



In vitro effects of lithium and nickel at different levels on Neuro-2a mouse Neuroblastoma cells

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Abstract

Lithium and nickel present low toxicity, but are able to cause alterations in different tissues. The toxic effects of lithium and nickel at different cellular levels were assessed using two inorganic chemical species: lithium chloride and nickel(II) chloride. Mouse neuroblastoma cell cultures (Neuro-2a) were exposed to both compounds for 24 h. The cytotoxic effects evaluated were cell proliferation by quantification of total protein content, cytoplasmic membrane integrity to cytosolic lactate dehydrogenase leakage, and lysosomal hexosaminidase release. Metabolic markers were lactate dehydrogenase activity and mitochondrial succinate dehydrogenase activity. Lysosomal markers were relative neutral red uptake by lysosomes, and lysosomal hexosaminidase sphingolipid degradation activity. Acetylcholinesterase activity on intact cells was also quantified. Nickel was found to be 36 times more toxic than lithium to neuroblastoma cell proliferation ($EC_{50} = 0.29$ and 10.5 mM, respectively), but the relative extent of other alterations differed. Lithium stimulated nearly all the indicators studied, particularly lactate dehydrogenase, mitochondrial succinate dehydrogenase and acetylcholinesterase activities, as well as hexosaminidase release. In contrast, nickel mainly stimulated hexosaminidase release and inhibited lactate dehydrogenase activity. The stabilization of the cytoplasmic membrane to lactate dehydrogenase leakage simultaneously with the secretion of lysosomal hexosaminidase for both compounds also shows that functional metabolic alterations produced by lithium and nickel are more important than cytoplasmic damage. © 2001 Published by Elsevier Science Ltd.

Keywords: Lithium; Nickel; Toxicity; Cytotoxicity; In vitro; Neuroblastoma

1. Introduction

Lithium and nickel present essential properties and low toxicity, but are able to cause alterations in different tissues (Nielsen, 1988). Although chronic lithium may cause toxic effects in a variety of organs, acute toxic effects are manifested mainly in the central nervous system. Nickel exposure was linked with genotoxic, immunotoxic, reproductive toxic, neurotoxic and carcinogenic effects (Wataha et al., 1999).

Lithium is widely used in the treatment of manic-depressive psychosis (Chan et al., 1981). Apart from organ and tissue occupational exposure in the manufacture of steel, other steel alloys, nickel/cadmium batteries and in the process of electroplating, the release of nickel ions from alloys commonly used in medical and

dental applications has been documented (Messer and Lucas, 1999).

There is increasing interest in the development of new methods for assessing chemical toxicity, and in the use of the knowledge of the mechanisms of toxic action to improve this assessment (Walum et al., 1993). The aim of this study was to compare the effects at different cellular levels of two inorganic chemicals, lithium chloride and nickel(II) chloride, on Neuro-2a mouse neuroblastoma cells, which were exposed for 24 h in vitro to different concentrations of each compound. Cytotoxic effects evaluated in the in vitro test system were cell proliferation by quantification of total protein content of the cultures, cytoplasmic membrane integrity to cytosolic lactate dehydrogenase leakage and lysosomal hexosaminidase release. Metabolic markers were lactate dehydrogenase activity and mitochondrial succinate dehydrogenase activity. Lysosomal markers were lysosomal function evaluated by the relative uptake of neutral red (RNRU) (Repetto and Sanz, 1993) and lysosomal hexosaminidase sphingolipid degradation activity. Neuronal acetylcholinesterase activity on intact cells was also quantified.

Abbreviations: AChE, acetylcholinesterase; DTNB, 5,5'-dithio-bis (nitrobenzoic acid); EC_{50} , mean effective concentration; HEX, hexosaminidase; LDH, lactate dehydrogenase; PBS, phosphate buffered saline; SDH, succinate dehydrogenase.

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2. Materials and methods

2.1. Toxicants and reagents

Test chemicals and reagents were of the highest purity available, and were purchased from Sigma (St Louis, MO, USA), and Merck (Darmstadt, Germany). Concentrated solutions of the test compounds (Merck) were prepared daily in deionized water and sterilized through 0.22 μm filters (Millipore Corp., Bedford, MA, USA). Exposure solutions were prepared before use in culture media by dilution of the concentrated solutions.

2.2. Cell culture

C1300 cell line, Neuro-2a clone was obtained from Flow (Irvine, UK) and maintained as continuous monolayer cultures in tissue culture flasks (Costar, Cambridge, MA, USA) at 37°C in a humidified atmosphere of 5% CO₂:95% air. Cells were grown in modified Eagle's medium, supplemented with 10% fetal calf serum, 100 U penicillin G/ml and 100 μg streptomycin/ml (Flow Laboratories, Irvine, UK).

2.3. Toxicant exposure

Cells were plated at a density of 10,000 cells/well in 96-well tissue culture plates (Costar). After 24 h, the culture medium was replaced with 0.2 ml medium containing the test chemical in solution at different concentrations and then incubated for another 24 h.

2.4. Cytotoxicity assessment

As previously stated, all the determinations were carried out in the same 96-well tissue culture plates where exposure originally took place (Repetto et al., 1994). Plates were assigned randomly to the different biomarkers, and the supernatant culture medium for LDH and HEX release was transferred from each well to the corresponding well of a second microtitre plate. All the culture plates were gently washed twice with phosphate buffered saline (PBS), and biochemical reagents for each colorimetric determination were added to the corresponding plates. The possibility of chemical interference on the spectrophotometric determination was assessed in parallel. Absorbency was read on a Multiskan RC plate reader (Labsystems, Finland).

Cell proliferation according to total cellular protein was quantified in situ using Coomassie Brilliant Blue G-250 by a modification of the Bradford method (1976) to assure good linearity, as previously stated (Repetto and Sanz, 1993). LDH (EC 1.1.1.27) activity in cells and in culture medium was determined according to Duffy and Flint (1987) by following the production of NADH during the conversion of lactate to pyruvate. HEX (*N*-

acetyl- β -D-hexosaminidase, EC 3.2.1.30) activity in cells and in culture medium was quantified by the Duffy and Flint method (1987), which measures the *p*-nitrophenol produced by hydrolysis of *p*-nitrophenol-*N*-acetyl- β -D-glucosaminide. Lysosomal function was evaluated by the relative uptake of neutral red (Borenfreund et al., 1988; Repetto and Sanz, 1993). This assay is based on the uptake of the supravital dye and its accumulation in the lysosomes expressing the results in relative form to cell culture protein content. The determination of SDH (EC 1.3.99.1) activity of intact cells, based on the reduction of MTT, was performed using the Mosmann method (1983) as modified by Repetto et al. (1994), in which 1 mM KCN and 2.5 μM antimycin A are added to the tetrazolium salt incubation media in order to inhibit other dehydrogenases. AChE (acetylcholine acetylhydrolase, EC 3.1.1.7) activity on intact cells was measured by adapting the method of Ellman et al. (1961), which follows the formation of the yellow 5-thio-2-nitrobenzoate anion produced by combination of dithiodinitrobenzoic acid (DTNB) with the thiocholine produced in the hydrolysis of acetylthiocholine iodide. The plates were washed twice with PBS and 200 μl of freshly prepared reaction mixture (160 μl 0.075 M acetylthiocholine iodide, 800 μl 0.01 M DTNB in 24 ml 0.1 M (pH 8.0) buffer phosphate at 25°C and 100 μl 2.5 mM tetraisopropylpyrophosphoramidate as butyrylcholinesterase inhibitor) were added to each well, after which the plate was agitated briefly. Absorbency was read at a wavelength of 414 nm at the initial time and after incubation in the dark at 25°C for 30 min. The increase in absorbency of the blanks without cells, due to non-enzymic hydrolysis was subtracted. AChE activity was consequently expressed as μmol of acetylthiocholine hydrolyzed/min/mg of total cellular protein (Repetto et al., 1994).

2.5. Calculations and statistical analysis

Values for enzyme activities and relative neutral red uptake were corrected for cell culture total protein content in order to avoid misinterpretation due to the influence of the chemicals tested on cell proliferation and cell detachment. In order to avoid interference of the chemicals in enzyme activities, the percentage of culture enzyme release (LDH and HEX) was defined as extracellular activity $\times 100$ divided by total culture (extracellular + intracellular), activity. The EC₅₀ values, defined as the concentration of test chemical that modified each biomarker by 50% in comparison with the appropriate untreated control cultures, were calculated from the dose-response curves. All experiments were performed at least three times, using six wells per concentration of test agent and biomarker studied. The results thus obtained are expressed in the figures as percentage change compared with unexposed cells. Statistical analysis was

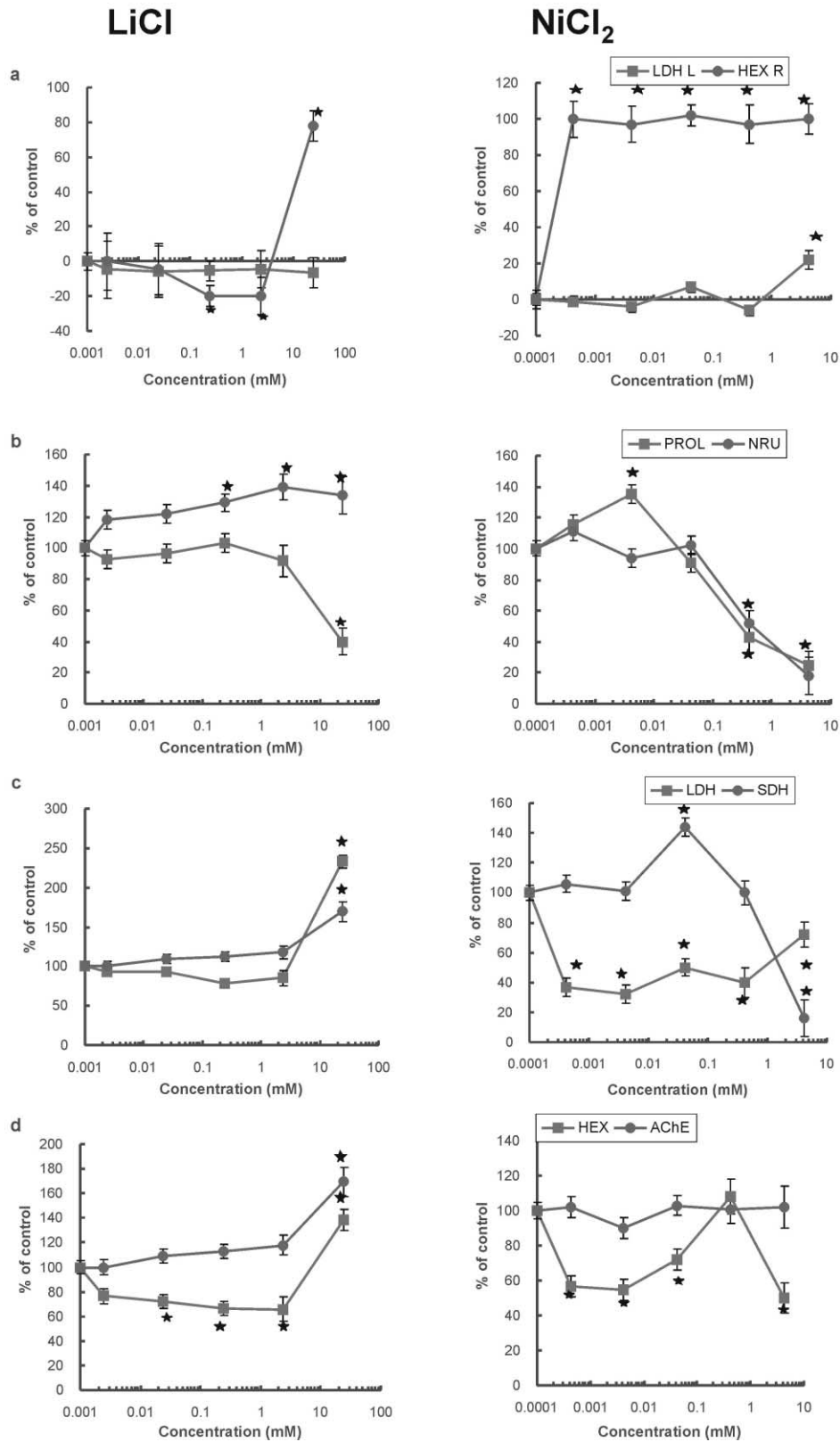


Fig. 1. In vitro effects of exposure for 24 h to different concentrations of lithium chloride or nickel (II) chloride on Neuro-2a cells (a) lactate dehydrogenase leakage (■) and hexosaminidase release (●), (b) cell proliferation (■) and relative neutral red uptake (●), (c) lactate dehydrogenase activity (■) and succinate dehydrogenase activity, and (d) hexosaminidase activity (■) and acetylcholinesterase activity (●). Data expressed relative to mean value in respective unexposed controls. In order to avoid interference of chemicals in enzyme activities, the percentage of enzyme release of a culture was calculated as extracellular activity×100 divided by total activity (extracellular+intracellular activity) of the culture. * Indicates significant difference from control value ($P < 0.01$).

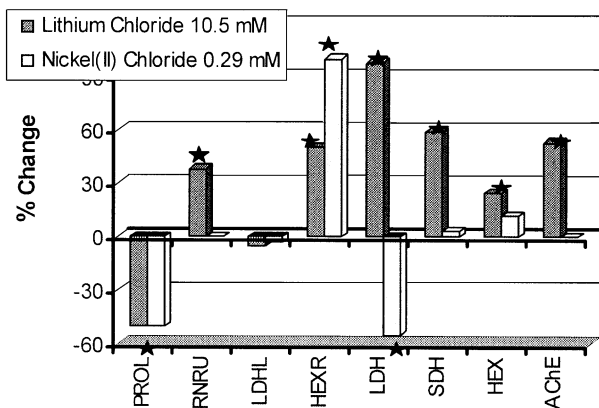


Fig. 2. Comparative cytotoxic effects on the different biomarkers produced by 24 h exposure of Neuro-2a cell cultures to (a) 10.5 mM lithium chloride or (b) 0.29 mM nickel (II) chloride. Toxicity indicators assessed in the in vitro test system were: cell proliferation (PROL), lysosomal function as relative neutral red uptake (RNRU), cytoplasmic membrane integrity to cytosolic lactate dehydrogenase leakage (LDHL), lysosomal hexosaminidase release (HEXR), lactate dehydrogenase (LDH) intracellular activity, mitochondrial succinate dehydrogenase (SDH) activity; lysosomal hexosaminidase (HEX) intracellular activity, and neuronal acetylcholinesterase (AChE) activity. Data expressed relative to mean value in respective unexposed controls. *Indicates significant difference from control value ($P < 0.01$).

carried out using analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. EC_{50} values were determined by linear interpolation.

3. Results and discussion

The exposure of actively dividing Neuro-2a mouse neuroblastoma cells to lithium for 24 h did not produce cell death, since cytoplasmic membrane permeability to LDH was not increased at the concentrations assayed (Fig. 1a). In the case of nickel, LDH leakage through the cytoplasmic membrane arose only at very high concentrations.

The release of the enzyme HEX differed for the two chemicals. Nickel produced total secretion of HEX at all concentrations assayed ($EC_{50} = 0.0002$ mM). This effect is produced by lysosomotropic agents that increase intralysosomal pH, which, in turn, causes oversecretion of newly-synthesized lysosomal enzymes (Lei et al., 1985). However, low concentrations of lithium significantly reduced the release of HEX, with enhanced release being observed only at high concentrations ($EC_{50} = 10.1$ mM).

Nickel was found to be 36 times more toxic than lithium to neuroblastoma cell proliferation ($EC_{50} = 0.29$ and 10.5 mM, respectively) but the relative extent of other alterations differed (Fig 1b). (Hasgekar et al., 1996) have reported that flow cytometric analysis revealed that neural precursor cells treated with lithium

were blocked in S phase. Nickel showed a biphasic pattern on Neuro-2a, with a stimulatory effect at the lowest concentrations tested (0.0005–0.01 mM) and an inhibitory effect on cell proliferation at higher concentrations ($EC_{50} = 0.29$ mM), as described by (Borella et al., 1990). According to Costa et al. (1982), cell growth is also selectively blocked by nickel in S phase.

Relative neutral red uptake was determined in order to evaluate lysosomal function (Repetto and Sanz, 1993). In this modification of the standard neutral red cytotoxicity assay (Borenfreund et al., 1988), the results of cellular lysosomal uptake of supravital dye are expressed relative to the cell culture protein content to avoid misinterpretation due to the influence of the chemical tested on cell proliferation and cell detachment. This useful marker of lysosomal function was stimulated by lithium and inhibited in a dose-dependent manner by nickel ($EC_{50} = 0.41$ mM). The stimulation of neutral red uptake within the range of concentrations assayed was previously detected for chromium (II) chloride, and is probably due to cytoplasmic membrane stabilization (Repetto and Sanz, 1993).

Metabolic function was also altered, with stimulation of LDH activity by lithium up to 125% at 24 mM ($EC_{50} = 7$ mM) (Fig. 1c), and up to 60% for SDH activity ($EC_{50} = 9.6$ mM). This agrees with previous in vivo studies showing that glucose utilization is inhibited by high lithium concentrations (197–295 mM) in the cerebrum and cerebellum of mice (Dixit and Smithberg, 1988).

LDH activity was inhibited very specifically by nickel, being the second most sensitive toxicity indicator used ($EC_{50} = 0.00032$ mM). However, SDH activity was slightly stimulated at 0.05 mM and inhibited at higher concentrations ($EC_{50} = 1.9$ mM). Monsees et al. (1998) have shown that non-cytotoxic concentrations (0.005–0.1 mM) of nickel significantly increased lactate production in cultivated Sertoli cells incubated for 24 h. Monnet-Tschudi et al. (1993) found that nickel increased the levels of all biochemical parameters (ChAT, GAD, glutamine synthetase) studied in aggregating cell cultures of foetal rat telencephalon. Whether the metabolic changes observed reflect the progress of poisoning and are due to enzyme induction or are related to non-specific ionic effects leading to changes in enzyme dissociation constants is under current investigation. Whatever the mechanisms involved, when the glycogen content is exhausted, the energy supply will be diminished. For organs highly dependent on carbohydrates, such as nervous tissue, the depletion or the resulting shortage of energy, respectively, might be deleterious to organ function.

HEX, an enzyme involved in sphingolipid degradation, was found to be a sensitive marker of toxicity for both compounds. HEX activity was inhibited at low concentrations of lithium and increased at 240 mM (up

to 40%). The activity of HEX decreased about 40% at all the doses of nickel, but was stimulated at 500 μM (Fig. 1d). The surprising increase in activity at high concentrations is difficult to understand; it might, however, be attributable to lysosomal redistribution of the enzyme (Lei et al., 1985). The metals are promoters of peroxidation of polyunsaturated fatty acids (Knight and Voorhees, 1990), and the altered molecules produced may be destroyed by enzymes such as HEX. Serum was used in the culture medium because nickel compounds form complexes with serum proteins. These complexes adsorb to the surface of the cell and enter by endocytosis. Within the cell, lysosomal proteinases hydrolyze carrier protein to release electrophilic metal ions. Nickel ions then bind with nucleic acids and cellular constituents (Venugopal and Luckey, 1978). The sensibility of lysosomal markers may be due to the interaction of the metal with the organelle.

Neural AChE activity was particularly stimulated at the highest concentration of lithium (up to 70%) ($\text{EC}_{50} = 10.2 \text{ mM}$), but was not modified by nickel.

To compare the extent of the most marked cytotoxic effects induced by the exposure of Neuro-2a cells for 24 h, the modification of the different bioindicators at the concentration that reduced cell proliferation by 50% was compared (Fig. 2), that is, 10.5 mM lithium chloride or 0.29 mM nickel (II) chloride. Lithium produced a pattern of stimulation, with important increases for LDH, SDH and AChE activities, non-significant HEX release, relative neutral red uptake, and a small stimulation of HEX activity. In contrast, nickel stimulated HEX release, reduced LDH activity, and produced non-statistically significant changes for the remaining indicators studied. The stabilization of the cytoplasmic membrane to LDH leakage simultaneously with the secretion of lysosomal HEX for both compounds also shows that functional metabolic alterations produced by lithium and nickel are more important than cytoplasmic damage.

In order to detect more subtle effects produced at low concentrations and to compare in vivo and in vitro data, the lowest statistically significant critical cellular toxic concentration was estimated (Walum et al., 1993). The critical cellular toxic concentration for lithium chloride found on neuroblastoma cells was 0.024 mM for HEX inhibition, followed at 0.24 mM by neutral red stimulation and hexosaminidase release inhibition. Marked simultaneous changes in other indicators occurred only at higher concentrations (24 mM). It has been reported that lithium is toxic to humans at 1.5 mM in serum, and becomes lethal from 2.5 mM in whole blood (Repetto and Repetto, 1999). In the case of nickel (II) chloride, the lowest statistically significant critical cellular toxic concentration found was 0.0004 mM for increasing HEX release, and for HEX and LDH activity inhibition. It has been reported that nickel (II) chloride

can produce toxicity from 0.00004 mM and become lethal to humans at 0.06 mM in whole blood (Repetto and Repetto, 1999). From the indicators considered, lysosomal functions were particularly sensitive to the opposite action of lithium and nickel. Although toxicokinetic factors and more specific endpoints may be required for a more complete interpretation of in vivo effects, the experimental model system seems to be useful for the investigation of cytotoxic effects on neuroblastoma cells.

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