A T-dependent antibody response evaluation in CD-1 mice after an acute whole-body inhalation exposure to nickel (II) chloride hexahydrate

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\textbf{ABSTRACT}

Nickel (Ni) in ambient air may vary regionally with contributions from both natural processes and anthropogenic activities. Exposure to Ni compounds in ambient air above a certain level is associated with acute adverse effects, such as upper respiratory tract irritation, pneumonitis, and chronic adverse effects, such as respiratory cancer. Inhalation reference exposure standards are enacted in different jurisdictions to minimize exposures to ambient Ni above levels that can elicit adverse effects. This paper reports a guideline/GLP-compliant study designed for setting inhalation exposure standards to protect from immunological effects associated with acute exposure to Ni. Female CD-1 mice were exposed via whole-body inhalation to aerosolized nickel chloride hexahydrate for 24-hr at nominal (vs. mean analyzed) concentrations of 20 (16), 50 (44) and 100 (81) \(\mu\)g Ni/m\(^3\). Host T-cell antibody immunological responses to intravenously-injected sheep red blood cells were then measured \textit{ex vivo} in an Antibody-Forming Cell (AFC) assay. Exposure to the Ni substance significantly decreased spleen cell levels by 33%, but this was within biological variability for outbred mice. No concurrent decreases in spleen, thymus, or body weights were noted. No immunosuppression was observed with the Ni substance in the context of Total Spleen Activity \((\text{IgM AFC/spleen} \times 10^6)\) and Specific Activity \((\text{IgM AFC/spleen cells} \times 10^6)\). Significant concentration-independent increases in Total Spleen Activity and Specific Activity seen with the nickel chloride hexahydrate were normal and within biological variability for outbred mice. In contrast, cyclophosphamide (positive control) significantly decreased spleen cell numbers, spleen and thymus weights, and abolished Specific Activity and Total Spleen Activity. Based on results here, an NOAEC of 81\(\mu\)g Ni/m\(^3\) for immunosuppressive effects from inhaled nickel chloride hexahydrate was identified. It is hoped this value can be used to derive a reference standard for human exposure to ambient Ni.

\textbf{Introduction}

Nickel (Ni) is an element that exists in nature as sulfides, oxides, and silicate minerals. Nickel is released into the atmosphere by natural processes such as weathering of rocks, volcanic eruptions, and forest fires, and through blowing of dusts; anthropogenic activities like metal mining and production, combustion of fossil fuels, and disposal of sludges and solids also contribute to the atmospheric levels of Ni (Buxton et al. 2019). Ambient Ni occurs mainly in particulate forms and aerosols, and in the United States, it can range in concentration from 7 to 12 ng Ni/m\(^3\), or up to 150 ng Ni/m\(^3\) near point sources. Indoor Ni air concentrations in the U.S. are reported to be on average 10 ng/m\(^3\) (ATSDR (Agency for Toxic Substances and Disease Registry) 2005). In some Canadian cities, the ambient Ni concentrations (measured as PM\(_{2.5}\)) range from < 0.1 to 4.5 ng Ni/m\(^3\) (Alberta Environment 2004). In most remote areas globally, ambient Ni levels range from 1 to 3 ng Ni/m\(^3\), but levels in rural and urban air can range from 5 to 35 ng Ni/m\(^3\) (WHO (World Health Organization) 2019). The average daily intake of Ni via inhalation is \(\approx 0.04\mu\)g Ni/m\(^3\) (ECHA (European Chemicals Agency) 2018).

There are acute and chronic toxic effects associated with inhalation of Ni-containing substances. One of the most important effects from chronic exposure to Ni compounds is an increase in respiratory cancers, as observed in workers involved in the processing/refining of sulfidic nickel ores or with historically-high exposures to Ni compounds (Doll et al. 1990). For metallic Ni, the epidemiological and animal studies by relevant exposure routes indicate a lack of respiratory carcinogenic effect (Doll et al. 1990; Sivulka 2005; Oller et al. 2008). The body of evidence has shown that in regard to cancers, inhalation is the only exposure route of concern since the increases in tumors associated with Ni compound exposure are most often of the type local to the respiratory tract (Heim et al. 2007; ECHA (European Chemicals Agency) 2018). The acute effects associated with Ni inhalation include upper respiratory tract irritation, pneumonitis, and even death following very high exposures (i.e. \(\geq 0.5\text{ mg Ni/m}^3\)) to fine Ni particles (Buxton et al. 2019).

In addition to the above-noted toxicities, Ni is known to cause a dermal immune reaction, i.e. a delayed Type 2 contact hypersensitivity reaction known as Allergic Contact Dermatitis (ACD) (Torres et al. 2009; Silverberg et al. 2020). It is important to note here that for Ni ACD to develop, an individual must...
have first been immunologically-sensitized to Ni via prior exposures on the skin. A few cases of occupational asthma have been reported among workers exposed primarily to soluble Ni compounds (Buxton et al. 2019). There are a few reports of Ni causing immunosuppression in rodents after injection (Smialowicz et al. 1984, 1985, 1987). For example, in rats injected intramuscularly (IM) with nickel chloride (NiCl₂), the primary antibody response to sheep red blood cells (SRBC) was not affected but natural killer (NK) cell activity was significantly reduced (Smialowicz et al. 1987). In rodents, the immunosuppression was transient. In a similar hemolytic plaque assay in CD-1 mice that underwent an IM injection with, or a 2-hr nose-only inhalation to, NiCl₂ prior to an SRBC immunization, significant immunosuppression – as measured by changes in number of plaques – was seen (Graham et al. 1975, 1978). In repeat-dose inhalation studies, nickel sulfate (NiSO₄) hexahydrate, nickel subulfide (Ni₃S₂), and nickel oxide (NiO) exposure stimulated lung macrophage recruitment to the lungs, but no suppression in macrophage activity was noted (NTP (National Toxicology Program)) 1996a,b,c).

To protect workers and the general public from the inhalation toxicities associated with Ni, occupational and ambient air standards have been set that take into account the acute or chronic nature of the exposure and the differences in potency among the various chemical forms of Ni. For chronic effects, respiratory cancer is always the driving force for setting standards for Ni compounds. For acute effects, different health endpoints were chosen by different agencies. The Graham et al. (1978) study, the only acute immunotoxicity study using a physiological route of exposure (i.e. nose-only inhalation), was selected by the California OEHHA in 2012 to set the acute 1-hr Ni Reference Exposure Level (REL) and as a supporting study for the 8-hr nickel REL. The same study was used in 2013 by the Government of Quebec (Canada) to set a 24-hr Ni REL under the Clean Air regulation. However, the 1978 Graham study was not suited for risk assessment in regulatory settings. It was conducted prior to issuance of the first immunotoxicology testing guideline by the Office of Prevention, Pesticides and Toxic Substances (OPPTS) of the U.S. Environmental Protection Agency (USEPA 1998). In the hemolytic plaque or Antibody-Forming Cell (AFC) assay, two endpoints, i.e. Specific Activity (AFC/10⁶ spleen cells) and Total Spleen Activity (AFC/spleen (× 10⁶)) – were recommended for evaluation of study results. Only Specific Activity was determined in the 1978 Graham study. The test guideline of 1998 indicated a need for use of a positive control, but this was not included in the 1978 study. Furthermore, regarding the REL from the Government of Quebec, the length of exposure (2-hr) in the Graham study was not comparable to the duration of the 24-hr air standard, thereby requiring duration of exposure extrapolations and increasing the uncertainty of the standard.

The report presented here describes an immunotoxicity study (i.e. an AFC assay) that addresses weaknesses of the previous Graham et al. study and can also be used by regulatory agencies to set 24-hr ambient air standards. The study was conducted under GLP, following the USEPA OPPTS 870.7800 immunotoxicity test guideline (Health Effects Test Guidelines, OPPTS 870.7800 Immunotoxicity). This is the first acute AFC immunotoxicity study with NiCl₂ to be conducted as guideline- and GLP-compliant, and the study was designed to derive dose-response information related to the immunotoxicity effects of Ni after inhalation. In brief, mice were exposed to either a negative or a positive control, or one of three concentrations of NiCl₂ (as hexahydrate) via whole-body exposure for 24-hr, and subsequently immunized with SRBC. All results are considered in the context of existing data on the immunotoxicity of Ni, and the applicability of the results for the setting of a 24-hr ambient air standard is discussed.

Materials and methods

Nickel chloride hexahydrate (NiCl₂·6H₂O, CAS 7791-20-0, Batch #BCBV1926, ≥98% purity) was purchased from Sigma (St. Louis, MO) and stored in a room with temperature and humidity controls. The positive control cyclophosphamide monohydrate (CAS 6055-19-2, Batch #MKBX1822V, 100% pure, 6% water content) was also purchased from Sigma and stored in a 5°C refrigerator. The positive control vehicle phosphate-buffered saline (PBS; USP sterile, Batch #18D0956177) was purchased from VWR Scientific (Radnor, PA). Sterile water for injection (USP, Batch #90-087-JT) was purchased from Hospira Inc. (Lake Forest, IL, USA). Both the PBS and water were stored in a room maintained at 18–24°C. Certified Rodent LabDiet 5002 blocks and meals were purchased from PMI Nutrition International, LLC (St. Louis). Other reagents and supplies were purchased from Sigma, Lampire Biological Laboratories (Pipersville, PA), and Cedarlane Laboratories (Burlington, ON, Canada), and stored as recommended by the supplier.

GLP compliance and guidelines

The study was conducted in accordance with the United Stated Code of Federal Regulations, Title 40, Parts 160 and 792: Good Laboratory Practice Standards and as accepted by OECD Principles of Good Laboratory Practice and Japan MAFF and METI.

The immunotoxicity phase of the study was conducted according to the United States EPA Office of Prevention, Pesticides and Toxic Substances (OPPTS) Harmonized Health Effects Test Guideline 870.7800 (1998). All studies were conducted at the Charles River Laboratory facility in Ashland, OH, with the exception of the Ni content analyses which were conducted at RTI International (Research Triangle Park, NC, USA).

Preparation of test substance and positive control

Cyclophosphamide monohydrate (CP; positive control) was prepared in PBS at 5 mg/ml, aliquoted, and stored at 5°C until needed. The dosing formulation was prepared once prior to administration. A stock solution of the test substance (Ni) was prepared by dissolving ≈575 mg of the test substance in 1000 ml deionized water and was used undiluted for the generation solution for Chamber 4 (Group 4, nominal 100 μg Ni/m³). Chamber 3 (Group 3, nominal 50 μg Ni/m³) exposure solution was prepared by diluting 125 ml of the stock with 125 ml deionized water, and the subsequent diluted test substance was used as the generation solution. For Chamber 2 (Group 2, nominal 20 μg Ni/m³) exposure, 62.5 ml stock was diluted with 187.5 ml deionized water and the subsequent diluted test substance was used as the generation solution.

Animals

Crl:CD1 (ICR) mice (female, ≈12 wk-old, 28.3–33.6 g at initiation of exposure) were received from Charles River Laboratories.
Inc. (Raleigh, NC). Female CD-1 mice were selected because this was the strain and gender used in the inhalation immunotoxicity study of Graham et al. (1978). Each mouse was identified with a subcutaneously-implanted electronic identification chip (BMDS: Bio Medic Data Systems, Seaford, DE). A detailed clinical observation was performed on each animal within 4 days of receipt, on the day of randomization, and on Days 1 and 6 of the study. The mice were acclimated for 7 days and then assigned to five groups with 10 animals per group (except Groups 1 and 4 which had 15 each). Assignment was done using a stratified randomization scheme to achieve similar group mean body weights. The mice were weighed within 4 days of receipt, and once daily throughout the study.

Throughout the study, all mice were individually-housed in clearly labeled solid-bottom cages in a room maintained at 20–26°C, with a 30–70% relative humidity and a 12-hr light/dark cycle. The mice were provided food and water ad libitum throughout the study. Rodent LabDiet 5002 blocks (oval pellets, 10 × 16 × 25 mm) were provided during the 24-hr inhalation exposure phase to minimize contamination of the feed; Rodent LabDiet 5002 meal (ground pellets) was provided at all other times. All feed and water were analyzed; Ni content was always found to be below the limit of detection. The mice were maintained in accordance with the Guide for the Care and Use of Laboratory Animals. The Charles River Ashland animal facilities are accredited by AAALAC International.

**Exposure duration and dose selection**

To minimize the uncertainty in duration of exposure extrapolation (e.g. to derive a 24-hr ambient air standard), the study was planned to last 24-hr. In a 2-hr nose-only inhalation study in CD-1 mice with exposures to 100, 250, 375, or 500 μg Ni/m³ (as NiCl₂; Graham et al. 1978), the no-observed and lowest-observed adverse effect concentration (NOAEC and LOAEC) identified were, respectively, 100 and 250 μg Ni/m³. Based on lung burdens after 2-hr inhalation exposure to 644 μg Ni/m³ also reported in the Graham study, deposited doses at 2- and 24-hr corresponding to the reported NOAEC and LOAEC for that study were calculated. The air concentrations that would result in 24-hr equivalent retained doses at the NOAEC and LOAEC (calculations not shown) were then calculated. Based on the calculations, the selected Ni exposure concentrations were 20, 50, and 100 μg Ni/m³ (as NiCl₂). It was assumed that when variabilities in exposure duration were accounted for, the whole-body and nose-only inhalations with the same test substance in the same animal test strain would result in similar particle deposition in the lung. In the current study, whole-body inhalation exposure was employed because a 24-hr nose-only inhalation would have caused undue discomfort and stress to the animals. During whole-body inhalation exposure, systemic Ni levels will reflect absorption through the respiratory tract as well as contributions from oral (through grooming) and dermal absorption. However, the latter routes contribute a minor fraction to the absorbed dose (≤ 2%) (Buxton et al. 2019) compared to that from inhalation (≥ 70%) (Carvalho and Ziemer 1982).

**Whole-body inhalation exposure**

Whole-body exposure chambers of appropriate size were used, one for the negative control group (filtered air), and one for each of the test substance-treatment groups. Mice were housed individually in compartments within the exposure chambers. Air supplied to the exposure chambers was provided as described below. All exposure chamber exhaust was directed to the facility exhaust system, which consisted of redundant exhaust blowers behind activated-charcoal and HEPA-filtration units.

Temperature and relative humidity within each exposure chamber was monitored using a Vaisala temperature and humidity probe connected to a digital display. Chamber airflow was monitored using a sharp edge orifice meter and pressure gauge. Each gauge was calibrated for conversion from pressure to airflow in standard liters per minute (LPM). Exposure chamber temperature, relative humidity, ventilation rate, and negative pressure were monitored and manually recorded at ≈ 60-min intervals during the 24-hr exposure period. Oxygen content of the exposure atmospheres was determined prior to the exposure using a calibrated O₂ sensor (20.9% for all groups).

For the filtered air control group (Group 1), humidified supply air was delivered from the facility temperature-/humidity-controlled supply air source to the chamber at a mean flow rate of 126 LPM. For the NiCl₂·6H₂O exposure groups (Groups 2–4), a liquid aerosol of the test substance was generated using a 6-jet Collision nebulizer (BGI Inc., Waltham, MA). Facility compressed air controlled using a Coihose pneumatics regulator (Model 8802 K; East Brunswick, NJ, USA) was supplied to the nebulizer. A Harvard syringe pump (ModelPHD 2000, Harvard Apparatus, Holliston, MA, USA) equipped with a syringe of appropriate size was used to deliver the test substance generation solutions to each nebulizer. The test substance aerosol was directed from the outlet of the Collison nebulizer through 1/2-inch I.D. anti-static tubing to a “T” fitting on the chamber inlet, where test substance aerosol was mixed with humidified supply air before entering the exposure chamber. Prior to the chamber inlet, a siphon was placed in-line to achieve a lower stable aerosol concentration. Using a rotameter-type flowmeter (Model VA20436 for Chambers 2 and 3, and Model VA22433 for Chamber 4; Dwyer Instruments, Michigan City, IN), a controlled portion of the aerosolized generation solution was removed using a house vacuum system as needed.

The target NiCl₂·6H₂O exposure concentrations were 20 μg Ni/m³ (Group 2, Chamber 2), 50 μg Ni/m³ (Group 3, Chamber 3) and 100 μg Ni/m³ (Group 4, Chamber 4). The mice were exposed via whole-body inhalation for 24-hr and then returned to their home cages. Total aerosol concentrations in the exposure atmospheres were determined using standard gravimetric methods and Ni concentrations achieved were subsequently measured by analyzing the filters. Samples of the exposure atmosphere were collected on pre-weighed glass fiber filters held in an open-face filter holder positioned in the animal-breathing zone of each exposure chamber. Following each sample collection, each filter was re-weighed and mass concentration (μg/m³) of test substance aerosol was calculated from the filter weight difference divided by the sample volume. Each filter was then placed individually into 50 ml digestion tubes (DigiTUBEs 50 ml Non RockLock, SCP Science, Champlain, NY), stored at ambient temperature, and shipped overnight under ambient conditions for nickel content analysis at RTI International.

Sample flows were controlled using a needle valve connected to the vacuum source and measured using a mini-BUCK Calibrator. A single sample was collected from the control exposure system. Approximately two blocks of rodent block feed were removed from the feeding tray from Groups 1 (air filter control) and 4 (high exposure dose of test substance) prior to and after the inhalation exposure. The block feed samples were placed in pre-weighed 50-ml digestion tubes (DigiTUBEs), the tubes with
block feed were re-weighed, and then shipped for Ni content analysis. Aerosol particle size measurements were conducted once during the 24-hr exposure period using cascade impactors. Atmosphere samples were collected for \( \approx 720, 300, \) and 150 min from Chambers 2, 3, and 4, respectively, onto pre-weighed filters. Following sample collection, all filters were re-weighed and particle size measurements were calculated. Particle size was expressed in terms of Mass Median Aerodynamic Diameter (MMAD) and Geometric Standard Deviation (GSD). The target range of MMAD and GSD were respectively, 1.0–3.0 \( \mu m \) and 1.5–3.5.

**SRBC immunization and necropsy**

On Day 2 after the exposure, 5 mice/group from Group 1 and Group 4 were euthanized by inhalation of CO\(_2\) and exsanguination, and blood and spleen samples collected for Ni content analysis. In brief, \( \approx 1 \) ml blood was collected from each animal via the inferior vena cava and processed for serum. The serum was then split into two aliquots in cryovials, stored at \(-80^\circ C\), and shipped on dry ice overnight for Ni content analysis at RTI International. The collected spleens were weighed, flash-frozen in liquid N\(_2\), stored at \(-80^\circ C\), and later shipped overnight on dry ice for Ni content analysis.

On the day of immunization (Day 2), SRBC were prepared for injection according to Charles River Ashland SOP. The remaining animals in Groups 1–4 received a single 0.2 ml injection of SRBC (containing \( \approx 10^8 \) cells) via a lateral tail vein. Following the immunization, the positive control animals in Group 5 were administered 50 mg CP/kg/day by intraperitoneal injection for 4 consecutive days (i.e. from Days 2–5). On Day 6, all mice were euthanized, and a gross necropsy conducted; this included examination of the external surface, all orifices, and the cranial, thoracic, abdominal, and pelvic cavities, including viscera. The spleen, thymus, and liver were removed and weighed. Representative samples of spleen and thymus were placed in 10% neutral-buffered formalin. The spleens collected for use in the AFC assay were placed in pre-weighed tubes containing Earle’s Balanced Salt Solution (EBSS) and 15 mM HEPES. Each tube was re-weighed and a “wet” spleen weight determined. The spleen samples from Groups 1–4 animals were randomized and blind-coded so the analyst was unaware of treatment. Group 5 spleens were labeled as positive controls.

**Spleen IgM AFC assay**

To prepare single-cell suspensions, each spleen was mashed between the frosted ends of two microscope slides while being held in a 100 \( \times \) 15 mm petri dish according to Charles River Ashland SOP documents). Total lytic spots were then counted using a Bellco plaque viewer and total Specific Activity \([\text{AFC}/10^6\text{ spleen cells}]\), Total Spleen Activity \([\text{AFC}/\text{spleen} (\times 10^3)]\) and total spleen cells \((\times 10^7)\) were then calculated.

**Nickel content analyses**

To each tube containing a filter sample, 25 ml of 5% HNO\(_3\)/1.5% HCl extraction solution was added; the tubes were then capped and heated in ultrasonic bath at 80°C for 3 hr. The samples were allowed to cool and brought to final volume of 50 ml with deionized water. An aliquot was taken for analysis by inductively coupled plasma - optical emission spectrometry (ICP-OES) using an iCAP6500 ICP-OES equipped with a CETAC ASX520 autosampler (ThermoFisher Scientific, Waltham, MA) enclosed in a HEPA filtered clean box.

To each tube containing feed sample, 5 ml concentrated HNO\(_3\) was added and the tubes were allowed to stand at room temperature for 30 min. The tubes were placed in a digestion block at 60°C for 1 hr, after which the samples were removed and cooled. One ml 30% H\(_2\)O\(_2\) was then added and the tubes were returned to the digestion block to incubate at 90°C for 1 hr. The samples were then cooled and deionized water added to bring the final volume to 50 ml. The samples were mixed by inversion and aliquots taken for analysis by inductively coupled plasma – mass spectrometry (ICP-MS) using a X-Series II ICP-MS equipped with a CETAC ASX520 autosampler (ThermoFisher Scientific) enclosed in a HEPA filtered clean box.

For analyses of biosamples, to 0.1 g of spleen and 50 \( \mu l \) serum was added 1.5 ml HNO\(_3\) before heating in the digestion block for 30 min at 95°C. After cooling, 0.5 ml 30% H\(_2\)O\(_2\) was added to each tube and the materials were returned to the digestion block for 30 min at 95°C. The samples were then cooled, deionized water added to bring the final volume to 15 ml, inverted to mix, and then aliquots taken for ICP-MS analysis.

**Statistical analysis**

Pair-wise comparisons were made between Groups 2–5 versus Group 1, the negative control. Datasets were compared using an overall one-way analysis of variance (ANOVA) \( F \)-test. If the overall \( F \)-test was found to be significant, the pair-wise comparisons were conducted using Dunnett’s test. Significance was set at \( p < 0.05 \). Data are presented as means ± standard error of mean (SEM), unless otherwise noted.

**Results**

**Optimization of CD-1 mice splenic AFC assay**

The Plaque Assay is a widely accepted method for determining immune responses to a T-cell-dependent immunogen (Jerne and Nordin 1963; Jerne et al. 1974; White et al. 2010). The AFC assay, a modification of the Jerne Plaque assay, is used to determine the response of IgM antibody-forming cells toward SRBC. The optimum level of Guinea Pig Complement (GPC) and SRBC, and the peak day of the IgM response for this study were determined in a method validation phase. CD-1 mice were selected for use as they are an accepted rodent species in immunotoxicity testing, and a similar study with the same test agent (Graham et al. 1978) used this strain of mice.

For the three GPC dilutions evaluated, i.e. 1:2, 1:4 and 1:8, the Specific Activity and Total Spleen Activity in male mice
increased as the GPC was diluted more. In female mice, the Specific Activity and Total Spleen Activity decreased at a GPC dilution of 1:8. Therefore, a GPC dilution of 1:2 was selected for use in the main experiment. Similarly, for mice immunized with 0.2 ml of a 1.25 × 10⁸, 5 × 10⁸, or 2 × 10⁹ SRBC/ml solution, Specific Activity (1008 ± 253) for males and 2013 ± 249) for females) and Total Spleen Activity (181 ± 52) for males and 254 ± 20) for females) outcomes in hosts immunized with 0.2 ml of the 5 × 10⁸ SRBC/ml solution were consistent with the published literature. Thus, the optimum SRBC concentration used in the main experiment was 5 × 10⁸ SRBC/mL (at a dose volume of 0.2 ml). For spleen samples collected 3-, 4-, 5- and 6-days post-immunization, Day 4 was determined as the peak day of IgM response, consistent with other reports (Graham et al. 1975, 1978).

Clinical observations
There were no unscheduled deaths in any of the five groups prior to study completion. No change in food consumption was noted throughout the study in any group. Body weights were not affected by inhalation exposure to the test substance. Less than a 10% change in body weight was recorded in all test substance-treated and positive control group mice compared to negative control mice throughout the study. Body weights of all mice decreased from Day 1 (exposure day) to Day 2; by Day 6 these had returned to near-Day 1 values (Table 1). Compared to in control mice, no test substance-related effects on liver, spleen, or thymus weights were noted in Group 2, 3, and 4 mice. However, as expected, in the positive control group (Group 5), the mice had significantly lower spleen (42%) and thymus (56%) weights compared to in control mice.

Characterization of exposure concentrations and aerosol particle size
The mean temperature and relative humidity in exposure chambers 1 to 4 ranged between 20.2 ± 0.40 to 20.6 ± 0.41°C and 49.7 ± 0.89 to 57.5 ± 1.02%, respectively. The chamber ventilation rate (LPM) for chambers 1, 2, 3, and 4 were, respectively, 125.6 ± 0.51, 125.5 ± 0.35, 126.8 ± 2.58, and 137.5 ± 10.34. Table 2 summarizes the target Ni and NiCl₂ levels for the treatment groups, and the aerosol particle parameters expressed as MMAD and GSD. The mean gravimetric concentrations (µg/m³) (not shown) were above the target levels (µg/m³) for the test substance exposure groups. This may have been due to long sampling times (240-600 min) used to get enough material on the filters at these low exposure levels. Potentially, this might have resulted in filters picking up animal dander, airborne urine, feces, and food particles in the chambers. Because the filter Ni content analyses were not completed prior to the initiation of exposures, only minimal adjustments were made to the aerosol generation system. These adjustments resulted in the analyzed concentrations of Ni in the test substance exposure groups being below (but no lower than 80%) from the target values for Groups 2 (16.0 vs. 20 µg Ni/m³), 3 (44.1 vs. 50 µg Ni/m³), and 4 (80.9 vs. 100 µg Ni/m³). The mean percent difference between the target and analyzed concentrations ranged from 12–20% and the mean MMAD and GSD were within the target values (with exception of MMAD in Group 2 that was 0.7 µm).

Nickel in feed, serum, and spleen
The Ni content of food samples taken from exposure chambers 1 and 4, prior to and after inhalation exposure, ranged between 1.32 and 1.60 µg/g. The Ni content of food samples was low and similar across exposure chambers, suggesting that the exposure of feed to the Ni-containing atmosphere contributed little to the feed Ni levels. In turn, the feed contributed negligible amounts to the overall Ni intake of the mice. The Ni content of food samples before and after the aerosol exposure did not differ by more than 0.28 µg/g.

Table 1. Organ weights at terminal necropsy and body weights from Day 1 to Day 6.

| Group | Organ weight (g) | *Nominal exposure concentration listed. |
|-------|-----------------|----------------------------------------|
| Liver (g) | Spleen weight (g) | Thymus weight (g) | Body weight (g) |
| Group 1 (Negative control) | 1.44 ± 0.04 | 0.13 ± 0.01 | 0.05 ± 0.00 | 30.56 ± 0.36 |
| *Group 2 (20 µg Ni/m³) | 1.49 ± 0.04 | 0.14 ± 0.01 | 0.06 ± 0.01 | 30.72 ± 0.37 |
| *Group 3 (50 µg Ni/m³) | 1.49 ± 0.02 | 0.14 ± 0.01 | 0.05 ± 0.00 | 30.95 ± 0.22 |
| *Group 4 (100 µg Ni/m³) | 1.51 ± 0.03 | 0.14 ± 0.01 | 0.05 ± 0.00 | 30.71 ± 0.40 |
| Group 5 (Positive control) | 1.43 ± 0.04 | **0.08 ± 0.00 | **0.02 ± 0.00 | 30.39 ± 0.44 |

Day 1 Day 2 Day 3 Day 4 Day 5 Day 6

Nickel effect on T-cell-dependent antibody response
The potential immunosuppressive effects of Ni were assessed via a T-cell-dependent antibody response to SRBC in a splenic AFC assay, one of the most extensively validated assays to assess immunotoxicity. When there is immunosuppression, decreases in values for spleen or thymus weights, spleen cell numbers, and/or Specific Activity or Total Spleen Activity (i.e. measures of IgM changes/10⁶ spleen cells and in total spleen, respectively) may be observed. In the current study, exposure to the NiCl₂ up to 81 ug Ni/m³ did not impact significantly on the spleen and thymus weights (Table 1). In contrast, the positive control cyclophosphamide (CP) caused significant decreases in both. The number of spleen cells were reduced as the level of exposure to NiCl₂ increased, with a significant decrease (33%) due to the highest
Table 2. aGravimetric concentrations, nickel concentrations and aerosol particle size.

| Group | Target concentration (µg Ni/m³) | bMean analyzed µg Ni/m³ | Mean MMAD (µm) | Mean GSD |
|-------|---------------------------------|-------------------------|----------------|----------|
| 1     | <0.1                            | --                      | --             | --       |
| 2     | 2.20                            | 81                      | 16.0 ± 1.5     | 0.7      | 3.33     |
| 3     | 5.0                             | 203                     | 44.1 ± 4.6     | 1.0      | 3.46     |
| 4     | 10.0                            | 405                     | 80.9 ± 12.4    | 1.1      | 2.96     |

aFor the filter from exposure chamber 1 (Group 1), Ni content was < 0.5 µg/filter. For chambers 2 (Group 2), 3 (Group 3) and 4 (Group 4) filter samples, Ni contents were, respectively, 101.7 (± 6.4) µg/filter (N = 2), 163.3 (± 10.4) µg/filter (N = 3), and 207.0 (± 14.9) µg/filter (N = 5).
bValues reported as mean ± SD (standard deviation).

Table 3. Summary of immunotoxicity findings in mice with 24-hr exposure to NiCl₂·6H₂O.

| Endpoint                          | 0°C | 20 | 50 | 100 | Cyclophosphamide |
|-----------------------------------|-----|----|----|-----|------------------|
| Spleen cells (× 10⁷)              | 4.55±0.35 | 4.22±0.15 | 3.86±0.19 | **×3.07±0.27 | **×1.54±0.36 [−66.2] |
| Total spleen activity             | 175.22±24.41 | **×131.70±29.01 [−78.3] | 202.20±19.51 [15.4] | **×282.10±27.74 [61.1] | **×0.00 [−100.0] |
| Specific activity (µg AFC/spleen) | 1365.11±225.18 | **×2510.30±250.62 [83.9] | 1763.80±180.64 [29.2] | **×3239.10±430.50 [137.3] | **×0.00 [−100.0] |

aNegative control. Female mice were exposed to filtered air. A Grubb’s test for outliers identified one control mouse with an unusually higher number of spleen cells (animal thus excluded from analyses).

Values in brackets represent percent difference from negative control group.

N = 10, unless otherwise noted.

* *p < 0.01 vs. Group 1.

Figure 1. Individual IgM antibody-forming cell responses to SRBC in female CD-1 mice following 24-hr whole-body inhalation exposure to nickel chloride hexahydrate. (A) Inter-leaved scatter with bars of individual spleen cell numbers in vehicle control, nominal nickel chloride hexahydrate treatment groups, and positive control (CP). (B) Inter-leaved scatter with bars of individual Total Spleen Activity [IgM AFC/spleen (10⁷)] values. (C) Inter-leaved scatter with bars of individual Specific Activity [IgM AFC/spleen cells (10⁶)] responses to SRBC on Day 4 after exposure. Scatter plots: biological variability of the responses in outbred mice, with low and high responders even in controls. N = 10 mice/group. *p < 0.05; **p < 0.01 vs. control (Group 1).
exposure concentration (Table 3, Figure 1(A)). In comparison, the CP caused a greater magnitude of decrease in the spleen cells, i.e. \( \approx 66\% \). While the decrease in spleen cells by the NiCl\(_2\) was not associated with a decrease in spleen or thymus weights, the decrease in spleen cells by the positive control CP was associated with significant decreases in spleen or thymus weights.

The exposure to NiCl\(_2\)-H\(_2\)O did not lead to any decrease in Specific Activity and Total Spleen Activity values in any of the Ni-exposed mice. On the contrary, significant increases in each parameter were seen with the lowest and highest exposure concentrations (Table 3, Figures 1(B-C)). These increases were not dose-dependent and were in the normal biological variability seen with outbred animals. This variability can be seen more clearly with the individual values plotted in Figures 1(B,C). The positive control CP completely abolished Total Spleen Activity and Specific Activity, consistent with its immunosuppressive activity and in agreement with the concurrent significant decreases in spleen/thymic weight, and spleen cell numbers.

**Discussion**

The Antibody-Forming Cell (AFC) assay or splenic antibody Plaque-Forming Cell (PFC) assay is regarded as a “gold standard” for the evaluation of immunotoxicity effects of chemicals. An analysis of tiered testing strategies to detect compounds of potential immunotoxicity in mice demonstrated that the antibody plaque forming cell assay was one of two tests that showed the highest predictability of immunotoxicity or “highest association with immunotoxicity” (Luster et al. 1992). Further, the PFC assay is predictive of altered host resistance (Luster et al. 1993).

The study here reports the effects of water-soluble inorganic nickel chloride hexahydrate (NiCl\(_2\)-6H\(_2\)O) on the inducibility of immunoglobulin IgM in an AFC assay following 24-hr whole-body inhalation exposures. Under the conditions of this study, NiCl\(_2\)-6H\(_2\)O appeared to increase the Total and Specific Activity of the spleen and decreased the number of spleen cells at the highest exposure level tested without causing concomitant decreases in spleen or thymus weights. The AFC assay with SRBC has been optimized for evaluating immunosuppression, but not for assessing induction of immunostimulation or immunoenhancement. Recent AFC assay studies in rats have demonstrated a substance agnostic biological variability in the Specific Activity and Total Spleen Activity (Banton et al. 2011; White et al. 2014). The present findings for NiCl\(_2\) were within the biological variability among outbred mice.

Taking all the data into consideration, it appears that a 24-hr whole body inhalation exposure to NiCl\(_2\)-6H\(_2\)O at a level up to 81 \( \mu \text{g Ni/m}^3 \) did not cause clear immunosuppression in Crl:CD-1 mice. This conclusion was based on an observed lack of decrease in spleen and thymus weights and lack of decrease in Specific and/or Total Spleen Activity, the two traditional most-used measures of induced effects in immunotoxicity evaluations (Banton et al. 2011; White et al. 2014; Anderson et al. 2016; Shipkowski et al. 2017). Reasons for the lower total spleen cell count as the NiCl\(_2\)-6H\(_2\)O exposure levels increased are not known; this was not associated with lower spleen weights nor with a concomitant decrease in Total Spleen or Specific Activity.

Increases in Specific Activity and Total Spleen Activity were noted in another study with NiCl\(_2\), albeit in that case the exposure was via injection and in a different rodent species. In F344 rats injected intramuscularly (IM) with 10, 15, or 20 mg NiCl\(_2\)/kg and then immunized with SRBC, there were non-significant non-dose-dependent increases in Specific Activity and Total Spleen Activity compared to controls (Smialowicz et al. 1987). The authors did not deem these increases to be of biological relevance. In another study, this time in mice injected IM with 18.3 and 27.5 mg NiCl\(_2\)/kg prior to the SRBC, significant suppression of the Specific Activity and Total Spleen Activity were observed, along with significant decreases in body, thymus, and spleen weights at the highest dose. On Day 8 after the NiCl\(_2\) injection, the Specific Activity and Total Spleen Activity responses returned to control levels (Smialowicz et al. 1984). This split in Ni-induced responses demonstrate that any immunosuppressive effects of NiCl\(_2\) were transient. The response to SRBC in another IM injection study in CD-1 mice given NiCl\(_2\) at 3.09, 6.17, 9.26 or 12.34 \( \mu \text{g Ni/g BW} \) was depressed in terms of Specific Activity (Graham et al. 1975). No Ni was detected in the spleen of mice immediately or 1, 2, 3 or 4 days post-injection. One could interpret this as an immunosuppression arising from an NiCl\(_2\) injection that was not due to direct effects of Ni on the spleen cells.

In the inhalation study here, on Day 2 after inhalation, a 2- to 7-fold higher levels of Ni in the spleen and serum was detected in mice exposed to NiCl\(_2\) (as hexahydrate) compared to in control counterparts. In the Graham et al. (1975) study, there was a significant decrease in body weight and spleen weight at the highest injected dose of NiCl\(_2\) (12.34 \( \mu \text{g Ni/g} \)); no significant changes in body weight and spleen weight at the highest analyzed inhalation exposure dose of 81 \( \mu \text{g Ni/m}^3 \) were seen here. In contrast to the immunosuppression noted in these injection studies, no immunosuppression was observed in the current whole-body inhalation study. This begs the question as to whether responses following the Ni injection was representative of a response that would occur following inhalation exposures in occupational settings or by the public.

While an IM injection study with nickel sulfate (NiSO\(_4\)) or NiCl\(_2\) showed there was induction of significant immunosuppression (decrease in plaques/10\(^6\) spleen cells), a similar injection with nickel oxide (NiO) caused no immunosuppression (Graham et al. 1978). All doses of NiSO\(_4\) (3.09-12.34 \( \mu \text{g Ni/g} \)) led to significant decrease in plaques/10\(^6\) spleen cells; for NiCl\(_2\), significant decreases in plaques/10\(^6\) spleen cells were noted only at the two highest (9.25 and 12.34 \( \mu \text{g Ni/g} \)) and not at the two lowest (3.09 and 6.17 \( \mu \text{g Ni/g} \)) doses tested. Those authors speculated a role for the two anions (chloride and sulfate) in the toxicity observed. It can be inferred that the lack of effects with the insoluble NiO may be due to a low bioavailability of nickel ion (Ni\(^{2+}\)) from this substance compared to Ni\(^{2+}\) from the soluble nickel salts.

Intramuscular injection with cadmium chloride (CdCl\(_2\)) or chromium (III) chloride (CrCl\(_3\)) caused no significant decrease in plaques/10\(^6\) spleen cells, while inhalation exposure to 190 \( \mu \text{g Cd/m}^3 \) caused significant decrease in plaques/10\(^6\) spleen cells (Graham et al. 1978). It is interesting that significant immunosuppression was seen with CdCl\(_2\) after inhalation exposure but not after IM injection. The injection studies used exposure routes that were not representative of how people are exposed to Ni in occupational or environmental settings. Though the differ-ence in exposure routes may not adequately account for the effects here, the importance of exposure routes on the immunotoxicity of Ni cannot be dismissed. It is also worth noting that none of these injection studies nor the nose-only inhalation study cited here used positive controls.

In a study where CD-1 mice were exposed via 2-hr nose-only inhalation to NiCl\(_2\), significant immunosuppression was observed with exposures \( \geq 250 \mu \text{g Ni/m}^3 \) (Graham et al. 1978). Here, no
immunosuppression was observed after 24-hr with a level of 81 µg Ni/m³. It is worth noting some differences and similarities between the Graham study and the one here. First, though both studies exposed CD-1 mice to NiCl₂ by inhalation, the Graham study was 2-hr via nose-only exposure while the study reported here was a 24-hr whole-body exposure. In the Graham et al. study, the SRBC was injected intraperitoneally but here it was intravenously. The effect of this latter variable on immunotoxicity is not immediately apparent, given that Day 4 after immunization was still the peak day of IgM response in both studies.

One noteworthy difference from the Graham study is that the immunotoxicity results there were expressed only as Specific Activity, but here, results were expressed as Specific Activity and Total Spleen Activity, plus the number of spleen cells, endpoints that more appropriately recapitulate any immunotoxic effects. A range of 1000–2000 PFC/10⁶ spleen cells have been observed as normal for controls in different laboratories testing different chemicals over several years (Luster et al. 1988). In the current study, the control IgM AFC/10⁶ spleen cells were within this normal range (>1000), but the control in the Graham et al. study was below the normal range (<1000 PFC/10⁶ spleen cells).

In a study where female B₆C₃:F₁ mice were exposed via inhalation to different Ni agents for 6 hr/d, 5 d/wk for 65 days, elevated responses to SRBC were observed (Haley et al. 1990). When expressed as Specific Activity and Total Spleen Activity of lung-associated lymph nodes instead of spleen, significant elevated responses to NiO and nickel subsulfide (Ni₃S₂) were seen, but elevated responses to NiSO₄ (hexahydrate) did not attain statistical significance. However, when expressed as Specific Activity and Total Spleen Activity of the spleen, the results were different. A significant decrease in Specific Activity of the spleen after exposure to the lowest concentrations of 0.47 and 2.0 mg Ni/m³ (NiO) was observed; however, the decrease was not significant at the highest NiO level tested, 7.9 mg Ni/m³. Also, a significant decrease in Total Spleen Activity of the spleen was observed with Ni₃S₂ at the highest level tested (1.8 mg Ni/m³) but not the lower ones (0.11 and 0.45 mg Ni/m³).

Duration of exposure and exposure route also appear to impact on immunomodulatory responses to Ni compounds. In a 4-wk (continuous, whole-body) inhalation exposure to NiO at levels up to 0.8 mg/m³, a significant concentration-dependent decrease in antibody titer of spleen cells to SRBC was observed starting at levels of 0.2 mg Ni/m³ (Spiegelberg et al. 1984). This group also noted that following a 4-mo (continuous, whole-body) inhalation exposure, a significant decrease in antibody titer was observed at 0.15 mg/m³. The upshot from those studies was that effects of Ni compounds on the humoral immune system after inhalation exposure appeared to vary with dose, duration of inhalation exposure, and species of Ni compound being tested. Previous studies have also determined Ni immunotoxicity via effects on macrophages, cytokines, and lymphocytes (e.g. Spiegelberg et al. 1984; Smialowicz et al. 1987; Bechera et al. 2017). It appears that no single measure of immunotoxicity is most sensitive to assess Ni immunotoxicity. Other complementary measures of immunotoxicity, like effects on cytokines, macrophages, and lymphocytes, may be assessed in the AFC assay. This is a limitation in the study reported here.

Derivation of a 24-hr reference exposure limit or reference value

Ambient air standards for Ni are established in various jurisdictions to protect against possible acute/chronic exposure effects. These ambient standards are promulgated for various exposure durations, i.e. from 1-, 8-, and 24-hr (acute) to 1-yr (chronic). The GLP guideline-compliant immunosuppression study reported here is suitable for setting a 24-hr reference value or reference exposure limit corresponding to PM₁₀. At actual inhalation exposures up to 81 µg Ni/m³, there was no decrease in the more relevant measures of immunosuppression, specifically, in Specific Activity and Total Spleen Activity. Thus, 81 µg Ni/m³ can be taken as a no-observed adverse effect concentration (NOAEC) for the endpoint of immunosuppression, and this could be a point of departure (POD) for estimation of a 24-hr reference exposure limit. In this case, there is no need to adjust for exposure duration, since exposure here was 24 hr.

One such 24-hr Ni air standard is 0.014 µg/m³ issued for Québec in 2013 (Galarneau et al. 2016; LégisQuébec 2020). The Quebec standard was derived using the same approach as for the derivation of the California EPA 1-hr reference exposure limit (0.2 µg Ni/m³; OEHHA (Office of Environmental Health Hazard Assessment) 2012). Quebec started from a benchmark dose (lower confidence limit) (BMDL) of 165 µg/m³ identified by the CalEPA based on the Graham et al. (1978) 2-hr study. The value was then converted to a 24-hr value of 14 µg/m³ (165 µg/m³ × [2 hr/24 hr] = 14 µg/m³). From there, the same uncertainty factors were applied as by CalEPA in derivation of their 1-hr standard.

The CalEPA cumulative uncertainty factor of 1000 included one of √10 to account for uncertainty associated with use of BMDL as a POD. This factor is not relevant for a POD based on a NOAEC, as in the present study. Therefore, one could lower the cumulative CalEPA/Quebec uncertainty factor to 300. Thus, a 24-hr reference exposure standard applying this modified uncertainty factor (300) to the NOAEC here of 81 µg Ni/m³ would be as follows:

\[
\frac{(81 \, \text{µg Ni/m}^3)}{300} = 0.27 \, \text{µg Ni/m}^3
\]

Based on the particle size distribution of the Ni aerosol in the current study, this ambient standard value corresponds to PM₁₀. This 24-hr value here is ~20-fold higher than the Quebec 24-hr standard of 0.014 µg/m³ and ~4-fold higher than a newly-proposed value of 0.070 µg/m³.

Alternatively, a dosimetric adjustment from animal-to-human can be applied to the POD value from this study to calculate a human equivalent concentration (HEC) using the approach of the Texas Commission on Environmental Quality (TCEQ) when deriving an acute 1-hr reference value for Ni (based on Graham et al. 1978 as a supporting study) (TCEQ (Texas Commission on Environmental Quality) 2011). Calculating an HEC allows for more direct comparisons of effect levels between humans and animals than external exposure concentration and reduces the required uncertainty factor for toxicokinetic differences. By applying the regional deposited dose ratio (RDDR) determined for particulate matter by TCEQ of 4.423 to the present study, a HEC of 358 µg Ni/m³ was calculated (81 µg Ni/m³ × 4.423 = 358 µg Ni/m³). This HEC can then be used as a POD in a reference level derivation. A 24-hr reference exposure standard could be then derived using the TCEQ total uncertainty factor of 100. This results in a 24-hr reference exposure standard of:

\[
\frac{(358 \, \text{µg Ni/m}^3)}{100} = 3.58 \, \text{µg Ni/m}^3
\]

While these example derivations use uncertainty factors from the previous assessments, any new derivation of a reference value...
could use the NOAEC from this study (81 μg Ni/m³) as a POD and independently evaluate what uncertainty factors need to be applied. The resulting standard would again correspond to an ambient air PM₁₀ particle size fraction. However, if the dose-related decrease in spleen cells was taken as adverse outcomes, although it was not a measure of immunosuppression by itself and did not correlate with concomitant decreases in Specific Activity and/or Total Spleen Activity, then the NOAEC will be 44 μg Ni/m³. Accordingly, this paper has presented alternate derivations of the 24-hr reference exposure standards using this NOAEC in the supplementary materials.

Conclusions

In this study, 24-hr whole-body inhalation exposure of female CD-1 mice to 81 μg Ni/m³ as NiCl₂·6H₂O decreased total numbers of host spleen cells but increased the IgM AFC/spleen (x 10³) and IgM AFC/10⁶ spleen cells. The increase in Specific Activity and Total Spleen Activity was not exposure concentration-dependent. The changes in spleen cell numbers, Specific Activity, and Total Spleen Activity due to the exposure were within biological activity noted among outbred animals. In contrast, the positive control CP abolished both Specific Activity and Total Spleen Activity due to the exposure were

Acknowledgements

The studies were performed at the Charles River Laboratory in Ashland, OH. The Authors wish to thank all the staff at CRL that helped with this study.

Disclosure statement

Authors Buxton, Taylor, and Oller are employees of the funding organization, NiPERA Inc. Authors Weinberg, Randazzo, and Peachee are employees of Charles River Laboratories and were involved in conducting the studies. No potential conflict of interest was reported by the author(s).

Funding

This research work was funded by NiPERA Inc., the science division of the Nickel Institute, an association of global nickel producing companies.

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