CYTOTOXICITY OF NICKEL OXIDE PARTICLES IN RAT ALVEOLAR MACROPHAGES CULTURED IN VITRO

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Abstract: The cytotoxicity of two types of nickel oxide particles was investigated in rat alveolar macrophages cultured in vitro. The trypan blue dye exclusion test and the release of lactate dehydrogenase into culture medium were used as cytotoxic indices. Although both types of nickel oxide particles were produced by the same manufacturer and were commercially sold under the same name of “nickel oxide”, one type of the nickel oxide particles which had black color was much more cytotoxic than the other type of green color. The black nickel oxide particles were more soluble in various kinds of solutions than the green nickel oxide particles. Therefore, it appears that the difference in the cytotoxicity of the black and green nickel oxide particles may be attributable to the difference in the solubility of the particles.

Key words: Cytotoxicity, nickel oxide, alveolar macrophages, rat.

INTRODUCTION

A variety of nickel compounds, including metallic nickel, nickel chloride, nickel subsulfide, nickel sulfate, and nickel oxide are encountered occupationally in the nickel refining and electroplating industries, with inhalation being a primary route of human exposure (Warner, 1984; Sunderman, 1986). A number of animal experiments have been carried out to evaluate the inhalation toxicology of these nickel compounds.
compounds. Inhalation of a variety of nickel compounds resulted in development of inflammation in rabbits, rats, hamsters and mice, fibrosis in mice, and emphysema, alveolar proteinosis and cancer in rats (Ottolenghi et al., 1974; Wehner et al., 1975; Takenaka et al., 1985; Benson et al., 1987, 1988; Dunnick et al., 1988). The type of response produced in exposed animals depended on the animal species, the nickel compound used, and on the exposure concentration.

Benson et al. (1989) administered three types of nickel compounds to rats by inhalation, and found a toxicity ranking of NiSO₄ > Ni₃S₂ > NiO, based on toxicities of the compounds at equivalent mg Ni/m³ exposure concentrations. Haley et al. (1990) also found that the toxicity of nickel oxide was lower than nickel sulfide and nickel sulfate in rodents using histopathological changes and immunodysfunctions as toxicological indices, and noted that the order of toxicity corresponded to the water solubility of the nickel compounds.

In the course of our studies on the chemical characteristics of nickel compounds, we found that the chemical composition as well as the water solubility of commercial nickel oxide particles differed depending on their manufacturer and product types (Yamada et al., 1992). Therefore, we suspected that the toxicity of nickel oxide particles varies with the manufacturers and product types, also. In this paper, we present data indicating that the cytotoxicity of nickel oxide particles is remarkably different between product types, even though they are from the same manufacturer and are commercially sold under the same name of "nickel oxide", and discuss the possible reasons for the difference in cytotoxicity.

**MATERIALS AND METHODS**

Two types of commercial nickel oxide particles produced by the same manufacturer (INCO, Co. Ltd., USA) were obtained from a domestic distributor. Although both types of particles were called "nickel oxide", their colors and chemical compositions were different from each other. One type had a dark green color and the other was black. We refer to them as green nickel oxide and black nickel oxide in the following text. Chemical analysis of these nickel oxide particles was performed with atomic absorption spectrometry for nickel and with an inert gas fusion method for oxygen, and showed a relatively larger content of oxygen in the black nickel oxide than in the green one. The scanning electron microscopic examination showed that the particle size distribution were essentially identical (count median diameter of 1.8–2.0 μm with geometrical standard deviation of 2.1–2.2) in both black and green nickel oxide particles.

To estimate the water solubility, these green and black nickel oxide particles were suspended in five kinds of solutions at concentrations of 200 and 800 μg/ml. The solutions used were distilled water, physiological saline, Hanks’ balanced buffered saline, and Eagle’s MEM tissues culture medium with and without 10% fetal calf serum. The particle suspension solutions were kept at 37°C in air.
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containing 5 % CO₂ for 24 hours. Then, nickel oxide particles and dissolved nickel were separated by filtration through a 0.22 μm membrane filter (Milipore, Co, Ltd.). The concentration of nickel in the filtrates was measured by atomic absorption spectrometry after removing other elements such as sodium by an ion exchange column using a chelating form resin.

The cytotoxicity of these two types of nickel oxide particles was determined in rat alveolar macrophages cultured in vitro. Wistar strain rats, 10–16 weeks old, weighing 240–270 g were used as donors of alveolar macrophages. The animals were sacrificed under halothane anaesthesia, and broncho-alveolar cells were collected by the conventional lung lavage method with phosphate-buffered saline (PBS). The lavage cells were separated by centrifugation at 1500 rpm for 5 min, washed twice with PBS and resuspended in Eagle’s MEM supplemented with 10 % fetal calf serum. The cells were incubated in a 35 mm petri dish at 37°C under 5 % CO₂ and 95 % air. After 2 hours, the culture supernatant and non-adherent cells were discarded. The adherent cells, consisting of more than 94 % macrophages, were washed with PBS, and cultured again in medium containing the nickel oxide particles at concentrations of 200, 400 and 800 μg/ml. After incubation for 18, 42 and 72 hours, the viability of the macrophages, the activity of lactic dehydrogenase (LDH) in the culture medium, and the amount of nickel oxide particles taken up by the macrophages were measured. To determine the macrophage viability, the culture dish was rinsed with PBS to remove non-adherent macrophages and free particles. The rinsing solutions were centrifuged and the non-adherent macrophages were recovered. The cell viability of the non-adherent and adherent macrophages was determined by a dye exclusion test with trypan blue. The activity of LDH in the culture medium was assayed by the method of Cabaud and Wroblewski (1958) using LDH test kits (Sigma, USA). In some of the samples, instead of the cell viability test, the adherent macrophages were lysed with 0.5 % SDS, and the amount of nickel contained in the cells was determined by atomic absorption spectrometry. Because separating the non-adherent macrophages from the non-phagocytosed free particles was difficult, the amount of nickel taken up by the non-adherent macrophages could not be determined.

RESULTS

The solubilities of both types of nickel oxide particles is shown in Table 1. The amount of nickel dissolved from the black nickel oxide particles suspended at 800 μg/ml ranged from 15 to 80 μg Ni/ml for 24 hours, depending on the type of solution. On the other hand, the solubility of the green nickel oxide was remarkably lower: approximately 0.1 μg/ml in the distilled water and saline, and an undetectable amount (less than 0.05 μg/ml) in the other solutions, when suspended at concentration of 800 μg/ml.
Table 1. Solubility of the black and green nickel oxide particles in the various kinds of solutions.

<table>
<thead>
<tr>
<th>Particles</th>
<th>Concentration (μg/ml)</th>
<th>Solubility (μg/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D. W.</td>
<td>Saline</td>
</tr>
<tr>
<td>Black</td>
<td></td>
<td></td>
</tr>
<tr>
<td>800</td>
<td>83.2±2.1</td>
<td>55.6±0.6</td>
</tr>
<tr>
<td>200</td>
<td>34.3±0.6</td>
<td>23.0±0.5</td>
</tr>
<tr>
<td>Green</td>
<td></td>
<td></td>
</tr>
<tr>
<td>800</td>
<td>0.1±0.0</td>
<td>0.1±0.1</td>
</tr>
</tbody>
</table>

* The amount of nickel dissolved in the distilled water (D. W.), physiological saline (Saline), Hanks’ balanced salt solution (H-BSS), Eagle’s minimum essential medium (E-MEM), and E-MEM supplemented with 10% fetal calf serum (MEM+FCS). n. d.” denotes the not detectable level, that is less than 0.05 μg/ml.

Fig. 1. The viability of rat alveolar macrophages exposed to the black nickel oxide particles. Vertical bars show the standard deviation of six to eight culture preparations. O, control; △, 200 μg/ml; □, 400 μg/ml; ■, 800 μg/ml.
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Fig. 1 shows the viability of alveolar macrophages co-cultured with the black nickel oxide particles at concentrations of 200, 400 and 800 μg/ml. The viability of the macrophages, which was determined by the trypan blue dye exclusion test, decreased with time after incubation and with increasing nickel oxide concentrations. On the other hand, the viability of macrophages cultured with the green nickel oxide particles was not significantly lower than those in the control groups at all time points after incubation and within the concentration ranges used here, except for the macrophages cultured for 72 hours with the green nickel particles at a concentration of 800 μg/ml (Fig. 2).

![Viability of macrophages over time](image-url)

**Fig. 2.** The viability of rat alveolar macrophages exposed to the green nickel oxide particles. Vertical bars show the standard deviation of six to nine culture preparations. ○, control; △, 200 μg/ml; □, 400 μg/ml; ■, 800 μg/ml.
Fig. 3 shows the release of LDH from the macrophages to the medium following administration of nickel oxide particles at a concentration of 200 μg/ml. The LDH release in the macrophages administered the black nickel particles was larger than that in the control group throughout the experimental period, and increased with time after incubation. The LDH activities in the culture medium of the macrophages loaded with the green nickel particles were slightly higher than those of the control groups at all time points examined, but a statistically significant difference was only observed at 72 hours after incubation.

![Graph showing LDH activity over time](image)

Fig. 3. The activity of LDH in the culture medium of alveolar macrophages exposed to the black or green nickel oxide particles at a concentration of 200 μg/ml. Vertical bars show the standard deviation of six culture preparations.
- ○, control;
- ▲, green nickel oxide;
- ■, black nickel oxide.

The amount of nickel taken up by the adherent macrophages was determined at doses of 200 and 800 μg/ml for the black and green nickel oxide particles. As mentioned in Materials and Methods, it was impossible to determine the amount of nickel in the non-adherent macrophages. Assuming that the non-adherent
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macrophages contained nickel in the same concentrations as that in the adherent macrophages, the total uptake of nickel by the cultured macrophages was calculated (Table 2). The total uptake of nickel by the macrophages administered the black nickel oxide particles were 1.5 and 13.0 μg per 10⁵ cells at concentrations of 200 and 800 μg/ml, respectively. For the macrophages cultured with the green nickel particles, the total uptake of nickel was similar to that in the macrophages with the black nickel particles.

Table 2. Uptake of nickel by alveolar macrophages cultured with the black and green nickel oxide particles for 24 hours.

<table>
<thead>
<tr>
<th>Particles</th>
<th>Dose (μg/ml)</th>
<th>Uptake by* adherent cells</th>
<th>Adherent/total macrophages</th>
<th>Total uptake** of nickel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black</td>
<td>200</td>
<td>1.0±0.2</td>
<td>0.65±0.06</td>
<td>1.5±0.3</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>8.3±1.7</td>
<td>0.64±0.13</td>
<td>13.0±3.7</td>
</tr>
<tr>
<td>Green</td>
<td>200</td>
<td>1.4±0.4</td>
<td>0.69±0.14</td>
<td>2.0±0.7</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>7.9±2.1</td>
<td>0.78±0.11</td>
<td>10.1±3.0</td>
</tr>
</tbody>
</table>

* The amount of nickel (μg) in the adherent macrophages cultured for 24 hours after incubation.

** The calculated amount of nickel (μg) in the total macrophages (10⁵ cells).

The nickel amount taken up by the non-adherent macrophages was assumed to be the same concentration as that in the adherent macrophages.

DISCUSSION

The present results clearly show that the toxicity of two types of nickel oxide particles on cultured rat alveolar macrophages was remarkably different, even though they are from the same manufacturer and are commercially sold the same name of "nickel oxide". Two indices were used to evaluate the cytotoxicity of the nickel oxide particles: the trypan blue dye exclusion test and the extracellular release of LDH. In both indices, the black nickel oxide particles were more cytotoxic than the green nickel oxide particles.

The reason why the black nickel particles were more cytotoxic is not readily apparent from the present study. A possible explanation may be difference in the solubility of the particles. Haley et al. (1990) compared the toxicity of several kinds of nickel compounds, and found that the order of toxicity corresponded to their water solubility. As shown in Table 1, the solubility of the black nickel oxide particles was much higher than that of the green nickel particles, regardless of the type of solvent. This suggests that the black nickel oxide particles may release ionic nickel more rapidly in the alveolar macrophages than the green nickel particles. It is known that some metal ions including nickel, chromium and manganese ions are very cytotoxic to rabbit alveolar macrophages (Waters and Gardner, 1975). Therefore, it appears that the difference in the cytotoxicity of the black and green nickel oxide particles may be attributable to the difference in the solubility of the particles.
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A second explanation for the difference in the cytotoxicity may be the amount of nickel particles taken up by the alveolar macrophages. The amount of nickel in the macrophages administered the black and green nickel oxide particles was determined at 24 hours after incubation (Table 2). To be more precise, the values presented in Table 2 do not show the actual amount of nickel oxide particles taken up by the macrophages in 24 hours. We had to assume that the non-adherent macrophages phagocytosed the particles at the same rate as the adherent macrophages did, since it was difficult to separate the non-adherent cells from the free particles in the medium. However, from light microscopic examination, the adherent and non-adherent macrophages seemed to contain similar amounts of nickel oxide particles. The values in Table 2 also do not take into account the amount of nickel which had been taken up by macrophages as particles and released into the culture medium before the measurement. However, we have reported that, even for soluble nickel oxide particles such as the black nickel particles used here, the release of nickel from the macrophages to the culture medium was less than 10% of the amount of nickel which had been ingested by the macrophages as particles (Yamada et al., 1992). Therefore, we believe that the total uptake of nickel shown in Table 2 represents the approximate amount of nickel oxide taken up by the macrophages in 24 hours. There was no significant difference in the amount of nickel taken up by macrophages at 24 hours after incubation with the black and green nickel oxide particles. This indicates that the uptake of the nickel oxide particles by macrophages occurred at a similar rate for the black and green nickel particles, and that the difference in cytotoxicity between the black and green nickel particles was not attributable to the amount of nickel taken up by the macrophages.

The American Conference of Governmental Industrial Hygienists (ACGIH, 1986) has recommended different levels for the maximum permissible concentrations of nickel compounds in the air, depending on their chemical states. However, the present study shows that the cytotoxicity in rat alveolar macrophages was remarkably different between the types of nickel oxide particles. Although the cytotoxicity in alveolar macrophages does not directly indicate the actual in vivo toxicity in the airways, it is well known that a large fraction of inhaled particles are phagocytosed by alveolar macrophages, and that the adverse effects of toxic particles on alveolar macrophages induce chronic pulmonary disorders. Therefore, the present study may indicate that even though the two types of particles used here are in the same category of "nickel oxide", their toxic effect on the respiratory tract after inhalation may be significantly different.

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REFERENCES


