospective studies.

4. Conclusions

Our study demonstrates the difference in cellular response by the neural cells for as-produced versus processed particles generated by hip implant simulator system. The findings shows that, the wear products generated from a hip-simulator by maintaining the *in vivo* characteristics as close as possible could be considered as an optimum model material to study the toxicity of wear particles to the cells other than independent ions or commercially available wear particles. In addition, the observations on the oxidative stress, axonal damage, and apoptosis by the neural cells particularly at lower concentrations of DP suggest the need to test clinically relevant doses to understand potential implications of neurodegenerative outcomes in THR patients. The study will be extended by considering other clinical aspects and potential solutions to overcome the toxicity challenges posed by the THR in orthopedic patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

The authors acknowledge financial support from Blazer foundation for the Regenerative Medicine and Disability lab at Department of Biomedical Sciences, UIC College of Medicine at Rockford. The authors acknowledge NIH R01 AR070181 funding for the financial support.

Nomenclature:

DP	Degradation products
PDP	Processed degradation products
CoCrMo	Cobalt Chromium Molybdenum
TJR	Total joint replacement
THR	Total hip replacement
MoM	Metal-on-metal
MoP	Metal-on-poly
MoC	Metal-on-ceramic
CoC	Ceramic-on-ceramic
iPSCs	Induced pluripotent stem cells
NOX	NADPH oxidase
SOD-2	Superoxide dismutase-2
OCP	Open circuit potential
ROS	Reactive oxygen species

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Figure 1:

Showing the Scanning electron microscopic image of a) PDP b) DP with inset showing high magnification image. c) Graph showing elemental analysis of DP confirming the presence of Co and Cr. d-e) High-resolution TEM images of PDP lower and higher magnifications. g-h) High-resolution TEM images of DP lower and higher magnifications. Red circles in (g) indicates the metal particles in protein medium and arrows in (h) clearly demonstrating the presence of thick protein corona around metal particles. f-i) DLS spectrum showing the particle size of PDP and DP.

Figure 2:

a) 12% SDS PAGE gel of protein obtained from 10µg/ml of DP and PDP and 1:10 dilutions of DP and PDP respectively and b) graph showing corresponding band intensity obtained from ImageJ software. c) Endotoxin levels in blank BCS (simulated synovial fluid), DP and PDP. d) ICP-MS analysis data of metal ion concentrations from the cell extract after 24 h of treatment with 100µg/ml of DP and PDP.

Figure 3:
Light microscopic images of a) iPSCs b) Phase contrast image of Neural cell differentiation
c) Fluorescence microscopic image showing the efficient generation of forebrain
glutamatergic neurons (Tbr1⁺, Red & βIII-tubulin⁺, Green). Blue indicates Hoechst-stained
nuclei. Scale bar 50 μm.

Figure 4: Graph showing neurotoxicity of DP and PDP after (a) 3 h, (b) 6 h, (c) 24 h and (d) 48 h, (e) Graph Showing TC₅₀ values of DP and PDP after 24 h of treatment with neural cells, Data were presented as Mean $\pm$ SD. * $P < 0.05$ versus DP.

Figure 5:

Particle Uptake by neurons using flow cytometry a) Graph showing side scatter events by the cells after particle (24 h) uptake at different concentrations of particles b) Confocal microscopic image showing particle uptake by the neurons (arrow shows the particles inside the cells) c&d) Showing corresponding histogram and dot plot obtained from flow cytometry. Data were presented as Mean $\pm$ SD. * $P < 0.05$ versus DP.

Figure 6:

a) Fluorescent microscopic images of ROS generation in neural cells using H₂DCFDA Assay with different concentrations of DP and PDP. b) Live cell phase contrast microscopic images of control, 0.1 µg/ml DP and 0.1 µg/ml PDP treated neural cells. Dotted circles indicated small axonal swelling and arrow indicates the damaged axonal region.

Figure 7:

a) Flow cytogram of Annexin V/PI staining on neural cells 24 h after treatment of DP at different concentrations and b) graph showing corresponding percentage of apoptosis and necrosis.

Figure 8:
 Graph showing gene expression a) NOX-1 b) NFκB c) SOD-2 d) CASPASE 3 and e) Grx₂ by neural cells after 24 h of incubation. Data were presented as Mean ± SD. * $P < 0.05$ versus DP.

Figure 9:

Schematic representation of the summary of the work. The right side of the figure showing the individual particle coated with protein and the cross section. It can be speculated that processed particles, being larger, might not be able to enter the cells. However, the ions released can diffuse into the cells. Whereas, DP can enter the cells due to their smaller size and can release a large number of ions due to lysosomal digestion. In addition, it can interact with mitochondria, leads to ROS generation and cause subsequent cytotoxicity and genotoxicity.