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Interactions of 1D- and 2D-layered inorganic nanoparticles with fibroblasts and human mesenchymal stem cells

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Abstract

Aim—This study investigates the effects of tungsten disulfide nanotubes (WSNTs) and molybdenum disulfide nanoplatelets (MSNPs) on fibroblasts (NIH-3T3) and mesenchymal stem cells (MSCs) to determine safe dosages for potential biomedical applications.

Materials & methods—Cytotoxicity of MSNPs and WSNTs (5–300 µg/ml) on NIH-3T3 and MSCs was assessed at 6, 12 or 24 h. MSC differentiation to adipocytes and osteoblasts was assessed following treatment for 24 h.

Results—Only NIH-3T3 cells treated with MSNPs showed dose or time dependent increase in cytotoxicity. Differentiation markers of MSCs in treated groups were unaffected compared with untreated controls.

Conclusion—MSNPs and WSNTs at concentrations less than 50 µg/ml are potentially safe for treatment of fibroblasts or MSCs for up to 24 h.

Keywords

cytotoxicity; differentiation; inorganic nanoparticles; stem cells; tissue engineering; uptake

Layered transition metal dichalcogenides such as the 1D tungsten disulfide nanotubes (WSNTs) and 2D molybdenum disulfide nanoplatelets (MSNPs), analogous to carbon nanotubes and graphene, exhibit interesting physiochemical properties that have been harnessed for tribiological, optical, and electronic applications [1–3]. For instance, these nanomaterials are excellent lubricants even in the absence of moisture or at high temperatures [4,5]. Therefore, when included as lubrication additives to motor oils, the combustion of these oils in motor vehicles and oil waste plants will lead to higher

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

environmental exposure of these nanoparticles. For biomedical applications, MSNPs and WSNTs have been proposed for potential use as additives to lubricants used on catheters and coatings for orthodontic or orthopedic implants and wires [5–8]. Recently, we have investigated these nanomaterials as reinforcing agents in polymer nanocomposites for bone tissue engineering [9,10]. For this application, the mechanical properties of the nanocomposites should ideally be similar to that of native bone tissue [11]. Our results indicate that, compared with carbon nanoparticles such as graphene and carbon nanotubes, these inorganic nanoparticles are more efficacious as reinforcing agents. Additionally, these inorganic nanoparticles due to their large surface area could serve as a versatile scaffold to append drugs, genetic material and imaging agents.

The possible environmental impact and potential biomedical applications of WSNTs and MSNPs necessitates thorough evaluation of their cyto- and bio-compatibility. Few studies have reported the toxicity of these inorganic nanoparticles compared with carbon nanoparticles such as carbon nanotubes and graphene [12–16]. These reports investigated *in vitro* the interaction and effects of WSNTs and MSNPs on cells environmental exposure through inhalation or ingestion [6,17]. Pardo *et al.* investigated the cytotoxicity of WSNTs and MSNPs toward lung fibroblasts, human liver cells and macrophages. Their results indicated no toxicity and minimal immune response from exposure to WSNTs or MSNPs at concentrations ranging from 0 to 100 µg/ml [17]. Additionally, MSNPs were found to be relatively nontoxic (~100% survivability) to human epidermal fibroblasts, lung adenocarcinoma cells, and leukemic cells at concentrations ranging from 0 to 3.52 mg/l [6]. While these studies provide some insights on the effects of these nanoparticles under environmental exposure conditions, additional investigations are needed to examine their response on biological systems (e.g., cells, tissues) to determine the potentially safe doses relevant for biomedical applications.

An important cell type these nanoparticles will interact with when utilized for biomedical applications is the mesenchymal stem cell (MSC). MSCs are multipotent adult or somatic stem cells that readily differentiate into cells of various tissues such as osteoblasts, adipocytes and chondrocytes, and are critical for the in the regeneration/restoration of these tissue types [18]. Recent studies also show that MSCs assist the expansion of hematopoietic stem cell or embryonic stem cell cultures [19]. These attributes of MSCs are being utilized in the development of therapies to repair, regenerate and restore damaged tissues. The *in vitro* interactions of MSCs with a variety of metallic, carbon, ceramic, and polymeric nanoparticles have also been reported for stem cell applications [20–23]. However, the effects of transition metal dichalcogenides on MSC viability and differentiation are yet to be reported.

In this study, we have assessed the *in vitro* response of NIH-3T3 cells and MSCs to treatment with WSNTs and MSNPs dispersed in 1,2-distearoyl-sn-glycero-3-phosphoethanolamine conjugated with PEG (DSPE-PEG). We report the dose- and time-dependent cytotoxicity of these inorganic nanoparticles on NIH-3T3 cells and MSCs, their effect on MSC differentiation capability, and characterize the intracellular distribution of these nanoparticles to determine potentially safe dosages for biomedical applications.

Materials & methods

Nanoparticle synthesis & characterization Nanoparticle synthesis

Molybdenum trioxide (MoO_3) and sulfur (S) powder was purchased from Sigma-Aldrich, MO, USA. MoS_2 nanoplatelets (MSNPs) were synthesized as mentioned previously [24]. Briefly, we added MoO_3 and sulfur to an alumina crucible and placed it in a horizontal tube furnace. Prior to heating, the tube was evacuated by nitrogen gas (N_2) flow for 30 min. The furnace was then heated to 700°C for 2.5 h under N_2 atmosphere. After 2.5 h, the furnace was allowed to cool back to room temperature under N_2 atmosphere. The product was further annealed in the furnace to 1000°C for 1 h under N_2 atmosphere. Once the furnace cooled back to room temperature, we collected the silvery black MSNPs from the crucible. WS_2 nanotubes (WSNTs) were purchased from APNano (NY, USA).

Nanoparticle characterization

Transmission electron microscopy (TEM) was done to characterize the morphology and structure of the nanoparticles. Samples for TEM were prepared as follows. Nanoparticles were added to a solution of water and 100% ethanol at a ratio of 1:1, to obtain a concentration of approximately 1 mg/ml. This solution was dispersed by probe sonication (Cole Parmer Ultrasonicator LPX 750, IL, USA) using a 1 s 'on', 2 s 'off' cycle for 1 min. The solution was centrifuged at 10,000 rpm for 5 min and the supernatant was collected and drop cast on a lacey carbon grid (300 mesh size, copper support, Ted Pella, CA, USA). TEM was performed on a JOEL 2100F high-resolution analytical transmission electron microscope with an accelerating voltage of 200 kV. Raman spectroscopy was employed to identify the nanoparticles based on characteristic spectral analysis. The nanoparticles were added to isopropanol at a concentration of about 1 mg/ml. The samples were sonicated for 30 min to disperse the particles and were then drop cast onto a silicon wafer. Raman spectra were obtained on a 532 nm Nd-YAG excitation laser equipped WITec alpha300R Micro-Imaging Raman Spectrometer (TN, USA). Spectra were recorded between $50\text{--}3750\text{ cm}^{-1}$ at room temperature.

Cell culture

Mouse embryo fibroblast cell line (NIH-3T3) and human adipose derived stem cells (MSCs; Lifeline Cell Technology Cat No. FC-0034, MD, USA) isolated from lipoaspirate were used for this study. Dulbecco's Modified Eagle's Medium (DMEM) media (Invitrogen, NY, USA, cat no. 12491-015) with 10% fetal bovine serum and 1% penicillin streptomycin was used for cell culture of NIH-3T3 cells, while Stem-Life™ MSC medium (Lifeline, Cat No. LL-0034) was used for stem cell cultures. Media was changed every 2–3 days and the cells were incubated at 37°C and 5% CO_2 throughout the experiment. Passages 4–8 of MSCs were used for the studies.

Cytotoxicity

Presto Blue® assay (Invitrogen), and lactate dehydrogenase assay (LDH; Sigma-Aldrich) were used to assess cytotoxicity of MSNPs and WSNTs on NIH-3T3 and MSCs. Cells were seeded in 96-well plates at a density of 10,000 cells/well. Nanoparticles were coated with

DSPE-PEG to impart water-dispersibility. MSNPs or WSNTs nanoparticle dispersions (10 vol%) were added 24 h after plating to bring the final concentrations of MSNPs and WSNTs in culture media to 0 (DSPE-PEG), 5, 10, 50, 100 and 300 µg/ml. For Presto Blue assay, we used untreated cells and cells treated with ice-cold methanol for 30 min as positive and negative controls respectively. Acellular dispersions of MSNPs and WSNTs were used to determine interference in fluorescence intensity by the presence of nanoparticles. We performed the assays at 6, 12 and 24 h following treatment ($n = 6$) with the nanoparticle dispersions. For Presto Blue assay at each time point the wells were washed with phosphate-buffered saline (PBS) and 90 µl of fresh media followed by 10 µl of Presto Blue reagent was added. The plates were incubated for 2 h at 37°C. The fluorescence was measured at 580 nm emission with 530 nm excitation.

We performed LDH assays as described in the protocol provided in the Tox-7 kit (Sigma-Aldrich). Briefly, following incubation with nanoparticle dispersions, the culture plates were centrifuged at 250 g for 5 min and aliquots of 100 µl of media from each well was removed and placed on a flat bottom 96-well plate. We prepared LDH assay mixture by mixing equal amounts of assay substrate, enzyme and dye solution and added 100 µl of assay mixture to each well containing 50 µl of culture media. Then we covered the plates with foil to protect them from light. Following 30 min of incubation, we added 15 µl of 1 N hydrochloric acid to each well to stop the reaction. We measured absorbance of each well at 490 nm and subtracted background measurements taken at 690 nm. For LDH assay, we used groups treated with lysis buffer and untreated cells as positive and negative controls, respectively.

MSC differentiation

MSCs were plated in 24-well plates at a density of 20,000 cells/well. Cells were treated with DSPE-PEG or nanoparticle solutions of 10 or 50 µg/ml concentrations. After 24 h of treatment, cells were washed and osteogenic or adipogenic differentiation media (hMSC Osteogenic/Adipogenic BulletKit™, Lonza, Basel, Switzerland) was added. MSCs were maintained with media changes every 2–3 days for 14 days for osteogenic media. Following incubation, media was removed; wells were washed with PBS and filled with 2 ml DI water. Cell lysate, prepared by sonicating the wells in a bath sonicator for 5 min, was used for analyzing cell number, alkaline phosphatase (ALP) activity, and calcium content. Alizarin Red S staining was performed in a separate set of wells for qualitative analysis. For adipogenic differentiation, adipogenic induction media was used for the first three media changes followed by incubation with adipogenic maintenance media for a total of 21 days. Once differentiation media cycles were complete, adipogenic differentiation was analyzed by Oil Red O staining and elution.

Oil Red O

Following incubation with adipogenic differentiation media, cells were washed with 1 ml of PBS. In total, 1 ml of 4% paraformaldehyde was added for fixation and incubated for 10 min at room temperature. Para-formaldehyde was removed and fresh 1 ml of 4% para-formaldehyde was added and incubated for 1 h. Once fixed, paraformaldehyde was removed and cells were washed twice with deionized water and then washed with 60% isopropanol for 5 min at room temperature. Cells were then allowed to dry completely before addition of

0.5 ml Oil Red O working solution, two parts Oil Red O stock solution (0.35% solution in isopropanol) with three parts isopropanol and incubation for 10 min. After incubation, Oil Red O solution was removed and cells were washed with deionized water four-times. Images of Oil Red O staining were acquired on BX-51 Olympus microscope (Hamburg, Germany). Oil Red O stain was then eluted by addition of 100% isopropanol and incubation for 10 min with shaking. Absorbance of aspirated isopropanol was measured at 500 nm (Varioskan Flash, Thermo Scientific, MA, USA) with 100% isopropanol serving as blank.

Alizarin Red S

To observe mineralization in MSCs differentiated to osteoblasts, we stained the culture wells with Alizarin Red S. We removed the osteogenic media and washed the wells with PBS before fixing and staining. We fixed the cells with 1 ml of 4% paraformaldehyde at room temperature for 15 min. After incubation, cells were washed with deionized water two-times and 40 mM Alizarin Red S dye (adjusted to pH of 4.1 using 0.5 N ammonium hydroxide) was added in each well. Plates were incubated while shaking at room temperature for 20 min. Dye was removed and MSCs were washed with deionized water four-times with shaking for 5 min. Cell staining was observed using a BX-51 Olympus microscope.

Cell number

DNA in cell lysate was used to determine the number of cells per well with QuantiFluor[®] Dye Systems (Promega, WI, USA). A standard curve of known number of MSCs was used to determine the number of cells per well. A hundred microliters of 1× TE buffer was added to 100 µl of a QuantiFluor dye working solution. The mixture was then added to 100 µl of cell lysate in a 96-well plate, covered with foil and incubated at room temperature for 10 min. Fluorescence was measured at an excitation wavelength of 480 nm and an emission wavelength of 570 nm and data were presented as average number of cells per well.

ALP activity

ALP activity was presented as ALP activity in µmol per minute per cell. To measure ALP activity, 100 µl of p-nitrophenyl phosphate (pNPP) was added to a 96-well plate containing 100 µl of 4 nitrophenol standard or cell lysate in triplicate and was incubated for 1 h at 37°C. Postincubation, 100 µl of 0.2M NaOH stop solution was added and absorbance was measured at 405 nm. Data for ALP were presented as µmol/min/cell.

Extracellular calcium

To quantify the extracellular calcium in each well, we added 100 µl of 1 M acetic acid to 100 µl of cell lysate and placed the mixture on a shaker overnight. We placed 20 µl of the sample mixture or calcium chloride standard in 96-well plate and added 280 µl of Arsenazo III Calcium Assay reagent (Sigma-Aldrich). Samples from each well were analyzed in triplicates. We measured absorbance of each well at 650 nm.

Nanoparticle uptake

Transmission electron microscopy—MSCs, cultured on ACLAR[®] films (Ted Pella), were treated with 10 volume percent of 50 µg/ml MSNP or WSNT dispersions for 24 h.

Media was then removed and cells were washed with PBS and fixed using 1 ml of 1% glutaraldehyde for 1 h after which 1% osmium tetroxide in 0.1 M PBS was added. After fixation, cells were dehydrated in graded ethanol washes and embedded in Durcupan™ resin (Sigma Life Science, MO, USA). The samples were cut into 80 nm sections using a Reichert–Jung UltracutE ultramicrotome (NY, USA) and were then placed on formvar coated slot copper grids. The sections were counterstained with uranyl acetate and lead citrate. We imaged the samples using a JOEL-JEM-1400 transmission electron microscope (MA, USA) with a Gatan CCD Digital Camera system (CA, USA).

Statistics

All graphs are presented as average $\pm$ standard deviation. For viability assays, one-way ANOVA with Tukey *post hoc* was used to analyze significance. Differences with $p < 0.05$ were considered significant. For Differentiation assays, Kruskal–Wallace test with Dunn *post hoc* was performed to analyze significance of differences between the groups with $p < 0.05$ considered statistically significant.

Results

Nanoparticle characterization

Figure 1 shows representative TEM images of MSNPs and WSNTs. MSNPs (Figure 1A) are circular platelet shaped particles with diameters ranging from 60–90 nm and 8 nm thickness [9]. WSNTs (Figure 1B) are smooth nanotube structures with diameters of 50–100 nm and lengths ranging from 1–15 μm . Raman spectra for the two nanomaterials, presented in Figure 1C, shows clear bands at 378, 404 and 476 cm^{-1} for MSNPs and bands at 344 cm^{-1} and 414 cm^{-1} for WSNTs. The two major bands in the spectra for both nanoparticles signify the $\text{E}_{2\text{g}}^1$ and $\text{A}_{1\text{g}}$ Raman active modes. The band at 476 cm^{-1} in the MSNP spectrum is due to unreacted MoO_3 .

Cytotoxicity

Presto Blue—Presto Blue is a resazurin-based viability assay that works due to reduction of nonfluorescent resazurin to fluorescent resorufin by live cells. Dispersion of WSNTs or MSNPs without cells did not have any fluorescence at 580 nm emission with 530 nm excitation. Figure 2A & B shows viability of NIH-3T3 and MSCs treated with MSNPs at 5, 10, 50, 100 and 300 $\mu\text{g}/\text{ml}$ concentrations for 6, 12 and 24 h normalized to untreated controls. All dispersions were stable and did not settle to the bottom of the plate during the entire study duration. At concentrations below 10 $\mu\text{g}/\text{ml}$, there were no significant differences compared with untreated controls. At concentrations 50, 100 and 300 $\mu\text{g}/\text{ml}$, there was a dose and time dependent toxicity with groups treated at 300 $\mu\text{g}/\text{ml}$ having viability as low as 20% after 24 h. MSCs treated with MSNPs show no decrease in viability at any concentration or time point. Interestingly, groups treated with low concentrations of 5 $\mu\text{g}/\text{ml}$ and 10 $\mu\text{g}/\text{ml}$ at 24 h had significantly higher viability compared with untreated groups. The increase in viability was highest for groups treated with 5 $\mu\text{g}/\text{ml}$; 37% greater than untreated controls.

Figure 2C & D shows viability of NIH-3T3 and MSCs treated with WSNTs at concentrations between 5–300 µg/ml for 6, 12 and 24 h. MSCs and NIH-3T3 treated with WSNTs showed no time dependent or dose dependent cytotoxicity. NIH-3T3 cells treated with 300 µg/ml WSNTs for 24 h showed significantly higher viability (about 17%) compared with untreated controls. MSCs showed significantly higher viability compared with untreated groups for all concentrations at the 24-h time point with viability 45% greater than untreated cells at 300 µg/ml concentration.

LDH Assay

LDH is a cell membrane integrity assay, measured by the absorbance of LDH released into the media by lysed cells [25]. Figure 3 shows the LDH released by cells treated with MSNPs and WSNTs dispersions at 5, 10, 50, 100 and 300 µg/ml concentrations for 6, 12 and 24 h. Data for each group are normalized to positive control group treated with lysis buffer in which all the cells were lysed and LDH contained within the cells were released into the culture media. Figure 3A indicates that NIH-3T3 cells treated with MSNPs exhibit a time dependent cytotoxicity with the highest normalized LDH level at 24 h for all concentrations. Figure 3B indicates that LDH released from MSCs treated with MSNPs have neither time nor dose dependence. MSCs treated with 5 µg/ml of MSNPs had significantly higher LDH release compared with DSPE-PEG treated groups at the 6-h time point. The increase in LDH release compared with DSPE-PEG groups was not observed in higher concentrations.

Figure 3C & D shows the LDH release from NIH-3T3 and MSCs treated with WSNTs respectively. The data suggest a time dependent increase in LDH release from NIH-3T3 at 24-h time points with no significant differences from DSPE-PEG treated groups at any time points. MSCs treated with WSNTs at 5–300 µg/ml had significantly higher LDH release compared with DSPE-PEG groups.

Differentiation

Oil Red O staining & elution—Oil Red O is a lipid-soluble dye that stains fat vacuoles in adipocytes and is used to determine the extent of adipogenic differentiation [22,26]. Figure 4A shows representative images of MSCs stained with Oil Red O following 21 days in adipogenic differentiation media after 24 h treatment with 0, 10 or 50 µg/ml of MSNPs or WSNTs. For all groups fat vacuoles were observed throughout the culture wells. In the groups treated with nanoparticles, aggregates were present around the cells in the images. Elution of the Oil Red O stain (Figure 4B) showed significantly higher staining in groups treated with 10 µg/ml of MSNPs and WSNTs compared with groups treated with 50 µg/ml concentrations and untreated controls. The increase in elution was approximately 50% for both MSNPs and WSNTs.

Alizarin Red S

Alizarin Red S stains extracellular calcium and is used as a marker for osteo-differentiation of stem cells [22,27]. Figure 5 shows representative images of MSCs stained with Alizarin Red S for calcium deposits after treatment with 0 (Control), 10, or 50 µg/ml of MSNPs or WSNTs for 24 h and incubation in osteogenic differentiation media for 14 days. For all groups red staining of calcium was observed. Nanoparticles could be seen around the cells

for the treated groups with larger aggregates seen in the groups treated with the higher concentration. In all the groups the cells were seen to be elongated and spread out as expected from healthy preosteoblast and osteoblast cultures. There were no observable differences in staining pattern or intensity between any of the groups.

Cell number

Number of cells per well was used to determine ALP activity. Figure 6A shows average number of cells per well in groups treated with 10 and 50 $\mu\text{g/ml}$ of MSNPs or WSNTs and untreated controls for 24 h and incubated for 14 days in osteogenic differentiation media. Groups treated with WSNTs had no significant differences from untreated controls. Groups treated with both low and high concentrations of MSNPs had significantly lower cells per well compared with untreated groups. The decrease in cell number was approximately 25% for both concentrations of MSNPs compared with untreated controls.

ALP activity

Figure 6B shows ALP activity of groups treated with 10 and 50 $\mu\text{g/ml}$ of MSNPs or WSNTs and untreated controls for 24 h and incubated for 14 days in osteogenic differentiation media represented as activity/ $\mu\text{mol/min/cell}$. ALP is an early stage marker for osteogenesis [22]. MSCs treated with 10 or 50 $\mu\text{g/ml}$ MSNPs or WSNTs for 24 h, and then incubated with osteogenic differentiation media for 14 days were examined for this marker. MSCs treated with MSNP dispersion of 10 $\mu\text{g/ml}$ concentration showed significantly higher ALP activity; approximately 50% greater compared with MSCs treated with 50 $\mu\text{g/ml}$ MSNP dispersion. However, the ALP activity of neither of these groups was significantly different from the untreated control group. Groups treated with 10 $\mu\text{g/ml}$ of WSNTs showed 27% higher ALP activity compared with groups treated with 50 $\mu\text{g/ml}$ of WSNTs. However, the ALP activity of both the groups was not significantly different from untreated controls.

Extracellular calcium

Extracellular calcium is a late stage marker for osteo-genesis [22]. Figure 6C shows calcium levels of MSCs treated with 10 or 50 $\mu\text{g/ml}$ MSNPs or WSNTs for 24 h and incubated for 14 days in osteogenic differentiation media. Extracellular calcium contents for MSCs treated with MSNPs at 10 and 50 $\mu\text{g/ml}$ were 69 and 65 mmol/l, respectively. Untreated control groups had a calcium concentration of 62 mmol/l. There were no statistical significant differences between the any groups. MSCs treated with WSNTs at 10 and 50 $\mu\text{g/ml}$ had 63 and 69 mmol/l calcium concentration, respectively. There were no statistically significant differences between the groups or with untreated controls.

Cell uptake

TEM was performed to investigate cellular uptake of MSNPs and WSNTs. Figure 7 shows representative TEM images of MSCs treated with 50 $\mu\text{g/ml}$ of MSNPs (A & B) and WSNTs (C & D). In Figure 7A & B MSNPs were seen within the cytoplasm, enclosed in endocytic vesicles (red arrows) and against the cell membrane (orange arrows). WSNTs, shown in Figure 7B & C, were seen in the cytoplasm within (red arrows) and outside (yellow arrow)

endocytic vesicles. Vesicles containing MSNPs and WSNTs were seen in close proximity to the nuclear membrane. Neither of the nanoparticles was present within the nucleus.

Discussion

The objective of this study was to investigate the response of WSNTs and MSNPs on NIH-3T3 fibroblasts and MSCs to identify potentially safe dosages for any eventual biomedical application. NIH-3T3 fibroblasts cells were chosen since they are widely used as model fibroblastic cells for cytotoxicity evaluation [28]. MSCs were chosen since they are routinely used for tissue engineering and stem cell applications [29–31]. We performed cytotoxicity screening over a broad range of concentrations (0–300 µg/ml) and time points (6, 12 and 24 h) on fibroblasts (NIH3T3) and stem cells (MSCs) to identify a range of potentially safe doses. We then examined the effect of potentially safe low (10 µg/ml) and high (50 µg/ml) doses of nanoparticles on the adipo- and osteo-differentiation capabilities of MSCs. Through the cytotoxicity and differentiation studies, we identified a range of doses for the two nanoparticle formulations that do not significantly affect viability of fibroblasts and MSCs as well as differentiation capabilities of MSCs.

Characterization of nanoparticles was done by TEM and Raman spectroscopy. Raman spectroscopy revealed the common Raman active modes E_{2g}¹ and A_{1g} for transition metal dichalcogenides. The E_{2g}¹ peak indicates the in-plane phonon mode of the two sulfur atoms opposite vibration from the molybdenum or tungsten atom while the A_{1g} peak indicates the out-of-plane phonon mode of the sulfur atoms opposite vibration between layers [32,33]. A slight shift in wavenumbers is observed as compared with previous studies. This shift is due to the number of layers of nanoparticles stacked together during the measurements [34].

The Presto Blue assay indicated a time and dose dependent cytotoxicity for NIH-3T3 cells treated with MSNPs. CD₅₀ values of MSCs were 578, 250 and 140 µg/ml, for 6, 12 and 24 h, respectively, calculated from concentration versus viability graphs. This trend was not seen in viability assessed by LDH. There were no significant differences in any of the groups compared with untreated controls. Live cells reduce nonfluorescent resazurin to fluorescent resorufin that is detected by Presto Blue assay; whereas, LDH assay detects LDH enzyme that is released when cell membrane integrity is compromised in dying cells. The difference in trends observed from these two assays could be due to the nanoparticles affecting the cellular machinery without affecting their membrane integrity. Further investigations are required to identify the cause of these differences in outcomes of the two assays. Unlike MSNPs, NIH-3T3 cells treated with WSNTs showed no time or dose dependent cytotoxicity. Results of both Presto Blue and LDH were similar and indicate that concentrations up to 300 µg/ml of WSNTs have no significant effect on viability of NIH-3T3 cells. CD₅₀ values at all time points for NIH-3T3 cells treated with MSNPs calculated from LDH assay results and for NIH-3T3 cells treated with WSNTs calculated from Presto Blue and LDH assay results were greater than 300 µg/ml.

MSCs treated with MSNPs or WSNTs show no time or dose dependent cytotoxicity in Presto Blue and LDH assays. Results from both the assays indicate that MSNPs or WSNTs at concentrations up to 300 µg/ml do not elicit a significant cytotoxic response. All CD₅₀

values for MSCs treated with MSNPs or WSNTs at all time points were greater than 300 $\mu\text{g}/\text{ml}$. An increase in proliferation of MSCs was noted at all WSNT treatment concentrations with the highest increase of 45% compared with DSPE-PEG treated controls at the 24-h time point. A previous report on carbon black and silica nanoparticles showed that these nanoparticles increase lung epithelial cell proliferation [35] via the activation of the protein kinase B pathway. However, this effect only occurred when the cells were treated with carbon black and silica nanoparticles with a median diameter of 14 nm. Larger particles did not elicit the same results [35]. Even though the WSNTs and MSNPs used in this study, had sizes several times larger than the carbon black and silica nanoparticles, similar pathways maybe responsible for the increased cell proliferation. Further investigations are required to determine the mechanism.

Adipogenic differentiation was analyzed by Oil Red O staining and elution quantification. The results indicate that MSCs incubated for 24 h with MSNPs or WSNTs at 10 or 50 $\mu\text{g}/\text{ml}$ concentrations maintain their differentiation potential. Significantly higher Oil Red O staining in groups treated with 10 $\mu\text{g}/\text{ml}$ concentrations compared with groups treated with the 50 $\mu\text{g}/\text{ml}$ concentration for both MSNPs and WSNTs could be due to higher cell numbers. Viability analysis indicated that MSCs treated with MSNPs at 10 $\mu\text{g}/\text{ml}$ had 52% higher viability compared with MSCs treated with 50 $\mu\text{g}/\text{ml}$. Since adipogenesis was induced using adipogenic induction media 24 h after the treatment with the nanoparticles, groups treated with 50 $\mu\text{g}/\text{ml}$ most likely had lower number of cells compared with groups treated with 10 $\mu\text{g}/\text{ml}$. Taking differences in cell numbers into consideration, the results imply that treatment with low and high concentrations of WSNTs and MSNPs should not hinder the adipogenesis.

Osteogenic differentiation was analyzed by ALP and calcium assays two weeks following treatment with nanoparticles. As a part of the ALP analysis, cell numbers were obtained for the treated and untreated groups. Cell number for groups treated with MSNPs was significantly lower than cell numbers for groups treated with WSNTs and untreated control. This result is in agreement with our viability assessment where we observed increased viability in groups treated with WSNTs and decreased viability in groups treated with MSNPs at 50 $\mu\text{g}/\text{ml}$ concentration. For both MSNPs and WSNTs, ALP activity was significantly higher in groups treated with a lower concentration compared with groups treated with a higher concentration. This difference in ALP activity at low and high doses may be due to variable propensity of the nanoparticles at these dosages to aggregate in cell media. We observed, at the high concentration, both MSNPs and WSNTs aggregate with time to form larger sized loosely held particles, and at the low concentrations, the particles remained well dispersed and did not aggregate during the incubation period. Thus, at higher concentration, the increased aggregation may prevent/reduce the uptake of these nanoparticles into cells, and at the lower concentration, the nanoparticles could be internalized into cells at higher amounts.

We used calcium content in the matrix as another indicator of osteo-differentiation [22]. There were no significant differences in calcium concentration levels between the groups. The assays for osteogenic differentiation markers were performed following 14 days of incubation with osteogenic differentiation media. At this stage of differentiation MSCs

produce increased amounts of organic extracellular matrix proteins that is closely followed by deposition of inorganic components [36]. Additional studies are needed to examine if these nanoparticles affect the gene expression of osteo-differentiation markers and the mechanical properties of deposited matrix. The results of the osteo-differentiation studies taken together imply that treatment at 10 or 50 µg/ml of WSNTs or MSNPs concentrations should not adversely affect the osteogenic differentiation potential of MSCs.

TEM analysis of the histological specimens of the MSCs treated with the nanoparticles was performed to further investigate their uptake characteristics. Since no significant differences in viability or differentiation of MSCs were observed between groups treated with WSNTs and MSNPs compared with untreated controls, TEM analysis also allowed us to determine whether this lack of difference was due to poor uptake of these nanoparticles into these cells. TEM images of histological sections of MSCs treated with MSNPs and WSNTs qualitatively showed significant uptake of both nanoparticles into cells. Additionally, the nanoparticles were present within and outside the cells. Inside the cell, MSNPs were seen only in vesicles within the cytoplasm whereas WSNTs were seen in vesicles as well as the cytoplasmic matrix. Outside the cell, MSNPs and WSNTs were observed on the cell membranes. However, cytoplasmic protrusions observed during micropinocytosis were seen only around the WSNT aggregates [37]. These observations suggest that WSNTs and MSNPs can enter the cells via different uptake mechanisms without affecting viability or differentiation potential of MSCs at concentrations up to 50 µg/ml.

Previous cytotoxicity studies on MSNPs and WSNTs mainly focused on their *in vitro* effect at low concentrations (0 to 3.52 mg/l and 0 to 100 µg/ml) on cells exposed during inhalation and oral ingestion [6,17]. The above results compliment the results of those studies and provide guidelines on potentially safe dosages for biomedical applications. For instance, recently MSNPs and WSNTs have shown promise as reinforcing agents for polymeric bone tissue engineering nanocomposites [9,10]. Biodegradable polymer matrices incorporated with low concentrations (0.2 weight%; 0.2 g of nanoparticles in 1 g of polymer) of MSNPs or WSNTs showed up to 108% enhancement in mechanical (compressive and flexural modulus) properties compared with the polymer alone [9,10]. Thus, the total concentration of the nanoparticles released per cm³ (or ml) of the 90% porous nanocomposite (nanocomposite volume: 10 cm³) would be 20 µg/ml nanoparticles upon degradation of the polymer. As the scaffolds degrade, these nanoparticles will be released into the extracellular matrix and interact with surrounding tissue. Our results suggest that the released nanoparticles should not significantly affect the viability of fibroblasts and viability or differentiation of MSCs at concentrations higher than the estimated 20 µg/ml concentration. Additionally, these inorganic nanoparticles are better at reinforcing biodegradable polymers employed for tissue engineering applications compared with carbon nanoparticles such as 1D carbon nanotubes or 2D graphene [9,10]. Furthermore, high concentrations of carbon nanotubes (>100 µg/ml) or graphene (300 µg/ml) affect MSC viability significantly (60–80% or 38–100% decrease, respectively) while treatment with similar high concentrations of MSNPs and WSNTs had minimal (~10%) to no decrease in MSC viability [22,38]. The results of our cytotoxicity study in conjugation with the previous efficacy studies identify optimal formulations of these inorganic nanoparticles to design and develop a new class of

mechanically robust and biocompatible tissue engineering implants. Furthermore, the significant uptake of these nanoparticles in MSCs at potentially safe doses also opens opportunities to introduce them as multifunctional agents for stem cell monitoring and therapeutics. These nanoparticles could, at potentially safe doses, serve as versatile platforms to attach drugs, genes and/or imaging agents and introduced *ex vivo* into MSCs. These labeled MSCs could then be employed for stem cell applications.

Conclusion

Treatment with MSNPs at concentrations up to 10 µg/ml does not significantly affect viability of NIH-3T3 cells. No dose or time dependent increase in cytotoxicity was observed for NIH-3T3 cells treated with WSNTs or MSCs treated with MSNPs or WSNTs. MSCs treated with low (10 µg/ml) and high (50 µg/ml) concentrations of MSNPs and WSNTs for 24 h maintain their differentiation potential to adipocytes and osteoblasts. MSNPs are internalized in vesicles in the cells while WSNTs are internalized in vesicles as well as cytoplasmic matrix. The results taken together indicate that concentrations less than 50 µg/ml of MSNPs and WSNTs should be potentially safe for incubation with MSCs and fibroblasts up to 24 h. The results provide preliminary safety guidelines to further explore the promise of these nanoparticles as multifunctional agents for biomedical applications.

Future perspective

The efficient reinforcement capabilities of these inorganic nanomaterials have thus far only been demonstrated for polymeric bone tissue engineering implants [9,10]; however, these nanoparticles at potentially safe and efficacious concentrations could also be employed to improve the mechanical properties of polymeric or ceramic implants and coatings for other tissue engineering applications. The significant uptake of these nanoparticles into cells indicates their potential as intercellular delivery agents for drugs, proteins or nucleic acids. These particles could also be covalently or noncovalently functionalized with various targeting moieties (e.g., peptides and antibodies) and imaging agents and thus, employed as multifunctional agents for targeted cellular or molecular therapy and imaging [39,40].

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Executive summary

Background

- 1D- and 2D-layered transition metal dichalcogenide nanoparticles, tungsten disulfide nanotubes (WSNTs) and molybdenum disulfide nanoplatelets (MSNPs), analogous to carbon nanotubes and graphene, have been proposed for various biomedical applications.
- The possible environmental impact and potential biomedical applications of WSNTs and MSNPs necessitate thorough evaluation of their cyto- and bio-compatibility.
- Few studies that have investigated *in vitro* the interactions of WSNTs and MSNPs on cells involved in environmental exposure through inhalation or ingestion provide some insights on the effects of these nanoparticles under environmental exposure conditions. However, additional investigations are needed to examine their response on biological systems (e.g., cells, tissues) to determine the potentially safe doses relevant for biomedical applications.
- In this study, we report the dose- and time-dependent cytotoxicity of these inorganic nanoparticles on NIH-3T3 cells and MSCs, their effect on mesenchymal stem cell (MSC) differentiation capability and characterize the intracellular distribution of these nanoparticles to determine potentially safe *in vitro* conditions for biomedical applications.

Materials & methods

- Cytotoxicity of MSNPs and WSNTs was assessed by Presto Blue[®] and lactate dehydrogenase (LDH) viability assays at concentrations ranging from 5 to 300 µg/ml for 6, 12 or 24 h.
- MSC differentiation to adipocytes was assessed by Oil Red O staining and elution, and to osteoblasts by Alizarin Red S staining, alkaline phosphatase activity and extracellular calcium assays after treatment with potentially safe low (10 µg/ml) and high (50 µg/ml) doses of MSNPs and WSNTs.
- Uptake of MSNPs and WSNTs by MSCs following 24 h treatment at 50 µg/ml was assessed by transmission electron microscopy.

Results

- No dose or time dependent increase in toxicity was observed for NIH-3T3 cells treated with WSNTs or MSCs treated with MSNPs or WSNTs. Viability of NIH-3T3 cells treated with MSNPs with concentrations above 50 µg/ml was significantly lower compared with untreated cells.
- MSC differentiation potential was not significantly affected after treatment with potentially safe low (10 µg/ml) and high (50 µg/ml) doses of MSNPs and WSNTs.
- Both MSNPs and WSNTs were uptaken by MSCs.

Conclusion & future perspective

- MSNPs are potentially safe for NIH-3T3 cells up to 50 µg/ml and for MSCs up to 300 µg/ml. WSNTs are potentially safe for NIH-3T3 cells and MSCs up to 300 µg/ml.
- These nanoparticles when incubated with MSCs at concentrations up to 50 µg/ml for 24 h do not affect their differentiation potential toward adipocytes and osteoblasts.
- The results provide preliminary safety guidelines to further explore the potential of these nanoparticles at potentially safe doses as multifunctional agents for biomedical applications.

Figure 1. Nanoparticle characterization

Representative high-resolution transmission electron microscopy images of MSNPs (A) and WSNTs (B). (C) Representative Raman spectra of MSNPs (i) and WSNTs (ii).

MSNP: Molybdenum disulfide nanoplatelet; WSNT: Tungsten disulfide nanotube.

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Figure 2. Assessment of viability by Presto Blue®

Presto Blue assay results at 6, 12 and 24 h after treatment with molybdenum disulfide nanoplatforms (A) and tungsten disulfide nanotubes (B) for NIH-3T3 fibroblasts; after treatment with molybdenum disulfide nanoplatforms (C) and tungsten disulfide nanotubes (D) for MSCs. For each nanoparticle, cells were treated with PEG-DSPE, 5, 10, 50 and 300 µg/ml concentrations. Data are presented as mean ± standard deviation of percentage viability compared with untreated cells (n = 4). Statistical significance (p = 0.05) with respect to untreated groups at 6, 12 and 24 h are denoted by (■), (), (▲), respectively. Statistical significance between time points within groups is denoted by (*).

MSC: Mesenchymal stem cell; PEG-DSPE: Distearoyl-sn-glycero-3-phosphoethanolamine-N-(amino[PEG]).

Figure 4. Adipogenesis results

(A) Histological specimens of mesenchymal stem cells incubated with MSNPs and WSNTs for 24 h, followed by incubation with adipogenic differentiation media for 21 day, stained by Oil Red O. (B) Elution of Oil Red O stain. Data are normalized to control values and presented as mean $\pm$ standard deviation ($n = 3$). Statistical significance ($p < 0.05$) was determined by the Kruskal–Wallis test with Dunn's *post hoc* compared with the control (▲) or within groups is denoted by (*).

MSC: Mesenchymal stem cell; MSNP: Molybdenum disulfide nanoplatelet; WSNT: Tungsten disulfide nanotube.

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Figure 5. Alizarin Red S stained mesenchymal stem cells

Mesenchymal stem cells were treated for 24 h with either 10 or 50 µg/ml of MSNPs or WSNTs respectively, followed by 14 days incubation with osteogenic differentiation media prior to staining.

MSNP: Molybdenum disulfide nanoplatelet; WSNT: Tungsten disulfide nanotube.

Figure 6. Osteogenesis results (see facing page)

(A) Cellularity for mesenchymal stem cells after treatment for 24 h with either 10 or 50 µg/ml of MSNPs or WSNTs, respectively, followed by 14 days incubation with osteogenic differentiation media. (B) Alkaline phosphatase activity for mesenchymal stem cells after treatment for 24 h with either 10 or 50 µg/ml of MSNPs or WSNTs, respectively, followed by 14 days incubation with osteogenic differentiation media. (C) Calcium content after treatment for 24 h with either 10 or 50 µg/ml of MSNPs or WSNTs, respectively, followed by 14 days incubation with osteogenic differentiation media. Data are presented as mean ±

standard deviation ($n = 3$). Statistical significance ($p < 0.05$) was determined by the Kruskal–Wallis test with Dunn’s *post hoc* as compared with the control (▲) or within groups (*).

MSNP: Molybdenum disulfide nanoplatelet; WSNT: Tungsten disulfide nanotube.

Figure 7. Nanoparticle uptake by mesenchymal stem cells

Representative transmission electron microscopy images of mesenchymal stem cells treated with molybdenum disulfide nanoplatelets (A & B) and tungsten disulfide nanotubes (C & D). Molybdenum disulfide nanoplatelets are seen in endocytic vesicles (red arrows), and on the membrane of the cells (orange arrows). Tungsten disulfide nanotubes are seen in the cytoplasm within (red arrows) and outside (yellow arrow) endocytic vesicles. Neither nanoparticle can be seen in the nucleus.