Mixtures of Nickel and Cobalt Chlorides Induce Synergistic Cytotoxic Effects: Implications for Inhalation Exposure Modeling

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INTRODUCTION

Many occupations involve exposure to mixtures of chemicals, yet most toxicology studies investigate adverse responses to individual chemicals, not mixtures of chemicals. The traditional assumption, in the absence of further information, has been that the chemical components of a mixture have mutually independent effects, and the toxic response to multiple chemicals is additive. The data presented here show that mixtures of NiCl₂ and CoCl₂ induce a synergistic (that is, greater than additive) toxic response in cell culture. Immortalized alveolar epithelial type II cells were incubated for 4 h with various concentrations of either NiCl₂, CoCl₂, or NiCl₂ and CoCl₂ together, and cell viability assessed 24 h later. The LD₅₀ for NiCl₂ was 5.7 mM, CoCl₂, with an LD₅₀ of 1.1 mM, was about five times more potent than NiCl₂. Mixtures of NiCl₂ and CoCl₂ decreased cell viability synergistically. For example, a mixture of 750 µM NiCl₂ and 750 µM CoCl₂ reduced cell viability by more than three times the value predicted by the additive approach. We used concentration-response data from these studies in a mathematical model; this model describes the equivalent inhalation exposure to an aerosol composed of a mixture of chemicals with different toxicities and also accounts for synergistic responses to these chemicals. Our results along with previous studies using an animal model suggest that these synergisms should be taken into account when conducting future exposure assessments. © 2001 British Occupational Hygiene Society. Published by Elsevier Science Ltd. All rights reserved

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cells are likely to be damaged when humans inhale mixtures of NiCl₂ and CoCl₂ was synergistic. We used concentration-response data from these studies to mixtures of nickel and cobalt chlorides affect certain aspects of pulmonary morphology in a synergistic manner. They conducted an inhalation study in which rabbits were exposed to either 0.5 mg/m³ CoCl₂ alone or to 0.5 mg/m³ CoCl₂ and 0.5 mg/m³ NiCl₂ together for 6 h per day, 5 days a week for 4 months. A previous inhalation study by the same authors investigated the toxic effects of 0.6 mg/m³ NiCl₂ alone under the same exposure regimen (Johansson et al., 1989). Data from these studies showed that mixtures of NiCl₂ and CoCl₂ induced a synergistic response in the respiratory tract, as indicated by type II cell aggregates and decreased cytochrome P450 levels (Bucher et al., 1999).

Although studies such as those mentioned above have investigated the toxicity of nickel and cobalt alone, little is known about the toxic effects of mixtures of nickel and cobalt on most tissues and organs. A study by Johansson et al. (1991) suggests that inhalation exposure to mixtures of nickel and cobalt chlorides affect certain aspects of pulmonary morphology in a synergistic manner. They conducted an inhalation study in which rabbits were exposed to either 0.5 mg/m³ CoCl₂ alone or to 0.5 mg/m³ CoCl₂ and 0.5 mg/m³ NiCl₂ together for 6 h per day, 5 days a week for 4 months. A previous inhalation study by the same authors investigated the toxic effects of 0.6 mg/m³ NiCl₂ alone under the same exposure regimen (Johansson et al., 1989). Data from these studies showed that mixtures of NiCl₂ and CoCl₂ induced a synergistic response in the respiratory tract, as indicated by type II cell aggregates and decreased cytochrome P450 levels (Bucher et al., 1999). Since the Johansson et al. (1991) study suggested that using the additive model may not always be valid, we hypothesized that concentration-response data describing the toxic responses induced by mixtures of compounds might be used to develop a mathematical model for estimating equivalent occupational exposures that accounts for synergistic responses to mixtures. We used a cell culture model with a binary-chemical exposure to generate the concentration-response data. We exposed immortalized alveolar epithelial type II cells to various concentrations of NiCl₂, CoCl₂, or mixtures of NiCl₂ and CoCl₂, and then measured cell viability. We chose an alveolar epithelial type II cell line because type II cells are likely to be damaged when humans inhale NiCl₂ and CoCl₂. The ratio of the LD₅₀ₐₜₖ (the concentrations at which there is a 50% decrease in cell viability) for NiCl₂ and CoCl₂ in this system was similar to the ratio of the TLVs for these metals. In this system, as in the rabbit model, the toxic response to mixtures of NiCl₂ and CoCl₂ was synergistic. We used concentration-response data from these studies in a mathematical model that describes the relationship between exposures to chemical mixtures and toxicity so that the model now accounts for synergistic responses to mixtures.

THEORETICAL FRAMEWORK

We modified a model developed by Ramachandran et al. (1996) which describes exposures to mixtures of chemicals with different toxicities, to incorporate the effects of synergistic interactions. A brief description of the model is given below.

If the mass of a single aerosol species i deposited in region j of the respiratory tract is Aᵢⱼ, then the internal exposure to this species, in relation to a specific health effect or toxic response, is a non-uniformly weighted sum of the masses deposited in the n different regions,

\[ E_h = \sum_{j=1}^{n} \alpha_{ij} A_{ij} \]  \hspace{1cm} (1)

where \( \alpha_{ij} \) represents weighting factors that account for differences in the degree to which a chemical species contributes to a biological response h; \( \alpha \) can range from 0 to 1. This model is based on the assumption that deposited particles, which are not removed by clearance mechanisms, contribute to the observed health effect; thus \( \alpha \) is a measure of biological uptake of the deposited particles. A later section describes how values for \( \alpha \) were obtained.

For a multi-species aerosol composed of m chemicals with differing toxicities that lead to the same toxic response, we need to consider the relative toxicities of the different species. The overall exposure \( E_h \) to the mixture is expressed as

\[ E_h = \sum_{i=1}^{m} \beta_i \left( \sum_{j=1}^{n} \alpha_{ij} A_{ij} \right) \]  \hspace{1cm} (2)

where \( \beta_i \) is the toxicity ratio of each chemical i in relation to a reference chemical.

A simplified description of the pulmonary system divides it into the extrathoracic (ET), tracheobronchial (TB), and alveolar (ALV) regions. Equation (2) can therefore be expressed by:

\[ E_h = \sum_{i=1}^{m} \beta_i \left( A_{ET,i} A_{ET,i} + \alpha_{TB} A_{TB,i} + \alpha_{ALV} A_{ALV,i} \right) \]  \hspace{1cm} (3)

where \( A_{ET,i} A_{TB,i} \) and \( A_{ALV,i} \) are the masses penetrating into the extrathoracic, tracheo-bronchial, and alveolar regions, respectively. The above equation is based on the assumption that the value for \( \beta \) is the same for all three pulmonary regions. Since \( \beta \) normalizes effects relative to a reference chemical, the


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Then Eq. (3) can be more simply written as

\[ E_h = \beta_{LD50,mixture} \sum_{i=1}^{m} \left[ \alpha_{ET,i}A_{ET,i} + \alpha_{TB,i}A_{TB,i} + \alpha_{ALV,i}A_{ALV,i} \right] \]  

(7)

Clearly, the value of \( \beta_{LD50,mixture} \) will depend on the relative proportion of the different chemicals in the mixture.

This more general expression encompasses the special case when the responses are purely additive when \( \beta_{LD50,mixture} \) is related to the \( \beta \) of the individual chemicals by the relation

\[ \beta_{LD50,mixture} = \frac{\sum_{i=1}^{m} \beta_i [\alpha_{ET,i}A_{ET,i} + \alpha_{TB,i}A_{TB,i} + \alpha_{ALV,i}A_{ALV,i}]}{\sum_{i=1}^{m} \alpha_{ET,i}A_{ET,i} + \alpha_{TB,i}A_{TB,i} + \alpha_{ALV,i}A_{ALV,i}} \]  

(8)

In other words, for the additive case \( \beta_{LD50,mixture} \) is a weighted average of the \( \beta \) of the individual chemical, with the weights being the amounts of each chemical being taken up biologically. If there are synergisms or antagonisms, then the value of \( \beta_{LD50,mixture} \) will be greater than or less than the right-hand side of Eq. (8), respectively.

METHODS

Cell culture

MP48 cells, originally isolated from a fetal rat lung (Mallampalli et al., 1992), were grown in a gassed (5% CO₂) humidified incubator at 37°C in Waymouth’s MB 752/1 medium (Life Technologies, Inc., Rockville, MD) containing 10% fetal bovine serum (FBS) (Life Technologies). We used cells between passages 40 and 80. Agents were added directly to the medium after cells had grown to confluency.

Cell viability measurements

Stock solutions (100 mM) of NiCl₂ (Sigma Chemical, St Louis, MO) and CoCl₂ (Spectrum Chemical Manufacturing Corp., Redondo Beach, CA) were prepared in serum-free medium and stored at 4°C. Cells were treated with 20 µM gramicidin (Sigma Chemical), NiCl₂, or CoCl₂ in the presence of 10% FBS. After the cells were incubated with the agents for 4 h, they were rinsed twice with phosphate-buffered saline (PBS). Fresh medium containing 10% FBS was added, and the cells were incubated at 37°C for an additional 24 h.

We used the CellTiter 96 Cell Proliferation Assay (Promega Corp., Madison, WI) to monitor cell viability. Briefly, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye was added to each well and incubated at 37°C for 1 h, 47 min
before adding the Stop Solution supplied with the kit. Absorbance was measured at 570 and 650 nm using a spectrophotometer. Observed cell viability (Obs %V) of cells exposed to a concentration of metal salt or a mixture of metal salts was determined by

$$\text{Obs } %V = \frac{(E_{570} - E_{650}) - (G_{570} - G_{650})}{(V_{570} - V_{650}) - (G_{570} - G_{650})} \times 100$$

where $A_{570}$ and $A_{650}$ are the absorbances at 570 and 650 nm, $E$ is the metal salt-exposed cells, $G$ is the positive-control cells treated with gramicidin, and $V$ is the vehicle control (treatment with serum-free media only). In live cells, MTT dye is converted to a formazan product which has a maximal absorbance at 570 nm ($A_{570}$). To reduce background from nonspecific absorbance, such as cell debris or fingerprints, the absorbance at a reference wavelength above 630 nm was measured and subtracted from the $A_{570}$ value.

Because the raw absorbance values may vary slightly between each experiment, negative and positive controls were performed with each trial. The negative control cells were untreated (exposed to the vehicle only, represented by $V$); the positive control cells were treated with a known cytotoxic agent, gramicidin, to ensure the death of all of the cells (these absorbance values are represented by $G$). Equation (9) normalizes the raw absorbance values of each treated sample such that they are represented as percentages of the untreated cell population. Cell viability equals 0% for the positive control and 100% for the negative control.

**Regression fits**

Cell viability data used in modeling was fit to the logistic equation

$$\% \text{ Cell viability} = \frac{1}{1 + e^{a + b \ln C_1}}$$

where

$$a = -1.658 \frac{\ln \text{[median]}}{\ln \sigma_2}$$

and

$$b = \frac{1.658}{\ln \sigma_2}$$

Agent concentration ($C$) on the abscissa was plotted against the percent cell viability function on the ordinate. The median of the curve represented the LD$_{50}$ concentration; $\sigma_2$ is a measure of the steepness of the concentration-response curve and is calculated by the ratio LD$_{84}$/LD$_{50}$.

**RESULTS AND DISCUSSION**

**Obtaining estimates of $\alpha$**

We used the modeling results of Hsieh et al. (1999) to derive a value for the biological uptake factor, $\alpha$. Their model includes an equation to describe changes in the lung mass burden over time due to soluble nickel (NiSO$_4$):

$$\frac{dM}{dr} = r - \lambda M$$

where $r$ is the rate of deposition, $M$ is the mass burden, and $\lambda$ is the overall clearance rate coefficient, defined as

$$\lambda = a \exp \left(-b \left(\frac{M}{M_{50}}\right)^c\right) \text{ (day}^{-1}\right)$$

In humans, the dimensionless clearance rate coefficients $a$, $b$, and $c$ for NiSO$_4$ are 10.285, 17.16, and 0.105, respectively (Hsieh et al., 1999). The term $m_S$ is equal to $M/S$ where $M$ is the mass burden of nickel in the lung and $S$ is the alveolar surface area (6.27x10$^5$ cm$^2$ for humans). For the application of Eqs (11) and (12) in our model, that is, Eq. (7), we assumed that the clearance kinetics of NiCl$_2$ and NiSO$_4$ were identical since both are soluble nickel compounds.

Andersen and Svenes (1989) autopsied employees who had worked in a nickel refinery and measured their lung nickel content. The mass burdens measured by them were the result of complex exposures to a number of nickel compounds of different particle sizes and solubilities. Recognizing these limitations in their data, while using their reported values to represent the mass burden $M$, we chose the lowest and highest values for our calculation of the nickel clearance rate. We used deposition rate calculations (0.143 and 0.297 $\mu$g min$^{-1}$ for ‘at rest’ and ‘at work’ breathing parameters, respectively) performed by Ramachandran et al. (1996) to calculate the degree of biological uptake, $\alpha$:

$$\alpha = \frac{\text{deposition rate} - \text{clearance rate}}{\text{deposition rate}}$$

Equation (13) assumes that any particles deposited in the lung that are not removed by clearance mechanisms are biologically absorbed by the organism and, therefore, contribute to the observed biological effect.

Using deposition rate values from Ramachandran et al. (1996) and nickel burden data from Andersen and Svenes (1989) and Eq. (13), we found that $\alpha = 1.00$ for every situation. The uniform $\alpha = 1.00$ values calculated for biological uptake mean that all of the deposited particles will be taken up by the organism almost immediately upon contact in the lung, regardless of the workers’ breathing parameters. This makes sense, since NiSO$_4$ and NiCl$_2$ are soluble compounds. We assume that $\alpha = 1.00$ for CoCl$_2$ as well, since it is also a soluble metal salt.
Obtaining estimates of $\beta$

We set out to determine the value of the toxicity parameter for our model, $\beta$, that we can use in Eqs (3) and (7). In the model we propose, we assume that the toxicity ratio, $\beta$, calculated from cellular assays can be extrapolated to human inhalation exposures.

As we have noted earlier, the parameter $\beta$ can be calculated in three different ways. We quantified the toxicity ratio, $\beta$, $\beta_{LD_{50}}$, in terms of NiCl$_2$/CoCl$_2$ mixtures in the following manner. After fitting logistic curves to the individual concentration-responses for NiCl$_2$ and CoCl$_2$ alone (Fig. 1), we found that the LD$_{50}$ for NiCl$_2$-induced decreased cell viability was 5.7 mM, whereas the LD$_{50}$ for CoCl$_2$-induced decreased cell viability was 1.1 mM. These values were substituted into Eq. (5) to calculate the toxicity ratio, $\beta$, of CoCl$_2$ relative to NiCl$_2$:

$$\beta_{LD_{50},CoCl_2} = \frac{LD_{50,NiCl_2}}{LD_{50,CoCl_2}} = \frac{5.7 \text{ mM}}{1.1 \text{ mM}} = 5.2$$

This value is very close to that calculated using Eq. (4):

$$\beta_{TLV,CoCl_2} = \frac{TLV_{NiCl_2}}{TLV_{CoCl_2}} = \frac{0.10 \text{ mg m}^{-3}}{0.02 \text{ mg m}^{-3}} = 5.0$$

This is consistent with an analysis performed by Suda et al. (1999) who found good correlation between TLV and LD$_{50}$ values derived from animal models. In their investigation, LD$_{50}$ and median lethal air concentration (LC$_{50}$) values as listed in the Registry of Toxic Effects of Chemical Substances (RTECS) were plotted against the ACGIH’s current TLVs (ACGIH, 1999). Although regression analyses revealed better correlations for LC$_{50}$ versus TLV (adjusted $R = 0.859-0.912$) compared to LD$_{50}$ versus TLV (adjusted $R = 0.616-0.717$), we feel that the favorable correlations justify our substitution.

However, the use of these values of $\beta$ calculated in Eqs (4) and (5) are limited to additive models. We therefore used the more general expression given in Eq. (6). For the special cases of pure NiCl$_2$ and CoCl$_2$ exposures, this general expression should yield the same result as Eq. (5), illustrated by the following calculation. Using LD$_{50,mixture}$ values stated in Table 1, and LD$_{50,NiCl_2} = 5.7$ mM, when [NiCl$_2$] = 0,

$$\beta = \frac{LD_{50,NiCl_2}}{LD_{50,mixture}} = \frac{5.7}{5.2} = 1$$

Similarly, when [CoCl$_2$] = 0, we obtain

$$\beta = \frac{LD_{50,NiCl_2}}{LD_{50,mixture}} = \frac{5.7}{5.7} = 1$$

We then investigated the effects of mixtures of NiCl$_2$ and CoCl$_2$ on cell viability. CoCl$_2$ was held constant at six different concentrations (0, 100, 300, 500, 600, and 750 mM) while the concentration of NiCl$_2$ varied (Fig. 2). The results of holding [NiCl$_2$] constant at 0, 100, 300, 500, 600, and 750 mM while varying [CoCl$_2$] are shown in Fig. 3. Figures 2 and 3 show only the logistic regression fits to the concentration-response data.

We chose to represent mixture composition as the fraction of one metal salt normalized to the total agent concentration. Although the fraction of either metal salt could have been chosen as data for testing the model, we chose to use the CoCl$_2$ fraction ($F_{Co}$). $F_{Co}$ was calculated by the following:

$$F_{Co} = \frac{[CoCl_2]}{[CoCl_2] + [NiCl_2]}$$

Table 1 lists the mixtures shown in Figs. 2 and 3 that induced 50% decreases in cell viability, along with the corresponding $F_{Co}$ values.

As stated earlier, exposure to nickel is linked with lung cancer in humans (Costa, 1991; Kaldor et al., 1986), and cobalt induces alveolar and bronchiolar tumor formation in animal models (Bucher et al., 1999). Consequently, Eq. (7) can be simplified to describe only the effects of NiCl$_2$ and CoCl$_2$ in the tracheobronchial and alveolar portions of the lung, so that $a_{ET} = 0$ and $a_{TB} = a_{ALV} = 1$:

$$E_h = \beta_{LD_{50,mixture}} \left( A_{TB} + A_{ALV} [NiCl_2] \right) \left( A_{TB} + A_{ALV} [CoCl_2] \right)$$

Fig. 1. Percentage viability of alveolar cells exposed to various concentrations of pure CoCl$_2$ (solid line) and NiCl$_2$ (dashed line). The solid and open circles are the actual experimental data±standard error (SE) and the curves are logistic regression fits to the data.
Table 1. Combinations of [NiCl$_2$] and [CoCl$_2$] from concentration-response curves in Figs. 3 and 4 that result in 50% cell viability. For each combination, the fractional cobalt concentration is calculated as $F_{Co} = [CoCl_2]/([CoCl_2] + [NiCl_2])$.

<table>
<thead>
<tr>
<th>CoCl$_2$ (mM)</th>
<th>LD$_{50}$, mixture</th>
<th>$F_{Co}$</th>
<th>$\beta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.709</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>0.1</td>
<td>3.243</td>
<td>0.030</td>
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<tr>
<td>0.3</td>
<td>1.100</td>
<td>0.214</td>
<td>5.19</td>
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<tr>
<td>0.5</td>
<td>0.774</td>
<td>0.393</td>
<td>7.38</td>
</tr>
<tr>
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<td>0.689</td>
<td>0.465</td>
<td>8.28</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CoCl$_2$ (mM)</th>
<th>NiCl$_2$ (mM)</th>
<th>$F_{Co}$</th>
<th>$\beta$</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
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<td>0.667</td>
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<tr>
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<td>0.516</td>
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<tr>
<td>0.556</td>
<td>0.75</td>
<td>0.426</td>
<td>10.26</td>
</tr>
</tbody>
</table>

*Calculated at the LD$_{50}$.

![Fig. 2. Percentage viability of alveolar cells exposed to mixtures of NiCl$_2$ and CoCl$_2$ with CoCl$_2$ held constant at the concentrations shown in the inset. Curves are logistic fits to data points (not shown). The arrow illustrates how an LD$_{50}$ value for a particular mixture (LD$_{50}$,mixture) was obtained. In this example, the LD$_{50}$ of NiCl$_2$ alone (no CoCl$_2$) was determined to be 5.7 mM.](image1)

![Fig. 3. Percentage viability of alveolar cells exposed to mixtures of NiCl$_2$ and CoCl$_2$ with NiCl$_2$ held constant at the concentrations shown in the inset. Curves are logistic fits to data points (not shown). The arrow illustrates how an LD$_{50}$ value was obtained. In this example, the LD$_{50}$ of CoCl$_2$ alone (no NiCl$_2$) was determined to be 1.1 mM.](image2)

The $\alpha$ terms have been removed from the above expression since they are all equal to one. To quantify the synergistic effects of NiCl$_2$/CoCl$_2$ mixtures, we first determined the cell viability values expected for an additive interaction. For the additive case, $\beta_{LD_{50},mixture}$ is a weighted average of the $\beta$ values of NiCl$_2$ and CoCl$_2$, with the weights being $(A_{TB} + A_{ALV})_{NiCl_2}$ and $(A_{TB} + A_{ALV})_{CoCl_2}$, respectively. In a plot of $\beta_{LD_{50},mixture}$ versus $F_{Co}$, using the $\beta$ values for pure NiCl$_2$ and pure CoCl$_2$ (obtained earlier) as the endpoints, a straight line was drawn to represent the additive interaction (dashed line, Fig. 4). The equation describing this line is

$$B_{LD_{50},mixture} = 4.2 \left[ \frac{[Co]}{[Co] + [Ni]} \right] + 1 \quad (16)$$

However, the actual measurements of $\beta_{LD_{50},mixture}$ at various values of $F_{Co}$ did not fall on this straight line.
and therefore did not match the proposed additive model (Fig. 4). The values of $\beta_{\text{LD50,mixture}}$ lay above the linear additive model, suggesting a synergistic response to the mixture. A second-order polynomial equation was found to fit the data well.

$$\beta = -22.778 (\frac{[\text{Co}]}{[\text{Co}]+[\text{Ni}]})^2 + 26.796 (\frac{[\text{Co}]}{[\text{Co}]+[\text{Ni}]}) + 0.9808$$ \hspace{1cm} (17)

While this equation is merely a regression fit and no physical significance should be attached to it, it can be used to quantitatively describe the synergistic cytotoxic effects of NiCl$_2$/CoCl$_2$ mixtures on MP48 cells. For a particular NiCl$_2$/CoCl$_2$ mixture composition, the equivalent NiCl$_2$ exposure is obtained by substituting Eq. (17) into Eq. (15).

**Incorporating synergism between chemicals while estimating equivalent exposures**

We use Eq. (17) to extrapolate to human inhalation exposures by using the amounts of airborne nickel and cobalt mass deposited in various parts of the respiratory tract (instead of concentrations of these metal salts in solution used in our cell culture experiments). Ramachandran et al. (1996) used personal cascade impactor data obtained at the copper electro-winning process of a nickel refinery to obtain particle mass size distributions of nickel and cobalt aerosols. These were used with results of particle deposition efficiency curves developed by Heyder et al. (1986) from experimental studies of inhalation with human volunteers. Table 2 identifies the values for the thoracic and alveolar mass concentrations of nickel and cobalt used to calculate the health-related nickel-equivalent exposure, $E_h$ (see Ramachandran et al. (1996) for details). We used these data to calculate equivalent exposures first assuming additivity, and then incorporating synergistic effects. For these two cases, we examined conditions that can be related to ‘at rest’ and ‘at work’, that is, 7.5 breaths per min with 1 l. per breath (or 7.5 l. min$^{-1}$), and 15 breaths per min with 1.5 l. per breath (or 22.5 l. min$^{-1}$), respectively. Table 3 lists the equivalent exposures and the dose rates for all these cases. As an example, substituting the mass concentrations for ‘at work’ deposition conditions into the additive model (Eq. (16)), we obtain: $E_h = 13.7$ µg m$^{-3}$. If we use the same mass concentration values to calculate $\beta$ for the synergistic model (Eq. (17)), we obtain: $\beta_{\text{LD50,mixture}} = 6.1$, leading to the nickel-equivalent exposure of $E_h = 41.5$ µg/m$^3$ which is about three times greater than the additive interaction.

The mass deposition rate, or dose, that an individual receives is more relevant to health. The exposure values calculated above, along with the corresponding breathing rates for ‘at work’ conditions, lead to a nickel equivalent dose according to the additive model of 0.308 µg min$^{-1}$, but the corresponding nickel equivalent dose ‘at work’ according to the synergistic model is 0.934 µg min$^{-1}$.

NiCl$_2$- and CoCl$_2$-induced cytotoxicity

Our observation that mixtures of NiCl$_2$ and CoCl$_2$ induce a synergistic toxic response in cell culture is remarkably consistent with Johansson et al.’s finding that mixtures of NiCl$_2$ and CoCl$_2$ induce a synergistic response *in vivo* (Johansson et al., 1989, 1991). Johansson et al.’s subchronic inhalation studies showed that mixtures of NiCl$_2$ and CoCl$_2$ induced a synergistic response in the respiratory tract of rabbits, as indicated by changes in the concentrations of phosphatidylcholine and phospholipid levels in the wet lung and formation of type II cell aggregates (Johansson et al., 1989, 1991). For example, exposure of rabbits for 6 h per day, 5 days a week for 4 months to aerosols of either NiCl$_2$ (0.5 mg m$^{-3}$) or CoCl$_2$ alone (0.5 mg m$^{-3}$) increased phosphatidylcholine levels in the lung by 20% and 30%, respectively, relative to the unexposed controls. Exposure of rabbits to a mixture of NiCl$_2$ and CoCl$_2$
(0.5 mg m⁻³ of each salt) increased the phospholipid levels by 130% — again, more than a three-fold increase over what would be expected from an additive response. In addition, Johansson et al. reported that CoCl₂ alone, but not NiCl₂ alone, induced the formation of aggregates of type II cells. The formation of these aggregates was more pronounced after exposure to CoCl₂ and NiCl₂ together than to CoCl₂ alone. This suggests that NiCl₂ potentiates the effects of CoCl₂ in the formation of these aggregates when these metal salts are presented together.

The mechanisms by which mixtures of NiCl₂ and CoCl₂ interact to induce a synergistic toxic response are not yet clear. One possibility is that each metal potentiates the cellular uptake of the other metal. Costa demonstrated that the carcinogenic potency of a nickel species is positively correlated with the degree to which it is phagocytosed (Costa and Mollenhauer, 1980). Insoluble nickel, which is carcinogenic, is phagocytosed to a greater extent than soluble nickel salts, such as NiCl₂, which are not carcinogenic. Further work is needed to determine if CoCl₂ increases the rate of cellular uptake of NiCl₂ through either phagocytosis or another transport mechanism. Little is known about cellular transport of cobalt or alternate transport mechanisms for NiCl₂. There is a bacterial transport system, called CorA, which can transport Mg²⁺, Co²⁺ and Ni²⁺ (Bijvelds et al., 1998). A similar transport system may also exist in mammalian cells. Interestingly, Mg²⁺ uptake is inhibited by Co²⁺ and Ni²⁺ in rat jejunal brush border membrane vesicles (Bijvelds et al., 1998). This suggests that Co²⁺ and Ni²⁺ may compete with magnesium for a common transport system in mammalian cells.

Once in the cell, NiCl₂ and CoCl₂ may cause toxicity by damaging DNA or modulating critical enzymatic reactions. For example, some investigators suggest that NiCl₂ causes single-strand breaks in DNA by generating hydroxyl radicals (OH) (Costa, 1991; Stohs and Bagchi, 1995). NiCl₂ also induces the formation DNA–protein adducts linking preferentially to the amino acids histidine, cysteine, and tyrosine (Costa et al., 1994). NiS₂ and NiSO₄ also induce DNA–protein adducts (Chakrabarti et al., 1999). Cobalt can also damage DNA; CoSO₄ exposure causes a G to T nucleic acid transversion (Bucher et al., 1999). A cell containing DNA damage may induce its own death, for example through apoptosis. In addition, cobalt can substitute for other divalent cations such as Mg²⁺ and Ca²⁺ in certain biological reactions (Jennette, 1981), which may also affect cell fate and function. The similarities between the synergistic response of our cell culture model and Johansson et al.’s in vivo model to mixtures of NiCl₂ and CoCl₂ suggest that our model system may be useful for investigating the cellular and molecular mechanisms underlying the synergistic toxic responses to mixtures of NiCl₂ and CoCl₂.

**CONCLUSIONS**

Our results suggest that the traditional assumption of an additive response to exposure to a mixture of chemicals is not always appropriate, and neglecting synergistic interactions may cause an underestimation of the equivalent exposure to mixtures. Experiments in a cell culture system showed that mixtures of NiCl₂ and CoCl₂ induced a synergistic response. These results were incorporated into a human inhalation model for calculating equivalent exposures to mixtures of different aerosol species. Using particle size-selective exposure data from an occupational environment, we calculated the equivalent exposure to a mixture of nickel and cobalt aerosols. Our calculations...
showed that not including synergisms between the different species in a mixture may underestimate the equivalent exposure to mixtures by a factor of three or more.

Although our studies used relatively high concentrations of NiCl$_2$ and CoCl$_2$, two lines of evidence suggest that our results may also apply to estimating human exposures to relatively low airborne concentrations. First, results from our studies regarding the synergistic response to mixtures of NiCl$_2$ and CoCl$_2$ are remarkably consistent with the results of Johansson et al.’s in vivo studies, in which rabbits were exposed to much lower concentrations of NiCl$_2$ and CoCl$_2$ (Johansson et al., 1989, 1991). These striking similarities suggest that the results from our cell culture system may indeed reflect the synergistic actions of NiCl$_2$ and CoCl$_2$ in vivo at lower concentrations. Second, our model uses the parameter $\beta$, which is the ratio of the toxicity of a chemical relative to a reference chemical. For the additive case, the value of $\beta_{1,\text{LD}_{50};\text{CoCl}_2} = (\text{LD}_{50;\text{NiCl}_2})/(\text{LD}_{50;\text{CoCl}_2})$ obtained from in vitro experiments done at high solution concentrations is 5.2. This is very close to the value of $\beta_{1;\text{TLV;CoCl}_2} = (\text{TLV}_{\text{CoCl}_2})/(\text{TLV}_{\text{NiCl}_2}) = 5.0$, obtained from relatively low TLVs that are derived from a synthesis of both epidemiology and toxicology studies. Recognizing that a toxicity ratio derived from in vitro experiments cannot be extrapolated to estimate human cancer risks, further research is required to confirm that the results from our cell culture studies can be applied directly to human inhalation exposures. In any case, our results, together with the results from Johansson et al.'s in vivo studies, underscore the importance of considering the potential synergistic actions of mixtures when conducting exposure assessment.

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