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4125 Usefulness of Evaluation System for Activation Potential of Various Nanoparticles Using Monocytic Cell Line THP-1 and Prediction of Toxicity

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Background and Purpose: In recent years, high-performance products like cosmetics have been developed using nanoparticles to reduce whitening effects. Nanomaterials, with their small sizes and unique shapes, have different physicochemical properties than traditional chemicals. Their benefits have gained attention, leading to energy, environment, medicine, and manufacturing applications. However, their unique properties raise health concerns, and there is insufficient toxicity data. Developing a rapid, accurate toxicity assessment method acceptable to regulatory authorities is crucial. The OECD (Organization for Economic Cooperation and Development) leads European and US efforts. Member countries are working through the OECD Working Group on Industrialized Nanomaterials (WPMN) and the OECD Test Guideline Working Group (WNT) to create test guidelines (TG) and guidance documents (GD) for nanomaterial regulation. Progress is being made, but one critical area, the effect on the immune system, still needs to be evaluated. Establishing a test method for immunotoxicity is urgent. This study aimed to validate the in vitro h-CLAT assay for evaluating nanomaterial immunotoxicity. We assessed the system's effectiveness in activating antigen-presenting cells in the THP-1 monocyte cell line, using statistical analysis to evaluate the reproducibility of various nanomaterials (SiO_2 NPs, CeO_2 NPs, ZnO NPs) and predict toxicity-related physical properties. **Methods:** The nanomaterials used in this study were provided by the Joint Research Centre (JRC) in Ispra, Italy, which collaborates with the OECD on nanomaterial activities. Additionally, CeO_2 NPs (S211575) and ZnO NPs (S677450, S544906) were purchased from Sigma-Aldrich. To investigate the effects of nanomaterials on immune cells, the h-CLAT assay (OECD TG442E), a skin sensitization test method, was used to evaluate the activation potential of antigen-presenting cells. Some data from this study are referenced from the literature (DOI: 10.3389/ftox.2024.1293147). For the h-CLAT test, THP-1 cells and test substances were added to a 24-well plate, incubated in a CO_2 incubator for 24 hours, then washed with PBS and treated with a blocking solution. The cells were then treated with CD54 and CD86 antibodies, and their expression was measured using flow cytometry. The expression of CD54 and CD86 was calculated as relative fluorescence intensity (RFI) compared to the control group, and cell viability was calculated. Based on preliminary results, exposure concentrations were set at eight levels, with a maximum of 1000 µg/ml. Three tests were conducted to confirm reproducibility. Favorable judgment criteria were based on the OECD Test Guidelines: in at least two of the three tests, the RFI of CD86 was 150% or more, or the RFI of CD54 was 200% or more, even at one concentration. Statistical analysis was used to determine the accuracy and precision of the assay and validate the analytical method for repeatability/intra-assay precision. Precision was determined by expressing the variation in measured values between each experiment as a standard deviation and the differences in the three negative/positive determinations. Additionally, we performed functional fitting on these measured values and evaluated the stability and predictive performance of the test method based on the constructed model. We also analyzed the characteristics of physicochemical properties using principal component analysis (PCA) with multivariate analysis software SIMCA17 (SIMCA-P+ by Umetrix). We examined the relationship between physical properties and the favorable judgment of h-CLAT test results using orthogonal partial least squares (OPLS). We identified the characteristics of nanomaterials that show toxicity. **Results:** The evaluation of antigen-presenting cell activation showed that four types of SiO_2 NPs (NM-200, NM-201, NM-202, NM-203) exceeded the standard value for CD54 expression, with NM-204 showing the lowest concentration (3.48 µg/ml). However, CD54 expression did not exceed the standard value in any case. For the three types of CeO_2 NPs (NM-211, NM-212, S211575), CD86 and CD54 expression did not exceed the standard value. For the four ZnO NPs (NM-110, NM-111, S677450, S544906), CD54 expression exceeded the reference value in all cases, but no significant differences were observed. CD86 expression exceeded the reference value only for NM-111 (10 µg/ml). Excluding the three types of CeO_2 NPs that did not show favorable results, the order of strength of favorable judgments for the nanomaterials was NM-204 > NM-200 > S677450 > NM-110 > S544906 > NM-111 > NM-202 > NM-203 > NM-201. Accuracy and precision were evaluated using the standard deviation of n=3 measurements and function fitting. A good model with a goodness of fit of 0.939 or more was obtained for SiO_2 NPs and ZnO NPs, but it was difficult to model CeO_2 NPs due to insufficient measurement intensity. The Pearson correlation coefficient between the EC200 from the model function and the median estimated EC200 was 0.851, and Spearman's rank correlation coefficient was 0.700. The property characteristic analysis could have been more favorable, as the cumulative prediction of the model based on PCA was negative and low, and cross-validation results were poor. The PCA results showed that the three ZnO NPs (NM-110, NM-111, S544906) had a significant positive change in the first principal component compared to other test materials. These characteristics included non-spherical particle size and high values for secondary particle size, polydispersity index, and zeta potential. The OPLS analysis of the relationship between physical properties and toxicity suggested that nanomaterials are characterized by high zeta potential and low EC200 values. Due to considerable variation, other physical properties could not be evaluated for their relationship with toxicity. **Conclusions:** This study gathered data on tests and analysis using an evaluation method for the antigen-presenting cell activation ability of THP-1 cells, using CD54 and CD86 as indicators for various nanomaterials. The h-CLAT test results for the three CeO_2 NPs showed no toxicity, suggesting CeO_2 NPs are safer than SiO_2 NPs and ZnO NPs. Additionally, the variation in concentration and sample standard deviation for n=3 measurements was stable, and the square root of unbiased variances and model values for accuracy were comparable, indicating high predictability for EC200 for SiO_2 NPs and ZnO NPs. These results suggest good intra-laboratory reproducibility of the in vitro h-CLAT test for nanomaterials. The OPLS loading plots from the multivariate analysis showed a relationship between the zeta potential values of the nanomaterials and their physical properties and toxicity. Although this study was not a validation study following OECD GD34, it was a pre-validation study. Therefore, the results are expected to contribute significantly to ensuring the safety of nanomaterials used in raw materials for cosmetics and other products. We thank the JRC Nanomaterials Repository (JRC in Ispra, Italy) of the European Commission's JRC for providing various nanomaterials for this study.

4126 *In Silico* Prediction of Nanoparticle Toxicity in Human Hepatoma HepaRG Cells Using Machine Learning

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Sponsor: N. Singhal, Society of Toxicologic Pathology

Background and Purpose: As the use of nanoparticles (NPs) continues to grow in consumer and industrial applications, evaluating their potential toxicity is critical for ensuring safety and meeting regulatory standards. The HepaRG cell line, a human hepatoma model, provides a relevant system for studying liver toxicity and generating dose-response data on the adverse effects of various nanoparticles, such as Ag, TiO2, and CuO. This study aims to develop an in silico hazard assessment model using logistic regression to predict HepaRG cell responses to different nanoparticles. A key focus of the model is the use of group-stratified splits to improve its ability to generalize across diverse nanoparticle types, ensuring that it can accurately predict toxicity outcomes for a broad range of materials. **Methods:** Data from cell-based assays were collected, including 14 distinct readouts such as cell viability, apoptosis, and mitochondrial membrane potential. The resulting dataset consists of 110 samples representing 11 unique nanoparticle (NP) types. A logistic regression model was trained using a group-stratified approach, ensuring that the model was both trained and validated on distinct groups of nanoparticles. This methodology was specifically designed to enhance the model's reliability and generalizability across different types of nanoparticles.Model performance was evaluated using a range of metrics, including ROC AUC, sensitivity, F1 score, negative predictive value (NPV), precision, and accuracy. **Results:** By employing the group-stratified approach, the logistic regression model demonstrated excellent predictive performance. The mean ROC AUC for training was 0.9545 ± 0.0117, while for validation it was 0.9552 ± 0.0600. For the blind test set, the model achieved an ROC AUC of 0.9355 ± 0.0020, indicating strong generalizability. The performance metrics are as follows: sensitivity of 83.33%, F1 score of 86.96%, negative predictive value (NPV) of 95.24%, precision of 90.91%, and accuracy of 90.91%. These results outperformed those of the comparative model presented in the referenced study, underscoring the robustness of the group-stratified approach in nanoparticle toxicity prediction. **Conclusions:** The group-stratified logistic regression model demonstrated robust predictive capabilities in assessing nanoparticle (NP) toxicity in HepaRG cells, effectively capturing the complex relationships between nanoparticle characteristics and toxicological responses. This in-silico approach not only offers reliable toxicity predictions but also provides a flexible framework for hazard assessment, enabling quicker and more cost-effective screening compared to traditional experimental methods. With its demonstrated high performance, this model holds substantial potential for integration with other in vitro toxicity systems, creating a more comprehensive and efficient approach to safety testing. By combining the strengths of different models, this approach could enhance predictive accuracy across a wide range of compounds, ultimately contributing to more effective regulatory decision-making and risk assessment processes.

4127 Evaluating the relationship between nano and microplastic size and composition in pulmonary genotoxicity and inflammation *in vitro*

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Background and Purpose: Nano and Microplastics are an environmental and occupational health concern due to the rapidly growing industry predicted to result in the production of over 34 billion tons of plastic by 2050. Recently, studies have found increasing concentrations of micro and nano sized plastic particles in the environment, including in the ambient air, food chain, and water supplies. Additionally, the inhalation of micro and nano plastics as an occupational concern has been raised, though little information of the extent of these exposures has been uncovered. Few studies have been able to determine occupational exposure levels due to limitations in methods and sampling techniques, though concerns for exposure in the textiles, fiber manufacturing, flocking, waste management, and more recently, additive manufacturing industries, have been noted. Several studies have linked microplastics to inflammation, cell injury, genotoxicity, and other mechanisms that may result in adverse health outcomes such as respiratory and allergic diseases. The lack of thorough data correlating microplastic particle size, surface reactivity, chemical

composition, and other physicochemical characteristics to these toxicity outcomes has resulted in a significant knowledge gap. The current study aimed to investigate two plastic nanoparticles, polystyrene (PS) and polymethyl methacrylate (PMMA) at two different particle sizes (15 nm and 1000nm diameters) for pulmonary genotoxicity and inflammation in vitro. **Methods:** Two sizes of two plastic microspheres, PS and PMMA, were used in the current study. These particles were fluorescently tagged (488/560) and were either 15 nm or 1000 nm in diameter. Human bronchiolar epithelial cells (BEAS-2B) were used to investigate the genotoxicity of the microplastics, while differentiated human monocytes (THP-1s) were used to assess inflammation. In both cell lines, cell viability and proliferation were assessed after 24-hour exposure to nano and microplastics at doses ranging 0-500 µg/ml using WST-1 and Alamar Blue in addition to lactate dehydrogenase (LDH) in THP-1 cells. DNA damage, cell cycle disruption, and oxidative stress were assessed in BEAS-2B cells while caspase-1 activity and IL-1β release was assessed in THP-1 cells. **Results:** Cytotoxicity occurred at lower doses after exposure to 15 nm particles when compared to their larger diameter counterparts. The 1000 nm diameter PS particle had minimal effect on cell viability, even at 500 µg/ml, while the 1000 nm diameter PMMA particle significantly reduced viability at approximately 300 µg/ml in the Alamar Blue assay. No significant changes in DNA damage or cell cycle occurred at 6.25 or 25 µg/ml for either particle; however, all materials and sizes induced varying degrees of oxidative stress with the 1000 nm PS particle inducing a more significant increase at the high dose. Each particle increased Caspase-1 activity at 6 hours beginning at the lower doses. Additionally, lower doses of 1000 nm PS increased IL-1B release at 24 hours of exposure. **Conclusions:** Overall, minimal to no change in cell viability occurs at doses lower than 180 µg/ml for both particles and sizes; however, the larger diameter particles were not as cytotoxic as the smaller particles at a given dose. Although there was minimal genotoxicity following exposure to any of the particles, oxidative stress was induced by all materials and inflammation occurred with all particles. These findings varied with chemical composition, size, and dose. Overall, PS1000 was the most toxic of the particles at the 25 and 6.25 µg/ml doses, though the smaller diameter particles affected cell viability and proliferation at lower doses compared to the larger diameter particles, while PMMA1000 was the least toxic. This data suggests that both particle size and chemical composition are prominent determinants of micro and nano plastic toxicity. Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention.

PS 4128 An Extensive Study Focusing on Quantum Dots and Their Interaction with Actin

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Background and Purpose: Quantum dots (QDs) are biocompatible nanoparticles with a unique range of optical and fluorescent properties which makes them a prospective diagnostic tool in nanomedicine applications for cell labeling, bioimaging, and drug administration. However, the integration of QDs in real life biological scenarios as a biomedicine carrier has been limited due to QDs' ability to non-specifically interact with the cells. As such, this study aims to study QDs' interaction with intracellular proteins, including actin and actin-binding proteins and the consequence of their interactions. **Methods:** We conducted spin-down assays to examine the effects of QDs on actin polymerization and depolymerization as well as the interaction between QDs and actin in the presence of alpha actinin. We then performed fluorometer-based assays to monitor pyrene labeled actin polymerization and depolymerization over time in the presence of varying concentrations of QD. We also carried out a wound-healing assay to investigate the effects of QD on the migration of HeLa cells over time. **Results:** Our spin-down experiment showed that high concentrations of QD hinder actin polymerization and promote depolymerization. Furthermore, our spin-down assay also demonstrated the ability of QD to cause bundling of actin filaments. However, in the presence of both alpha actinin and high QD concentration, actin bundling does not occur. This indicates an interaction between QD and alpha actinin. In addition, our fluorometer assay revealed that high concentrations of QD inhibit actin polymerization while lower concentrations stimulate polymerization. Correlating with these results, the wound-healing assay revealed that QD was able to hinder cell migration, likely due to the disruption of the actin cytoskeleton. **Conclusions:** Our study showcased a novel discovery in terms of QD' functionality: it interacts with actin and actin-binding proteins, resulting in defects in actin dynamics. These results provide insight and further emphasize on the importance of understanding the interactions between QDs and intracellular proteins to maximize their potential in biomedical applications while mitigating any side effects.

PS 4129 The Transcription Factor PU.1 Mediates Multi-Walled Carbon Nanotubes-Induced Arachidonate 5-Lipoxygenase Expression and Proinflammatory Responses in Macrophages

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Background and Purpose: Exposure to fibrogenic multi-walled carbon nanotubes (MWCNTs) induces the production of proinflammatory lipid mediators (LMs) in myeloid cells to instigate inflammation. The molecular underpinnings of LM production in nanotoxicity remain unclear. **Methods:** Murine (J774A.1 cells) or human monocyte derived macrophages were treated with MWCNTs (Mitsui-7) or Carbon black (CB, Printex90), an amorphous carbon material control, at 2.5 or 10 µg/mL for 24 hours. The molecular induction of arachidonate 5-lypoxygenase (Alox5) and production of LMs were assessed by quantitative RT-PCR or ELISA assay, respectively, and the key transcription factor was identified by a series of luciferase reporter assay and chromatin immunoprecipitation assay after the exposure in macrophages. **Results:** MWCNTs induced the expression of Alox5 at both mRNA and protein levels in murine and human macrophages, accompanied by marked elevation of chemotactic LM leukotriene B4 (LTB4). Induction is comparable to those by potent M1 inducers. CB did not increase Alox5 expression or LTB4 production at equivalent doses. MWCNTs induced the expression of a heterologous luciferase reporter under the control of the murine *Alox5* promoter. Deletional analysis for the 5' upstream regulatory region of *Alox5* promoter uncovered multiple activation regions of which the activities were further increased by MWCNTs. We also found that the *Alox5* promoter contains four binding sites for PU.1, a ETS domain-containing master regulator of hematopoiesis, which was further increased by MWCNTs. Knockdown of PU.1 using specific small hairpin-RNA blocked the basal and induced expression of *Alox5* mRNA and the production of LTB4 as well as prostaglandin E2. **Conclusions:** The results demonstrate a critical role of PU.1 in mediating MWCNTs-induced expression of Alox5 and production of proinflammatory LMs, revealing a molecular framework where the hematopoietic transcription factor PU.1 is activated to orchestrate multiple proinflammatory responses to sterile particulate stimuli.

PS 4130 Physicochemical Properties and Functionalization Effects on the Bioactivity of Nickel (II) Oxide Nanoparticles in Macrophages

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Background and Purpose: Nickel (II) oxide nanoparticles (NiO NPs) have a wide array of uses, including applications in industry and medicine. The increased use of engineered nanomaterials such as NiO NPs is an emerging health concern as these particles can also cause chronic inflammation in a similar fashion to other environmental and endogenous particles such as crystalline silica or calcium pyrophosphate (CPP) crystals. Currently, there is evidence that particle surface properties contribute to the bioactivity of nanoparticles and define particle interactions with lysosomal membranes. Because lysosomal membrane permeability is a central factor in particle induced inflammation in macrophages, the analysis of NiO NPs' ability to initiate inflammation is necessary. Furthermore, the effect of Ni^{2+} on mitochondrial function should be considered as it relates to additional contributions to lysosome dysfunction and inflammation. In this study, nickel (II) oxide nanoparticles from three different manufacturers were compared. To further refine the understanding of functionalization to bioactivity of nickel oxide nanoparticles (NP), the three NiO NPs were functionalized with -COOH groups. **Methods:** Bare NiO NPs were analyzed with transmission electron microscopy to understand the morphology of the differing particles. Zeta potentials were calculated for each NiO NP to Bare NiO NPs (SIG, CHEM, and AA) were assessed for free Ni^{2+} ions after incubation for 1 hr or 72 hr at 37° C in synthetic lysosomal fluid (pH 4.7) using dimethylglyoxime (DMG), which forms a pink complex with free Ni^{2+} ions that can then be quantified with absorbance readings on a microplate reader. Bare and functionalized particles were assessed for cytotoxicity and inflammatory cytokine IL-1β in a macrophage model. Additionally, DMG was used to pretreat macrophages prior to NiO NP application to assess the role of Ni^{2+} ions in NiO NP bioactivity. Reactive oxygen species (ROS) were assessed in macrophages after 2 hours using a fluorescent indicator of ROS, MitoROS. Lysosomal membrane permeability due to NiO NPs was evaluated in macrophages after 4 hours. **Results:** TEM analysis of the three nickel oxide nanoparticles showed differences in morphology. SIG nanoparticles displayed a disordered mesoporous structure comprised of nickel oxide nanocrystals (~10 nm in diameter). CHEM nanoparticles ranged in size from 30-210 nm in diameter with an overall avg diameter of ~90 nm. AA nanoparticles had an average diameter of 12 nm. The Zeta potentials were measured at -12.7 (CHEM), -12.6 (SIG), and -9.08 (AA). NiO NPs incubated in synthetic lysosomal fluid for 1 hour demonstrated Ni^{2+} content that increased from CHEM to SIG to AA indicated by absorbance readings of 0.048, 0.143, and 7.88 respectively. At 72 hours incubation in synthetic lysosomal fluid, NiO NPs demonstrated a similar pattern of Ni^{2+} content with absorbance readings of 0.132 (CHEM), 1.49 (SIG), and 9.20 (AA). Functionalization of bare SIG and AA with -COOH groups reduced the respective Ni^{2+} ions released after 1 hr incubation with synthetic lysosomal fluid. Murine ex vivo alveolar macrophages treated with NiO NPs for 24 hr and assessed for cell death and IL-1β release demonstrated the greatest response with SIG particles, with AA and CHEM particles showing less cell death or IL-1β release in comparison. Functionalization with -COOH reduced the bioactivity of NiO NPs. Macrophages pre-treated with DMG Ni^{2+} chelator for 2 hr prior