Université de Montréal

Évaluation des risques cytotoxiques et génotoxiques de certains dérivés de nickel suite à l'exposition des lymphocytes humains *in vitro*

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Thèse présentée à la Faculté des études supérieures
en vue de l'obtention du grade de Philosophiae Doctor (Ph D)
en Santé publique
option Toxicologie de l'environnement

Septembre, 2004

Université de Montréal

Faculté des études supérieures

Cette thèse intitulée:

Évaluation des risques cytotoxiques et génotoxiques de certains dérivés de nickel suite à l'exposition des lymphocytes humains *in vitro*

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Thèse acceptée le: 1 décembre 2004

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Les objectifs de ce travail sont (1) d'évaluer les risques cytotoxiques et génotoxiques du sulfate ($NiSO_4$), du sous-sulfure (Ni_3S_2), de l'oxyde (NiO) et de l'hydroxyde carbonate (NiCH) de nickel chez l'humain suite à l'exposition *in vitro* des lymphocytes du sang périphérique en suspension et en culture, (2) d'examiner le rôle du stress oxydatif, des mitochondries et du calcium intracellulaire dans l'induction des dommages génotoxiques et de la mortalité des lymphocytes (cytotoxicité).

Dans la première partie de notre étude, les effets dépendant de la concentration (0-4 mM) et du temps (0-4 h), des différents composés de nickel sur la mortalité des lymphocytes en suspension ont été étudiés. Nos résultats montrent que la mortalité des lymphocytes diminue selon l'ordre des composés de nickel suivants: le NiCH > le Ni_3S_2 > le $NiSO_4$. Ces composés ont induit une surproduction de peroxyde d'hydrogène (H_2O_2) et ont causé la déplétion de la réserve cellulaire en groupements sulfhydryles liés (SH-P) et non liés (SH-NP) aux protéines, ainsi que la peroxydation des lipides (POL). Par contre, une surproduction de l'anion superoxyde (O_2^-) n'a été observée qu'avec le NiCH et le Ni_3S_2. La catalase (piégeur de H_2O_2), la deferoxamine (DFO) (chélateur du fer) et le glutathion (GSH) (antioxydant) ont non seulement inhibé

significativement la surproduction de H_2O_2 et l'induction de la POL, mais ont aussi amélioré le niveau cellulaire en SH-NP et en SH-P. La superoxyde dismutase (SOD) (piégeur de O_2^-) n'a pas réduit de façon significative l'induction de la POL, mais a réduit significativement la mortalité des lymphocytes causée par le NiCH et le Ni_3S_2. La catalase, le diméthylthiourea (DMTU)/mannitol (piégeurs de radical hydroxyle), la DFO et le GSH/la *N*-acétylcystéïne (NAC) ont aussi significativement réduit la mortalité des lymphocytes. Ces résultats montrent que divers types d'espèces radicalaires oxygénées (ERO) jouent un rôle important dans l'induction de la mortalité des lymphocytes par les composés de nickel. D'autre part, le NiCH, le Ni_3S_2, et le $NiSO_4$ ont causé la perte de l'imperméabilité des pores mitochondriaux (PIPM). La cyclosporine A (CsA) (un inhibiteur spécifique de la PIPM) a non seulement inhibé la PIPM, mais a aussi réduit significativement la mortalité des lymphocytes. En outre, ces composés de nickel ont aussi causé la déstabilisation de l'homéostasie cellulaire du calcium. Les inhibiteurs de l'afflux intracellulaire du calcium tels le verapamil, le nifedipine, le diltiazem, et d'autres types d'inhibiteurs des canaux calciques qui agissent au niveau du stockage intracellulaire du calcium comme le dantrolene, le CsA et le ruthénium rouge, également le chélateur d'ions calcium comme le BAPTA, ont amélioré l'équilibre de l'homéostasie intracellulaire du calcium et ont aussi réduit la mortalité des

lymphocytes. Ainsi, ces résultats indiquent que les mécanismes responsables de l'activation des signaux entraînant la mort des lymphocytes par les composés de nickel impliquent non seulement la surproduction des différents types d'ERO, mais impliquent aussi une induction de la PIPM et de la déstabilisation de l'homéostasie intracellulaire du calcium.

Dans la deuxième partie de notre étude, les effets cytogénotoxiques de $NiSO_4$, NiCH, Ni_3S_2 et NiO sur les lymphocytes sanguins humains en culture ont été étudiés. Après 2 h d'exposition, à une concentration de 15 μM, tous ces composés de nickel ont induit significativement une augmentation des bris simple-brin à l'ADN (BSB ADN) sur la chromatine des chromosomes et des noyaux en comparaison avec le contrôle. Cependant, en comparant ces composés entre eux, seuls le NiCH et le NiO ont induit une augmentation significative des BSB ADN sur la chromatine nucléaire. Le NiCH a causé plus de BSB ADN sur la chromatine nucléaire que le NiO. Le NiCH et le NiO ont causé plus de BSB ADN sur la chromatine chromosomique que le Ni_3S_2 et le $NiSO_4$. Nos résultas montrent que le potentiel génotoxique diminue comme suit: NiCH > NiO ≥ Ni_3S_2 > $NiSO_4$. Nos résultats montrent aussi que le NiCH (30 μM), le Ni_3S_2 et le NiO (120 μM) ont significativement augmenté la fréquence des échanges entre chromatides-sœurs (ECS) et ont aussi ralenti de manière significative, comparés au contrôle, le cycle (IP) et la

croissance cellulaire (IM) mais seulement à une concentration de 60 μM pour le Ni_3S_2 et le NiO. Par contre, le $NiSO_4$ (60 et 120 μM) n'a eu aucun effet significatif sur l'induction des ECS et la IM. La catalase, la SOD, le DMTU, la NAC et la DFO réduisent de façon significative l'induction des BSB ADN et des ECS par le NiCH et améliorent aussi le IM et le IP. Ces résultats suggèrent que divers types de ROS sont impliqués dans la génotoxicité des composés de nickel. Le verapamil, le dantrolene et le BAPTA ont inhibé significativement les BSB ADN et les ECS, et ont aussi amélioré le IM et le IP. Ces résultats suggèrent que la déstabilisation de l'homéostasie cellulaire du calcium est aussi impliquée dans l'induction des BSB ADN et des ECS par le nickel.

Donc, notre étude a permis de franchir une nouvelle étape dans la compréhension des mécanismes cellulaires et moléculaires de toxicité des composés de nickel. Les résultats de cette recherche fournissent des informations importantes pour la prévention des dommages causés à l'ADN des personnes travaillant dans l'industrie du nickel. Ces dommages peuvent, potentiellement, à long terme être à l'origine d'une panoplie des effets néfastes à la santé incluant les effets héréditaires (cassures de chromosome et réparation irrégulière du matériel génétique), les anomalies congénitales et le développement du cancer.

Mots-clés: lymphocytes sanguins humains, composés de nickel, bris simple-brin à l'ADN, chromatine chromosomique et nucléaire,

échanges entre chromatides-sœurs, index prolifératif et mitotique, mortalité des lymphocytes, stress oxydatif, dysfonction mitochondriale, homéostasie du calcium, *in vitro*, culture cellulaire, marquage terminal *in situ*.

SUMMARY

The objectives of this work are (1) to value *in vitro* the cytotoxic and genotoxic risk of nickel sulfate ($NiSO_4$), nickel subsulfide (Ni_3S_2), nickel oxide (NiO) and nickel carbonate hydroxide (NiCH) in the human peripheral blood lymphocytes in suspension and in culture, (2) to examine the role of the oxidative stress, mitochondrial permeability transition, and calcium homeostasis in nickel compound-induced genotoxic damages and lymphocyte death (cytotoxicity).

In the first part of our study, isolated human lymphocytes were treated with various concentrations (0-4 mM) of nickel compounds for 4 h, both concentration-and time-dependent effects of these Ni-compounds on lymphocyte death were observed. As such, the lymphocyte death potency decreased in the following order: NICH > Ni_3S_2 > $NiSO_4$. Increased generation of hydrogen peroxide (H_2O_2), depletion of both nonprotein (NP-) and protein (P-) sulfhydryl (SH) contents and lipid peroxidation (LPO) were induced by NiCH, Ni_3S_2 and $NiSO_4$ studied, whereas excess generation of superoxide anion (O_2^-) was observed only with NiCH and Ni_3S_2. Pretreatment of lymphocytes with either catalase (H_2O_2 scavenger), or deferoxamine (DFO) (iron chelator), or excess GSH (antioxidant) not only significantly reduced the Ni-compound-induced generation of H_2O_2

and LPO, but also increased the NP-SH and P-SH contents initially reduced by Ni-compounds. NiCH-, or Ni_3S_2-induced generation of excess O_2^- but not excess LPO was significantly reduced by pretreatment with superoxide dismutase (SOD). Ni-compound-induced lymphocyte death was significantly prevented by pretreatment with either catalase, or dimethylthiourea (DMTU)/mannitol (hydroxyl radical scavengers), or DFO, or excess GSH/*N*-acetylcysteine (NAC) (antioxidant). NiCH-, and Ni_3S_2-induced lymphocyte death was also significantly prevented by pretreatment with excess SOD. Thus, various types of oxidative stress play important role in Ni-compounds-induced lymphocyte death. Cotreatment with cyclosporin A (CsA) (a specific inhibitor of MPT) not only inhibited Ni-compound-induced mitochondrial permeability transition (MPT), but also significantly prevented Ni-compound-induced lymphocyte death. Furthermore, these Ni-compounds induced destabilization of cellular calcium homeostasis. As such, Ni-compound-induced lymphocyte death was significantly prevented by modulating intracellular calcium fluxes using both Ca^{2+} channel blockers and intracellular Ca^{2+} antagonist. Thus, the mechanism of different Ni-compound-induced activation of lymphocyte death signalling pathways involves not only the excess generation of different types of oxidative stress by Ni-compounds, but also Ni-compound-induced MPT and destabilization of cellular calcium

homeostasis as well.

In the second part of our study, the effects of $NiSO_4$, NICH, Ni_3S_2 and NiO on the induction of DNA single-strand breaks in chromosomal and nuclear chromatin in human blood lymphocytes in culture were studied. After 2 h exposure, the chromosomal chromatin showed significantly higher DNA SSBs due to all four Ni-compounds at 15 μM concentration as compared to the control value. However, exposure to only NiCH and NiO showed significantly higher DNA SSBs in nuclear chromatin when compared to the control value. Both NiCH and NiO produced significantly higher induction of SSBs than those of Ni_3S_2 and $NiSO_4$ in chromosomal chromatin. NiCH-induced SSBs were found to be significantly higher than those due to NiO in nuclear chromatin. Overall, the genotoxic potency seems to be decreased as follows: NiCH > NiO ≥ Ni_3S_2 > $NiSO_4$. Ours results show also that NiCH at 30 μM but Ni_3S_2 and NiO at 120 μM produced moderate but significant increase in the SCE frequency per cell compared to the control value, whereas $NiSO_4$ failed to produce any such significant increase. Except $NiSO_4$, NiCH, Ni_3S_2 and NiO produced significant cell-cycle delay (as measured by inhibition of RI) as well as significant inhibition of mitotic index (MI) at respective similar concentrations mentioned above. Pretreatment of human blood lymphocytes with either catalase, or SOD, or DMTU, or NAC, or DFO significantly reduced DNA SSBs and significantly prevented changes in SCEs, RI

and MI induced by NiCH, suggesting that various types of oxidative stress are involved in such genotoxicity. Simultaneous treatment with either verapamil, or dantrolene, or BAPTA, significantly prevented NiCH-induced both SSBs and changes in SCEs, RI and MI. These results indicate that $[Ca^{2+}]_i$ is also implicated in such genotoxicity.

Thus our study may provide some new insights into the cellular and molecular mechanisms of Ni-compound-induced both DNA single-strand breaks in human blood lymphocytes and human lymphocyte death as well as contribute some important information for the prevention of DNA damage among workers in nickel-related industrial environments. This damage can, potentially, lead to a range of long-term adverse health effects including heritable effects, developmental defects, and cancer initiation.

Key words: Human blood lymphocytes, nickel compounds, DNA single-strand breaks, chromosomal and nuclear chromatin, sister-chromatid exchanges, replication index, mitotic index, lymphocyte death, oxidative stress, mitochondrial permeability transition, calcium homeostasis, *in vitro*, cell culture, *in situ* end-labeling.

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CHAPITRE I

Article I

Listes des symboles et des abréviations

ADN	acide désoxyribonucléique
Al	aluminium
ARN	acide ribonucléique
As	arsenic
ATP	adénosine triphosphate
BAPTA	1,2-bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acide, tetraacetoxymethyl ester
BSB	bris simple-brin à l'ADN
Ca^{2+}	ions calcium
$[Ca^{2+}]_i$	concentration intracellulaire de Ca^{2+}
$CaCl_2$	chlorure de calcium
CEHC	cellules embryonnaires de hamster Chinois
CEHS	cellules embryonnaires de hamster Syrien
CHO	Chinese hamster ovary
COHC	cellules ovariennes de hamster Chinois
Cr	chrome
CsA	cyclosporin A
CsCl	chlorure de césium
DFO	deferoxamine
DMT-1	duodenal metal-transporter
DMTU	dimethylthiourea

DPCs	DNA-protein cross-links
ECS	échanges entre chromatides-soeurs
EM-ISEL	electron microscopy *in situ* end-labeling
ERO	espèces radicalaires oxygénées
ISEL	*in situ* end-labelling
Fe	fer
GGGG	tétraglycine
GGH	glycyl-glycyl-histidine
Gpt	glutamic pyruvic transaminase
GSH	glutathion
Hg	mercure
H_2O_2	peroxyde d'hydrogène
HGPRT	hypoxanthine-guanine-phosphoribosyl-transférase
HPRT	hypoxanthine phosphoribosyl-transférase
IHKE	immortalized human kidney epithelial cell line
IM	index mitotique
IP	index prolifératif
j	jour
kg	kilogramme
L	litre
LCAP	liaisons croisées ADN-protéines
LPO	lipid peroxidation

MCA	méthylchlolanthrene
MDA	malondialdéhyde
$MgCl_2$	chlorure de magnésium
ml	millilitre
Mn	manganèse
MPT	mitochondrial permeability transition
NAC	*N*-acetyl-L-cysteine
NAG	*N*-acétyle-β_2-D-glucosaminidase
Ni	nickel
Ni^{2+}	ions nickel
NiCH	hydroxyde carbonate de nickel
$NiCl_2$	chlorure de nickel
$2NiCO_3.3Ni(OH)_2.$	hydroxyde carbonate de nickel
Ni_3S_2	sous-sulfure de nickel
Ni_3Se_2	sous-séléniure de nickel
NiO	oxyde de nickel
NiS	sulfure de nickel
$NiSO_4.$	sulfate de nickel
N-OH-AAF	*N*-hydroxy-*N*-2-acetylaminofluorène
NP-SH	nonprotein sulfhydryl groups
NQO	1-oxyde-4-nitroquinoline
Nramp 2	intestinal iron transporter
O_2	oxygène

8-OH-dG	8-hydroxydeoxyguanosine
PAH	ion *para*-aminohippurate
Pb	plomb
PB-SH	protein-bound sulfhydryl groups
PIPM	perte de l'imperméabilité des pores mitochondriaux
POL	peroxydation des lipides
PPIase	isomérase du peptidylprolyl-cis-trans
PRibPP	5-phosphoribosyl-1pyrophosphate
ROS	reactive oxygen species
SCEs	sister-chromatid exchanges
SHE	syrian hamster embryo
SH-P	groupements sulfhydryles liés aux protéines
SH-NP	groupements sulfhydryles non liés aux protéines
SOD	superoxide dismutase
SSBs	DNA single-strand breaks
TdT	terminal deoxynucleotidyl transferase
TPA	tétradécanoyl-phorbol-acétate
TUNEL	TdT-mediated bio-dUTP nick end-labeling
TWAs	8-hour total weight average
μg	microgramme
μl	microlitre

μmol	micromole
V_{max}	vitesse maximale intrinsèque de raccourcissement dans la contraction isotonique

À mes parents, David et Julienne.

À mon épouse et mes enfants,

pour le temps qu'ils

ont partagé avec la science

avec grande patience.

Remerciements

Je tiens tout d'abord à manifester ma reconnaissance à mes directeurs de thèse, les Docteurs Saroj Chakrabarti et Nicole Lemieux, tous deux professeurs à la Faculté de médecine de l'Université de Montréal, qui ont bien voulu m'accueillir chacun dans leur laboratoire en me confiant ce projet et en assurant ma formation en toxicologie génétique. Leur support et leur aide, les conseils fructueux qu'ils m'ont prodigués et leur encadrement scientifique ont conduit à la réalisation de ce projet.

Ma reconnaissance aux Docteurs Geneviève Renier et Jean-Claude Mamputu de l'Hôpital Notre-Dame du CHUM et leur équipe pour nous avoir approvisionné en cellules sanguines humaines.

Je remercie Monsieur Anwar Malik pour son assistance technique et sa sympathie, madame Fléchère Fortin, pour sa contribution à la réalisation de la partie cytogénétique de ce projet, ainsi que le Département de Pathologie de l'Hôpital Sainte Justine pour son assistance technique.

Mes remerciements vont également à la Faculté des études supérieures de l'Université de Montréal, au groupe de recherche en

toxicologie humaine (TOXHUM) et à Rédicy Inc. pour leur appui financier apporté durant mes études.

Ma profonde gratitude à mon épouse, Jacqueline Ntumba-Nzongo et à nos enfants, Lord, Chris, Marie-Ève et Noëlie pour leur soutien inestimable, leurs encouragements et leur disponibilité. Ma profonde reconnaissance à mes parents qui m'ont non seulement donné leurs chromosomes mais m'ont également supporté et encadré dans différentes étapes de ma formation scolaire. Je vous aime.

Avant-propos

Partout dans le monde, les effets du nickel sur la santé humaine font l'objet de préoccupations fort compréhensibles, étant donné l'importance de ce métal et de ses dérivés sur le plan économique. Leurs multiples utilisations industrielles sont à l'origine d'une exposition professionnelle à laquelle sont soumis un nombre important de travailleurs. Les risques associés à l'exposition à certains dérivés de nickel, tel le cancer, ont suscité beaucoup d'études.

Malgré de nombreux efforts visant à améliorer l'environnement de travail dans l'industrie du nickel, un grand nombre d'ouvriers est constamment exposé aux composés de nickel (solubles et insolubles). Ces travailleurs sont exposés de façon répétée ou à long terme dans divers milieux de travail tels le nickelage, la teinture et le lustrage des céramiques, l'industrie minière, les réacteurs chimiques (catalyseurs), la préparation d'aciers spéciaux résistant à la corrosion et à la chaleur, la fabrication d'alliages, des batteries Ni-Cd et des joailleries. La plupart de ces composés de nickel sont toxiques et cancérogènes chez l'animal et l'humain. L'exposition à ces dérivés en milieu de travail peut donc constituer des risques potentiels pour la santé humaine. Cependant, les mécanismes menant à ces toxicités sont

encore indéterminés et moins compris. Il s'avère alors important de comprendre ces mécanismes pour prévenir des dommages génétiques afin de réduire les risques de maladies professionnelles dues aux composés de nickel comme les cancers. C'est la raison pour laquelle nous avons effectué cette étude sur les lymphocytes humains pour évaluer les risques cytotoxiques et génotoxiques des composés de nickel exploités en milieu de travail.

Ce travail est divisé en quatre grandes parties. D'abord, on retrouve une revue générale de la littérature sur le nickel et ses composés, la rationalité, l'hypothèse et les objectifs de travail. Ensuite, la présentation des résultats sous forme d'articles à soumettre dans des journaux scientifiques. Enfin, on retrouve la discussion, une conclusion générale, puis la bibliographie.

I. INTRODUCTION

I.1. Généralité sur le sujet

I.1.1 Génotoxicité de nickel

Les effets génotoxiques peuvent être classés dans trois grandes catégories:

1. Les mutations géniques (au niveau des gènes)
2. Les mutations chromosomiques (au niveau de la structure et du nombre des chromosomes)
3. Les signes précoces d'altération de l'ADN et les mécanismes de réparation (par exemple: les ECS).

Les aberrations chromosomiques représentent la non-réparation des dommages induits à l'ADN. Le taux normal de ces aberrations est d'environ 0 à 4%. Les agents mutagènes induisent des lésions à l'ADN qui, si elles ne sont pas réparées rapidement ou si elles le sont avec des erreurs, se traduisent par des aberrations chromosomiques ou des mutations ponctuelles. Les aberrations chromosomiques sont des modifications qui portent sur les régions euchromatiques. Celles produites dans les régions hétérochromatiques mèneront à l'apparition de variants sans effet phénotypique. Le test d'évaluation des aberrations chromosomiques

met en évidence les cassures, les anomalies de structure et de nombre et localise les régions qui sont le plus souvent impliquées (les points chauds). Les anomalies numériques peuvent porter sur l'ensemble du complément chromosomique (polyploïde) ou sur un ou quelques chromosomes (aneuploïdie: hyper ou hypodiploïdie).

En fonction du stade du cycle cellulaire au moment du traitement, les anomalies de structure induites peuvent être:

1. **Chromatidiennes**, lorsque les cellules sont exposées aux agents mutagènes en phase S ou G_2 (après réplication). Les aberrations chromatidiennes sont localisées sur une des deux chromatides et se concrétisent par des gaps (seulement une partie de la chromatide est cassée), des bris et des échanges. Les échanges peuvent se produire au niveau des chromatides d'un seul chromosome (intra-bras ou inter-bras comme l'union entre chromatides-sœurs) ou de plusieurs chromosomes (interchange symétrique, triradial, interchange incomplet, etc.).
2. **Chromosomiques**, lorsque les cellules sont exposées aux agents mutagènes en phase G_0 ou G_1 (avant la réplication), mais aussi pendant la phase de synthèse (avant la réplication des différentes régions chromosomiques). Les anomalies chromosomiques se traduisent par des cassures (avec l'apparition de délétions terminales ou interstitielles, de

fragments acentriques, de minutes ou de double-minutes) ou des échanges (entre un ou plusieurs chromosomes). Les échanges qui se produisent à l'intérieur d'un seul chromosome peuvent intéresser un seul bras (duplication, anneau acentrique, inversion paracentrique) ou les deux bras (anneau avec centromère, inversion péricentrique). Quand plusieurs chromosomes sont impliqués dans les échanges, il peut en résulter des insertions, des translocations, des chromosomes dicentriques, tricentriques ou plus rarement acentriques.

I.1.1.1. Études expérimentales chez l'animal

Les composés de nickel sont considérés faiblement clastogènes. Il a été montré que les composés de nickel causent particulièrement des dommages chromosomiques structuraux. En effet, on a observé de la décondensation régionale, de fréquentes délétions et d'autres aberrations dans le bras long du chromosome X des cellules ovariennes de hamster Chinois (COHC) en culture exposées au Ni_3S_2 et au $NiCl_2$ (Sen et coll., 1987). Des dommages chromosomiques similaires ont aussi été observés dans les cellules embryonnaires de hamster Chinois (CEHC) transformées par le nickel (Conway et Costa, 1989).

Plusieurs études ont montré les effets génotoxiques du nickel chez l'animal (Nishimura and Umeda, 1979; Ohno et coll., 1982; Larramendy et coll., 1981; Ciccarelli et Wetterhahan, 1984a et 1984b; Dhir et coll., 1991; Chakrabarti et coll., 1999; Ohshima, 2003). Le chlorure de nickel, l'acétate, le cyanonickelate de potassium et le sulfure de nickel ont produit une variété des lésions chromosomiques (cassures, anomalies chromosomiques de structure, échanges, fragmentations, etc.) dans les cellules de carcinome mammaire (FM3A) de la souris C3H en culture (Nishimura et Umeda, 1979; ATSDR, 2002). Une augmentation significative de la fréquence des échanges entre chromatides-sœurs (ECS) a été observée après exposition des cellules de hamster chinois au sulfate (Ohno et coll., 1982; ATSDR, 2002) et des cellules embryonnaires de hamster syrien (CEHS) au chlorure de nickel (Larramendy et coll., 1981; ATSDR, 2002). Dans une autre étude, Dhir et collaborateurs (1991) ont rapporté qu'une injection intrapéritonéale du chlorure de nickel produit des aberrations chromosomiques chez des souris de manière dose-dépendante.

Chakrabarti et collaborateurs (1999) ont étudié la génotoxicité des composés de nickel chez le rat. Leurs travaux ont montré la formation des liaisons croisées ADN-protéine (LCAP) dans les cellules corticales rénales de rats exposés au sous-sulfure et au sulfate de

nickel.

Dans une étude sur la génotoxicité de nickel, Ciccarelli et Wetterhahan (1984a et 1984b; ATSDR, 2002) ont observé qu'une injection intrapéritonéale de carbonate de nickel induit des bris simple-brin d'ADN (BSB ADN) dans le rein et le poumon du rat Sprague-Dawley.

Dans une autre étude plus récente, Ohshima (2003) a montré une induction de l'instabilité génétique et chromosomique chez l'animal par les composés de nickel en évaluant la fréquence d'aberrations chromosomiques et de mutations au locus hypoxanthine phosphoribosyl-transférase (HPRT). Les cellules V79 de hamster chinois ont été exposées au sulfate de nickel (320 μM) et les clones dérivant des cellules exposées ont été isolés. Cinq des 37 clones (13.5%) ont montré une augmentation de la fréquence de mutations au locus HPRT, 17 (45.9%) ont montré une augmentation de la fréquence d'aberrations chromosomiques de structure.

I.1.1.2. Études de génotoxicité chez l'humain

Peu d'études humaines sont disponibles sur la génotoxicité du nickel et de ses composés. La fréquence d'anomalies chromosomiques

(gaps, cassures, fragments, remaniements structuraux) et des ECS dans les lymphocytes sanguins des travailleurs exposés au nickel en milieu de travail, a été étudiée (Werfel et coll., 1998; Waksvik et Boysen, 1982; Deng et coll., 1983, 1988; Cai et coll., 1987; Decheng et coll., 1987; Elias et coll., 1989; Senft et coll., 1992). Les résultats de ces études sont contradictoires. La majorité d'entre elles ne comprennent qu'un petit nombre des travailleurs qui sont aussi exposés à d'autres métaux tels le chrome, le manganèse et le fer pendant les travaux de soudage, le cuivre et d'autres métaux dans une raffinerie de nickel. Werfel et collaborateurs (1998) ont analysé les lymphocytes sanguins des soudeurs et d'un groupe contrôle. Les soudeurs ont montré un taux significativement élevé des bris simple-brin d'ADN et des ECS compareé au groupe contrôle. L'étude indique que les bris simple-brin d'ADN et la formation des liaisons croisées protéine-ADN (LCPA) augmentent chez les soudeurs de façon différentielle selon le niveau d'exposition au chrome et au nickel. Elias et coll. (1989) ont rapporté l'existence d'une corrélation significative entre la durée du travail et la fréquence des cassures chromosomiques chez un soudeur. Pourtant, Waksvik et Boysen (1982) n'avaient pas trouvé d'association entre la durée du travail dans une raffinerie du nickel et l'augmentation significative du nombre des cassures dans les chromosomes. Deng et collaborateurs (1983 et 1988) ont montré une augmentation du niveau d'aberrations

chromosomiques (gaps, cassures, fragments) et d'ECS chez des travailleurs d'une usine de nickelage, contrairement à Waksvik et Boysen (1982) (revue dans ATSDR, 2002) qui n'avaient pas observé. Ces effets n'ont pas été observés chez les travailleurs exposés au carbonyle de nickel dans l'étude de Decheng et coll., (1987). De la même façon, Cai et collaborateurs (1987) n'ont montré aucune différence significative dans la fréquence des aberrations chromosomiques et des ECS entre les travailleurs exposés et le groupe contrôle. Dans une étude antérieure, Waksvik et collaborateurs (1984), ont analysé la génotoxicité du nickel chez 9 travailleurs retraités 8 ans après leur retraite. Ces ouvriers ont été exposés au nickel sur une période allant de 8 à 25. Les résultats ont montré une persistance d'un faible niveau de gaps et de cassures chromosomiques dans les lymphocytes sanguins. Dans une autre étude plus récente (Perminova et collaborateurs, 1997) réalisée chez les travailleurs exposés aux composés de nickel, les résultats ont montré une augmentation significative des ECS parmi les fumeurs comparés aux non-fumeurs. L'étude a révélé aussi une augmentation significative de l'inhibition de réparation de la synthèse d'ADN chez les travailleurs exposés.

I.1.1.3. Effets de l'ion Ni^{2+} sur la chromatine

L'exposition des cellules de souris et de hamsters aux composés de nickel induit des dommages à la chromatine des chromosomes (Costa et coll., 1988). Patierno et collaborateurs (1985) ont démontré que le $NiCl_2$ induit la formation des liaisons croisées ADN-proteines (LCAD) sur la chromatine des COHC. Les bris simple-brin à l'ADN induits par l'ion Ni^{2+} ont été rapidement réparés, alors que les LCAP sont restées persistantes.

Dans une étude de Sen et Costa (1986b), les COHC ont été exposées au $NiCl_2$ et aux particules de NiS afin d'étudier leurs effets chromosomiques. Le NiS a augmenté la fréquence des ECS et a causé une augmentation des chromosomes dicentriques. Contrairement au NiS, les effets causés par le $NiCl_2$ ont été statistiquement non significatifs. Les analyses cytogénétiques faites sur les cellules provenant de hamster chinois mâle ont montré que quatre des cinq lignées cellulaires étudiées présentent une délétion de la chromatine du chromosome X.

Christie et collaborateurs (1988) ont étudié l'induction d'aberrations chromosomiques par les particules cristallines de NiS dans les lignées cellulaires C3H/10$T^{1/2}$ dérivées de la souris. La durée

du traitement a été de 6 ou 24h et les cellules ont bénéficié d'une période de 24h pour se remettre, cette période étant nécessaire pour obtenir des mitoses après blocage de la réplication induit par le nickel.

I.1.1.4. Interactions entre l'ion Ni^{2+} et les protéines-ADN

La force de l'interaction du nickel-protéines dépend de l'identité des acides aminés présents. Une très grande affinité est observée avec l'histidine. Cette propriété du nickel est largement utilisée pour la purification des protéines recombinantes contenant six marquages d'histidine sur agarose pour immobiliser les ions nickel (Crowe et coll., 1994).

Les métaux carcinogènes incluant le nickel et ses composés peuvent être associés à de l'aneuploïdie résultant d'interactions avec les protéines chromosomiques telle que la topoisomérase II (Gaulden, 1987; Denkhaus et Salnikow, 2002). Une perturbation du nombre de liaison ADN-protéine peut entraîner une interférence avec le mouvement des chromosomes à l'anaphase et créer une adhésion des chromosomes. Des effets secondaires peuvent être des cassures et la production d'aberrations chromosomiques.

Le nickel peut aussi modifier l'activité enzymatique des

protéines autres que l'ADN et l'ARN polymérase. Par exemple, l'hypoxanthine-guanine-phosphoribosyl-transférase (HGPRT) provenant de la levure (un produit génique lié au chromosome X) peut être activée par l'ion Ni^{+2} en l'absence de Mg (Ali et Sloan, 1986; Denkhaus et Salnikow, 2002). Tous les ions métal pouvant activer cet enzyme, incluant les ions Mg^{+2}, Mn^{+2}, Co^{+2} et Ni^{+2}, agissent via le même mécanisme, c'est-à-dire une fixation de deux ions bivalents au site actif de la HGPRT par la molécule 5-phosphoribosyl-1-pyrophosphate (PRibPP). L'activation de cet enzyme par l'ion Ni^{+2} est faible. La vitesse intrinsèque de raccourcissement dans la contraction isotonique (V_{max}) en présence de la concentration optimale de nickel (0.4 à 1 mM) est de 10 à 25% alors qu'elle est de 100% en présence d'autres métaux bivalents activateurs. L'affinité relative des ions métal pour la HGPRT suggère l'existence d'une coordination des ions métal, les résidus d'acide aminé, le phosphate et le groupe pyrophosphate de PRibPP.

Il est clair, qu'à partir de la spécificité de ses effets et de sa force de liaison relative aux ligands protéines par rapport aux ligands ADN, que le nickel exerce une grande partie de ses effets génotoxiques via les interactions avec les protéines.

Lee et collaborateurs (1982) ont mesuré la formation des

complexes stables protéines-ADN-Ni(II) dans l'ADN thymique de veau et dans les microsomes hépatiques de rat après exposition au Ni_3S_2. La concentration de la solution stock de Ni(II) soluble obtenue à partir des particules de Ni_3S_2 a été déterminée entre 1 et 9 mM, dépendant de la présence d'autres composants dans la solution. La solubilité de Ni_3S_2 pourrait dépendre en partie de la présence de base Tris. L'union de Tris avec le Ni(II) et le O_2 pourrait être impliquée dans la formation de Ni(V).

Costa et ses collaborateurs ont étudié la formation des liaisons croisées ADN-protéines (LCAP) dans les cultures cellulaires (Costa et coll., 1988; Patierno et Costa, 1985, 1987). Les COHC ont été traitées avec divers composés de nickel (solubles et insolubles) et analysées par élution d'alcalin pour la production des bris simple-brin et des LCAP. Le traitement avec la forme cristalline de NiS (24h, 10 μg/ml) résulte en une faible mais significative production des LCAP. La production des LCAP a augmenté en temps-dépendant (jusqu'à 48h), mais pas en dose-dépendante. En ce qui concerne les bris simple-brin à l'ADN (BSB ADN), ils ont été observés seulement après 48h. Les formes solubles de nickel ($NiCO_3$, $NiSO_4$ et $NiCl_2$) ont aussi produit une faible quantité des BSB ADN mais significative en fonction de la dose et à court terme (3h). Ces résultats ont été documentés par Denkhaus et Salnikow (2002).

I.1.2. Mécanismes d'action toxique du nickel

I.1.2.1. Rôle du stress oxydatif

L'implication possible d'espèces réactives oxygénées dans la toxicité du nickel a été examinée dans le passé (Nieober et coll., 1984; Donskoy et coll., 1986; Klein et coll., 1991; Kasprzak, 1995). Le nickel produit des radicaux libres dans les cellules à des niveaux bas mais mesurables tels que détectés par le dichlorofluorescence (Huang et coll., 1994; Salnikow et coll., 1994; Salnikow et coll., 2000). Il a été observé plus de radicaux libres dans le noyau des cellules exposées aux cristaux de Ni_3S_2 (Huang et coll., 1994). En plus de la mesure directe de radicaux libres, la déplétion du glutathion (GSH) représente un autre marqueur de stress oxydatif. Après injection de nickel chez la souris, les niveaux *in vivo* de GSH hépatique ont diminué considérablement (Herrero et coll., 1993). De la même façon, il a été établi que le GSH peut être déplété dans les cellules en culture exposées au nickel (Salnikow et coll., 1994; Li et coll., 1993).

Sunderman et coll., (1987), Knight et coll., (1987) et Donskoy et coll., (1986) ont démontré la participation d'espèces radicalaires oxygénées dans la pathogenèse de la toxicité aiguë du nickel, telle que mise en évidence par l'induction de la peroxydation des lipides dans

les tissus cibles des rats traités au chlorure de nickel. Nieboer et al (1984) ont démontré, qu'en présence d'albumine ou de certains peptides, le couple redox Ni^{3+}/Ni^{2+} peut participer aux réactions d'espèces radicalaires oxygénées *in vitro*. En initiant la formation d'espèces radicalaires oxygénées dans les cellules cibles, on peut suggérer que les traces d'ion Ni^{3+} de l'oxyde de nickel jouent un rôle crucial dans la pathogenèse de la génotoxicité et de la cytotoxicité (Sunderman et coll., 1987). Schlatter (1986) a suggéré que les métaux cancérogènes puissent être génotoxiques via un mécanisme par lequel les espèces radicalaires oxygénées causent des dommages oxydatifs à l'ADN.

L'administration parentérale de chlorure de nickel chez les rats produit une toxicité hépatique aiguë avec un accroissement de la peroxydation lipidique et de la stéatose (Shirai et coll., 1984). Le nickel peut inhiber la communication intercellulaire par l'intermédiaire de la peroxydation lipidique et des radicaux libres. Ces derniers réagissent avec les protéines jonctionnelles et affectent leur conformation. Ce mécanisme est aussi impliqué dans l'interruption de la communication intercellulaire par les promoteurs organiques tels le 12-0-tétradécanoyl-phorbol-13 acétate (TPA) et le peroxyde de benzoyle (Miki et coll., 1987).

Les effets des sels de nickel sur le niveau de glutathion hépatique ont été étudiés par plusieurs équipes. Les résultats démontrent une augmentation dose-dépendante du niveau de glutathion hépatique, de glutathion-réductase et de gluthation S-transférase associée à une diminution de l'activité de gluthation peroxydase et de gamma-glutamyltranspeptidase (Athar et coll., 1987; Sunderman et coll., 1984; Marzouk et Sunderman, 1984; Behari et coll., 1984; Sunderman et coll., 1985; ATSDR, 2002). L'administration sous-cutanée de chlorure de nickel induit la peroxydation lipidique, l'augmentation du niveau de glutathion hépatique et du fer, et la diminution de glutathion peroxydase. Les auteurs ont proposé un mécanisme par lequel le fer produit le radical hydroxyle à partir du peroxyde d'hydrogène qui cause la baisse de glutathion (Behari et coll., 1984; Sunderman et coll., 1985; Donskoy et coll., 1986) (Denkhaus et Salnikow, 2002).

Les effets oxydatifs du nickel dépendent de sa capacité à former le couple redox Ni(III)/Ni(II) associé à un pH autour de 7.4. Ce processus est possible lorsque le Ni(II) est complexé par certains ligands naturels incluant les peptides et les protéines, par exemple, le glycyl-glycyl-histidine (GGH) ou la tétraglycine (GGGG) (Bal et coll., 2000; Margerum et Anliker, 1988). Une conséquence importante de la réaction de tels complexes Ni(II) avec les espèces radicalaires

oxygénées (ERO) (par exemple: O_2^- ou H_2O_2 endogènes) est la production non seulement du radical hydroxyle (ou un oxo-cation NiO^{2+}), mais aussi d'autres radicaux concentrés en oxygène, carbone et soufre provenant des ligands (Kasprzak, 1996; Kasprzak et Buzard, 2000).

Une variété d'espèces réactives intermédiaires peuvent aussi être produites dans le processus oxydatif cellulaire de la solubilisation des sulfures de nickel, Ni_3S_2 et NiS. Les deux sont sensibles à l'oxydation par l'oxygène ambiant qui facilite leur dissolution dans les liquides biologiques, bien qu'avec une cinétique différente (Sunderman, 1984) (Denkhaus et Salnikow, 2002). En fait, les réactions oxydatives sont plus complexes et produisent des espèces réactives intermédiaires. Il a été démontré que l'interaction de O_2 avec le Ni_3S_2 peut être réduite en H_2O_2 (Costa et coll., 1989) et l'oxydation du soufre entraîne les espèce réactives du soufre incluant l'anion sulfite (Lee et coll., 1982); des telles espèces réactives intermédiaires sont capables d'induire des dommages pro-mutagènes à l'ADN par l'intermédiaire de l'oxydation ou de la désamination (Kasprzak, 1995; Kasprzak, 1996). Cela rend les sulfures de nickel capables de produire de plus grands et divers dommages oxydatifs que ceux produits par les autres composés de nickel. On doit cependant se rappeler que si le dommage est trop étendu, comme dans le cas du

couple redox des métaux hautement actifs tels que le cuivre, le fer, ou le cobalt, le résultat peut être plutôt la mort des cellules que la survie des cellules endommagées (Kasprzak, 1995; Kasprzak, 1996). Tkeshelashvili et collaborateurs (1993) ont observé que le ratio de mutagenèse augmente en fonction des dommages causés à l'ADN par les ERO qui sont produits beaucoup plus par le Ni(II) que par le Fe(II) ou le Cu(II).

I.1.2.2. Implication des mitochondries et de l'homéostasie du calcium

Il a été établi que les mitochondries représentent une cible majeure de cytotoxicité dépendant du calcium. En effet, une étude a suggéré que le mercure induit la nécrose et l'apoptose des lymphocytes et des monocytes humains suite à la perte de l'imperméabilité de la membrane mitochondriale (Close et coll., 1999). L'ouverture des pores mitochondriaux permet un passage des particules à travers la membrane interne mitochondriale (Gunter et Pfeiffer, 1990; Zoratti et Szabo, 1995). Cet état dépolarise, découple et gonfle les mitochondries. Cependant, peu d'études ont été faites sur la liaison entre le nickel et les mitochondries, et aucune sur l'implication précise des mitochondries dans les mécanismes toxiques des composés de nickel. Il a été démontré que le nickel inhibe l'activité

succinodéshydrogénase mitochondriale dans les cellules épithéliales du poumon chez le rat (Riley et coll., 2003). Il est établi aussi que le Ni_3S_2 se lie préférentiellement aux mitochondries des cellules du poumon, mais se lie faiblement aux mitochondries des lymphocytes (Shirali et coll., 1992). L'existence de la liaison entre le sous-sulfure de nickel et certaines structures sous cellulaires des lymphocytes humains et ceux du rat a également été rapportée (Hildebrand et coll., 1987). Cependant, on ne sait pas présentement comment la perturbation de l'activité mitochondriale induite par le nickel peut affecter la toxicité cellulaire.

Le rôle de la perturbation de l'homéostasie du calcium dans la mortalité ou la transformation cellulaire n'est pas bien compris. Le Ca^{2+} est reconnu comme étant un des plus importants messagers secondaires intracellulaires. En raison de la faible concentration du calcium dans le cytosol, il y a toujours un gradient important pour la diffusion du calcium vers le cytosol dans toutes les cellules des mammifères (Pozzan et coll., 1994; Rosen et coll., 195). Bien que l'hypothèse de l'implication du calcium dans l'oncogenèse a été formulée par Jaffe en 1982 (revue par Kasprzak et Waalkes, 1986), peu d'études ont été réalisées sur l'effet cancérigène et/ou toxique du nickel en relation avec la perturbation du métabolisme du calcium. Une des premières observations faites est celle des cellules

transformées par le nickel montrant une capacité exceptionnelle de prolifération rapide in vitro dans un milieu pauvre en calcium. Cela suggère qu'il y existe une altération du métabolisme du calcium intracellulaire dans les cellules transformées par le nickel (Swierenga et coll., 1976; Swierenga et coll., 1978). L'administration *in vivo* d'acétate de Ca(II) prévient la formation d'adénomes pulmonaires causés par l'acétate de nickel ou par l'acétate de plomb chez la souris (Poirier et coll., 1984). Par ailleurs, le carbonate de Ca(II) n'a pas démontré d'effet sur la carcinogenèse induite par le Ni_3S_2 dans le muscle du rat (Kasprzak et coll., 1985). Cependant, lorsqu'il est administré seul, l'acétate de Ca(II) augmente l'incidence d'adénomes pulmonaires. En utilisant l'expression du gène *Cap43* comme marqueur pour identifier le second messager impliqué dans la transcription d'ADN induite par le nickel, Salnikow et collaborateurs (1999) ont démontré que l'expression du gène *Cap43* dans les cellules pulmonaires humaines A549 est induite de la même façon par le chlorure de nickel et par l'ionophore Ca^{2+} A23187. L'induction de cette expression génique par ces deux métaux est supprimée lorsque le Ca^{2+} intracellulaire est chélaté par le 1,2-bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*',*N*'-tetraacetic acide, tetraacetoxymethyl ester (BAPTA-AM). Ces observations confirment l'hypothèse de l'augmentation des concentrations intracellulaires de calcium dans les cellules traitées par le nickel (Salnikow et coll., 1999). Les mécanismes qui mènent à

cette augmentation de calcium dans les cellules traitées ne sont pas actuellement bien compris. En outre, les mécanismes impliqués dans l'expression du gène *Cap43* qui augmente en tandem avec les concentrations intracellulaires de Ca^{2+} ne sont pas clairs non plus. Les composés solubles de nickel entrent probablement dans la cellule via les canaux calciques puisque l'ionophore du calcium ionomycine (3 µM) augmente la captation de nickel de quatre à cinq fois (Refvik et Andreassen, 1995; Funakoshi et coll., 1997). De plus, la captation de nickel par la lignée des cellules épithéliales rénales humaines immortalisées (IHKE) est inhibée par le calcium. Le Ni^{2+} est connu comme étant un inhibiteur des canaux calciques (Zamponi et coll., 1996). Il est possible qu'une baisse initiale du niveau intracellulaire de Ca^{2+} en réponse au traitement des cellules avec le Ni^{2+} ait été suivie d'une augmentation compensatoire de Ca^{2+} libre résultant de sa libération du stockage intracellulaire. En fait, il est établi que le nickel provoque la libération du calcium intracellulaire par un mécanisme impliquant un récepteur cellulaire de surface (Smith et coll., 1989). Une autre possibilité est que les ions Ni^{2+} interagissent avec un détecteur de Ca^{2+} ou un récepteur de Ca^{2+} sur la membrane plasmatique pour activer la libération intracellulaire de Ca^{2+}. Cependant, la modulation des niveaux extracellulaires du Ca^{2+} de 0 à 7 mM n'a affecté ni l'expression du gène *Cap43* induit par le calcium et le nickel, ni l'induction de *Cap43* par 1 mM de $NiCl_2$ (Salnikow et

coll., 1999). Ces résultats indiquent que si les ions Ni^{2+} interagissent avec une surface réceptrice de Ca^{2+}, ce n'est probablement pas un mode de liaison de Ca^{2+}, puisque c'est improbable que 1 mM de $NiCl_2$ entre en compétition avec 7 mM de $CaCl_2$ pour se lier à un site spécifique de la protéine.

I.1.3. Principes des outils de travail

I.1.3.1. Principes d'analyse de l'intégrité de la membrane cytoplasmique (nécrose).

Les méthodes standard habituellement utilisées pour mesurer l'intégrité de la membrane cytoplasmique sont basées sur la captation ou l'exclusion des molécules avec photoabsorption ou ayant des propriétés fluorescentes. La captation des colorants comme le bleu de Trypan ou le propidium iodé; la libération des isotopes radioactifs tels [^{51}Cr], [^{3}H]-thymidine, [^{3}H]-proline, [^{35}S]- ou [^{75}Se]-methionine, 5-[^{125}I]-2-deoxy-uridine, ou des colorants fluorescents comme bis-carboxyethyl-carboxyfluorescent (BCECF) ou calcéine-AM. Un autre groupe d'essais standard est basé sur la mesure des enzymes cytoplasmiques (LDH) libérés par les cellules endommagées. Pour notre étude, nous avons choisi le test d'exclusion au bleu de Trypan. Le principe d'analyse de la nécrose par le test au bleu de Trypan consiste à exposer les cellules à l'agent toxique dans des conditions

optimales d'exposition (température, durée d'exposition, etc.). Immédiatement après l'exposition, un colorant vital, le bleu de Trypan, est ajouté. Dans les cellules dont la membrane a été endommagée, le colorant est retenu à l'intérieur du cytoplasme et la membrane cytoplasmique ne rejette plus le colorant de l'extérieur. Les cellules non colorées représentent donc les cellules viables par opposition à celles où la coloration est présente. La viabilité cellulaire est déterminée par le comptage des cellules à l'aide d'un hémacytomètre. Elle est calculée en utilisant la formule suivante: nombre de cellules viables x 100 / le nombre total de cellules (vivantes et mortes). L'exclusion de ce colorant vital observé en microscopie photonique est un critère bien établi d'intégrité membranaire et de viabilité cellulaire (Muller, 1984). Cependant, le désavantage de cet essai est l'évaluation subjective de la part de l'expérimentateur. En effet, l'observation de la zone de lyse et celle des changements morphologiques des cellules peuvent avoir une certaine teinte de subjectivité.

I.1.3.2. Principes d'analyse de la génotoxicité

Le potentiel génotoxique peut être évalué par la mesure entre autres des différents paramètres de cytogénétique. Pour notre étude, nous avons choisi les paramètres suivant: la détection des bris

simple-brin à l'ADN en utilisant la technique du marquage terminal *in situ* en microscopie électronique (EM-ISEL), l'évaluation de la fréquence des échanges entre chromatides-soeurs (ECS), de la fréquence du cycle (IP) et de la croissance cellulaire (IM).

I.1.3.2.1. Marquage terminal *in situ* en microscopie électronique (EM-ISEL)

Le marquage terminal *in situ* en microscopie électronique est une méthode enzymatique d'identification et de quantification des dommages simple-brin à l'ADN résultant de l'action d'un agent toxique ou probablement de réactions redox et/ou d'interférences ioniques à la réparation du génome. Elle a l'avantage de pouvoir être effectuée sur des tissus frais ou archivés (une simple perméabilisation à l'aide d'une protéase étant suffisante dans ce dernier cas), sur des cellules isolées ou sur des coupes histologiques. La sensibilité de cette méthode dépasse celle des méthodes standards (Fujita et coll., 1997; Migheli et coll., 1994). Les techniques « *in situ* end-labeling » (ISEL), détectant les terminaux hydroxyles 3' aux abords des lésions, peuvent en effet mettre en évidence des cassures simple-brin à l'ADN induites par les métaux. Pour ce faire, les techniques ISEL utilisent un marqueur analogue à une des bases azotées: la thymidine est remplacée par la biotine-dUTP, bien que certains auteurs remplacent aussi l'adénine par la biotine-dATP alternativement (Chen et coll.,

1997). Tous ces analogues de bases azotées sont détectables via des réactions immunocytochimiques (Inoki et coll., 1997). Les techniques ISEL utilisent des anticorps anti-biotine conjugués à l'or colloïdal à titre de rapporteur moléculaire (de l'or immunologique pour l'observation en microscopie électronique à transmission). Un des avantages est donc certainement la capacité de visualiser et de compter ces particules d'or par analyse d'images en utilisant le programme informatique « NIH image » de " U.S National Institute of Health ". En résumé, les techniques "ISEL" consistent en:

-l'induction des lésions bris simple-brin à l'ADN par l'exposition des cellules en culture à un agent toxique;

-l'amplification des bris simple-brin à l'ADN à l'aide de l'enzyme exonucléase III qui catalyse l'hydrolyse séquentielle des nucléotides dans le sens 3´→5´ à partir d'une extrémité 3´ OH libre;

-le marquage des régions BSB en utilisant le fragment Klénow de l'ADN polymérase I qui polymérise les nucléotides fournis (incluant la biotine-11-dUTP en remplacement de dTTP) dans le sens 5´→3´;

-la détection immunologique des nucléotides marqués par un anticorps couplé à l'or colloïdal;

-l'observation en microscopie électronique des BSB sur des noyaux en interphase et sur des chromosomes en métaphase;

-le comptage des particules d'or immunologique par μm^2 sur les noyaux ou sur les chromosomes;

-l'analyse statistique des résultats comparés aux contrôles correspondants.

I.1.3.2.2. Échanges entre chromatides-sœurs (ECS), index mitotique (IM) et index prolifératif (IP)

Les échanges entre chromatides-sœurs est un test standard utilisé pour déterminer le potentiel génotoxique d'une substance chimique (Lin et coll., 1994). Le principe du test des ECS est basé sur la réplication semi-conservatrice de l'ADN (Heddle, 1982) après l'exposition des cellules aux agents chimiques dans la phase G_0, de telle façon qu'après la synthèse (Wolff, 1982), l'ADN de chaque chromatide contient toujours un ancien brin de la cellule parentale et un nouveau brin qui incorpore un analogue de la thymidine, le 5 bromo-2-déoxyuridine (BrdU) (Perry et Wolff, 1974) ajouté pendant la synthèse. Les chromatides bi-substituées (après un deuxième ou troisième cycle de division) sont moins condensées à cause de l'incorporation de la molécule du BrdU (contenant du brome qui est un atome volumineux) et, elles sont donc plus susceptibles à la dégradation par photolyse (exposition à la lumière noire et hydrolyse alcaline). Par conséquent, ces régions seront moins colorées par le Giemsa. Cette technique, permet alors de différencier les chromatides bi-substituées (de couleur pâle) de celles mono-substituées (de

couleur foncée).

Les ECS sont comptés sur des chromosomes après le deuxième cycle cellulaire (deuxième génération) en présence du BrdU (les chromosomes arlequins ont l'une des chromatides de couleur pâle et l'autre de couleur foncée). Ainsi sont dénombrés les SCE produits au cours des deux générations précédentes ($F_1 + F_2$). Chaque point où se situe un changement de coloration correspond à un ECS. Les comptages sont effectués par deux examinateurs indépendants, chacun évaluant 50 cellules de deuxième génération dans chaque culture, pour un total de 200 cellules par donneur pour l'analyse des ECS. Les résultats sont analysés statistiquement et comparés aux contrôles correspondants.

L'index mitotique est un indice de la croissance cellulaire. Il indique la fréquence des cellules en division. Il représente le nombre de mitoses rapportées au nombre total de noyaux et de mitoses exprimé en pourcentage. Il se calcule après l'analyse de 1000 cellules par la formule suivante (Choudhury et coll., 2000):

$$IM = \frac{\text{Nombre de cellules en mitose}}{\text{Nombre de cellules en mitose + nombre de noyaux}} \times 100$$

L'index prolifératif est un indice de la fréquence du cycle cellulaire. L'arrêt du cycle cellulaire peut être provoqué par l'induction des ponts ADN-protéines ou par des ponts ADN-ADN irréversibles. Il est mesuré en calculant le pourcentage de cellules ayant complété un cycle (M_1), deux cycles (M_2) ou trois cycles (M_3) de réplication. 100 cellules en métaphase sont analysées à partir de deux échantillons (50 cellules chacun) et le IP est calculé en utilisant la formule suivante (Lamberti et coll., 1983):

$$IP = 1M_1 + 2M_2 + 3M_3 / 100.$$

M_1 = nombre de mitoses en F_1 (première génération); M_2 = nombre de mitoses en F_2 (deuxième génération); M_3 = nombre de mitoses en F_3 (troisième génération).

I.1.4. Rationalité, Hypothèse, Objectifs et plan de travail

I.1.4.1. Rationalité

La toxicité et la carcinogénicité des composés de Ni chez les animaux de laboratoire ont été rapportées (Coogan et coll., 1989; Arsalane et coll., 1994; Nieboer and Nriagu, 1992; Obone et coll.,

1999; Chakrabarti et coll., 1999; Chakrabarti et coll., 2001; Kasprzak et coll., 2003; Denkhaus and Salnikow, 2002; Chakrabarti et coll., 2001; M'Bemba-Meka et Chakrabarti, 2001). Le nickel est aussi connu comme étant mitostatique et clastogène, représentant ainsi un risque génotoxique pour la santé humaine. De plus, les études épidémiologiques et expérimentales ont établi que le nickel est un cancérogène humain (Vyskocil et coll., 1994; Oller et coll., 1997; Antico et Soana, 1999; Wozniak et Blasiak, 2002; Kasprzak et coll., 2003; Denkhaus et Salnikow, 2002; Kawanishi et coll., 2002; ICNCM, 1990). Cependant, les mécanismes menant à de telles toxicités sont encore indéterminés et moins compris. En outre, il a été suggéré que la toxicité des composés de nickel puisse dépendre de leur ligand chimique (qui module leur biodisponibilité et réactivité avec les cibles biochimiques). L'étude comparative de toxicité des différents types de composés du nickel chez les animaux est très limitée jusqu'à présent (Benson et coll., 1992, Dunnick et coll., 1995; M'Bemba-Meka and Chakrabarti, 2001; Chakrabarti et coll., 2001), et est presque inexistante chez l'humain (M'Bemba-Meka et Chakrabarti, 2003).

Il a été également rapporté que l'exposition aux composés de nickel produit des effets hématologiques chez les animaux et les humains. En effet, une augmentation transitoire du niveau des réticulocytes sanguins a été observée chez les ouvriers après

consommation de l'eau contenant du sulfate et du chlorure de nickel (Sunderman et coll., 1988). Bien que l'augmentation du stress oxydatif induit par le chlorure de nickel ait été trouvée dans le plasma (Chen et coll., 2002) et les lymphocytes humains (Chen et coll., 2003), la relation entre le stress oxydatif dû au chlorure de nickel et la toxicité cellulaire/mortalité cellulaire est inconnue.

L'augmentation de la peroxidation des lipides (POL) et la baisse simultanée de l'activité du glutathion peroxydase dans le foie ont été observées dans le foie et le rein des rats traités avec le $NiCl_2$ *in vivo* (Sunderman et coll., 1985; ATSDR, 2002). L'induction de la POL a été inhibée par le traitement des rats avec l'inhibiteur du radical hydroxyle (diméthylthiourea), mais pas avec le superoxide dismutase (SOD) (un inhibiteur d'anion superoxyde) ni avec la catalase (un inhibiteur de peroxyde d'hydrogène) (Athar et coll., 1987; ATSDR, 2002). La mortalité cellulaire et la POL hépatique causées par le $NiCl_2$ ont été corrélées à la diminution du niveau de glutathion (GSH) (Andersen and Andersen, 1989; ATSDR, 2002). Bien que des mécanismes oxydatifs sont impliqués dans le développement de la carcinogenèse du nickel (Kasprzak, 1991; Klein et coll., 1991), les études visant à élucider le rôle des divers types de stress oxydatif dans le développement des lésions cellulaires ou de la mortalité cellulaire induites par le Ni(II) sont encore très limitées. Dans une

autre étude, la mortalité cellulaire et la POL hépatique chez les souris traitées au $NiCl_2$ ont été corrélées à une réduction du niveau de GSH (Kasprzak, 1991). Les études antérieures, dans notre laboratoire, ont montré que la cytotoxicité induite par le $NiCl_2$ dans les cellules rénales du rat n'est pas causée par la POL mais par l'induction de la réaction Fenton, générant le radical hydroxyle (Chakrabarti and Bai, 1999). En étudiant les effets de différents composés de nickel sur le transport de l'ion para-aminohippurate (PAH) à travers les tranches du cortex rénal chez le rat, il a été observé que le mannitol (un modulateur du radical hydroxyle) ou le glutathion a réduit considérablement l'inhibition de la captation du PAH par la forme soluble de sous-sulfure de nickel, alors qu'aucun effet n'a été observé avec l'hydroxyde carbonate de nickel (M'Bemba-Meka et Chakrabarti, 2001). L'exposition *in vitro* des lymphocytes de rat au sous-sulfure de nickel augmente la formation d'espèces radicalaires oxygénées (ERO) et le co-traitement au sous-sulfure de nickel avec le catalase, ou le diméthylthiourea, ou le mannitol, a diminué considérablement la formation des LCAP suggérant ainsi que ces lésions pourraient être causées par la surproduction d'ERO initiée par le sous-sulfure de nickel (Chakrabarti et coll., 2001).

Il a été démontré que le nickel inhibe l'activité de la déshydrogénase succinate mitochondriale dans les cellules

épithéliales du poumon chez le rat (Riley et coll., 2003). Il est établi que le Ni_3S_2 se lie préférentiellement aux mitochondries, à l'appareil de Golgi et aux peroxysomes des cellules pulmonaires, mais se lie faiblement aux mitochondries des lymphocytes (Shirali et coll., 1992). L'existence de plusieurs liaisons entre les protéines et le nickel dans le poumon et les structures intracellulaires du foie a été documentée (Herlant-Peers et coll., 1983). La liaison entre le sous-sulfure de nickel et les structures intracellulaires des lymphocytes humains et ceux du rat, telles que l'euchromatine, les mitochondries et aussi la liaison avec le système de Golgi, a également été rapportée (Hildebrand et coll., 1987). Cependant, on ne sait pas présentement comment la perturbation de l'activité mitochondriale induite par le nickel peut affecter la toxicité cellulaire.

L'homéostasie du calcium cellulaire et les fonctions à médiation calcique sont de plus en plus reconnues comme des cibles sensibles et critiques de l'action des métaux toxiques (Beuters et coll., 1997; Rossi et coll., 1991; Yamagami et coll., 1998). Les métaux sont connus pour leur liaison et interaction avec les récepteurs sur la surface cellulaire, ou avec les protéines intracellulaires contrôlant l'homéostasie du calcium. Quelques études ont rapporté l'effet toxique du nickel avec perturbation de l'homéostasie du calcium. Par exemple, une augmentation des concentrations du calcium dans le

pancréas de souris a été observée suite à l'administration de nickel (Funakoshi et coll., 1996). Malgré la pertinence de cette étude, la forme chimique exacte du nickel responsable de l'effet n'est pas connue. Une libération significative du calcium intracellulaire a été observée après une exposition des cellules épithéliales rénales A6 aux ions Ni^{2+} (Faurskov et Bjerregaard, 2002). Les niveaux du calcium intracellulaire libre sont augmentés dans les cellules traitées au nickel comparés au contrôle (Salnikow et coll., 1999). Cependant, la relation entre l'augmentation de la concentration du calcium intracellulaire causée par le nickel et la toxicité ou la mortalité cellulaire est inconnue. Est-il possible qu'une perturbation de l'homéostasie du calcium due aux composés de nickel puisse jouer un rôle important dans cette toxicité induite par le nickel? Les différents canaux calciques peuvent-ils influencer cette toxicité? Les mécanismes menant à l'augmentation du $[Ca^{2+}]_i$ dans les cellules traitées au nickel ne sont pas aussi connus pour l'instant.

Donc, bien que le stress l'oxydatif soit impliqué vraisemblablement, les rôles du peroxyde d'hydrogène, de l'anion superoxide et du radical hydroxyle dans la mortalité cellulaire induite par les différents composés de nickel n'ont pas complètement été établis. De plus, il n'est pas encore connu si les mitochondries et l'homéostasie du calcium cellulaire sont impliquées dans les lésions

cellulaires induites par le Ni(II) dans les lymphocytes sanguins humains. Aussi, le rôle du calcium intracellulaire dans l'induction des ECS, de l'index prolifératif et mitotique dans les lymphocytes sanguins humains n'est pas présentement connu.

Une augmentation de la fréquence d'aberrations chromosomiques et des échanges entre chromatides-sœurs (ECS) dans les lymphocytes sanguins des personnes exposées au nickel en milieu de travail comparée au groupe contrôle a été rapportée (Werfel et coll., 1998; Myslak et Kosmider, 1997; Elias et coll., 1989; Popp et coll., 1991; Senft et coll., 1992; Deng et coll., 1988; Decheng et coll., 1987; Waksviks et Boysen, 1982; Waksviks et coll., 1984; Perminova et coll., 2001). Ces résultats montrent que les lymphocytes sont des cellules cibles des composés de nickel. Bien que ces études montrent une augmentation d'aberrations chromosomiques et ou des ECS associée à l'exposition professionnelle au nickel, ces études sont incomplètes et renferment peu d'informations sur l'historique de l'exposition des travailleurs, sur les protocoles expérimentaux, la concentration du nickel dans le sang, la toxicité du mélange et les mécanismes d'action de ces métaux combinés. En outre, les travailleurs d'industries du nickel sont généralement exposés à un mélange non seulement des composés de nickel mais aussi de plusieurs autres métaux (Oller et coll., 1997), comme par exemple

dans les usines de raffinerie du nickel on trouve le chrome et le cuivre, dans la soudure on trouve le fer, le manganèse et le chrome. Il est donc difficile dans ces conditions d'estimer clairement les risques génotoxiques associés individuellement à chacun de ces composés. D'autre part, les études d'évaluation du potentiel cytogénotoxique des différentes formes chimiques de nickel sur les lymphocytes sanguins humains sont rares encore présentement. Elles sont réalisées avec une seule forme chimique de nickel soluble dans l'eau soit le chlorure ou le sulfate de nickel (Sahu et coll., 1995; Conway et coll., 1986; Christie et coll., 1991; Larramendy et coll., 1981; Newman et coll., 1982; Wolf, 1980). C'est pourquoi, le choix d'une étude *in vitro* d'analyse du potentiel cytogénotoxique et cytotoxique et des mécanismes d'action toxique sur les lymphocytes a été effectué afin d'évaluer les risques cytotoxiques et génotoxiques des différentes formes chimiques de nickel chez l'humain. Il est donc important de faire avancer nos connaissances sur les risques génotoxiques encourus suite à une exposition au nickel puisqu'il est présent dans plusieurs milieux industriels.

Par conséquent, la présente étude a été entreprise pour (1) évaluer le potentiel cytotoxique et génotoxique du sulfate de nickel (composé soluble dans l'eau), de l'oxyde, du sous-sulfure et de l'hydroxyde carbonate de nickel (composés insolubles dans l'eau), (2)

étudier le rôle des différentes espèces réactives oxygénées, des mitochondries et du calcium intracellulaire en utilisant les lymphocytes humains du sang périphérique.

À la lumière des connaissances actuelles, nous avons utilisé dans le présent travail des lymphocytes sanguins en suspension et en culture. Les lymphocytes humains du sang périphérique sont facilement obtenus après une simple prise de sang. Ils sont utilisés pour effectuer la surveillance biologique auprès des personnes exposées aux agents chimiques en milieu de travail. Dans la circulation sanguine, il existe deux populations distinctes de lymphocytes: les *lymphocytes T*, actifs dans les réactions d'immunité telles que le rejet de greffes et l'activité cytotoxique contre les cellules cancéreuses, et les *lymphocytes B* qui, excités par un antigène étranger, se différencient en cellules productrices d'anticorps. Les deux types sont donc responsables et indispensables à l'ensemble des réactions immunitaires (Berenblum, 1975; Cline et Golde, 1979). Dans les cultures de lymphocytes, les cellules qui répondent par prolifération à la phytohémagglutine (PHA) sont de type T. C'est donc, cette faculté qui est exploitée et étudiée dans les expériences de toxicologie génétique. La cytogénétique permet la compréhension de phénomènes génétiques par l'observation des chromosomes (Siou et coll., 1982). En effet, après exposition des cellules à des substances

chimiques, toute altération décelée à ce stade révèle un bouleversement du comportement cellulaire tant biochimique (par exemple: synthèse d'enzymes modifiés) que morphologique (index mitotique altéré, noyaux endommagés, etc.). La transformation des cellules en culture peut donc servir de modèle à ce qui se passe *in vivo* après un dommage induit à l'ADN (Weinstein et coll., 1979). Le passage des lésions dans les cellules filles peut être détecté et évalué au niveau chromosomique, les aberrations mises en évidence étant le reflet de toutes ces déviations (Evans et O'Riordan, 1975) et aussi un moyen pour évaluer les risques de cancer chez l'humain. De plus, ce modèle *in vitro* exclut les phénomènes de biodisponibilité et de métabolisme et possède par conséquent comme avantage, le contrôle des concentrations des différents composés de nickel en contact avec les lymphocytes, ce qui est rarement le cas lors d'études *in vivo*. L'établissement de la relation dose-effet et temps-effet est alors plus facile à réaliser, car les concentrations des composés de nickel au contact avec les lymphocytes ne sont pas limitées par des phénomènes de transport ou de métabolisme. Le fait de ne pas être limité par la toxicité systémique des différents composés de nickel permet d'étudier leur potentiel génotoxique en utilisant une large gamme de concentrations non cytotoxiques.

Les travailleurs de l'industrie du nickel ont été exposés à des

concentrations de nickel atmosphérique qui varient entre 5 et 15 mg/m^3 dans la raffinerie de Falconbridge en Norvège (Doll, 1990) et entre 70 et 699 mg/m^3 dans la raffinerie hydrométallurgique de Sherritt Gordon à Fort Saskatchewan en Alberta (Egedahl et coll., 1991). Nieboer et collaborateurs (1992) ont également examiné un diagramme de dispersion montrant la relation linéaire entre les concentrations du nickel dans l'atmosphère en milieu de travail et dans le plasma de sept travailleurs exposés au sous-sulfure de nickel dans une industrie de raffinerie de nickel. Ils ont estimé les concentrations plasmiques moyennes de nickel à 60 $\mu g/L$ et 400 $\mu g/L$ correspondant respectivement à des niveaux d'exposition de 15 mg/m^3 et 100 mg/m^3. Dans une étude de Chakrabarti et collaborateurs (2001), les lymphocytes isolés du sang périphérique de rat ont été exposés à une concentration de 2 mM de sous-sulfure de nickel pendant 2 h à 37°C. Ils ont établi que la captation du nickel par les lymphocytes est de 4.38 $\mu g/ml$. Alors, si nous nous basons sur ces données, la captation du nickel par les lymphocytes humains est estimée à 33 et 264 $\mu g/L$ correspondant respectivement à des concentrations d'exposition *in vitro* de 15 et 120 μM de sous-sulfure de nickel pendant 2 h. Ces valeurs sont bien inférieures à l'échelle des concentrations plasmiques de nickel trouvées chez certains travailleurs mentionnés ci-haut. Les concentrations (de 15 à 120 μM) que nous avons utilisées dans nos expériences de génotoxicité se

justifient bien par rapport à ces études.

Pour observer les dommages causés à l'ADN par les composés de nickel, nous avons utilisé la technique de marquage terminal *in situ* en microscopie électronique (EM-ISEL), qui possède la capacité de détecter les terminaux hydroxyles 3´ aux abords des lésions. Nous avons utilisé à cet effet des anticorps anti-biotine conjugués à l'or colloïdal à titre de rapporteur moléculaire (de l'or immunologique pour l'observation en microscopie électronique à transmission). Un des avantages de l'EM-ISEL est certainement de permettre une quantification des particules d'or par un analyseur d'images. Cette technique nous a permis de détecter les bris simple-brin dans la chromatine des chromosomes en métaphase et dans la chromatine des noyaux interphasiques. L'EM-ISEL utilise la polymérase I de l'ADN (ou simplement le fragment Klénow pourvu de l'activité de polymérisation dans le sens 5´ → 3´). D'autres techniques qui marquent les terminaux 3´ OH sont discutées dans la litterature. En effet, il y a la technique "Terminal deoxynucleotidyl Transferase" (TdT) qu'on appelle aussi TUNEL ("TdT-mediated bio-dUTP nick end-labeling"). La grande différence avec l'EM-ISEL, est le fait que l'enzyme TdT employé dans la technique TUNEL, ne requiert pas de matrice ("template") pour transférer les nucléotides en solution. Cette technique a donc la capacité de marquer tous les terminaux hydroxyles 3´ OH qu'ils soient libres ou non (Didenko and Hornsby,

1996; Mundle and Raza, 1995). Par conséquent, cela confère à la technique TUNEL une capacité de marquage légèrement supérieure à celui obtenu avec la technique EM-ISEL (Migheli et coll., 1995; Fujita et coll., 1997). Cependant, la technique TUNEL semble avoir une moins grande facilité à détecter des changements plus subtiles de génotoxicité, en plus d'occasionner un marquage non-spécifique plus grand (Fujita et coll., 1997).

Cette étude contribue à l'avancement de l'état actuel des connaissances dans le domaine des mécanismes cellulaires et moléculaires de l'action toxique du nickel. Elle permettra le développement de bioindicateurs de la cancérogénicité du nickel en utilisant des lymphocytes sanguins circulants pour la prévention des dommages génotoxiques chez les personnes exposées en milieu de travail.

I.1.4.2. L'Hypothèse de notre travail

Les différentes formes chimiques de nickel pourraient causer de façon différentielle des dommages génotoxiques et de la mortalité des lymphocytes humains du sang périphérique.

I.1.4.3. Les Objectifs de notre recherche et plan de travail

Le but de notre recherche est d'évaluer, après exposition *in vitro* des lymphocytes humains du sang périphérique au sous-sulfure, à l'hydroxyde carbonate, à l'oxyde et au sulfate de nickel, les risques potentiels de cytotoxicité et de génotoxicité de ces composés chez l'humain.

Nos protocoles expérimentaux ont été approuvés par le Comité d'Éthique sur la Recherche Humaine de l'Université Montréal. Le sang entier humain a été obtenu de 4 donneurs sains avec l'assistance de l'Hôpital Sainte-Justine. Les donneurs âgés entre 27 et 42 ans étaient des non-fumeurs sains qui n'ont pas été exposés préalablement à des radiations, aux composés de métaux lourds, à la drogue, ou à une thérapie médicamenteuse et qui n'ont pas pris d'alcool pendant au moins deux jours avant la prise du sang. Les produits que nous avons utilisés dans les expériences des mécanismes de toxicité du nickel sont des principes actifs pharmaceutiques utilisés en clinique pour soigner les patients.

Nous avons effectué cette recherche en l'étayant sur des objectifs spécifiques qui sont décrits dans les chapitres I et II.

Dans le chapitre I, nous avons d'abord établi les conditions optimales d'exposition *in vitro* pour les lymphocytes humains isolés du sang périphérique. Nous avons par la suite exposés les cellules en suspension à l'hydroxyde carbonate (0-1 mM), au sous-sulfure (0-2 mM) et au sulfate de nickel (0-4 mM) à différents temps (0-4 h), afin de caractériser le potentiel cytotoxique (la nécrose cellulaire) de ces trois composés. Ce potentiel a été vérifié en mesurant la mortalité des lymphocytes par le test d'exclusion au bleu de Trypan. Ensuite, nous avons étudié les différents mécanismes cellulaires possiblement impliqués dans l'induction de la mortalité des lymphocytes. Nous avons alors exposé les cellules à ces trois composés et nous avons mesuré en temps-dépendant la surproduction des différentes ERO (l'anion superoxyde (O_2^-), le peroxyde d'hydrogène (H_2O_2)), la déplétion des thiols (les groupements sulfhydryles liés (SH-P) et non liés (SH-NP) aux protéines) et l'induction de la peroxydation des lipides. Pour valider ces mécanismes, nous avons pré-traité les cellules (avant de les exposer à nouveau à ces composés) avec les piégeurs de radicaux libres spécifiques à chaque type (la catalase (1000-2000 U/ml) (un piégeur de peroxyde d'hydrogène), la superoxyde dismutase (2500-5000 U/ml) (un piégeur de l'anion superoxyde), le diméthylthiourea (10-20 mM) et le mannitol (25-50 mM) (des piégeurs de radical hydroxyle), le glutathion (4-8 mM) et la *N*-acétylcystéïne (4-8 mM) (des antioxydants) et la deferoxamine (100

μM) (un chélateur du fer)). Dans d'autres expériences, nous avons exposé les cellules et mesuré à temps-dépendant la perte de l'imperméabilité des pores mitochondriaux. Ceci a été validé par un co-traitement des cellules avec les composés de nickel et la cyclosporine A (2 μM) (le modulateur de l'intégrité de la membrane mitochondriale). Finalement, nous avons exposé les cellules à ces trois composés pour étudier en temps-dépendant la déstabilisation de l'homéostasie intracellulaire du calcium. Pour valider ce dernier mécanisme, les cellules ont subi un traitement simultané avec les trois composés de nickel et les inhibiteurs de l'afflux du calcium (le verapamil (12-25 μM), le nifedipine (12-25 μM), le diltiazem (50 μM), le dantrolene (25-50 μM), le ruthénium rouge (2-5 μM)) et aussi le chélateur du calcium (BAPTA (3-6 mM)). Les résultats de ces objectifs sont rapportés dans les articles 1, 2 et 3:

Article 1 Rôle du stress oxydatif, des mitochondries et de l'homéostasie du calcium dans l'induction *in vitro* de la mortalité des lymphocytes humains par l'hydroxyde carbonate de nickel.

"Role of oxidative stress, mitochondrial permeability transition, and calcium homeostasis in human lymphocyte death induced by nickel carbonate hydroxide

in vitro."

Article 2 Rôle du stress oxydatif, des mitochondries et du calcium intracellulaire dans l'induction *in vitro* de la mortalité des lymphocytes humains par le sous-sulfure de nickel.

"Role of oxidative stress, mitochondrial permeability transition, and calcium homeostasis in nickel subsulfide-induced human lymphocyte death *in vitro*."

Article 3 Rôle du stress oxydatif, des mitochondries et du calcium intracellulaire dans l'induction *in vitro* de la mortalité des lymphocytes humains par le sulfate de nickel.

"Role of oxidative stress, mitochondrial permeability transition, and calcium homeostasis in nickel sulfate-induced human lymphocyte death *in vitro*."

Dans le chapitre II, nous avons d'abord établi les conditions optimales pour l'obtention des chromosomes en culture. Ensuite, nous avons exposé les cellules à l'hydroxyde carbonate (0-60 μM), au sous-sulfure (0-120 μM), à l'oxyde (0-120 μM) et au sulfate de nickel (0-120 μM) pendant 2 h pour évaluer le potentiel génotoxique de ces

composés en utilisant la technique EM-ISEL (pour identifier et quantifier les bris simple-brin sur la chromatine), le test des ECS et celui des index mitotique et prolifératif. Comme l'hydroxyde carbonate de nickel s'est montré le plus génotoxique, nous l'avons utilisé comme modèle pour étudier les mécanismes moléculaires d'induction des dommages génétiques. Nous avons donc examiné le rôle de l'ERO en pré-traitant les cellules avant de les exposées à l'hydroxyde carbonate de nickel (15-30 μM), avec les inhibiteurs du stress oxydatif (la catalase (1000 U/ml), la superoxyde dismutase (2500-5000 U/ml), le diméthylthiourea (20 mM), la *N*-acétylcystéïne (8 mM) et la deferoxamine (100 μM)). Finalement, nous avons étudié la possibilité que le calcium soit impliqué dans ces mécanismes. Pour ce faire, les cellules ont subi un traitement simultané avec l'hydroxyde carbonate de nickel (15-30 μM) et les inhibiteurs de l'afflux du calcium (le verapamil (25 μM), le dantrolene (50 μM)) et le chélateur du calcium (BAPTA (3 mM)). Les résultats de ces objectifs font l'objet des articles 4 et 5 suivants:

Article 4 Rôle du stress oxydatif et du calcium intracellulaire dans l'induction *in vitro* des bris simple-brin à l'ADN sur la chromatine chromosomique et nucléaire des lymphocytes sanguins humains par les composés de nickel en utilisant la technique du marquage terminal *in situ* en microscopie

électronique.

"Role of oxidative stress and intracellular calcium in nickel compound-induced DNA single-strand breaks in chromosomal and nuclear chromatin in human blood lymphocytes *in vitro* using electron microscopy *in situ* end-labeling."

Article 5 Rôle du stress oxydatif et du calcium intracellulaire dans l'induction *in vitro* des échanges entre chromatids-sœurs, de l'index prolifératif et mitotique par les composés de nickel sur les lymphocytes humains du sang périphérique en culture.

"Role of oxidative stress and intracellular calcium in nickel compound-induced sister-chromatid exchanges, replication index and mitotic index in human peripheral blood lymphocytes in culture."

II. CHAPITRE I: Mécanismes cellulaires de la mortalité des lymphocytes humains induite par les composés de nickel.

Role of oxidative stress, mitochondrial permeability transition, and calcium homeostasis in human lymphocyte death induced by nickel carbonate hydroxide *in vitro*.*[1]

Role of oxidative stress, mitochondrial permeability transition, and calcium homeostasis in nickel subsulfide-induced human lymphocyte death *in vitro*. *[1]

Role of oxidative stress, mitochondrial permeability transition, and calcium homeostasis in nickel sulfate-induced human lymphocyte death *in vitro*. *[1]

*Cette recherche a été présentée en partie lors du 42me Congrès Annuel de la Societé Américaine de Toxicologie à Salt Lake City, UT, du 9 au13 mars 2003.

[1]Articles soumis pour publication.

II.1. ARTICLE I

Role of oxidative stress, mitochondrial permeability transition, and calcium homeostasis in human lymphocyte death induced by nickel carbonate hydroxide in vitro.

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Abstract

When isolated human lymphocytes were treated *in vitro* with various concentrations of soluble forms of nickel carbonate hydroxide (NiCH) (0-1 mM), at 37°C for 4 h, both concentration-and time-dependent effects of NiCH on lymphocyte death were observed. Increased generation of hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), depletion of both nonprotein (NP-) and protein (P-) sulfhydryl (SH) contents and lipid peroxidation (LPO) were induced by NiCH. Pretreatment of lymphocytes with either catalase (H_2O_2 scavenger), or deferoxamine (DFO) (iron chelator), or excess glutathione (GSH) (an antioxidant) not only significantly reduced the NiCH-induced generation of H_2O_2 and LPO, but also increased the NP-SH and P-SH contents initially reduced by NiCH. NiCH-induced generation of excess O_2^- but not excess LPO was significantly reduced by pretreatment with superoxide dismutase (SOD). NiCH-induced lymphocyte death was significantly prevented by pre-treatment with either catalase, or dimethylthiourea/mannitol (hydroxyl radical scavengers), or DFO, or excess GSH/N-acetylcysteine. NiCH-induced lymphocyte death was also significantly prevented by pretreatment with excess SOD. Thus, various types of oxidative stress play important role in NiCH-induced lymphocyte death. Cotreatment with cyclosporin A (a specific inhibitor of mitochondrial permeability

transition (MPT)) not only inhibited NiCH-induced MPT, but also significantly prevented Ni-compound-induced lymphocyte death. Furthermore, NiCH-induced destabilization of cellular calcium homeostasis. As such, NiCH-induced lymphocyte death was significantly prevented by modulating intracellular calcium fluxes using both Ca^{2+} channel blockers and intracellular Ca^{2+} antagonist. Thus, the mechanism of different NiCH (soluble form)-induced activation of lymphocyte death signalling pathways involves not only the excess generation of different types of oxidative stress, but also the induction of MPT and destabilization of cellular calcium homeostasis as well.

Key words: Human lymphocytes; lymphocyte death; oxidative stress; nickel carbonate hydroxide; mitochondrial permeability transition; calcium homeostasis.

Introduction

Occupational exposure to nickel compounds occurs principally through mining, smelting, refining operations, alloy production, electroplating and welding operations, during manufacture of steel and other alloys and batteries. In 1990, the International Committee on Nickel Carcinogenesis in Man suggested that respiratory cancer

risks are primarily related to exposure to soluble nickel concentrations above 1mg/m^3 and to exposure to less soluble forms at concentrations above 10 mg/m^3 (IARC, 1990). Nickel carbonate hydroxide is used in nickel plating, in colours and glazes for ceramics and in high pure form it is used in electronic components. Thus, this nickel compound possesses potentials risks to occupational and environmental health.

Epidemiological studies have confirmed that water-insoluble Ni-compouds such as crystalline NiS and NiO are suspected agent for the induction of human lung and nasal cancers (IARC, 1990). The increased frequencies of chromosomal aberrations and sister-chromatid exchanges (SCEs) (compared to the control group) in the peripheral lymphocytes of workers occupationally exposed to nickel have been reported in many studies (Werfel et al., 1998; Myslak and Kosmider, 1997; Elias et al., 1989; Popp et al., 1991; Senft et al., 1992; Deng et al., 1988; Decheng et al., 1987; Waksviks and Boysen, 1982; Waksviks et al., 1984; Perminova et al., 2001). These results have shown that the lymphocytes are also the target cells for nickel compounds. Although the majority of these studies have shown an increased incidence of chromosomal aberrations, or of SCEs associated with occupational exposure to nickel, these reports did not provide enough details about exposure history of workers,

experimental protocols and blood concentrations of nickel. Furthermore, in most cases the workers in nickel industries are generally exposed to a number of other metals as well (e.g. chromium in electroplating refinery, copper and other metals in a nickel refinery, and iron, manganese and chromium in welding operations) in addition to nickel. Since the exposure in nickel industrial environments is always mixed, it is difficult to clearly estimate various toxic risks associated with individual nickel compounds whether water-soluble or water-insoluble forms (Oller et al., 1997).

The toxicity and carcinogenicity of Ni-compounds including the oxidative mechanism in experimental animals (Coogan et al, 1989; Arsalane et al, 1994; Nieboer and Nriagu, 1992; Obone et al, 1999; Chakrabarti et al, 1999; Chakrabarti et al, 2001; review by Kasprzak et al, 2003; review by Denkhaus and Salnikow, 2002; Kawanishi et al., 2002; Chakrabarti et al, 2001; M'Bemba-Meka and Chakrabarti, 2001) and humans (Vyskocil et al, 1994; Oller et al, 1997; Antico and Soana, 1999; Wozniak and Blasiak, 2002; Kasprzak et al, 2003) have been well established. However, the mechanisms underlying such toxicities involving insoluble Ni-compounds are still not completely defined and hence poorly understood. The toxicity of Ni-compounds is believed to be dependent on their chemical ligand (speciation) which modulates their bioavailability and reactivity with biochemical targets.

As such, mechanistic toxicity studies of water-insoluble Ni-compounds such as NiCH in animals are very limited at present (Arsalane et al., 1992, 1994; M'Bemba-Meka and Chakrabarti, 2001). Besides, toxicity studies including the mechanism of NiCH in humans are hardly available. A transient increase in the level of blood reticulocytes was reported among workers after consuming water containing nickel sulfate and nickel chloride (Sunderman et al, 1988). Although $NiCl_2$-induced increase in oxidative stress has been observed in human plasma (Chen et al, 2002) and lymphocytes (Chen et al, 2003), how such oxidative stress relates to cellular toxicity/cell death due to $NiCl_2$ is not known.

Although oxidative mechanism(s) may be involved in Ni-carcinogenesis (Kawanishi et al., 2002; Kasprzak, 1991; Klein et al, 1991), studies involving the roles of various types of oxidative stress in the development of NiCH-induced cellular injury or cell death (cytotoxicity) are at present very limited and hardly available in human lymphocytes which are also considered as target cells for Ni-compounds.

Nickel has been shown to inhibit mitochondrial succinate dehydrogenase activity in rat lung epithelial cells (Riley et al, 2003). Ni_3S_2 derived particles were found to be bound preferentially, though

less markedly than lymphocytes, to mitochondria, Golgi apparatus, and peroxisomes from lung cells (Shirali et al, 1992). The existence of several nickel-binding proteins in lung and liver subcellular fractions has been demonstrated (Herlant-Peers et al, 1983). Similarly, the binding of nickel sulfides to both rat and human lymphocyte subcellular structures, such as the cell membranes, euchromatin, mitochondria, and Golgi system has also been reported (Hildebrand et al, 1987). However, the information how such Ni-induced disturbances in mitochondria could affect Ni-induced cellular toxicity or cell death is presently unavailable.

Cellular calcium homeostasis and calcium-mediated functions are being increasingly recognized as sensitive and critical targets for the action of toxic metals (Beuters et al, 1997; Rossi et al, 1991; Yamagami et al, 1998). Metals are known to bind to and interact with receptor proteins on the cell surface, with ion channel proteins, or with intracellular proteins controlling Ca^{2+} homeostasis. Very few studies have been reported relating to the toxic effect of nickel with disturbance in calcium homeostasis. For example, an increase in calcium concentrations in mice pancreas has been observed following nickel administration (Funakoshi et al, 1996). Ni^{2+}-treated renal epithelial A6 cells have been shown to release significantly $[Ca^{2+}]_i$ (Faurskov and Bjerregaard, 2002). Levels of free intracellular calcium

$[Ca^{2+}]_i$ were increased in nickel-treated cells (Salnikow et al, 1999). However, how such increase in intracellular calcium concentration $[Ca^{2+}]_i$ relates to Ni-induced toxicity or cell death is not known. In other words, is it possible that a disturbance in calcium homeostasis due to nickel compound could play an important role in Ni-induced cytotoxicity or lymphocyte death? Furthermore, the mechanism leading to the elevation of $[Ca^{2+}]_i$ in nickel-treated cells is also presently not known. Besides, the possibility whether different calcium channels could influence nickel-induced toxicity or cell death is yet to be verified.

Thus, although the oxidative stress is likely involved, the role of the oxidative stress involving hydrogen peroxide, superoxide anion (O_2^-) and hydroxyl radical in cell death induced by NiCH have not yet been established. Furthermore, it is not known yet whether the mitochondrial permeability transition and cellular calcium homeostasis could also be involved in such Ni(II)-induced cellular injury or cell degeneration. Therefore, the present investigation was carried out to study the *in vitro* cytotoxic action of soluble form of NiCH in human lymphocytes and its ability to induce different types of oxidative stress, changes in intracellular Ca^{2+} homeostasis and mitochondrial permeability transition in isolated human lymphocytes. Hence, the roles of oxidative stress, mitochondrial permeability

transition and intracellular Ca^{2+} homeostasis in NiCH-induced lymphocyte death has been examined and the results are presented in this paper. For developing cell death in a short duration of exposure (3-4 h) using human lymphocytes in suspension, we had to use higher concentrations (0-1 mM) of nickel carbonate hydroxide; the principal objective here was to study the mechanisms of such cell death.

Materials and methods

Materials

Nickel carbonate hydroxide tetrahydrate ($2NiCO_3.3Ni(OH)_2.4H_2O$, green) (NiCH) was obtained from Sigma–Aldrich Canada Ltd, Ont., Canada. All other chemicals and reagents were obtained from Sigma, unless otherwise mentioned. NiCH are insoluble in water or physiological saline. Therefore, it was dissolved in 0.05 M Tris-HCl buffer, pH 7.4, by incubation at 37° C for 12h with shaking using the method of Lee et al. (1982). Substantial amount of NiCH was dissolved, leading to soluble nickel concentrations of 10-16 mM. The concentration of nickel was determined by electrothermal atomic absorption spectrometry.

The protocols described below were approved by the Ethics

Committee on Human Research, Université de Montréal.

Measurement of cell viability in isolated human lymphocytes

Human whole blood was obtained from 4 healthy donors provided by Sainte-Justine Hospital, Montréal. Peripheral blood samples from 4 healthy non-smoking donors between ages 27 and 42 and who were not previously exposed to any radiation or heavy metal compounds or drug therapy and who did not take any alcohol at least two days before this experiment were used in the present experiments. Questionnaires were obtained from each blood donor to evaluate exposure history. Methodology for the handling of human blood was followed according to the strict guidelines as devised for clinical workers at Sainte-Justine Hospital, Montréal. Human peripheral blood was collected in sodium-heparinized vacutainers. Separation of peripheral blood lymphocytes was carried out under sterile conditions on Ficoll-Paque (Pharmacia) gradients by the method of Boyum (1976). Aliquots of the heparinized whole blood diluted with an equal volume of ice-cold physiological saline (1:1) were gently applied on an equal volume of Ficoll-Paque in centrifuge tubes. The samples were then centrifuged at 400 g for 30 min. The resultant interface (buffy coat) was then carefully aspirated from the gradient, washed twice in ice-cold Dulbecco's phosphate-buffered saline by centrifugation at 200 g for 10 min. The subsequent pellet of purified

lymphocytes was finally resuspended in RPMI 1640 medium and adjusted to the desired lymphocyte concentration (1 to $5x10^6$ cells per ml) in RPMI 1640 medium (pH 7.4). This range of cell concentrations was necessary depending on the type of study protocol used. The approximate yield of lymphocytes was determined by counting the cells on a hemocytometer. The viability of the cells was determined by trypan blue dye exclusion.

Determination of NiCH-induced human lymphocyte death

The stock solution of nickel carbonate hydroxide (NiCH) was diluted to the appropriate concentrations using RPMI 1640 medium. Isolated human lymphocytes were exposed for 0-4 h at 37°C to either control, or $2NiCO_3.3Ni(OH)_2$ (0-1 mM) in a humidified atmosphere of 95% oxygen and 5% carbon dioxide. At the end of such exposure, lymphocytes from each group were washed twice with RPMI 1640 medium. An equal volume of 0.4% trypan blue dye reagent was then added to the lymphocyte suspension and the percentage of dead lymphocytes was determined under a field microscope, using trypan blue exclusion. To determine the time-dependent effect of NiCH-induced lymphocyte death, isolated human lymphocytes were exposed at 37°C NiCH (0.75 mM), or control at different times, e.g. 30, 60, 90, 120, 180 and 240 min followed by measurement of lymphocyte death by trypan blue dye exclusion at each time.

Measurements of NiCH-induced generation of reactive oxygen species/oxidative stress

The production of hydrogen peroxide (H_2O_2) in control and Ni-treated lymphocytes was assayed following a colorimetric method developed by Graf and Penniston (1980). The method is based on the oxidation of iodide in the presence of ammonium molybdate and photometry of the resulting blue starch-iodine complex (which is stable for several hours) was carried out at 570 nm. The concentration of H_2O_2 was finally estimated from a standard curve. The formation of cellular superoxide anion (O_2^-) was determined by the nitro blue tetrazolium (NBT) reduction assay as described by Rauen et al., (2000) with minor modifications. NBT was added to RPMI 1640 medium (pH 7.4) in a final concentration of 1 mg/ml and lymphocytes were incubated in this medium for 0-3 h at 37°C without and with nickel carbonate hydroxide (0.75 mM). At the end of the incubation period, lymphocytes were separated and carefully washed with Hanks' Balanced Salt Solution and then lysed at 37°C with 5% sodium dodecyl sulfate in phosphate buffer (80 mM, pH 7.8) containing 0.45% gelatin. The samples were centrifuged for 5 min at 13,000 g. The absorbance due to formazan at 540 nm was determined against a lysis buffer blank immediately and within 2 min after such formation. The concentration of superoxide anion (O_2^-) was evaluated following the method of Rauen et al., (2000). Lipid peroxidation was

determined indirectly by measuring the production of malondialdehyde (MDA) followed by its reaction with thiobarbituric acid as described by Schnellmann (1988). Briefly, aliquots of the cell suspension (control and Ni-treated) corresponding to 1 x 10^6 lymphocytes (0.5 ml) were deproteinized with 0.5 ml TCA (10%) and centrifuged. The supernatant (0.9 ml) was then mixed with 0.5 ml 2-thiobarbituric acid reagent (0.76%), heated in a boiling waterbath for 10 min, and allowed to cool. Sample absorbance was then measured at 535 nm. Protein content was estimated by the method of Bradford, using bovine serum albumin as a standard (Bradford, 1976).

To evaluate the effects of various scavengers of reactive oxygen species (ROS), or oxidative stress on Ni-compound-induced generation of ROS/oxidative stress, isolated human lymphocytes were first pretreated for 15 min with either catalase (0, 1,000-2,000 U/ml) (scavenger of H_2O_2), or superoxide dismutase (0, 2,500-5,000 U/ml) (scavenger of superoxide anion, O_2^-), or dimethylthiourea (DMTU) (0-20 mM) (scavenger of hydroxyl radical), or deferoxamine (DFO) (0-100 μM) (scavenger of iron-mediated oxidative damage), or glutathione (GSH) (0-8 mM) (scavenger of oxidative stress) before treatment of lymphocytes with 0.75 mM NiCH and the generation of H_2O_2, or O_2^-, or lipid peroxidation (LPO) was then measured as mentioned above after different periods of such treatment, e.g. 0, 10, 15, 30, 60, 120

and 180 min at 37°C.

Effects of scavengers of reactive oxygen species on NiCH-induced lymphocyte death

In order to study the involvement of reactive oxygen species in the induction of Ni-compound-induced lymphocyte death, scavengers of superoxide anion (O_2^-) such as superoxide dismutase (SOD) (0, 2,500-5,000 U/ml), scavengers of H_2O_2 such as catalase (0, 1,000-2,000 U/ml) and scavengers of hydroxyl radical such as dimethyl thiourea (DMTU) (0-20 mM) and mannitol (0-50 mM), glutathione (GSH) (0-8 mM) and N-acetylcysteine (NAC) (0-8 mM) (scavengers of ROS) were first separately incubated for 15 min before treatment human lymphocytes with 0.75 mM NiCH for 3 h at 37°C. The lymphocyte death was determined as described above. To determine the iron-mediated oxidative damage in Ni-compound-induced lymphocyte death, deferoxamine (DFO) (0-100 μM) was incubated either alone or pretreated for 15 min before incubation with NiCH for 3 h at 37°C and the lymphocyte death was determined as mentioned above.

Effect of NiCH on total, nonprotein, and protein-bound sulfhydryl contents

Both protein-bound (P-SH) and nonprotein sulfhydryl group

(NP-SH) contents in lymphocytes were determined using the method of Sedlak and Lindsay (1968). Determination of total sulfhydryl (T-SH) contents: Briefly, aliquots of 0.25 ml of the cell suspension (Ni-treated) were mixed with 0.75 ml of 0.2 M Tris buffer, pH 8.2, and 0.05 ml of 0.01 M 5,5'-dithiobis-(2-nitrobenzoic acid (DTNB). A reagent blank (without sample) and a sample blank (without DTNB) were prepared in a similar manner. The test tubes after stoppered with rubber caps were immediately agitated and the reaction mixtures centrifuged at approximately 3,000 g at room temperature for 15 min. The absorbance of the supernatants was read in a Beckman model DU-7 Spectrophotometer at 412 nm. Determination of NP-SH groups: Aliquots of 0.5 ml of the lymphocyte suspension, (control and Ni-treated) were mixed in 15 ml test tubes with 0.4 ml of distilled water and 0.1 ml of 50% trichloroacetic acid (TCA). The tubes were immediately agitated and centrifuged at 3,000 g at room temperature for 15 min. 0.5 ml of the supernatant was mixed with 1 ml of 0.4 M Tris buffer, pH 8.9, and 0.025 ml DTNB and the sample was shaken. The absorbance was read within 5 min of the addition of DTNB at 412 nm against a reagent blank. The contents of different sulfhydryl groups were quantified by comparison of the results from a standard curve. The content of PB-SH is calculated by subtracting the content of NP-SH from that of T-SH.

Determination of the mitochondrial permeability transition (MPT)

Rhodamine 123, a cationic fluorescent dye whose mitochondrial fluorescence intensity decreases quantitatively in response to dissipation of the mitochondrial membrane potential, was used to evaluate perturbations in mitochondrial membrane potential (Wu et al. (1990). Both control and Ni-treated lymphocytes as described above were incubated at 37°C with 1.5 μM rhodamine 123 in an incubator for 10 min with gentle shaking, followed by washing the lymphocytes with the culture medium. Thereafter, lymphocytes were suspended in culture media prior to fluorescence measurement with excitation at 493 nm and emission at 522 nm using a Perkin-Elmer MPF 3 L spectrofluorimeter.

To determine the effect of cyclosporin A on MPT, lymphocytes were cotreated at 37°C for 3 h with 2 and 5 μM cyclosporin A (specific inhibitor of MPT, and calcineurin and Ca^{2+} release from mitochondria) and 0.75 mM NiCH, or without Ni-compound (control). First, lymphocyte death was estimated as described above, and immediately thereafter incubated at 37°C with 1.5 μM rhodamine 123 as described above. The uptake of rhodamine in both control and Ni-treated lymphocytes was measured fluorometrically as described above.

Measurement of intracellular Ca^{2+}, $[Ca^{2+}]_i$

At first isolated human lymphocytes were treated at 37°C for 0-2 h with 0.75 mM NiCH, as described above. The lymphocytes were then washed from their original medium with Hanks' Balanced Salts Solution (HBSS) (137 mM NaCl, 5.4 mM KCl, 8 mM $MgSO_4$, 0.4 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$ and 15 mM glucose) and resuspended in oxygenated (95% O_2, 5% CO_2) HBSS supplemented with 1% of FCS (loading medium) and incubated with 5 μM Fura-2 AM at 37°C for 30 min. Then the cells were centrifuged for 5 min at 1800 rpm, washed twice with HBSS, and resuspended in the HBSS at a concentration of 1.5 x 10^6 cells/ml. The cells were transferred to a cuvette in a Perkin-Elmer MPF 3 L spectrofluorometer. Changes in $[Ca^{2+}]_i$ were evaluated at 25°C by measuring the intensity of the fluorescence emission at 500 nm with the excitation at 336 nm and 366 nm as described by Grynkiewicz et al. (1985). The Fura-2 AM fluorescence ratio signal was calibrated in terms of $[Ca^{2+}]_i$ as described by Grynkiewicz et al. (1985) and according to the following equation: $[Ca^{2+}]_i = K_d(R-R_{min})/(R_{max}-R)(F_o/F_s)$ where K_d is 184 nM (a value for the Fura-2 AM-Ca^{2+} complex), R the ratio 336/366 nm of fluorescence of the indicator, R_{min} the ratio 336/366 nm of Fura-2 AM in the Ca^{2+}-free HBSS, R_{max} the ratio of Fura-2 AM in the presence of saturating Ca^{2+} concentration (1 mM $CaCl_2$) and F_o/F_s the ratio of 366 nm excitation fluorescence at zero and saturating Ca^{2+} levels.

To determine the role of intracellular calcium, $[Ca^{2+}]_i$ in NiCH-induced human lymphocyte death, isolated lymphocytes were treated at 37°C for 3 h with NiCH (0.75 mM) alone, or simultaneously with each of the following calcium channel blockers/antagonist such as verapamil (12 and 25 μM), nifedipine (12 and 25 μM), diltiazem (50 μM), ruthenium red (inhibitor of Ca^{2+} uptake by mitochondria) (2 and 5 μM), dantrolene (inhibitor of Ca^{2+} release from sacroplasmic reticulum) (25 and 50 μM), cyclosporin A (inhibitor of Ca^{2+} release from mitochondria) (2 μM) and BAPTA (Ca^{2+} chelator) (3 and 6 mM). At the end of the incubation period, the lymphocyte death was estimated in control and various treated groups as described above. Such concentrations of different modulators of $[Ca^{2+}]_i$ used in this study were found to be non-toxic. To study the effect of Ca^{2+} channel blocker (diltiazem), or calcium ion antagonist (ruthenium red) on $[Ca^{2+}]_i$, human lymphocytes were treated at 37°C for different periods of time (15, 30, 60 and 120 min) with NiCH alone at fixed concentration, or simultaneously with either diltiazem (50 μM), or ruthenium red (2 μM). At the end of each incubation period, the $[Ca^{2+}]_i$ was determined as described above.

Data analysis

The data are presented as the mean ± SEM for four or five separate experiments. Data were analysed by analysis of variance. Difference

between treatment means was tested by Tukey-Kramer multiple comparisons test. For time-dependent effects, the treatment means were compared with corresponding control including a zero-time control value. The level of significance was set at $p < 0.05$.

Results

Concentration- and time-dependent effects of NiCH on human lymphocyte death *in vitro*.

Freshly isolated human lymphocytes were treated *in vitro* with various concentrations of soluble forms of NiCH (0-1 mM), at 37°C for 4 hours. The results of such concentration-dependent effects on human lymphocyte death (as measured by the trypan blue exclusion) are presented in Fig. 1A. It is seen that the concentrations of NiCH required for significant minimum and maximum (100%) lymphocyte death were found to be 0.5 and 1mM. NiCH has shown also the time-dependent effect on human lymphocyte death as shown in Fig. 1B.

Effects of NiCH on the generation of reactive oxygen species (ROS), or oxidative stress.

The excess generation of H_2O_2 due to exposure of human lymphocytes to soluble form of nickel carbonate hydroxide (0.75 mM) at different time periods (0-3 h) has been presented in Fig. 2.

Significant generation of H_2O_2 due to the Ni-compound started after 15 min of such exposure and attained a maximum value after 60 min of such exposure followed by gradual diminution but still significantly high during later time periods. The time-dependent effect of exposure of human lymphocytes to 0.75 mM NiCH on the excess generation of superoxide anion (O_2^-) as measured by NBT reduction is shown in Fig. 3. It is seen that NiCH was capable of generating significant amounts of superoxide anion compared to the control starting after 60 min of such exposure. Since the generation of ROS is often accompanied by a change in the thiol status, we also determined the effects of Ni-compounds on lymphocyte nonprotein (NP)- and protein-sulfhydryl (P-SH) contents. Significant time-dependent diminution of both nonprotein- and protein sulfhydryl contents has been observed starting after 15 min of exposure to nickel carbonate hydroxyde (0.75 mM). Such diminution of NP-SH reached a maximum value of about 3% of the control due to NiCH following 60-180 min of exposure of human lymphocytes to the Ni-compound (Fig. 4). Similarly, a maximum decrease of about 10-12% of the control value for protein-SH (P-SH) contents was observed following similar exposure (Fig. 4).

Time-dependent effect of the Ni-compound on the production of lipid peroxidation (LPO) (as measured by malondialdehyde (MDA)

formation) in human lymphocytes are shown in Fig. 5. Significant elevation of LPO started after 30 min of exposure to NiCH and increased to a maximum at 60 min of such exposure followed by gradual diminution of such increase in LPO but still significantly high even after 120 and 180 min of such exposure.

Effects of ROS scavengers and antioxidants on the formation of Ni-compound-induced various oxidative stress.

To determine whether the increase of LPO induced by Ni-compound was associated with the generation of ROS, the effects of scavengers of ROS and other antioxidants on the formation of LPO were studied. It is seen that catalase, a H_2O_2 scavenger, attenuated the production of LPO (Fig. 5). Since acute treatment with Ni-compound caused a decrease in cellular NP-SH contents in our present study, we have therefore examined the effects of adding excess GSH on the generation of LPO in human lymphocytes induced by Ni-compound. It is seen that excess GSH prevented significantly the production of LPO induced by NiCH (Fig. 5). Similarly NiCH-induced increase in oxidative stress as measured by production of LPO, was significantly reduced by DMTU (Fig. 5). Thus, Ni^{2+} causes an increase in intracellular ROS which have the potential to develop oxidative damage to the isolated human lymphocytes. Similarly, Ni^{2+}-induced increase in oxidative stress as measured by LPO was

significantly reduced by pretreatment with DFO which was used to block iron-mediated LPO (Fig. 5). However, SOD failed to inhibit LPO production induced by NiCH (Fig. 5).

The effects of ROS scavengers on Ni-compound-induced generation of H_2O_2 (as measured by blue colored complex formation), depletion of NP-SH content and superoxide anion (O_2^-) as measured by NBT reduction were also examined and the results are shown in Figs. 2, 3 and 4 respectively. It is seen that pre-treatment of lymphocytes with either catalase (a H_2O_2 scavenger), or deferoxamine (DFO), or excess GSH significantly reduced the NiCH-induced generation of excess H_2O_2 (Fig. 2). Similarly, NiCH-induced generation of excess superoxide anion was significantly reduced by pre-treatment with either SOD, or DFO (Fig. 3). Pretreatment with either excess GSH, or catalase, or DFO significantly increased the NP-SH contents initially reduced by nickel carbonate hydroxide (Fig. 4).

Effects of ROS scavengers and antioxidants on NiCH-induced human lymphocyte death

To examine the role of ROS on Ni-compound-induced human lymphocyte death, we have evaluated the effects of various ROS antagonists on such lymphocyte death. Since significant depletion of nonprotein sulfhydryl content (the reductive reserve) occurred much

earlier than the lymphocyte death due to NiCH, we have therefore examined whether this was associated with Ni-induced lymphocyte death. It is seen that pre-treatment of lymphocytes for 15 min with excess glutathione (GSH) significantly reduced such cell death induced by NiCH (Fig. 6). Similarly, pretreatment of lymphocytes with excess NAC significantly reduced Ni-compound-induced lymphocyte death (Figs. 6). Similarly, pretreatment of human lymphocytes with catalase for 15 min significantly reduced lymphocyte death induced by NiCH (Fig. 6). However, significant protection against the lymphocyte death induced only by NiCH was achieved following such pretreatment with superoxide dismutase (a scavenger of superoxide anion, O_2^-) (Fig. 6). Pretreatment of lymphocytes with either dimethylthiourea (DMTU), or mannitol (hydroxyl radical scavenger) for 15 min significantly prevented cell death induced by NiCH (Fig. 6).

It is also hypothesized that Ni-compound may induce lymphocyte death through iron-mediated oxidative damage. Therefore, the effect of deferoxamine (DFO), a potent iron-chelator, on Ni-induced lymphocyte death was studied. It is seen that when lymphocytes were pretreated with each Ni-compound in the presence of deferoxamine (DFO) the percentage of cell death induced by NiCH was significantly reduced (Fig. 6). These findings provide evidence that iron-catalyzed oxygen radical producing reactions also play a role in

Ni-induced cell death.

Time-dependent effects of NiCH on the mitochondrial permeability transition in isolated human lymphocytes.

We have examined the effect of NiCH on the induction of MPT in isolated human lymphocytes and hence, to find out the relationship between Ni-induced induction of MPT and Ni-induced lymphocyte death. The MPT was measured by estimating Rhodamine 123 uptake using fluorescence method. Thus, when isolated human lymphocytes were exposed to 0.75 mM NiCH for different periods of time, e.g. 15, 30, 60, 130, 180 min, significant induction of MPT compared to the control value, was observed starting after 30 min of exposure to NiCH (Fig. 7). Furthermore, the MPT continued to be significantly induced due to nickel compound till 180 min of exposure used in this study (Fig. 7). We have also utilized a specific MPT pore inhibitor, cyclosporin A (CsA) in order to examine whether the MPT pore is directly involved in the lymphocyte death induced by NiCH. Thus when lymphocytes were treated for 3h with 0.75 mM NiCH in the presence of 2 μM cyclosporin A, the reduced Rhodamine 123 uptake caused by NiCH was significantly ameliorated, and reached a nearly control value due to cyclosporin A cotreatment (Fig. 8). At the same time, such cotreatment with CsA significantly prevented the lymphocyte death induced by the Ni-compound (Fig. 8).

Effects of NiCH on the intracellular calcium homeostasis and its relationship to Ni-induced lymphocyte death.

This study was designed to seek evidence of any impaired calcium regulation whether occurring early during Ni^{2+}-induced lymphocyte death and signs of a close temporal relationship between the onset of lymphocyte death and any impaired calcium ion (Ca^{2+}) regulation due to NiCH. At first changes in the free intracellular Ca^{2+} influx and release from intracellular stores were investigated with NiCH and the results are presented (Fig. 9). Using the fluorescent probe Fura-2 AM, significant increase in free intracellular Ca^{2+} level, $[Ca^{2+}]_i$ started after 30 min of exposure to NiCH and continued to increase with time and reached a maximum increase of about four times the control value after 120 min of such treatment (Fig. 9). Therefore, the effects of Ni^{2+}-induced lymphocyte death were investigated using different calcium channel blockers involving $[Ca^{2+}]_i$ influx and release as well as Ca^{2+} uptake. As pharmacologic tools, we used primarily compounds that were known to act on cell membranes. However, since elevated $[Ca^{2+}]_i$ can also originate from intracellular stores in mitochondria, or smooth endoplasmic reticulum (Trump et al, 1989), and since mitochondria were shown to be affected by exposure to NiCH in this study, we therefore tested the possibility that modulating intracellular calcium fluxes using both Ca^{2+} channel blockers and intracellular Ca^{2+} antagonist might prevent

the Ni-induced lymphocyte death and the results are presented in Fig. 10. It is seen that cotreatments of lymphocytes with Ni-compound and different Ca^{2+} channel blockers, such as diltiazem, nifedipine and verapamil considerably and significantly prevented in concentration-dependent manner the lymphocyte death induced by Ni-compound. Similarly cotreatments of lymphocytes with Ni-compound and different intracellular Ca^{2+} antagonists, such as dantrolene, cyclosporin A, ruthenium red significantly and considerably protected in concentration-dependent manner against lymphocyte death induced by Ni-compound (Fig. 10). Furthermore, cotreatment of human lymphocytes with Ni-compound and 1,2-bis (2-Aminophenoxy) ethane-*N*,*N*,N´,*N*´-tetraacetic acid tetrapotassium salt (BAPTA) (an intracellular Ca^{2+} chelator) significantly protected in concentration-dependent manner against Ni-compound-induced lymphocyte death (Fig. 10).

Effects of Ca^{2+} channel blocker and intracellular Ca^{2+} antagonist on free intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$ were also examined. The results show that cotreatments of human lymphocytes with NiCH and either diltiazem (Ca^{2+} channel blocker) or ruthenimum red (intracellular Ca^{2+} antagonist), significantly reduced the increase of $[Ca^{2+}]_i$ induced by this nickel compound (Fig. 9). The effects of catalase (a H_2O_2 scavenger), and excess GSH (an antioxidant) as well

as cyclosporin A (a specific inhibitor of MPT) on NiCH-induced increase in $[Ca^{2+}]_i$ were also studied and the results are shown in Fig. 9. Both catalase and excess GSH as well as cyclosporine A are able to significantly reduce the increase of $[Ca^{2+}]_i$ induced by this Ni-compound. The effects of Ca^{2+}-channel blockers on the generation of H_2O_2 were also evaluated and the results are shown in Fig. 2. It is seen that Ca^{2+}-channel blockers such as diltiazem and ruthenium red failed to reduce the excess generation of H_2O_2 induced by NiCH.

Evidence indicating that initial oxidative stress or ROS altered MPT was obtained by studying the effect of catalase and excess GSH on Ni-induced MPT. The effects of pretreatment of lymphocytes with either catalase, or, excess GSH on NiCH-induced MPT are shown in Fig. 7. It is seen that both catalase and excess GSH are able to block the Ni-compound-induced MPT, suggesting that initial oxidative stress is responsible for induction of MPT which appeared significantly at the earliest time, e.g., after 30 min of exposure to NiCH (Fig. 7). To examine whether increase in MPT induced by Ni-compound is due to free intracellular Ca^{2+} $[Ca^{2+}]_i$, we have studied the effects of two Ca^{2+} channel blockers on NiCH-induced MPT and the results are presented in Fig. 7. It is seen that cotreatment of lymphocytes with either diltiazem or verapamil (Ca^{2+} channel blocker) failed to block the Ni-compound induced MPT (Fig. 7) as verified by

the fact that reduced Rhodamine 123 uptake (compared to control) caused by NiCH was not ameliorated towards the control value by the two Ca^{2+} channel blockers.

Discussion

Information regarding the toxic potency of nickel carbonate hydroxide compared to other Ni-compounds is at present very limited. Nickel carbonate hydroxide (NiCH) has been found to induce different forms of DNA damage (Ciccarelli et al, 1981; 1982; 1984). Arsalane et al (1994) have studied the cytotoxicity of nickel carbonate hydroxide ($NiCO_3.Ni(OH)_2.2H_2O$) using guinea pig alveolar macrophages (AMs) in culture. A good correlation between ATP decrease and lactate dehydrogenase release, consistent with dose-dependent cytotoxic effect of this Ni-compound characterizing the depletion of cellular energy reserves has been observed. Furthermore, the effects of NiCH on alveolar macrophage functions were characterized by large generation of free radicals and a depletion of cellular energy reserves (Arsalane et al, 1992).

Evidence has been presented in this study to suggest that oxidative damage-inducing free radicals may play an important role in

the underlying biochemical mechanisms involved in human lymphocyte death due to NiCH. Increased generation of various types of ROS (e.g. H_2O_2, superoxide anion, hydroxyl radical) as well as depletions of nonprotein-sulfhydryl (NP-SH) and protein-bound sulfhydryl (P-SH), and activation of lipid peroxidation due to Ni-compound are all believed to be the initiators of pathogenesis of lymphocyte death, as they appeared before any occurrence of Ni-compound-induced lymphocyte death. These are confirmed as follows. The percentage of Ni-compound-induced dead cells is significantly reduced by treatment with excess GSH, or excess N-acetylcysteine (a precursor of GSH), suggesting that intracellular GSH level is one of the determining factors in the susceptibility of human lymphocytes to the cytotoxic effects of NiCH. Excess GSH may be involved in the metabolism of free radicals and hydroperoxides. NiCH-induced generation of H_2O_2 is significantly reduced not only by catalase and DFO, but also by excess of GSH (Fig. 2). This suggests that the loss of cellular thiol reserve also resulted in significant generation of H_2O_2 and hydroxyl radical. However, since catalase and/or DFO also significantly prevented the depletion of NP-SH induced by Ni-compound (Fig. 4), it indicates that generation of ROS such as H_2O_2 and $^{\cdot}OH$ are also responsible for the loss of cellular thiols. This is not unusual as both the generation of H_2O_2 and depletion of cellular NP-SH contents started at the same time, e.g. after 15 min of exposure to

Ni-compound. The inhibition of human lymphocyte death induced by NiCH by catalase suggests that H_2O_2 participates in such lymphocyte death. Similarly, the significant prevention of Ni-compound-induced lymphocyte death by mannitol and dimethylthiourea indicates that Ni-compound-induced cell death also occurs via generation of excess reactive hydroxyl radical species in isolated human lymphocytes. Significant prevention of soluble forms of NiCH-induced lymphocyte death by superoxide dismutase (SOD) suggests the participation of superoxide anion (O_2^-) as well in such lymphocyte death process. Significant prevention of lymphocyte death induced by NiCH has also been observed following pretreatment with DFO. Iron acts as a Fenton catalyst to produce hydroxyl radical from H_2O_2. Deferoxamine (DFO) being an iron chelator has the potential to inhibit the process of Ni^{2+}-induced ROS, resulting in prevention against Ni-compound-induced lymphocyte death. Thus each Ni-compound may express its cytotoxic (cell death) potential by way of iron-mediated oxidative damage as well. Furthermore, these findings provide support for iron chelator therapy in protection against Ni-compound induced oxidative damage. Besides, other studies from our laboratory have also shown that pre-treatment of human blood lymphocytes with either catalase, or superoxide dismutase, or dimethylthiourea, or N-acetylcysteine, or deferoxamine significantly inhibited the induction of either sister chromatid exchanges, or DNA single strand breaks by NiCH in human

blood lymphocytes *in vitro*. This suggests that various types of reactive oxygen species (ROS) play significant role in such Ni-compound induced genotoxicity (manuscript submitted).

The mechanism by which Ni(II)-compounds increase generation of ROS in human lymphocytes is not clear. Ni_3S_2 is believed to activate neutrophils and cause substantial production of H_2O_2 levels (Zhong et al, 1990). It has been shown that peptides containing the glycyl-glycyl-L-histidyl sequence trigger nickel-dependent production of oxygen radicals through reaction with H_2O_2. When chelated with peptides containing the glycyl-glycyl-L-histidyl sequence, Ni^{2+} could also peroxidize lipids either through H_2O_2 disproportionation and hydroxyl radical production, or directly by reaction with the lipid peroxides (Torreilles and Guérin, 1990). However, Ni^{2+} ions themselves have been shown to induce the formation of oxidized DNA bases (Kawanishi et al, 1989; 2002; Nackerdien et al, 1991). Similarly, nickel subsulfide (soluble form)-induced formation of reactive oxygen species has been detected by dichlorofluorescein fluorescence in isolated rat renal cortical cells *in vitro* (Chakrabarti et al, 1999). Both nickel subsulfide (insoluble) and nickel chloride (soluble) have been shown to induce increased oxidants in intact cultured mammalian cells as detected by dichlorofluorescein fluorescence (Huang et al, 1993), and by nickel chloride in isolated human lymphocytes (Chen et

al, 2003). In view of above considerations, whether the soluble form of NiCH are capable of directly inducing increased generation of H_2O_2, or indirectly through some biological chelators cannot be determined from this study and warrants further investigation. Similarly, whether the soluble form of NiCH is capable of directly inducing superoxide anion (O_2^-) in isolated human lymphocytes, or indirectly through some biological chelators cannot be ascertained from this study and therefore warrants further evaluation.

Following the method of Lee et al (1982) significant concentrations (≈10mM) of Ni(II) were found in solution following incubation of insoluble nickel carbonate hydroxide (NiCH) up to 12 hr in 0.05M Tris-HCl buffer, pH 7.4. However, the nature of the chemical form of this Ni-compound after solubilizing in Tris-HCl buffer, pH 7.4 was not determined in this study. On the other hand, this increase in solubility for Ni_3S_2 for example, can be accounted for by the ability of Tris to coordinate nickel (II) ion and form soluble charged complexes (Dotson, 1972). The solubilized nickel exhibited electronic absorption spectra and magnetic moments characteristic of an octahedral nickel (II). So, it is assumed that insoluble nickel carbonate hydroxide (NiCH) may be changed to a soluble form with Tris involving some type of Ni^{2+}-coordinated complex. As such, whether the soluble forms of nickel carbonate hydroxide and nickel subsulfide in Tris-HCl are

capable of directly or indirectly generating excess reactive oxygen species warrant further investigation.

Cyclosporin A (CsA) is often considered as a specific blocker of the mitochondrial permeability transition (MPT) as well as a specific inhibitor of mitochondrial membrane potential (Lemasters et al, 1998a, b; Quian et al, 1997). Since CsA cotreatment markedly reduced the lymphocyte death induced by the Ni-compound (Fig. 11), it indicates that (a) the Ni-compound has the potential to induce mitochondrial dysfunction in human lymphocytes and (b) the MPT plays equally an important role in Ni-compound-induced lymphocyte death signalling process. CsA affects the mitochondria by inhibiting the induction of MPT, thereby may prevent disruption of the transmembrane potential caused by Ni-compounds. Studies with isolated mitochondria show that MPT pore favors a closed state, but some physiological and pathological signals trigger pore opening (Bernardi and Petronilli, 1996). The fully opened state creates a channel for ≤ 1.5 kDa molecules, resulting in dissipation of the H^+ gradient across the membrane and uncoupling of the respiratory chain (Green and Amarante-Mendes, 1998). Thus opening of high conductance pores in the mitochondrial inner membrane triggered by Ni-compounds precipitates the onset of the MPT, which could lead to membrane depolarization and release of ions and other physiological

effects. The MPT has been linked to significant depletion of cellular ATP (Duchen, 2000; Qian et al, 1999). It has been postulated that the MPT uncouples mitochondria and causes consumption of ATP by mitochondrial ATPases. Thus, studies by Arsalane et al (1992; 1994) on the cytotoxicity of nickel carbonate hydroxide using guinea pig alveolar macrophages in culture have shown significant generation of free radicals and a depletion of cellular energy reserve particularly ATP.

The mechanism of pore blockage by cyclosporin A has not yet been resolved, but may involve binding with the mitochondrial matrix protein, cyclophilin family protein associated with MPT pore (also called peptidylprolyl-cis-trans isomerase (PPIase) rather than direct interaction with the pore itself (Nicolli et al, 1996; Connern et al, 1992).

Evidence indicating that Ni-compound-induced initial ROS or oxidative stress is responsible for Ni-compound-induced MPT was obtained from the observation that the pretreatments of human lymphocytyes with either catalase (a H_2O_2 scavenger), or excess GSH significantly and effectively inhibited the Ni-compound-induced MPT (Fig. 7). The present study has established a temporal relationship between the generation of ROS, depletion of thiol reserves which was

then followed by a mitochondrial event such as MPT.

Our present study has also provided evidences both directly and indirectly that the mechanism of NiCH-induced activation of death signalling pathways involves not only the excess generation of various types of ROS and oxidative stress, and mitochondrial permeability transition (MPT), but also destabilization of cellular calcium homeostasis, resulting in significant elevation of free intracellular calcium concentration $[Ca^{2+}]_i$. Furthermore, the present study has shown that Ni-compound-induced such destabilization of cellular calcium homeostasis is caused not only by Ni-compound-induced ROS/oxidative stress but also by NiCH-induced MPT. This is substantiated by the observation that not only excess catalase and GSH (scavengers of ROS) but also cyclosporin A (a specific blocker of the MPT and a specific inhibitor of mitochondrial membrane potential) significantly reduced NiCH-induced elevation of $[Ca^{2+}]_i$ (Fig. 9). Consequently, both ROS/oxidative stress and mitochondria were causally involved in NiiCH-induced destabilization of cellular calcium homeostasis.

The present study has also identified several calcium sites as additional targets by which Ni^{2+} may perturb cellular Ca^{2+} homeostasis which may therefore result in human lymphocyte death.

Thus, analyses of the different mechanisms involving the effects of different modulators of calcium channel blockers governing the increase of $[Ca^{2+}]_i$ suggest that the protective effects of nifedipine, verapamil and diltiazem against NiCH-induced increase of $[Ca^{2+}]_i$ and the consequent lymphocyte death are due to inhibition of calcium movements through voltage-operated calcium channel protein involving plasma membranes. Furthermore, the present study has also indicated that other calcium antagonists are also able to inhibit calcium movements through other mechanisms as well. Thus, NiCH-induced lymphocyte death can also be significantly prevented by inhibiting Ca^{2+} release from sarcoplasmic reticulum by dantrolene cotreatment as well as inhibiting Ca^{2+} release from mitochondria by cyclosporin A treatment and inhibiting Ca^{2+} uptake by mitochondria by ruthenium red treatment. Thus, the soluble form of NiCH may increase $[Ca^{2+}]_i$ via release of Ca^{2+} from voltage-operated calcium channel sensitive protein, as well as from mitochondria, sarcoplasmic reticulum and cellular oxidative stress. Whether there is/are more sensitive target(s) for Ni^{2+} remains unclear and warrants further study. It is possible that soluble form of NiCH apparently competes with the essential metals like calcium to use the same pathways, thus disrupting the intracellular balance of Ca^{2+} and resulting in cell death. Soluble nickel has been shown to compete with calcium for channels and to be taken up through calcium channels (Refvik and

Andreassen, 1995; Funakoshi et al, 1997). It has been shown that Ni uptake by rat hepatocytes occurs, at least in part, through the Ca channel transport processes. Pretreatment with nicardipine or verapamil, potent inhibitors of Ca channels, decreased Ni uptake by 20% (Funakoshi et al, 1997). However, other mechanisms of Ni uptake might also be involved which requires further study. Ca^{2+} is considered as one of the most important intracellular second messengers and is maintained at a very steep gradient between the outside and the inside of all mammalian cells (Pozzan et al, 1994; Rosen et al, 1995). Very few studies have related the carcinogenic and/or toxic effects of nickel with disturbances in calcium metabolism (see review by Denkhaus and Salnikow, 2002). Thus, an increase in calcium concentration has been observed in mice pancreas after nickel administration (Funakoshi et al, 1996). Nickel-transformed cells could rapidly proliferate in a low-calcium media suggesting an alteration of intracellular calcium metabolism in nickel-transformed cells (Swierenga et al, 1976). Cytoplasmic Ca^{2+} pulses signal gene expression associated with cell growth, differentiation, and apoptosis of many different types of cells in the body (Rosen et al, 1995; Nicotera and Orrenius, 1988). Since exogenous addition of BAPTA, a specific chelator of free intracellular calcium, $[Ca^{2+}]_i$ also attenuated NiCH-induced lymphocyte death (Fig. 10), it indicates that elevation of intracellular calcium was essential for such death.

Studies from our laboratory have also shown that either intracellular Ca^{2+} channel blocker, or intracellular Ca^{2+} antagonist, or Ca^{2+} chelator is capable of reducing either sister chromatid exchanges, or DNA strand breaks induced by nickel carbonate hydroxide in human blood lymphocytes, suggesting that Ni-compound-induced destabilization of calcium homeostasis plays an important role in such DNA damage (manuscript submitted).

Our study has further demonstrated that Ca^{2+} does not promote the onset of Ni^{2+}-induced MPT, nor initiate Ni^{2+}-induced ROS, as verified by the observation that treatment with either calcium channel blocker such as verapamil, diltiazem or ruthenium red failed to inhibit NiCH-induced accumulation of excess ROS (Fig. 2) and the induction of MPT (Fig. 7). In conclusion, it is unlikely that any single biochemical event is only responsible for Ni^{2+} (from NiCH)-induced activation of lymphocyte death signalling process. Ni^{2+}-(derived from NiCH)-induced disruptions in cellular biochemical processes including various oxidative stress, mitochondrial permeability transition and calcium homeostasis are equally critical events in the activation of death signalling process in which human lymphocytes are irreversibly committed to death. Based on our data a proposed schematic mechanism has been presented in Fig. 11. The present data may provide some new insights into the mechanisms of Ni-compound-

induced human lymphocyte death, as well as contribute important information for the treatment and prevention of adverse health effects in workers of nickel-related industries.

ACKNOWLEDGMENTS

The authors sincerely thank Sainte-Justine Hospital, for obtaining blood samples from donors and from the laboratory of vascular immunology of Dr. Jean-Claude Mamputu of CHUM, Research Center, Notre-Dame Hospital, Montreal.

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FIGURE CAPTIONS

FIG. 1. (A) Concentration-dependent effects of nickel carbonate hydroxide (NiCH) on human lymphocyte death. Freshly isolated human lymphocytes were treated *in vitro* with various concentrations of soluble form of NiCH at 37°C for 4 hours. (B) Time-dependent effects of nickel carbonate hydroxide (NiCH) on human lymphocyte death. Isolated lymphocytes were exposed *in vitro* to 0.75 mM of NiCH

for different time periods at 37°C. Cell death was determined by trypan blue dye exclusion. Values represent mean ± SEM from four separate experiments involving four separate blood donors. *Significantly different from control, $p < 0.05$. [a] Significantly different between NiCH-treated group, $p < 0.05$. If the error bar is not shown, the SEM is within the symbol.

FIG. 2. The generation of hydrogen peroxide (H_2O_2) following exposure of isolated human lymphocytes to 0.75 mM of nickel carbonate hydroxide (NiCH) at various time periods (0-180 min). For details, see Materials and Methods. Effects of various scavengers of ROS on NiCH-induced generation of hydrogen peroxide. Human lymphocytes were either first pretreated for 15 min, or not with various scavengers of ROS followed by exposure to NiCH for various time periods (0-180 min). Effects of diltiazem and ruthenium red on time-dependent increase in generation of hydrogen peroxide (H_2O_2) in NiCH-treated human lymphocytes *in vitro*. Isolated lymphocytes were exposed *in vitro* at various times to NiCH alone, or with NiCH plus 50 μM diltiazem, or with NiCH plus 2 μM ruthenium red and the generation of H_2O_2 was measured using the colorimetric method. The values represent mean ± SEM of four separate experiments from four separate blood donors. *Significantly different from control, $p < 0.05$. •Significantly different from NiCH-alone-treated group, $p < 0.05$. If the

error bar is not shown, the SEM is within the symbol.

FIG. 3. The generation of superoxide anion (O_2^-) (as measured by NBT reduction) following exposure of isolated human lymphocytes to nickel carbonate hydroxide (NiCH) at various times (0-180 min). Effects of superoxide dismutase and deferoxamine on NiCH-induced generation of O_2^-. Human lymphocytes were either first pretreated for 15 min, or not with either superoxide dismutase, or deferoxamine followed by exposure to 0.75 mM NiCH for various time periods (30-180 min). The results are mean ± SEM for 4 separate experiments. *Significantly different from control, $p < 0.05$ and •significantly different from NiCH-alone-treated group, $p < 0.05$. If the error bar is not shown, the SEM is within the symbol.

FIG. 4. Time-dependent effects of nickel carbonate hydroxide (NiCH) on (A) nonprotein sulfhydryl (NP-SH) and (B) protein sulfhydryl (P-SH) contents following exposure of human lymphocytes to NiCH for various times. (A) Effects of various ROS scavengers on NiCH-induced depletion of NP-SH contents. Human lymphocytes were either first pretreated for 15 min, or not with various ROS scavengers followed by exposure to 0.75 mM NiCH for various time periods (0-180 min). The values represent mean ± SEM from four different experiments. *Significantly different from control, $p < 0.05$. •Significantly different

from NiCH-alone-treated group, $p < 0.05$. If the error bar is not shown, the SEM is within the symbol.

FIG. 5. The production of lipid peroxidation (as measured by MDA formation) following exposure of human lymphocytes to nickel carbonate hydroxide (NiCH) for various time periods. Effects of various scavengers of ROS on NiCH-induced lipid peroxidation. Human lymphocytes were either first pretreated for 15 min, or not with various scavengers of ROS followed by exposure to 0.75 mM NiCH for various time periods (0-180 min). The results are mean ± SEM for 4 separate experiments. *Significantly different from control, $p < 0.05$ and •significantly different from NiCH-alone-treated group, $p < 0.05$. If the error bar is not shown, the SEM is within the symbol.

FIG. 6. Effects of various scavengers of ROS on nickel carbonate hydroxide (NiCH)-induced human lymphocyte death. Human lymphocytes were either first pretreated for 15 min, or not followed by exposure to 0.75 mM NiCH for 3 h at 37°C. Lymphocyte death was measured by trypan blue dye exclusion. Results are mean ± SEM for four separate experiments. A= NiCH (0.75 mM), B= Catalase pretreated (1000 U/ml), C= Catalase pretreated (2000 U/ml), D= SOD pretreated (2500 U/ml), E= SOD pretreated (5000 U/ml), F= GSH pretreated (4 mM), G= GSH pretreated (8 mM), H= NAC pretreated (4

mM), I= NAC pretreated (8 mM), J= Mannitol pretreated (25 mM), K= Mannitol pretreated (50 mM), L= DMTU pretreated (10 mM), M= DMTU pretreated (20 mM), N= DFO pretreated (100 μM). *Significantly different from NiCH-alone-treated group, $p < 0.01$.

FIG. 7. Time-dependent effects of nickel carbonate hydroxide (NiCH) on the mitochondrial permeability transition (or, the mitochondrial membrane potential). Effects of catalase, GSH, diltiazem and verapamil on NiCH-induced mitochondrial permeability transition (MPT) at various times in isolated human lymphocytes. Human lymphocytes were treated at 37°C for 2 h with either 0.75 mM NiCH alone, or with NiCH plus catalase (2000 U/ml), or NiCH plus GSH (8 mM), or NiCH plus 50 μM diltiazem, or NiCH plus 25 μM verapamil. The MPT was evaluated by the capacity of lymphocytes to take up the fluorescent cationic dye rhodamine 123. The results expressed as percentage of the total fluorescence of rhodamine 123 in the incubation medium and are means ± SEM of four separate experiments. *Significantly different from control, $p < 0.05$. •Significantly different from NiCH-alone-treated group, $p < 0.01$. If the error bar is not shown, the SEM is within the symbol.

FIG. 8. Effects of cyclosporin A on nickel carbonate hydroxide (NiCH)-induced mitochondrial permeability transition (MPT) in isolated

human lymphocytes. Human lymphocytes were cotreated at 37°C for 3 h with 2 μM cyclosporin A and 0.75 mM NiCH, or without NiCH (control). The MPT was evaluated by the capacity of lymphocytes to take up the fluorescent cationic dye rhodamine 123. The results expressed as percentage of the total fluorescence of rhodamine 123 in the incubation medium. Effects of cyclosporin A on NiCH-induced human lymphocyte death. Lymphocytes were treated at 37°C for 3 h with or without 0.75 mM NiCH in the presence, or absence of 2 μM cyclosporin A. Lymphocyte death was determined by trypan blue dye exclusion. Results are means ± SEM of four separate experiments. *Significantly different from NiCH-alone-treated group, $p < 0.05$.

FIG. 9. Time-dependent effect of nickel carbonate hydroxide (NiCH)-induced increase in intracellular calcium ion concentration, $[Ca^{2+}]_i$. For details, see Materials and Methods. Time-dependent increase in intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$ in NiCH-treated human lymphocytes *in vitro*, and effects of diltiazem and ruthenium red on such increase in $[Ca^{2+}]_i$. Isolated lymphocytes were exposed *in vitro* to either 0.75 mM NiCH alone, or with diltiazem (50 μM), or with ruthenium red (2 μM). Effects of catalase, GSH and cyclosporin A on time-dependent increase in $[Ca^{2+}]_i$ in NiCH-treated human lymphocytes *in vitro*. Isolated lymphocytes were exposed *in vitro* at various times to either 0.75 mM NiCH alone, or with NiCH plus

catalase (2000 U/ml), or NiCH plus GSH (8 mM), or NiCH plus cyclosporin A (2 μM) and $[Ca^{2+}]_i$ was determined after loading the lymphocytes with Fura-2AM. For details, see Materials and Methods. Results are mean ± SEM of four separate experiments. *Significantly different from control, $p < 0.05$. •Significantly different from corresponding time-matched NiCH-alone-treated group, $p < 0.05$. If the error bar is not shown, the SEM is within the symbol.

FIG. 10. Effects of various Ca^{2+} antagonists on nickel carbonate hydroxide (NiCH) (0.75 mM)-induced human lymphocyte death. For details, see Materials and Methods. (A) NiCH alone, (B) NiCH plus 50 μM diltiazem, (C) NiCH plus 12 μM nifedipine, (D) NiCH plus 25 μM nifedipine, (E) NiCH plus 12 μM verapamil, (F) NiCH plus 25 μM verapamil, (G) NiCH plus 3 mM BAPTA, (H) NiCH plus 6 mM BAPTA, (I) NiCH plus 2 μM ruthenium red, (J) NiCH plus 5 μM ruthenium red, (K) NiCH plus 25 μM dantrolene, (L) NiCH plus 50 μM dantrolene, (M) NiCH plus 2 μM cyclosporin A. Results are mean ± SEM of four separate experiments. *Significantly different from Ni-compound-alone-treated group, $p < 0.001$.

FIG. 11. Proposed schematic mechanism involving the roles of ROS, MPT and destabilization of calcium homeostasis in nickel carbonate hydroxide-induced human lymphocyte death.

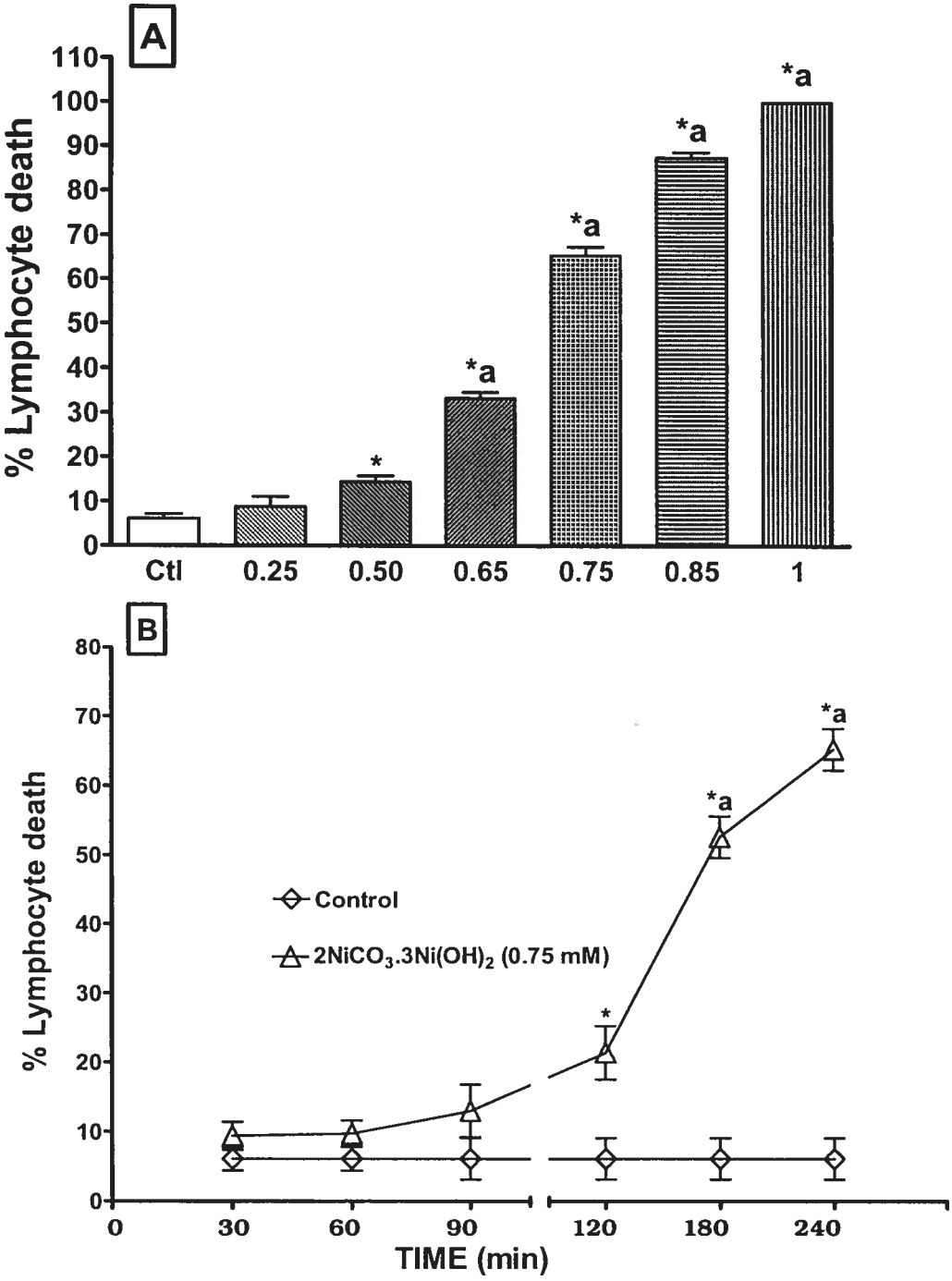
A
% Lymphocyte death
110
100
90
80
70
60
50
40
30
20
10
0
Ctl
0.25
0.50
0.65
0.75
0.85
1
*
*a
B
% Lymphocyte death
80
70
60
50
40
30
20
10
0
Control
2NiCO3.3Ni(OH)2 (0.75 mM)
0
30
60
90
120
180
240
TIME (min)

FIG. 1

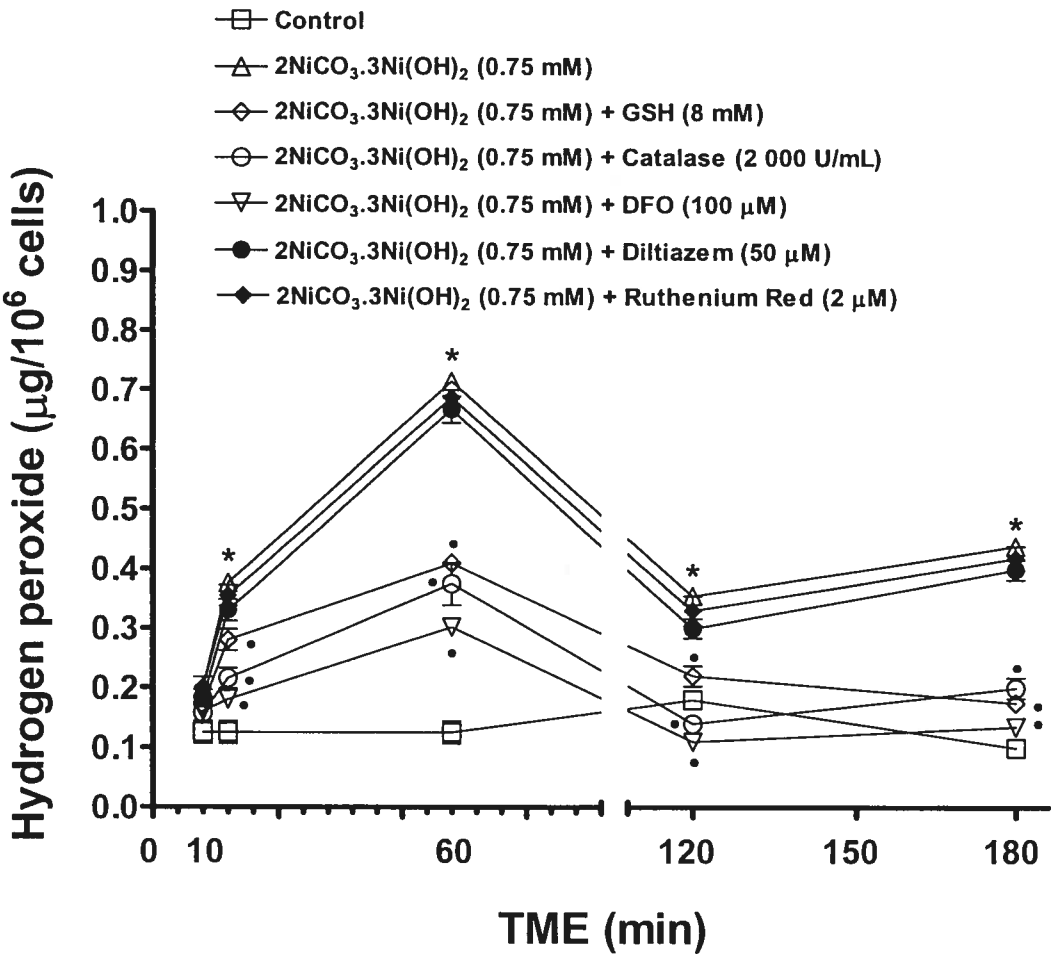
Control
2NiCO3.3Ni(OH)2 (0.75 mM)
2NiCO3.3Ni(OH)2 (0.75 mM) + GSH (8 mM)
2NiCO3.3Ni(OH)2 (0.75 mM) + Catalase (2 000 U/mL)
2NiCO3.3Ni(OH)2 (0.75 mM) + DFO (100 µM)
2NiCO3.3Ni(OH)2 (0.75 mM) + Diltiazem (50 µM)
2NiCO3.3Ni(OH)2 (0.75 mM) + Ruthenium Red (2 µM)
Hydrogen peroxide (µg/10^6 cells)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
60
120
150
180
TME (min)

FIG. 2

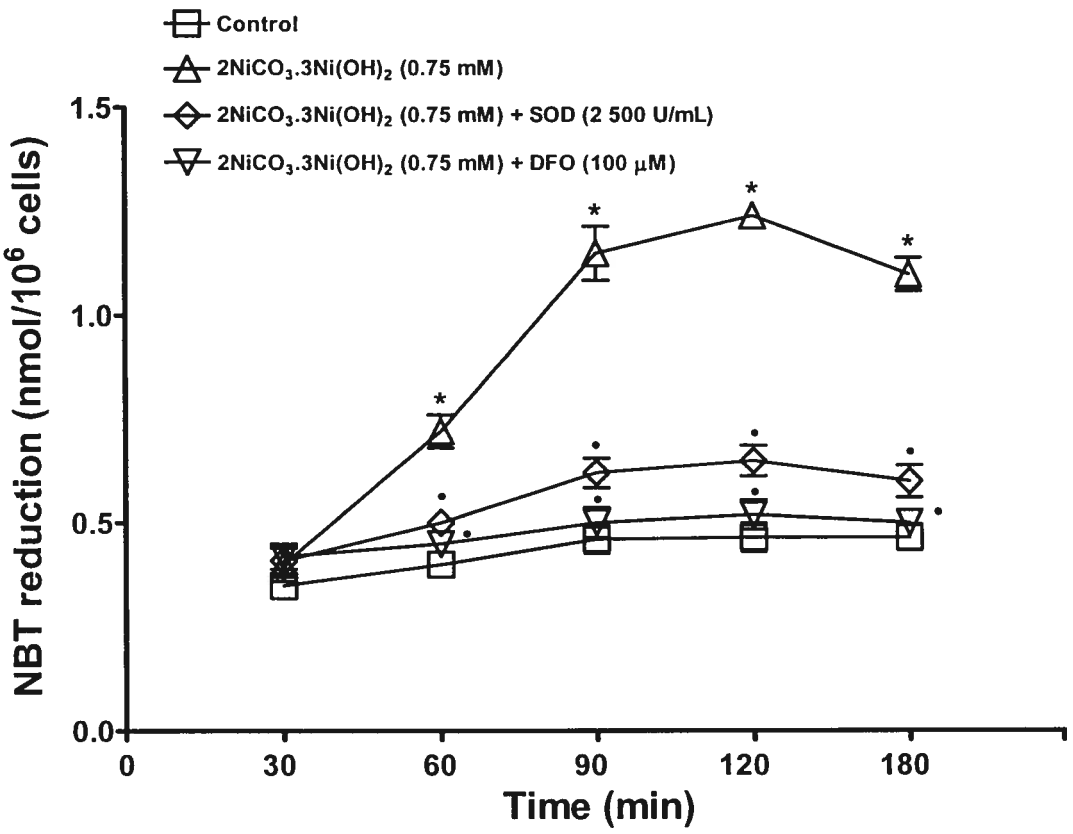
Control
2NiCO3.3Ni(OH)2 (0.75 mM)
2NiCO3.3Ni(OH)2 (0.75 mM) + SOD (2 500 U/mL)
2NiCO3.3Ni(OH)2 (0.75 mM) + DFO (100 µM)
NBT reduction (nmol/10^6 cells)
1.5
1.0
0.5
0.0
0
30
60
90
120
180
Time (min)

FIG. 3

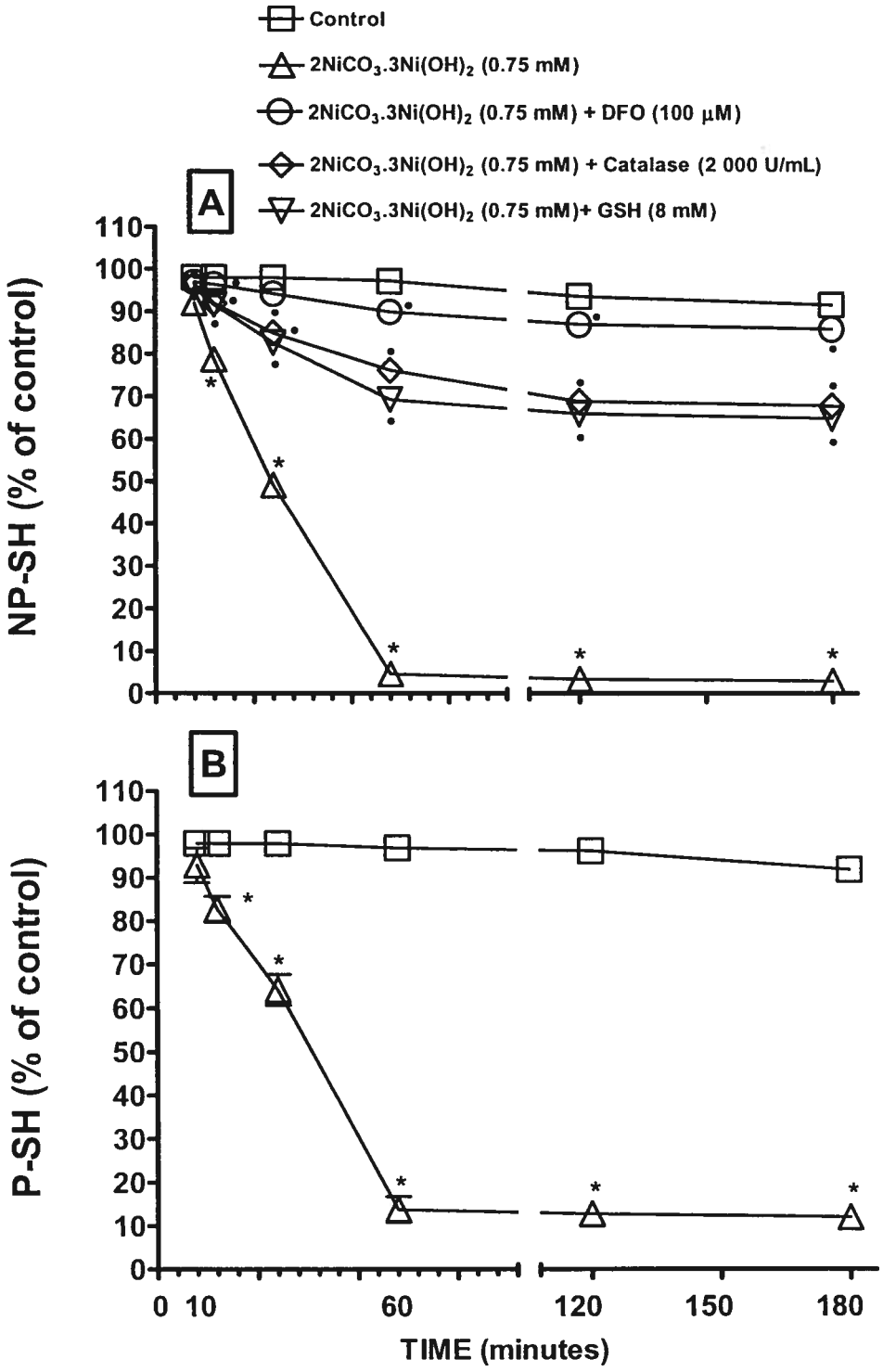
Control
2NiCO3.3Ni(OH)2 (0.75 mM)
2NiCO3.3Ni(OH)2 (0.75 mM) + DFO (100 µM)
2NiCO3.3Ni(OH)2 (0.75 mM) + Catalase (2 000 U/mL)
2NiCO3.3Ni(OH)2 (0.75 mM)+ GSH (8 mM)
A
B
NP-SH (% of control)
P-SH (% of control)
0
10
20
30
40
50
60
70
80
90
100
110
0 10
60
120
150
180
TIME (minutes)

FIG. 4

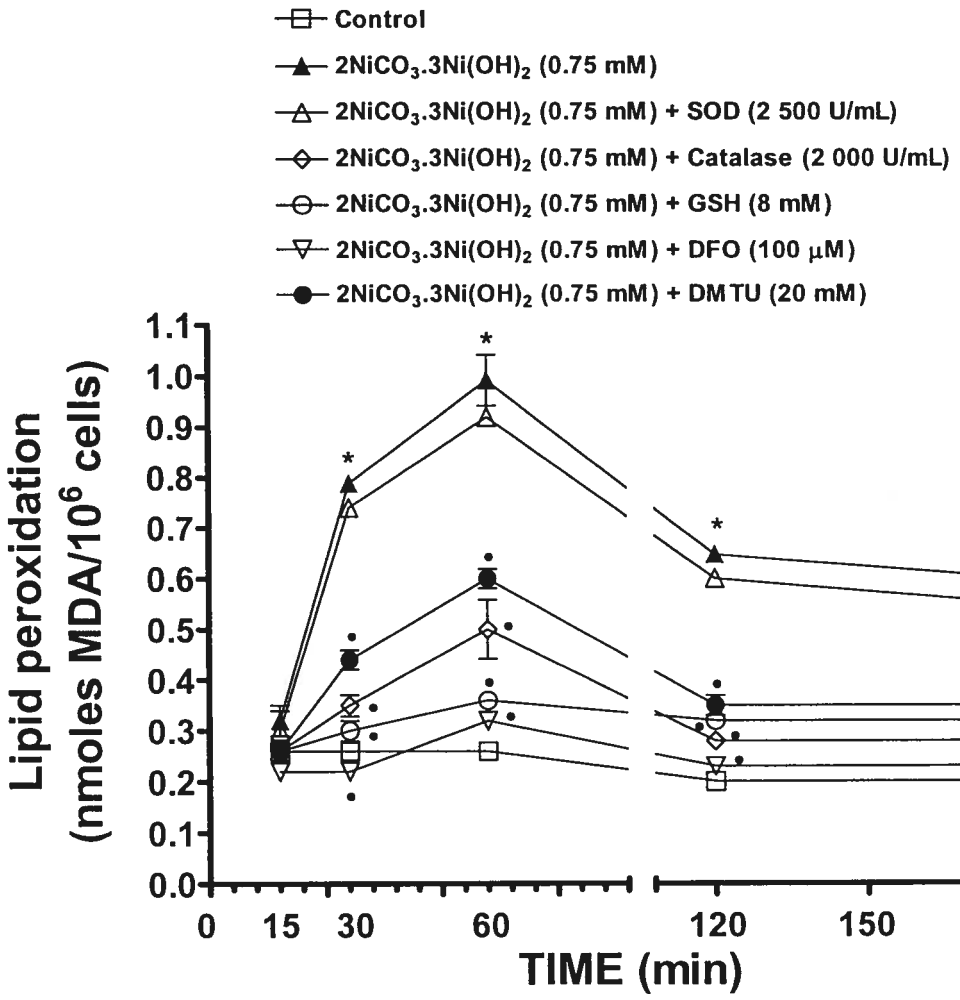
Control
2NiCO3.3Ni(OH)2 (0.75 mM)
2NiCO3.3Ni(OH)2 (0.75 mM) + SOD (2 500 U/mL)
2NiCO3.3Ni(OH)2 (0.75 mM) + Catalase (2 000 U/mL)
2NiCO3.3Ni(OH)2 (0.75 mM) + GSH (8 mM)
2NiCO3.3Ni(OH)2 (0.75 mM) + DFO (100 μM)
2NiCO3.3Ni(OH)2 (0.75 mM) + DMTU (20 mM)
Lipid peroxidation (nmoles MDA/10^6 cells)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
15
30
60
120
150
180
TIME (min)

FIG. 5

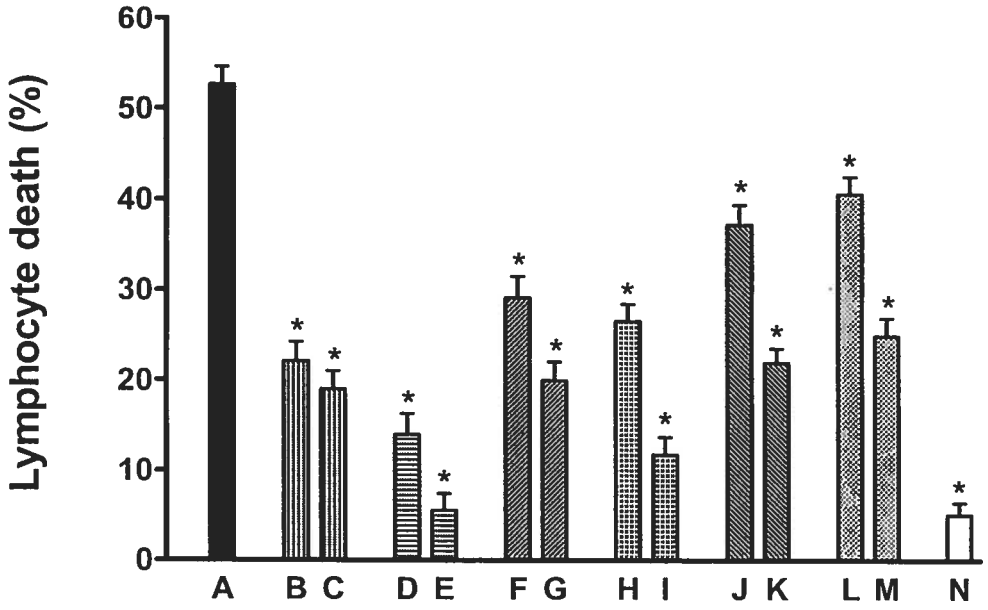

FIG. 6

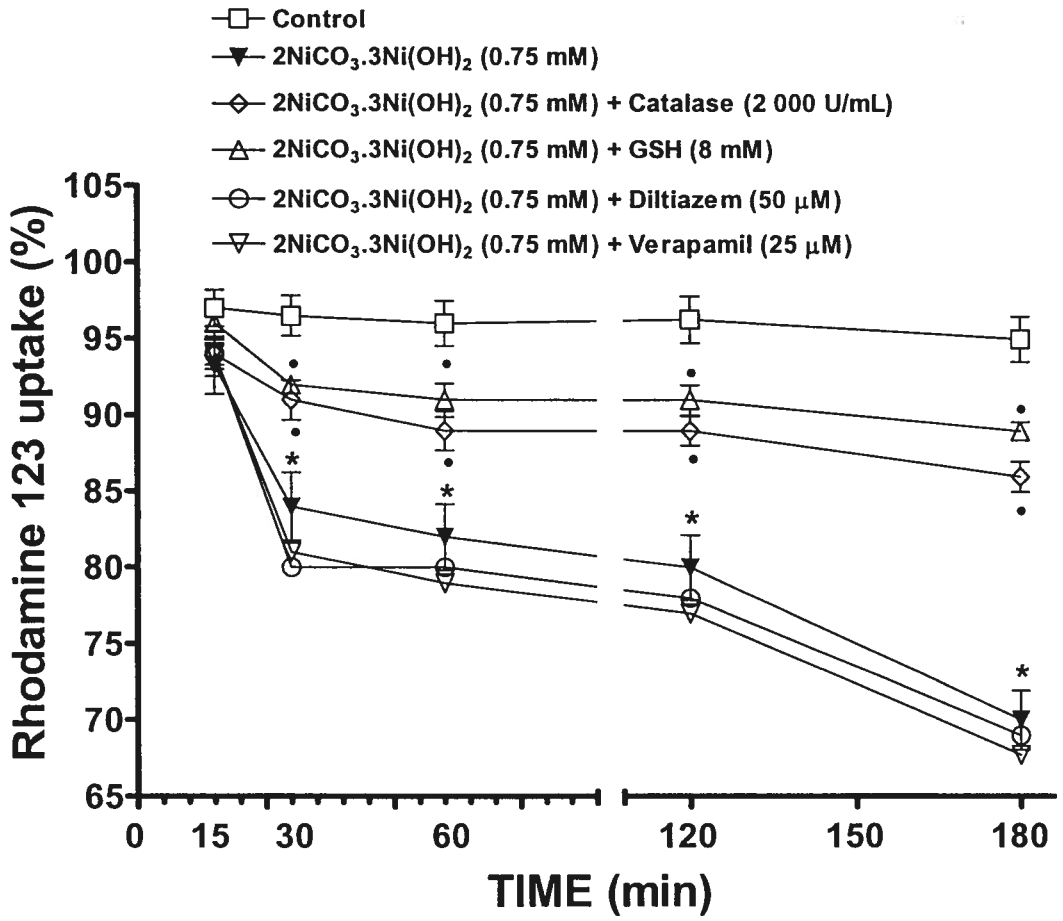
Control
2NiCO3.3Ni(OH)2 (0.75 mM)
2NiCO3.3Ni(OH)2 (0.75 mM) + Catalase (2 000 U/mL)
2NiCO3.3Ni(OH)2 (0.75 mM) + GSH (8 mM)
2NiCO3.3Ni(OH)2 (0.75 mM) + Diltiazem (50 μM)
2NiCO3.3Ni(OH)2 (0.75 mM) + Verapamil (25 μM)
Rhodamine 123 uptake (%)
105
100
95
90
85
80
75
70
65
0
15
30
60
120
150
180
TIME (min)

FIG. 7

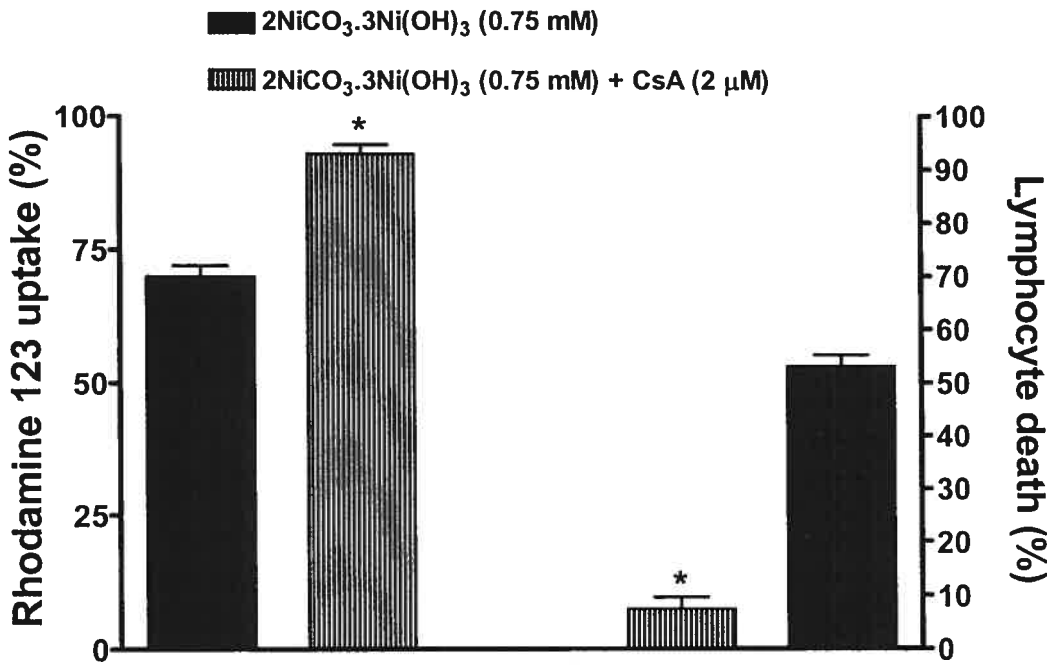
2NiCO3.3Ni(OH)3 (0.75 mM)
2NiCO3.3Ni(OH)3 (0.75 mM) + CsA (2 μM)
Rhodamine 123 uptake (%)
Lymphocyte death (%)
100
75
50
25
0
100
90
80
70
60
50
40
30
20
10
0
*
*

FIG. 8

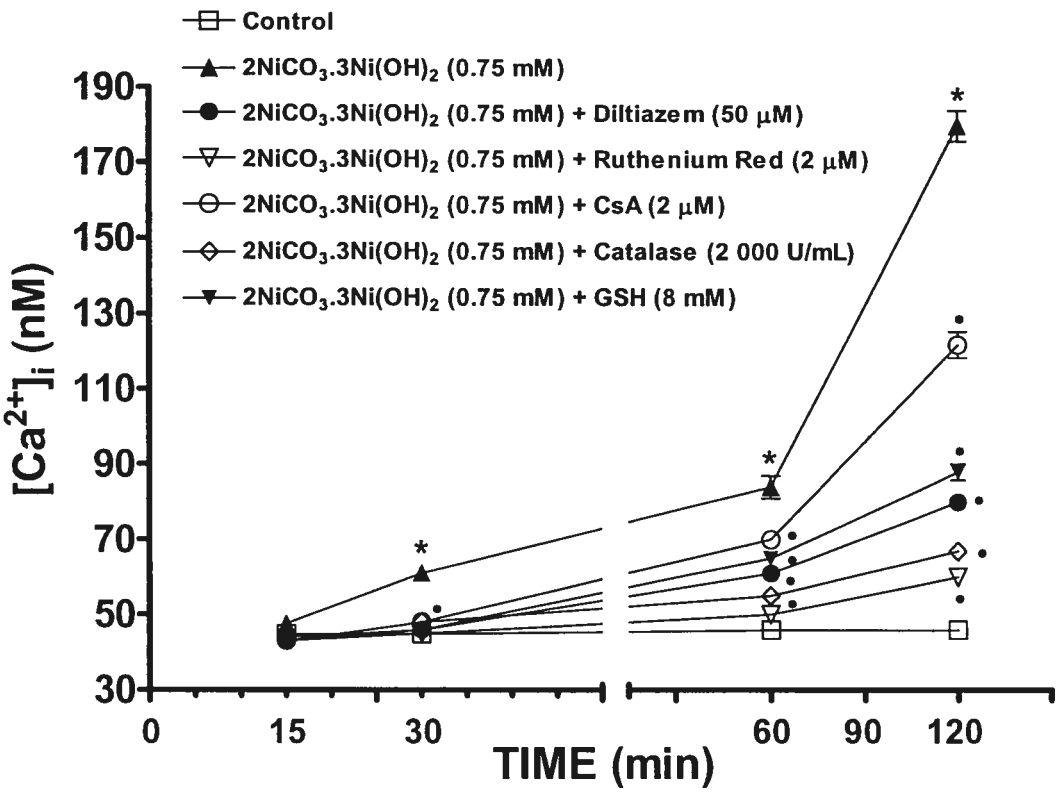
Control
2NiCO3.3Ni(OH)2 (0.75 mM)
2NiCO3.3Ni(OH)2 (0.75 mM) + Diltiazem (50 μM)
2NiCO3.3Ni(OH)2 (0.75 mM) + Ruthenium Red (2 μM)
2NiCO3.3Ni(OH)2 (0.75 mM) + CsA (2 μM)
2NiCO3.3Ni(OH)2 (0.75 mM) + Catalase (2 000 U/mL)
2NiCO3.3Ni(OH)2 (0.75 mM) + GSH (8 mM)
[Ca2+]i (nM)
190
170
150
130
110
90
70
50
30
0
15
30
60
90
120
TIME (min)

FIG. 9

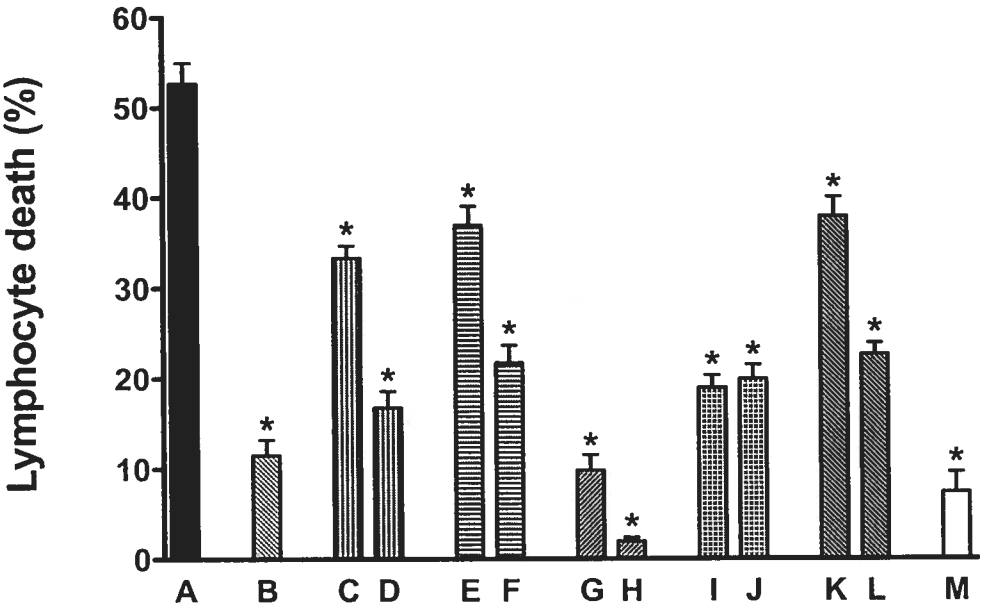

FIG. 10

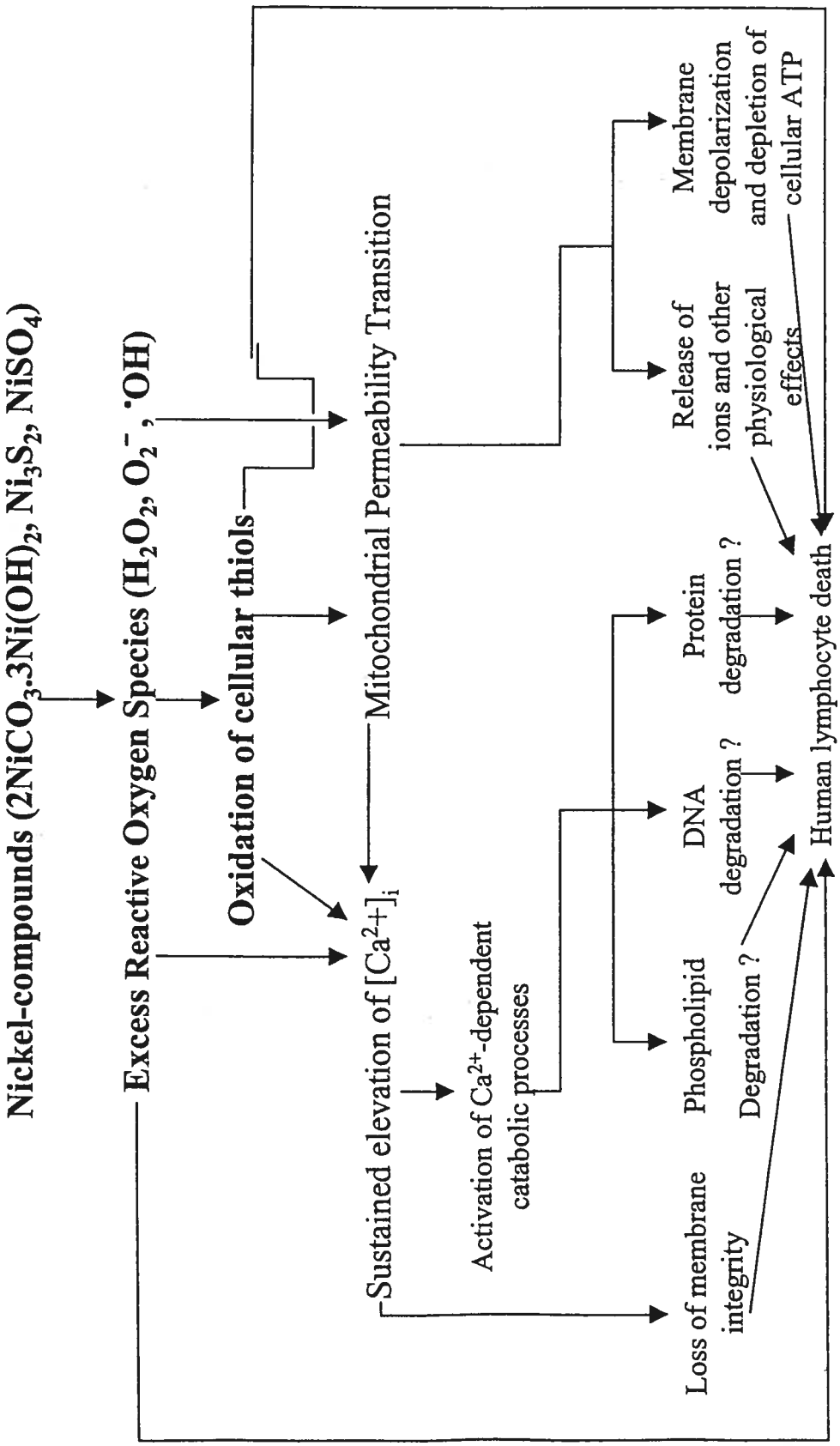
Nickel-compounds ($2NiCO_3.3Ni(OH)_2$, Ni_3S_2, $NiSO_4$)
Excess Reactive Oxygen Species (H_2O_2, O_2^-, $^{\cdot}OH$)
Oxidation of cellular thiols
Mitochondrial Permeability Transition
Sustained elevation of $[Ca^{2+}]_i$
Activation of Ca^{2+}-dependent catabolic processes
Loss of membrane integrity
Phospholipid Degradation ?
DNA degradation ?
Protein degradation ?
Release of ions and other physiological effects
Membrane depolarization and depletion of cellular ATP
Human lymphocyte death

FIG. 11

II.2. ARTICLE II

Role of oxidative stress, mitochondrial permeability transition, and calcium homeostasis in nickel subsulfide-induced human lymphocyte death *in vitro*.

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Abstract

When isolated human lymphocytes were treated *in vitro* with either various concentrations (0-2 mM) of soluble form of nickel subsulfide (Ni_3S_2) at 37°C for 4 h, or at various times (30-240 min.) both concentration-and time-dependent effects of Ni_3S_2 on lymphocyte death were observed. Increased generation of hydrogen peroxide (H_2O_2), and superoxide anion (O_2^-), lipid peroxidation and depletion of both nonprotein (NP-) and protein (P-) sulfhydryl (SH) contents were induced by Ni_3S_2. Ni_3S_2-induced lymphocyte death was significantly prevented by pre-treatment with either catalase (a H_2O_2 scavenger), or excess superoxide dismutase (scavenger of O_2^- radical), or dimethylthiourea/mannitol (hydroxyl radical scavengers), or deferoxamine, or excess glutathione/N-acetylcysteine. Cotreatment with cyclosporin A (a specific inhibitor of mitochondrial permeability transition (MPT)) not only inhibited Ni_3S_2-induced MPT, but also significantly prevented Ni-compound-induced lymphocyte death. Ni_3S_2-induced lymphocyte death was also significantly prevented by modulating intracellular calcium fluxes using both Ca^{2+} channel blockers and intracellular Ca^{2+} antagonist. Thus, the mechanism of Ni_3S_2 (soluble form)-induced activation of lymphocyte death signalling pathways involves not only the excess generation of different types of oxidative stress by Ni_3S_2, but also Ni_3S_2-induced mitochondrial

permeability transition and destabilization of cellular calcium homeostasis as well.

KEY WORDS: Human lymphocytes; lymphocyte death; oxidative stress; nickel subsulfide; mitochondrial permeability transition; calcium homeostasis.

Introduction

Occupational exposure to nickel compounds occurs principally through mining, smelting, refining operations, alloy production, electroplating and welding operations, during manufacture of steel and other alloys and batteries. Respiratory cancer risks are primarily related to exposure to soluble nickel concentrations above 1mg/m^3 and to exposure to insoluble forms at concentrations above 10 mg/m^3 (IARC, 1990). Nickel matte refining has been associated with high exposure to dusts of nickel subsulfide and nickel oxide, whereas in electrolytic refining operations workers are exposed to aerosols of nickel sulfate and nickel chloride. The increased frequencies of chromosomal aberrations and sister-chromatid exchanges (SCEs) (compared to the control group) in the peripheral lymphocytes of workers occupationally exposed to nickel have been reported in many studies (Perminova et al., 2001; Werfel et al., 1998; Waksviks et al., 1984). These results have shown that the human lymphocytes are

also the target cells for nickel toxicity.

The toxicity and carcinogenicity of Ni-compounds including the oxidative mechanism in experimental animals (review by Kasprzak et al, 2003; Kawanishi et al., 2002; M'Bemba-Meka and Chakrabarti, 2001; Chakrabarti et al, 2001; Chakrabarti and Bai, 1999; Obone et al, 1999) and humans (Oller et al, 1997; Wozniak and Blasiak, 2002; review by Kasprzak et al, 2003) have been well established. However, the studies relating the roles of various types of oxidative stress as well as other biochemical mechanisms in the development of Ni-compound-induced cellular injury/cell death involving human lymphocytes as target cells are at present very limited or hardly available. A transient increase in the level of blood reticulocytes was reported among workers after consuming water containing nickel sulfate and nickel chloride (Sunderman et al, 1988). Although $NiCl_2$-induced increase in oxidative stress has been observed in human plasma (Chen et al, 2002) and lymphocytes (Chen et al, 2003), how such oxidative stress relates to cellular toxicity/cell death due to $NiCl_2$ is not known.

Nickel has been shown to inhibit mitochondrial succinate dehydrogenase activity in rat lung epithelial cells (Riley et al, 2003). Ni_3S_2 derived particles were found to be bound preferentially, though

less markedly than lymphocytes, to mitochondria, Golgi apparatus, and peroxisomes from lung cells (Shirali et al, 1992). Similarly, the binding of nickel sulfides to both rat and human lymphocyte subcellular structures, such as the cell membranes, euchromatin, mitochondria, and Golgi system has been reported (Hildebrand et al, 1987). However, the information regarding how such Ni-induced disturbances in mitochondria could affect Ni-induced cellular toxicity or cell death is presently unknown.

Studies are very limited relating to the toxic effect of nickel with disturbance in calcium homeostasis. An increase in calcium concentrations in mice pancreas has been observed following nickel administration (Funakoshi et al, 1996). Ni^{2+}-treated renal epithelial A6 cells have been shown to release significantly $[Ca^{2+}]_i$ (Faurskov and Bjerregaard, 2002). Levels of free intracellular calcium $[Ca^{2+}]_i$ were increased in nickel-treated cells (Salnikow et al, 1999). However, how such increase in $[Ca^{2+}]_i$ relates to Ni-induced toxicity or cell death is not known. Furthermore, the mechanism leading to the elevation of $[Ca^{2+}]_i$ in nickel-treated cells is also presently unknown. Besides, it is yet to be verified whether different calcium channels could influence nickel-induced toxicity. Therefore, the present investigation was carried out to study the *in vitro* cytotoxic action of soluble form of Ni_3S_2 and its ability to induce different types of oxidative stress,

changes in intracellular Ca^{2+} homeostasis and mitochondrial permeability transition in isolated human lymphocytes. Hence, the role of various oxidative stress, mitochondrial permeability transition and intracellular Ca2+ homeostasis in Ni3S2-induced lymphocyte death has been evaluated.

Materials and methods

Materials

Nickel subsulfide (Ni_3S_2, 150 mesh, black) was obtained from Sigma–Aldrich Canada Ltd, Ont., Canada. All other chemicals and reagents were obtained from Sigma, unless otherwise mentioned. Ni_3S_2 is insoluble in water or physiological saline. Therefore, it was dissolved in 0.05 M Tris-HCl buffer, pH 7.4, by incubation at 37° C for 12h with shaking using the method of Lee et al. (1982). Substantial amount of Ni_3S_2 was dissolved, leading to soluble nickel concentrations of 10-12 mM. The concentration of nickel was determined by electrothermal atomic absorption spectrometry.

The protocols described below were approved by the Ethics Committee on Human Research, Université de Montréal.

Measurement of cell viability in isolated human lymphocytes

Human whole blood was obtained from 4 healthy donors provided by Sainte-Justine Hospital, Montréal. Peripheral blood samples from 4 healthy non-smoking donors between ages 27 and 42 and who were not previously exposed to any radiation or heavy metal compounds or drug therapy and who did not take any alcohol at least two days before this experiment were used in the present experiments. Questionnaires were obtained from each blood donor to evaluate exposure history. Methodology for the handling of human blood was followed according to the strict guidelines as devised for clinical workers at Sainte-Justine Hospital, Montréal. Human peripheral blood was collected in sodium-heparinized vacutainers. Separation of peripheral blood lymphocytes was carried out under sterile conditions on Ficoll-Paque (Pharmacia) gradients by the method of Boyum (1976). Aliquots of the heparinized whole blood diluted with an equal volume of ice-cold physiological saline (1:1) were gently applied on an equal volume of Ficoll-Paque in centrifuge tubes. The samples were then centrifuged at 400 g for 30 min. The resultant interface (buffy coat) was then carefully aspirated from the gradient, washed twice in ice-cold Dulbecco's phosphate-buffered saline by centrifugation at 200 g for 10 min. The subsequent pellet of purified lymphocytes was finally resuspended in RPMI 1640 medium and adjusted to the desired lymphocyte concentration (1 to $5x10^6$ cells per ml) in RPMI 1640 medium (pH 7.4). This range of cell concentrations

was necessary depending on the type of study protocol used. The approximate yield of lymphocytes was determined by counting the cells on a hemocytometer. The viability of the cells was determined by trypan blue dye exclusion.

Determination of Ni_3S_2-induced human lymphocyte death

For developing cell death in a short duration of exposure (3-4 h) using human lymphocytes in suspension, we had to use higher concentrations (0-2 mM) of nickel subsulfide; the principal objective here was to study the mechanisms of such cell death. The stock solution of Ni_3S_2 was diluted to the appropriate concentrations using RPMI 1640 medium. Isolated human lymphocytes were exposed for 0-4 h at 37°C to either control, or Ni_3S_2 (0-2 mM) in a humidified atmosphere of 95% oxygen and 5% carbon dioxide. At the end of such exposure, lymphocytes from each group were washed twice with RPMI 1640 medium. An equal volume of 0.4% trypan blue dye reagent was then added to the lymphocyte suspension and the percentage of dead lymphocytes was determined under a field microscope, using trypan blue exclusion. To determine the time-dependent effect of Ni_3S_2-induced lymphocyte death, isolated human lymphocytes were exposed at 37°C with 1 mM Ni_3S_2, or control at different times, e.g. 30, 60, 90, 120, 180 and 240 min followed by measurement of lymphocyte death by trypan blue dye exclusion as described above.

Measurements of Ni_3S_2-induced generation of reactive oxygen species/oxidative stress

The production of hydrogen peroxide (H_2O_2) in control and Ni-treated lymphocytes was assayed following a colorimetric method developed by Graf and Penniston (1980). The method is based on the oxidation of iodide in the presence of ammonium molybdate and photometry of the resulting blue starch-iodine complex (which is stable for several hours) was carried out at 570 nm. The concentration of H_2O_2 was finally estimated from a standard curve. The formation of cellular superoxide anion (O_2^-) was determined by the nitroblue tetrazolium (NBT) reduction assay as described by Rauen et al., (2000) with minor modifications. NBT was added to RPMI 1640 medium (pH 7.4) in a final concentration of 1 mg/ml and lymphocytes were incubated in this medium for 0-3h at 37°C without and with (1 mM) Ni_3S_2. At the end of the incubation period, lymphocytes were separated and carefully washed with Hanks' Balanced Salt Solution and then lysed at 37°C with 5% sodium dodecyl sulfate in phosphate buffer (80 mM, pH 7.8) containing 0.45% gelatin. The samples were centrifuged for 5 min at 13,000 g. The absorbance due to formazan at 540 nm was determined against a lysis buffer blank immediately and within 2 min after such formation. The concentration of superoxide anion (O_2^-) was evaluated following the method of Rauen et al., (2000). Lipid peroxidation was determined

indirectly by measuring the production of malondialdehyde (MDA) followed by its reaction with thiobarbituric acid as described by Schnellmann (1988). Briefly, aliquots of the cell suspension (control and Ni-treated) corresponding to 1 x 10^6 lymphocytes (0.5 ml) were deproteinized with 0.5 ml TCA (10%) and centrifuged. The supernatant (0.9 ml) was then mixed with 0.5 ml 2-thiobarbituric acid reagent (0.76%), heated in a boiling waterbath for 10 min, and allowed to cool. Sample absorbance was then measured at 535 nm. Protein content was estimated by the method of Bradford, using bovine serum albumin as a standard (Bradford, 1976).

Effects of scavengers of reactive oxygen species on Ni_3S_2-induced lymphocyte death

In order to study the involvement of reactive oxygen species in the induction of Ni_3S_2-induced lymphocyte death, scavengers of superoxide anion (O_2^-) such as superoxide dismutase (SOD) (0, 2,500-5,000 U/ml), scavengers of H_2O_2 such as catalase (0, 1,000-2,000 U/ml) and scavengers of hydroxyl radical such as dimethyl thiourea (DMTU) (0-20 mM) and mannitol (0-50 mM), glutathione (GSH) (0-8 mM) and N-acetylcysteine (NAC) (0-8 mM) (scavengers of ROS) were first separately incubated for 15 min before treatment human lymphocytes with 2 mM Ni_3S_2 for 3 h at 37°C. The lymphocyte death was estimated as described above. To determine the iron-

mediated oxidative damage in Ni-compound-induced lymphocyte death, deferoxamine (DFO) (0-100 μM) was incubated either alone or pretreated for 15 min before incubation with nickel compound for 3 h at 37°C and the lymphocyte death was determined as mentioned above.

Effect of Ni_3S_2 on total, nonprotein, and protein-bound sulfhydryl contents in human lymphocytes.

Both protein-bound (P-SH) and nonprotein sulfhydryl group (NP-SH) contents in lymphocytes were determined using the method of Sedlak and Lindsay (1968). Determination of total sulfhydryl (T-SH) contents: Briefly, aliquots of 0.25 ml of the cell suspension (Ni-treated) were mixed with 0.75 ml of 0.2 M Tris buffer, pH 8.2, and 0.05 ml of 0.01 M 5,5'-dithiobis-(2-nitrobenzoic acid (DTNB). A reagent blank (without sample) and a sample blank (without DTNB) were prepared in a similar manner. The test tubes after stoppered with rubber caps were immediately agitated and the reaction mixtures centrifuged at approximately 3000 g at room temperature for 15 min. The absorbance of the supernatants was read in a Beckman model DU-7 Spectrophotometer at 412 nm. Determination of NP-SH groups: Aliquots of 0.5 ml of the lymphocyte suspension were mixed in 15 ml test tubes with 0.4 ml of distilled water and 0.1 ml of 50% trichloroacetic acid (TCA). The tubes were immediately agitated and

centrifuged at 3000 g at room temperature for 15 min. 0.5 ml of the supernatant was mixed with 1 ml of 0.4 M Tris buffer, pH 8.9, and 0.025 ml DTNB and the sample was shaken. The absorbance was read within 5 min of the addition of DTNB at 412 nm against a reagent blank. The contents of different sulfhydryl groups were quantified by comparison of the results from a standard curve. The content of PB-SH is calculated by subtracting the content of NP-SH from that of T-SH.

Determination of the mitochondrial permeability transition (MPT)

Rhodamine 123, a cationic fluorescent dye whose mitochondrial fluorescence intensity decreases quantitatively in response to dissipation of the mitochondrial membrane potential, was used to evaluate perturbations in mitochondrial membrane potential (Wu et al. (1990). Both control and Ni-treated lymphocytes as described above were incubated at 37°C with 1.5 μM rhodamine 123 in an incubator for 10 min with gentle shaking, followed by washing the lymphocytes with the culture medium. Thereafter, lymphocytes were suspended in culture media prior to fluorescence measurement with excitation at 493 nm and emission at 522 nm using a Perkin-Elmer MPF 3 L spectrofluorimeter.

To determine the effect of cyclosporin A on MPT, lymphocytes

were cotreated at 37°C for 3 h with 2 and 5 μM cyclosporin A (specific inhibitor of MPT, and calcineurin and Ca^{2+} release from mitochondria) and 1 mM Ni_3S_2, or without Ni-compound (control). First, lymphocyte death was estimated as described above, and immediately thereafter incubated at 37°C with 1.5 μM rhodamine 123 as described above. The uptake of rhodamine in both control and Ni-treated lymphocytes was measured fluorometrically as described above.

Measurement of intracellular Ca^{2+}, $[Ca^{2+}]_i$

At first isolated human lymphocytes were treated at 37°C for 0-2 h with 1 mM Ni_3S_2, as described above. The lymphocytes were then washed from their original medium with Hanks' Balanced Salts Solution (HBSS) (137 mM NaCl, 5.4 mM KCl, 8 mM $MgSO_4$, 0.4 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$ and 15 mM glucose) and resuspended in oxygenated (95% O_2, 5% CO_2) HBSS supplemented with 1% of FCS (loading medium) and incubated with 5 μM Fura-2 AM at 37°C for 30 min. Then the cells were centrifuged for 5 min at 1800 rpm, washed twice with HBSS, and resuspended in the HBSS at a concentration of 1.5 x 10^6 cells/ml. The cells were transferred to a cuvette in a Perkin-Elmer MPF 3 L spectrofluorometer. Changes in $[Ca^{2+}]_i$ were evaluated at 25°C by measuring the intensity of the fluorescence emission at 500 nm with the excitation at 336 nm and 366 nm as described by Grynkiewicz et al. (1985). The Fura-2 AM

fluorescence ratio signal was calibrated in terms of $[Ca^{2+}]_i$ as described by Grynkiewicz et al. (1985) and according to the following equation: $[Ca^{2+}]_i = K_d(R-R_{min})/(R_{max}-R)(F_o/F_s)$ where K_d is 184 nM (a value for the Fura-2 AM-Ca^{2+} complex), R the ratio 336/366 nm of fluorescence of the indicator, R_{min} the ratio 336/366 nm of Fura-2 AM in the Ca^{2+}-free HBSS, R_{max} the ratio of Fura-2 AM in the presence of saturating Ca^{2+} concentration (1 mM $CaCl_2$) and F_o/F_s the ratio of 366 nm excitation fluorescence at zero and saturating Ca^{2+} levels.

To determine the role of intracellular calcium, $[Ca^{2+}]_i$ in Ni_3S_2-induced human lymphocyte death, isolated lymphocytes were treated at 37°C for 3 h with (1 mM) Ni_3S_2 alone, or simultaneously with each of the following calcium channel blockers/antagonist such as verapamil (12 and 25 μM), nifedipine (12 and 25 μM), diltiazem (50 μM), ruthenium red (inhibitor of Ca^{2+} uptake by mitochondria) (2 and 5 μM), dantrolene (inhibitor of Ca^{2+} release from sacroplasmic reticulum) (25 and 50 μM), cyclosporin A (inhibitor of Ca^{2+} release from mitochondria) (2 μM) and BAPTA (Ca^{2+} chelator) (3 and 6 mM). At the end of the incubation period, the lymphocyte death was estimated in control and various Ni-treated groups as described above. Such concentrations of different modulators of $[Ca^{2+}]_i$ used in this study were found to be non-toxic.

Data analysis

The data are presented as the mean ± SEM for four separate experiments. Data were analysed by analysis of variance. Difference between treatment means was tested by Tukey-Kramer multiple comparisons test. For time-dependent effects, the treatment means were compared with corresponding control including a zero-time control value. The level of significance was set at $p < 0.05$.

Results

Concentration- and time-dependent effects of nickel subsulfide (Ni_3S_2) on human lymphocyte death *in vitro*.

Freshly isolated human lymphocytes were treated *in vitro* with various concentrations of soluble form of Ni_3S_2 (0-2 mM) at 37°C for 4 hours. The results of such concentration-dependent effects on human lymphocyte death (as measured by the trypan blue exclusion) are presented in Fig. 1A. The respective minimum and maximum concentrations required for significant minimum and maximum (100%) lymphocyte death due to exposure to Ni_3S_2 are 0.75 and 2 mM Ni_3S_2 respectively. Ni_3S_2 has shown also the time-dependent effects on human lymphocyte death (Fig. 1B).

Effects of Ni_3S_2 on the generation of reactive oxygen species (ROS), or oxidative stress.

The excess generation of H_2O_2 due to exposure of human lymphocytes to 1 mM Ni_3S_2 at different time periods (0-3 h) has been presented in Fig. 2A. Significant generation of H_2O_2 due to Ni_3S_2 started after 15 min of such exposure and attained a maximum value after 60 min of such exposure followed by gradual diminution but still significantly high during later time periods. The time-dependent effects of exposure of human lymphocytes to 1 mM Ni_3S_2 on the excess generation of superoxide anion (O_2^-) as measured by NBT reduction are shown in Fig. 2B. Ni_3S_2 were capable of generating significant amounts of superoxide anion compared to control starting after 60 min of such exposure. Since the generation of ROS is often accompanied by a change in the thiol status, we also determined the effects of Ni_3S_2 on lymphocyte nonprotein (NP)- and protein-sulfhydryl (P-SH) contents. Significant time-dependent diminution of both nonprotein- and protein sulfhydryl contents has been observed starting after 15 min of exposure to 1 mM Ni_3S_2. Such diminution of NP-SH reached a maximum value of about 10-12% of the control value following 60-240 min of exposure of human lymphocytes to Ni_3S_2 (Fig. 3). Similarly, a maximum decrease of about 10-12% of the control value for protein-SH (P-SH) contents was observed following

60-240 min of exposure of lymphocytes to this Ni-compound (Fig. 3). Time-dependent effect of Ni_3S_2 on the production of lipid peroxidation (LPO) (as measured by malondialdehyde (MDA) formation) in human lymphocytes is shown in Fig. 4. Significant increase of LPO due to Ni_3S_2 started to occur following 60 min of such exposure, and stayed significantly high till 180 min of such exposure.

Effects of ROS scavengers and antioxidants on Ni_3S_2-induced human lymphocyte death

To examine the role of ROS on Ni_3S_2-induced human lymphocyte death, we have evaluated the effects of various ROS antagonists on such lymphocyte death. Since significant depletion of nonprotein sulfhydryl content (the reductive reserve) occurred much earlier than the lymphocyte death due to Ni_3S_2 we have therefore examined whether this was associated with Ni-induced lymphocyte death. It is seen that pre-treatment of lymphocytes for 15 min with excess glutathione (GSH) significantly reduced such cell death induced by nickel subsulfide (Fig. 5). Pretreatment of lymphocytes with excess *N*-acetylcysteine significantly reduced Ni-compound-induced lymphocyte death (Fig. 5). Similarly, pretreatment of human lymphocytes with catalase for 15 min significantly reduced lymphocyte death induced by Ni_3S_2 (Fig. 5). Significant protection against Ni-induced lymphocyte death was achieved following such

pretreatment with superoxide dismutase (a scavenger of superoxide anion, O_2^-) (Fig. 5). Pretreatment of lymphocytes with either dimethylthiourea (DMTU), or mannitol (hydroxyl radical scavenger) for 15 min significantly prevented Ni_3S_2-induced cell death induced (Fig. 5). The effect of deferoxamine (DFO), a potent iron-chelator, on Ni-induced lymphocyte death was also studied. When lymphocytes were treated with Ni-compound in the presence of deferoxamine (DFO) the percentage of cell death induced by Ni-compound was significantly reduced (Fig. 5).

Role of mitochondrial permeability transition (MPT) on Ni_3S_2-induced lymphocyte death.

We have examined the effect of Ni_3S_2 on the induction of MPT in isolated human lymphocytes and hence, to find out the relationship between Ni-induced induction of MPT and Ni-induced lymphocyte death. The MPT was measured by estimating Rhodamine 123 uptake using fluorescence method. Thus, when isolated human lymphocytes were exposed to 1 mM nickel subsulfide for different periods of time, e.g. 15, 30, 60, 130, 180 min, significant induction of MPT compared to the control value was observed starting after after 60 min exposure to nickel subsulfide (Fig. 6A). Furthermore, the MPT continued to be significantly induced due to the nickel compound till 180 min of exposure used in this study (Fig. 6A). We have also utilized a specific

MPT pore inhibitor, cyclosporin A (CsA) in order to examine whether the MPT pore is directly involved in the lymphocyte death induced by Ni_3S_2. Thus when lymphocytes were treated for 3h with 2 mM nickel subsulfide in the presence of 1 μM cyclosporin A, the reduced Rhodamine 123 uptake caused by the Ni-compound was significantly ameliorated, and reached a nearly control value due to CsA cotreatment (Fig. 6B). At the same time, such cotreatment with CsA significantly prevented the lymphocyte death induced by Ni_3S_2 (Fig. 6B).

Effects of intracellular calcium homeostasis on Ni_3S_2-induced lymphocyte death.

This study was designed to seek evidence of any impaired calcium regulation whether occurring early during Ni^{2+}-induced lymphocyte death and signs of a close temporal relationship between the onset of lymphocyte death and any impaired calcium ion (Ca^{2+}) regulation due to soluble form of Ni_3S_2. Therefore, the effects of Ni^{2+}-induced lymphocyte death were investigated using different calcium channel blockers involving $[Ca^{2+}]_i$ influx and release as well as Ca^{2+} uptake. As pharmacological tools, we used primarily compounds that were known to act on cell membranes. However, since elevated $[Ca^{2+}]_i$ can also originate from intracellular stores in mitochondria, or smooth endoplasmic reticulum (Trump et al, 1989), and since

mitochondria were shown to be affected by exposure to soluble form Ni_3S_2 in this study, we therefore tested the possibility that modulating intracellular calcium fluxes using both Ca^{2+} channel blockers/antagonist, inhibitor of Ca^{2+} release/uptake, and Ca^{2+} chelator might prevent the Ni-induced lymphocyte death and the results are presented in Fig. 7. It is seen that cotreatments of lymphocytes with Ni_3S_2 and different Ca^{2+} channel blockers, such as diltiazem, nifedipine and verapamil considerably and significantly prevented in concentration-dependent manner the lymphocyte death induced by Ni_3S_2. Similarly cotreatments of lymphocytes with Ni_3S_2 and different inhibitor of Ca^{2+} release, such as dantrolene, cyclosporin A, and inhibitor of Ca^{2+} uptake such as ruthenium red significantly and considerably protected in concentration-dependent manner against lymphocyte death induced by Ni-compound (Fig. 7). Furthermore, cotreatment of human lymphocytes with Ni_3S_2 and 1,2-bis (2-Aminophenoxy) ethane-*N*,*N*,*N*´,*N*´-tetraacetic acid tetrapotassium salt (BAPTA) (an intracellular Ca^{2+} chelator) significantly protected in concentration-dependent manner against Ni-compound-induced lymphocyte death (Fig. 7).

Discussion

Increased generation of various types of ROS (e.g. H_2O_2, superoxide anion, hydroxyl radical) as well as depletions of

nonprotein-sulfhydryl (NP-SH) and protein-bound sulfhydryl (P-SH), and activation of lipid peroxidation due to Ni_3S_2 are all believed to be the initiators of pathogenesis of lymphocyte death, as they appeared before any occurrence of Ni-compound-induced lymphocyte death. These are further confirmed as follows. The percentage of Ni-compound-induced dead cells is significantly reduced by treatment with excess GSH, or excess N-acetylcysteine (a precursor of GSH), as cellular depletion of NP-SH contents appeared initially at 15 min and the lymphocyte death occurred 120 min after Ni-treatment. It suggests that intracellular GSH level is one of the determining factors in the susceptibility of human lymphocytes to the cytotoxic effect of Ni_3S_2. Excess GSH may be involved in the metabolism of free radicals and hydroperoxides. Ni-compound (for example, nickel carbonate hydroxide)-induced generation of H_2O_2 is significantly reduced not only by catalase and DFO, but also by excess GSH (unpublished results). This suggests that the loss of cellular thiol reserve also resulted in significant generation of H_2O_2 and hydroxyl radical. The inhibition of Ni_3S_2-induced human lymphocyte death by catalase suggests that H_2O_2 participates in such lymphocyte death. Similarly, the significant prevention of Ni-compound-induced lymphocyte death by mannitol and dimethylthiourea indicates that Ni-compound-induced cell death also occurs via generation of excess reactive hydroxyl radical species in isolated human lymphocytes. Significant

prevention of soluble form of nickel subsulfide-induced lymphocyte death by superoxide dismutase (SOD) suggests the participation of superoxide anion (O_2^-) as well in such lymphocyte death process. Significant prevention of lymphocyte death induced by this Ni-compound has also been observed following pretreatment with DFO. Iron acts as a Fenton catalyst to produce hydroxyl radical from H_2O_2. Deferoxamine (DFO) being an iron chelator has the potential to inhibit the process of Ni^{2+}-induced ROS, resulting in prevention against Ni-compound-induced lymphocyte death. Thus this Ni-compound may express its cytotoxic (cell death) potential by way of iron-mediated oxidative damage as well. Furthermore, these findings provide support for iron chelator therapy in protection against Ni-compound induced oxidative damage.

The mechanism by which Ni_3S_2 increases generation of ROS in human lymphocytes is not clear. Ni_3S_2 is believed to activate neutrophils and cause substantial production of H_2O_2 levels (Zhong et al, 1990). The peptides containing the glycyl-glycyl-L-histidyl sequence may trigger nickel-dependent production of oxygen radicals through reaction with H_2O_2. When chelated with peptides containing the glycyl-glycyl-L-histidyl sequence, Ni^{2+} could also peroxidize lipids either through H_2O_2 disproportionation and hydroxyl radical production, or directly by reaction with the lipid peroxides (Torreilles

and Guérin, 1990). However, Ni^{2+} ions themselves have been shown to induce the formation of oxidized DNA bases (Kawanishi et al., 2002; Nackerdien et al., 1991). Similarly, nickel subsulfide (soluble form)-induced formation of reactive oxygen species has been detected by dichlorofluorescein fluorescence in isolated rat renal cortical cells *in vitro* (Chakrabarti et al, 1999). Both nickel subsulfide (insoluble) and nickel chloride (soluble) have been shown to induce increased oxidants in intact cultured mammalian cells as detected by dichlorofluorescein fluorescence (Huang et al, 1993), and by nickel chloride in isolated human lymphocytes (Chen et al, 2003). In view of above considerations, whether the soluble form of Ni_3S_2 is capable of only directly inducing increased generation of H_2O_2, or indirectly through some biological chelators cannot be determined from this study and warrants further investigation. Similarly, whether the soluble form of nickel subsulfide is capable of directly inducing superoxide anion (O_2^-) in isolated human lymphocytes, or indirectly through some biological chelators cannot be ascertained from this study and therefore warrants further evaluation.

Following the method of Lee et al (1982) significant concentrations (≈10mM) of Ni(II) were found in solution following incubation of insoluble Ni_3S_2, up to 12 hr in 0.05M Tris-HCl buffer, pH 7.4. However, the nature of the chemical form of this Ni-

compound after solubilizing in Tris-HCl buffer, pH 7.4 was not determined in our present study. On the other hand, this increase in solubility for Ni_3S_2 for example, can be accounted for by the ability of Tris to coordinate nickel (II) ion and form soluble charged complexes (Dotson, 1972). The solubilized nickel exhibited electronic absorption spectra and magnetic moments characteristic of a octahedral nickel (II).

Cyclosporin A (CsA) is often considered as a specific blocker of the mitochondrial permeability transition (MPT) as well as a specific inhibitor of mitochondrial membrane potential (Lemasters et al, 1998a, b; Quian et al, 1997). Since CsA cotreatment markedly reduced the lymphocyte death induced by Ni_3S_2 (Fig. 6B), it indicates that (a) Ni-compound has the potential to induce mitochondrial dysfunction in human lymphocytes and (b) the MPT plays equally an important role in Ni-compound-induced lymphocyte death signalling process. CsA affects the mitochondria by inhibiting the induction of MPT, thereby may prevent disruption of the transmembrane potential caused by Ni-compound. Studies with isolated mitochondria have shown that MPT pore favors a closed state, but some physiological and pathological signals trigger pore opening (Bernardi and Petronilli, 1996). The fully opened state creates a channel for $\leq$ 1.5 kDa molecules, resulting in dissipation of the H^+ gradient across the

membrane and uncoupling of the respiratory chain (Green and Amarante-Mendes, 1998). Thus opening of high conductance pores in the mitochondrial inner membrane triggered by Ni_3S_2 may precipitate the onset of the MPT, which could lead to membrane depolarization and release of ions and other physiological effects. The MPT has been linked to significant depletion of cellular ATP (Duchen, 2000; Qian et al, 1999). It is postulated that the MPT uncouples mitochondria and causes consumption of ATP by mitochondrial ATPases. Thus, studies by Arsalane et al (1994) on the cytotoxicity of nickel carbonate hydroxide using guinea pig alveolar macrophages in culture have shown significant generation of free radicals and a depletion of cellular energy reserve particularly ATP. The mechanism of pore blockage by cyclosporin A has not yet been resolved, but may involve binding with the mitochondrial matrix protein, cyclophilin family protein associated with MPT pore (also called peptidylprolyl-cis-trans isomerase (PPIase) rather than direct interaction with the pore itself (Nicolli et al, 1996; Connern et al, 1992).

Evidence indicating that Ni-compound-induced initial ROS or oxidative stress is responsible for Ni-compound-induced MPT was obtained from the observation that the pretreatments of human lymphocytyes with either catalase (a H_2O_2 scavenger), or excess GSH significantly and effectively inhibited nickel carbonate hydroxide-

induced MPT (unpublished results). The present study has also established a temporal relationship between the generation of ROS, depletion of thiol reserves which was then followed by a mitochondrial event such as MPT, which in turn appeared before any cell death.

Our previous study has shown that nickel carbonate hydroxide-induced destabilization of cellular calcium homeostasis is caused not only by Ni-compound-induced oxidative stress but also by Ni-compound-induced MPT. This is verified by the observation that not only excess catalase and GSH (scavengers of ROS) but also cyclosporin A (a specific blocker of the MPT and a specific inhibitor of mitochondrial membrane potential) significantly reduced nickel carbonate hydroxide-induced elevation of $[Ca^{2+}]_i$. Consequently, both ROS and mitochondria were causally involved in such destabilization of cellular calcium homeostasis (manuscript submitted). The present study has identified several calcium sites as additional targets by which Ni^{2+} may perturb cellular Ca^{2+} homeostasis which may therefore result in human lymphocyte death. Thus, analyses of the different mechanisms involving the effects of different modulators of calcium channel blockers governing the increase of $[Ca^{2+}]_i$ suggest that the protective effects of nifedipine, verapamil and diltiazem against Ni-compound-induced increase of $[Ca^{2+}]_i$ and the consequent lymphocyte death are due to inhibition of calcium movements

through voltage-operated calcium channel protein involving plasma membranes. Furthermore, the present study has also indicated that other calcium antagonists are also able to inhibit calcium movements through other mechanisms as well. Thus, Ni_3S_2-induced lymphocyte death can also be significantly prevented by inhibiting Ca^{2+} release from sacroplasmic reticulum by dantrolene cotreatment as well as inhibiting Ca^{2+} release from mitochondria by cyclosporin A treatment and inhibiting Ca^{2+} uptake by mitochondria by ruthenium red treatment. Thus, the soluble form of Ni_3S_2 may increase $[Ca^{2+}]_i$ via release of Ca^{2+} from voltage-operated calcium channel sensitive protein, as well as from mitochondria, and sarcoplasmic reticulum. Soluble nickel has been shown to compete with calcium for channels and to be taken up through calcium channels (Refvik and Andreassen, 1995; Funakoshi et al, 1997). It has been shown that Ni uptake by rat hepatocytes occurs, at least in part, through the Ca channel transport processes. Pretreatment with nicardipine or verapamil, potent inhibitors of Ca channels, decreased Ni uptake by 20% (Funakoshi et al, 1997). It is possible that soluble form of Ni_3S_2 apparently competes with the essential metals like calcium to use the same pathways, thus disrupting the intracellular balance of Ca^{2+} and resulting in cell death. However, other mechanisms of Ni uptake might also be involved which requires further study. Since exogenous addition of BAPTA, a specific chelator of free intracellular calcium,

$[Ca^{2+}]_i$ also attenuated Ni^{2+}-induced lymphocyte death (Fig. 7), it indicates that elevation of intracellular calcium was essential for such death. Overall, the present data may provide some new insights into the mechanisms of Ni_3S_2-induced human lymphocyte death, as well as contribute important information for the treatment and prevention of adverse health effects in workers of nickel-related industries.

ACKNOWLEDGMENTS

The authors sincerely thank Sainte-Justine Hospital, for obtaining blood samples from donors and from the laboratory of vascular immunology of Dr. Jean-Claude Mamputu of CHUM, Research Center, Notre-Dame Hospital, Montreal.

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FIGURE CAPTIONS

FIG. 1. (A) Concentration-dependent effects of nickel subsulfide (Ni_3S_2) on human lymphocyte death. Freshly isolated human lymphocytes were treated *in vitro* with various concentrations of soluble form of Ni_3S_2 at 37°C for 4 hours. (B) Time-dependent effects of nickel subsulfide (Ni_3S_2) on human lymphocyte death. Isolated lymphocytes were exposed *in vitro* to 1 mM of Ni_3S_2 for different time periods at 37°C. Cell death was determined by trypan blue dye exclusion. Values represent mean ± SEM from four separate experiments involving four separate blood donors. *Significantly different from control, $p < 0.05$. [a]Significantly different between Ni_3S_2-treated group, $p < 0.05$. If the error bar is not shown, the SEM is within the symbol.

FIG. 2. (A) The generation of hydrogen peroxide (H_2O_2) and (B) superoxide anion (O_2^-) following exposure of isolated human lymphocytes to 1 mM of Ni_3S_2 at various time periods (0-180 min). For

details, see Materials and Methods. The values represent mean ± SEM of four separate experiments from four separate blood donors. *Significantly different from control, $p < 0.05$.

FIG. 3. Time-dependent effects of nickel subsulfide (Ni_3S_2) on (A) nonprotein sulfhydryl (NP-SH) and (B) protein sulfhydryl (P-SH) contents following exposure of human lymphocytes to Ni_3S_2 for various times. For details, see Materials and Methods. The values represent mean ± SEM from four different experiments. *Significantly different from control, $p < 0.05$.

FIG. 4. The production of lipid peroxidation (as measured by MDA formation) following exposure of human lymphocytes to nickel subsulfide (Ni_3S_2) for various time periods. The results are mean ± SEM for 4 separate experiments. *Significantly different from control, $p < 0.001$.

FIG. 5. Effects of various scavengers of ROS on nickel subsulfide (Ni_3S_2)-induced human lymphocyte death. Human lymphocytes were either first pretreated for 15 min, or not followed by exposure to 1 mM Ni_3S_2 for 3 h at 37°C. Lymphocyte death was measured by trypan blue dye exclusion. Results are mean ± SEM for four separate experiments. Control (Ctl), A= Ni_3S_2 (1 mM), B= Catalase pretreated

(1000 U/ml), C= Catalase pretreated (2000 U/ml), D= SOD pretreated (2500 U/ml), E= SOD pretreated (5000 U/ml), F= GSH pretreated (4 mM), G= GSH pretreated (8 mM), H= NAC pretreated (4 mM), I= NAC pretreated (8 mM), J= Mannitol pretreated (25 mM), K= Mannitol pretreated (50 mM), L= DMTU pretreated (10 mM), M= DMTU pretreated (20 mM), N= DFO pretreated (100 μM). *Significantly different from Ni_3S_2-alone-treated group, $p < 0.01$.

FIG. 6. (A) Time-dependent effects of Ni_3S_2 on the mitochondrial permeability transition (MPT) (or, the mitochondrial membrane potential). (B) Effects of cyclosporin A on Ni_3S_2-induced MPT and lymphocyte death in isolated human lymphocytes. Human lymphocytes were treated at 37°C for 3 h with either 1 mM Ni_3S_2 or 1 mM Ni_3S_2 plus 2 μM cyclosporin A (CsA). The MPT was evaluated by the capacity of lymphocytes to take up the fluorescent cationic dye rhodamine 123. The results expressed as percentage of the total fluorescence of rhodamine 123 in the incubation medium. Lymphocyte death was determined by trypan blue dye exclusion. Results are means ± SEM of four separate experiments. *Significantly different from control, $p < 0.001$. [a]Significantly different from Ni_3S_2-alone-treated group, $p < 0.05$.

FIG. 7. Effects of various Ca^{2+} antagonists on nickel subsulfide (Ni_3S_2)

(1 mM)-induced human lymphocyte death. For details, see Materials and Methods. (A) Ni_3S_2 alone, (B) Ni_3S_2 plus 50 μM diltiazem, (C) Ni_3S_2 plus 12 μM nifedipine, (D) Ni_3S_2 plus 25 μM nifedipine, (E) Ni_3S_2 plus 12 μM verapamil, (F) Ni_3S_2 plus 25 μM verapamil, (G) Ni_3S_2 plus 3 mM BAPTA, (H) Ni_3S_2 plus 6 mM BAPTA, (I) Ni_3S_2 plus 2 μM ruthenium red, (J) Ni_3S_2 plus 5 μM ruthenium red, (K) Ni_3S_2 plus 25 μM dantrolene, (L) Ni_3S_2 plus 50 μM dantrolene, (M) Ni_3S_2 plus 2 μM cyclosporin A. Results are mean ± SEM of four separate experiments. *Significantly different from Ni_3S_2-alone-treated group, $p < 0.001$.

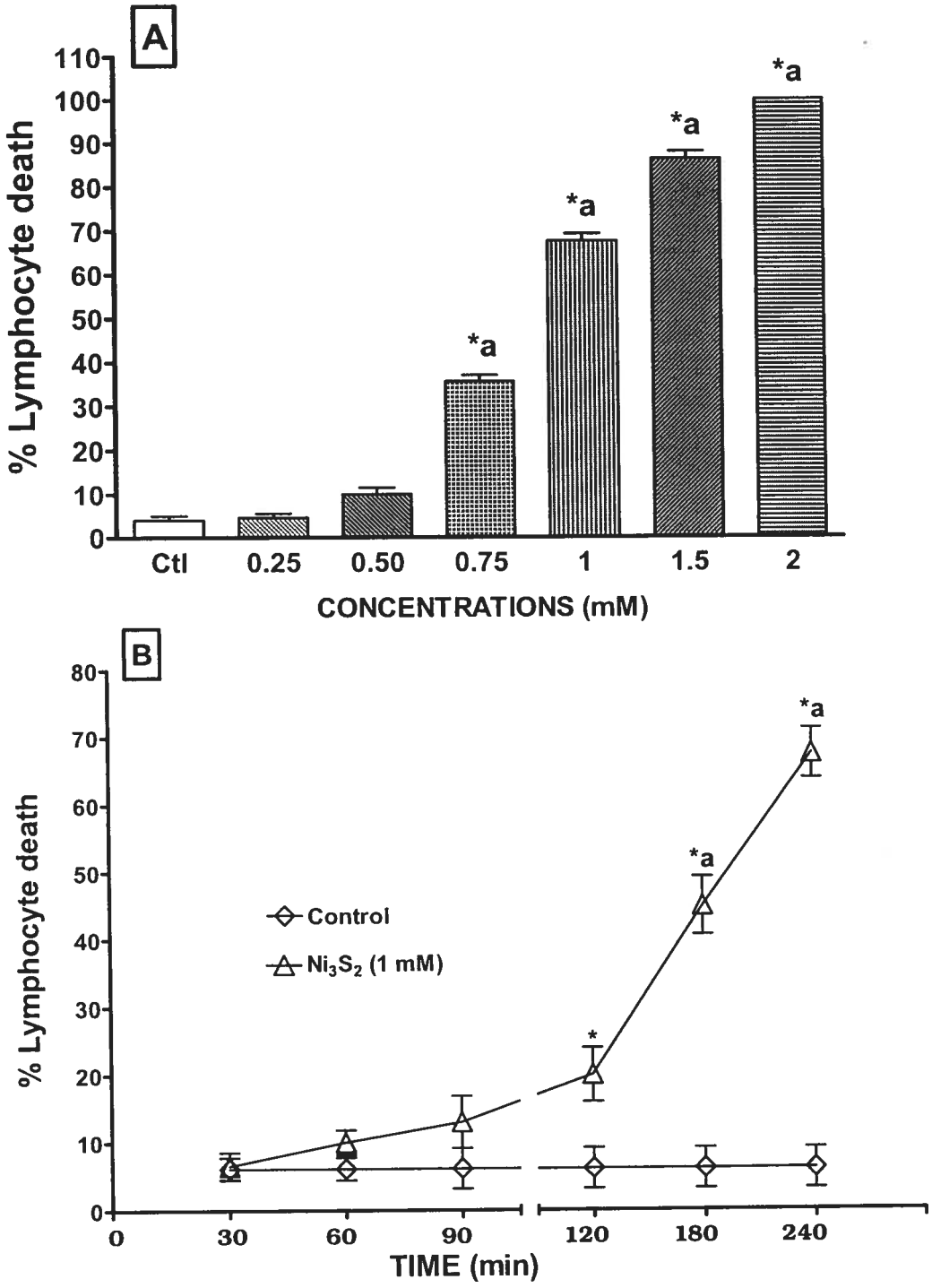
A
% Lymphocyte death
110
100
90
80
70
60
50
40
30
20
10
0
*a
*a
*a
*a
*a
Ctl
0.25
0.50
0.75
1
1.5
2
CONCENTRATIONS (mM)
B
% Lymphocyte death
80
70
60
50
40
30
20
10
0
*a
*a
*
Control
Ni3S2 (1 mM)
0
30
60
90
120
180
240
TIME (min)

FIG. 1

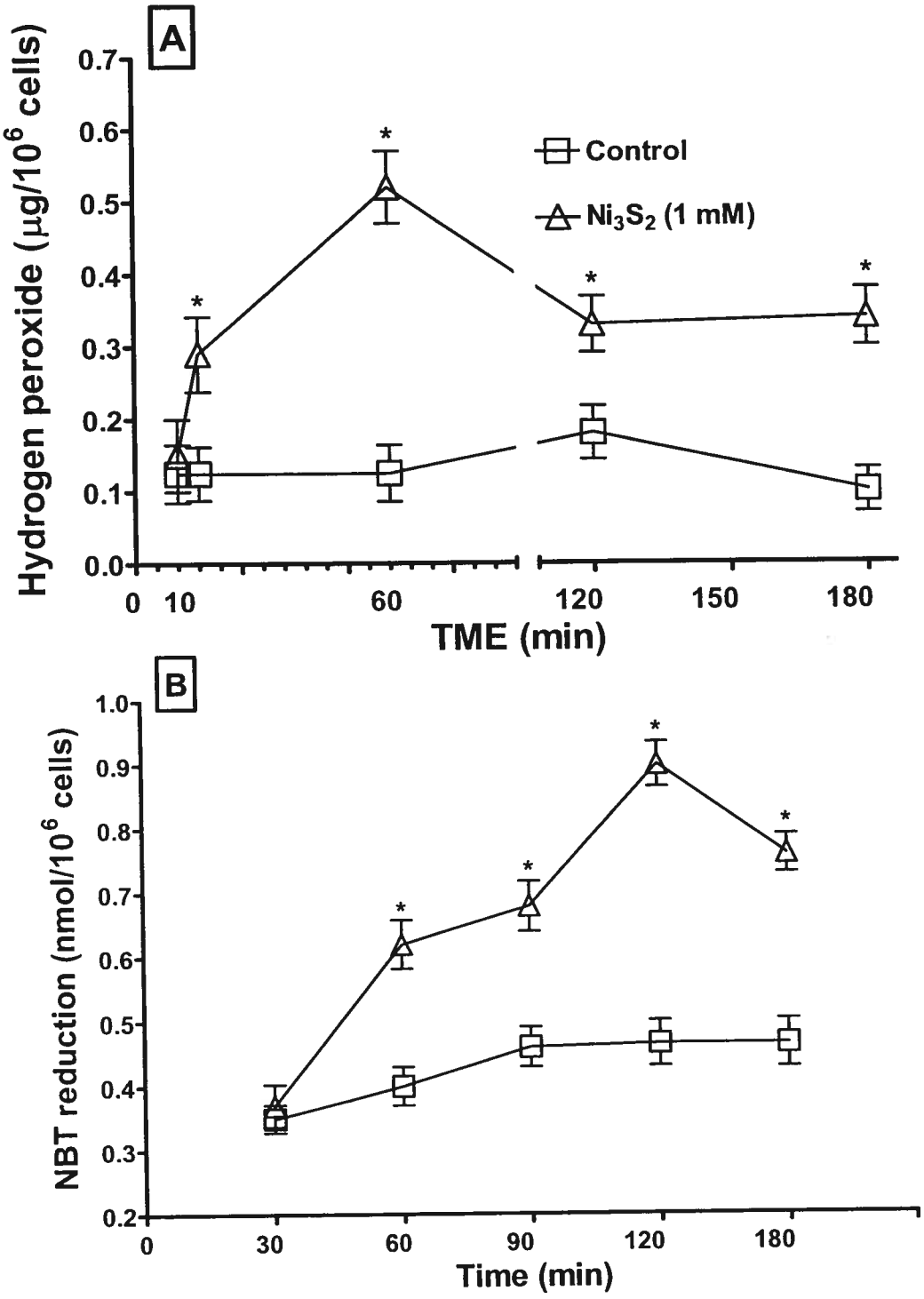
A
Hydrogen peroxide (μg/10^6 cells)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Control
Ni3S2 (1 mM)
0
10
60
120
150
180
TME (min)
B
NBT reduction (nmol/10^6 cells)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0
30
60
90
120
180
Time (min)

FIG. 2

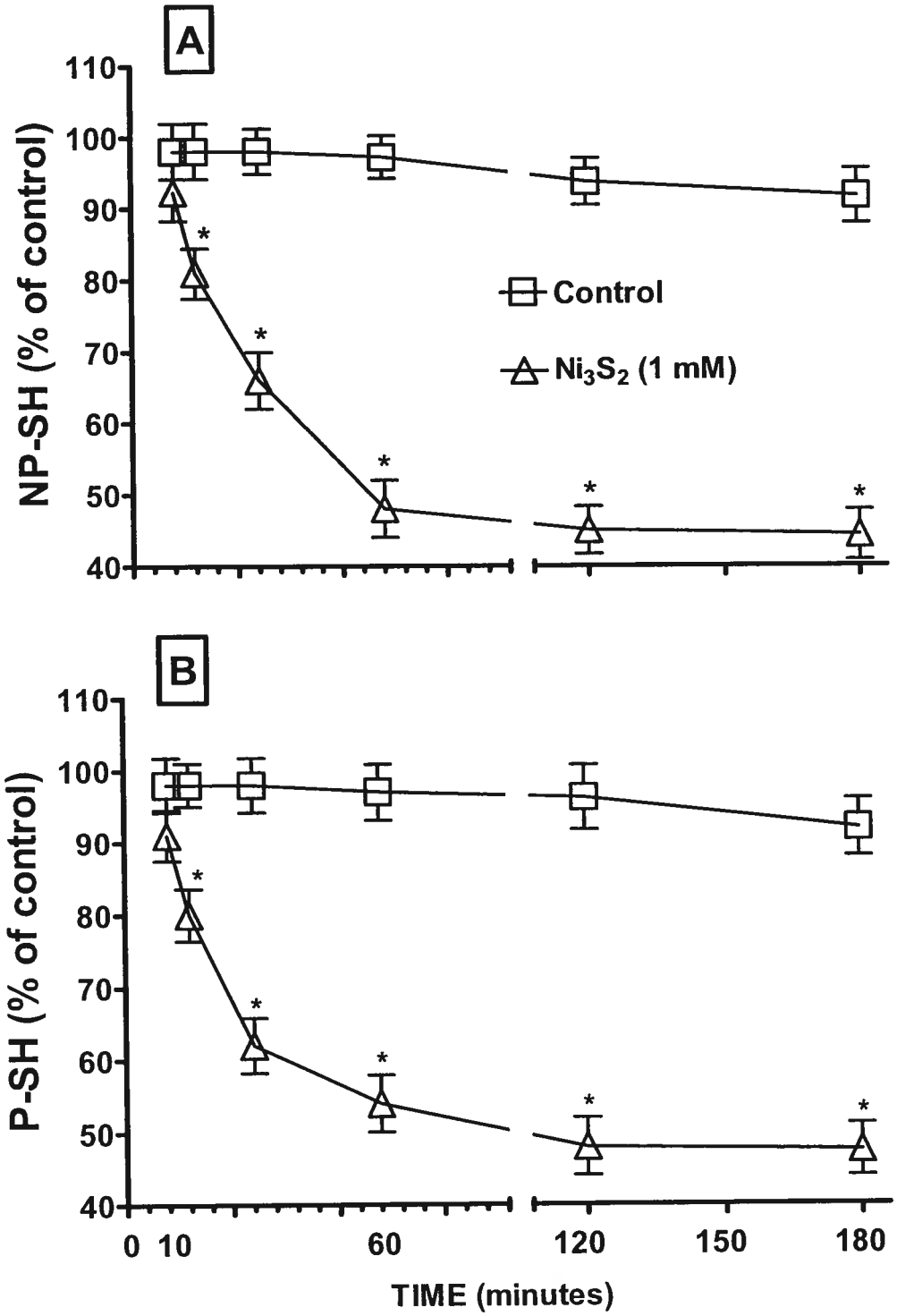
A
NP-SH (% of control)
110
100
90
80
70
60
50
40
Control
Ni_3S_2 (1 mM)
*
B
P-SH (% of control)
0 10
60
120
150
180
TIME (minutes)

FIG. 3

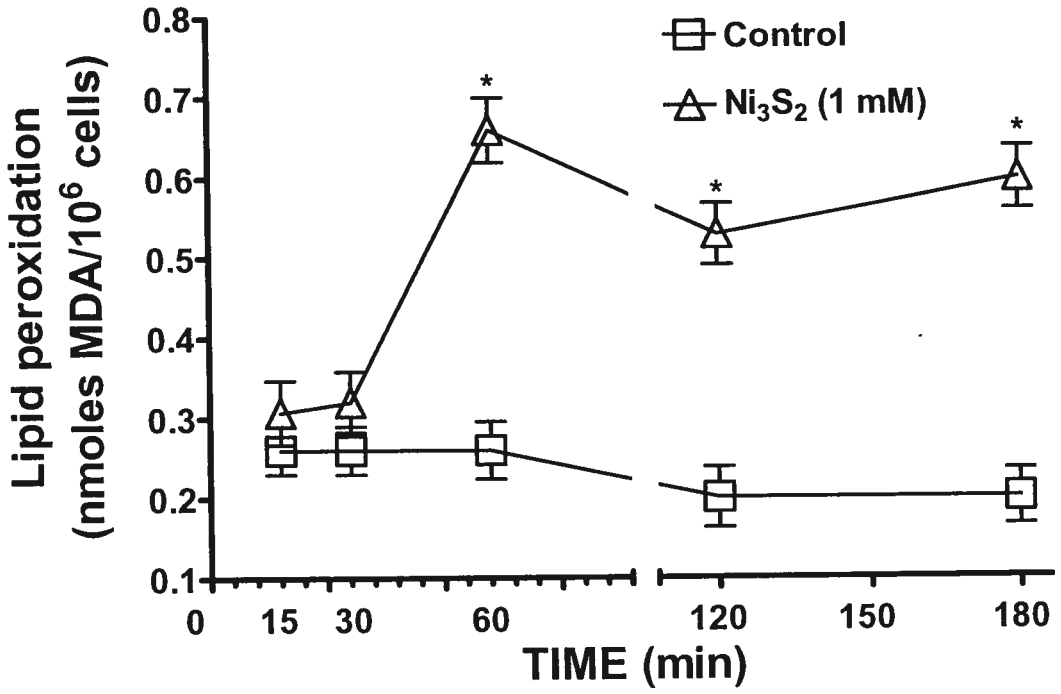
Lipid peroxidation
(nmoles MDA/10^6 cells)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
15
30
60
120
150
180
TIME (min)
Control
Ni3S2 (1 mM)
*
*
*

FIG. 4

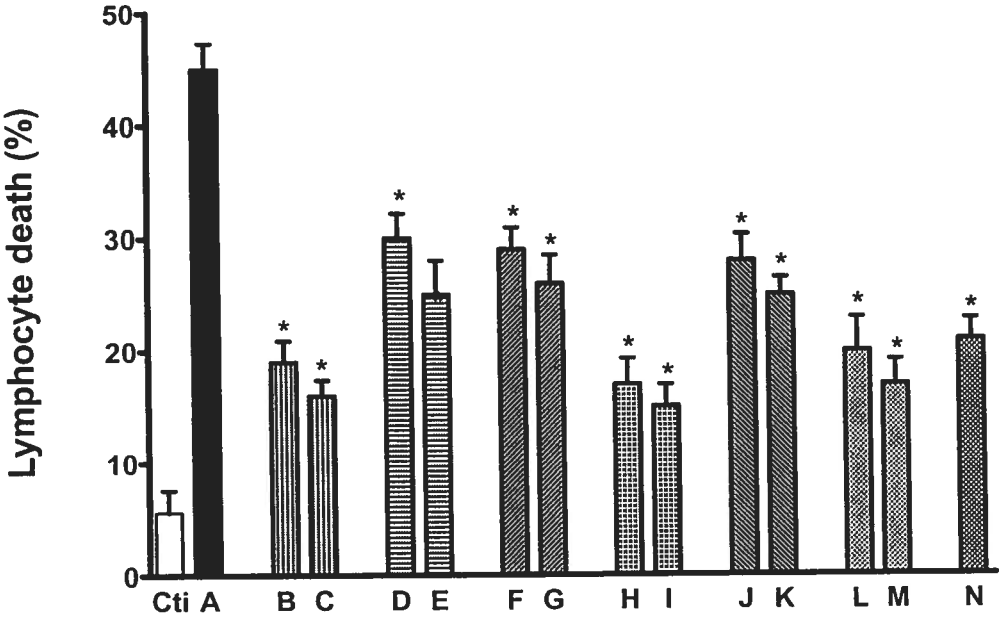

FIG. 5

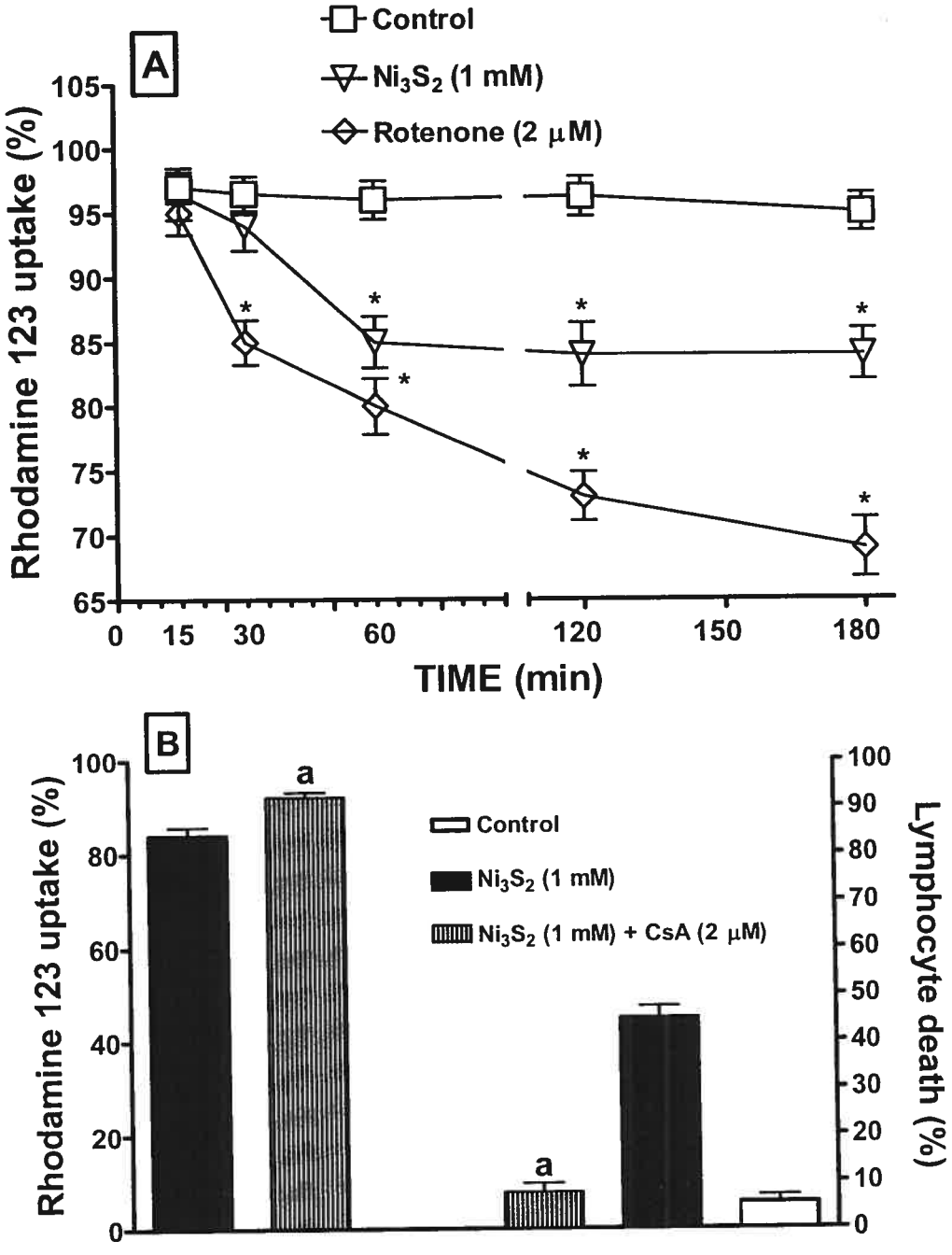
A
Control
Ni3S2 (1 mM)
Rotenone (2 μM)
Rhodamine 123 uptake (%)
105
100
95
90
85
80
75
70
65
0
15
30
60
120
150
180
TIME (min)
B
Rhodamine 123 uptake (%)
100
80
60
40
20
0
a
a
Control
Ni3S2 (1 mM)
Ni3S2 (1 mM) + CsA (2 μM)
Lymphocyte death (%)
100
90
80
70
60
50
40
30
20
10
0

FIG. 6

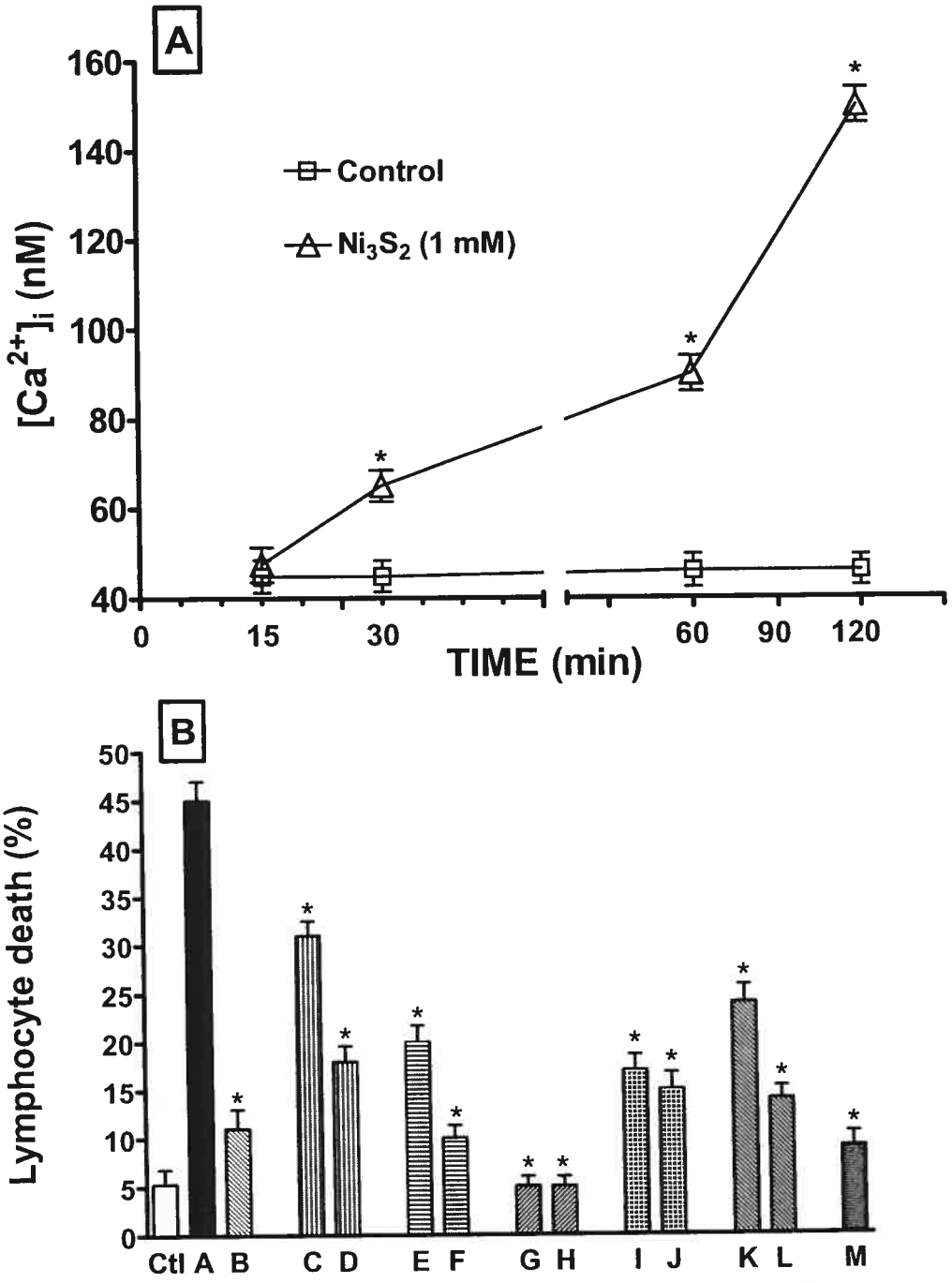
A
160
140
120
100
80
60
40
$[Ca^{2+}]_i$ (nM)
Control
Ni_3S_2 (1 mM)
0
15
30
60
90
120
TIME (min)
B
50
45
40
35
30
25
20
15
10
5
0
Lymphocyte death (%)
Ctl A B C D E F G H I J K L M

FIG. 7

II.3. ARTICLE III

Role of oxidative stress, mitochondrial permeability transition, and calcium homeostasis in nickel sulfate-induced human lymphocyte death *in vitro*.

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Abstract

When isolated human lymphocytes were treated *in vitro* with various concentrations of nickel sulfate ($NiSO_4$) (0-4 mM) at 37°C for 4 h, both concentration-and time-dependent effects of $NiSO_4$ on lymphocyte death were observed. Increased generation of hydrogen peroxide, and superoxide anion, depletion of both nonprotein and protein sulfhydryl contents and lipid peroxidation were induced by $NiSO_4$. $NiSO_4$-induced lymphocyte death was significantly prevented by pre-treatment with either catalase, or excess superoxide dismutase, or dimethylthiourea/mannitol, or deferoxamine, or excess glutathione/N-acetylcysteine. Cotreatment with cyclosporin A (a specific inhibitor of mitochondrial permeability transition) not only inhibited $NiSO_4$-induced mitochondrial permeability transition, but also significantly prevented Ni-compound-induced lymphocyte death. $NiSO_4$-induced lymphocyte death was also significantly prevented by modulating intracellular calcium fluxes using both Ca^{2+} channel blockers and intracellular Ca^{2+} antagonist. Thus, the mechanism of $NiSO_4$-induced activation of lymphocyte death signalling pathways involves not only the excess generation of different types of oxidative stress but also $NiSO_4$-induced mitochondrial permeability transition and destabilization of cellular calcium homeostasis as well.

KEY WORDS: Human lymphocytes; lymphocyte death; oxidative stress; nickel sulfate; mitochondrial permeability transition; calcium homeostasis.

Introduction

Occupational exposure to nickel compounds occurs principally through mining, smelting, refining operations, alloy production, electroplating and welding operations, during manufacture of steel and other alloys and batteries. In 1990, the International Committee on Nickel Carcinogenesis in Man suggested that respiratory cancer risks are primarily related to chronic exposure to soluble nickel concentrations above 1mg/m^3 and to exposure to less soluble forms at concentrations above 10 mg/m^3 (IARC, 1990). In electrolytic refining operations workers are exposed to aerosols of nickel sulfate and nickel chloride. Thus, these nickel compounds possess potentials risks to occupational and environmental health. The increased frequencies of chromosomal aberrations and sister-chromatid exchanges (SCEs) (compared to the control group) in the peripheral lymphocytes of workers occupationally exposed to nickel have been reported in many studies (Werfel et al., 1998; Waksviks et al., 1984; Perminova et al., 2001). These results have shown that the lymphocytes are also the target cells for nickel compounds.

The toxicity and carcinogenicity of Ni-compounds including the oxidative mechanism in experimental animals (review by Kasprzak et al, 2003; Kawanishi et al., 2002; Chakrabarti et al, 2001; M'Bemba-Meka and Chakrabarti, 2001; Obone et al, 1999) and humans (Antico and Soana, 1999; Wozniak and Blasiak, 2002;review by Kasprzak et al, 2003) have been well established. However, the studies relating the roles of various types of oxidative stress as well as other biochemical mechanisms in the development of Ni-compound-induced cellular injury/cell death involving human lymphocytes as target cells are at present very limited or hardly available. A transient increase in the level of blood reticulocytes was reported among workers after consuming water containing nickel sulfate and nickel chloride (Sunderman et al, 1988). Although $NiCl_2$-induced increase in oxidative stress has been observed in human plasma (Chen et al, 2002) and lymphocytes (Chen et al, 2003), how such oxidative stress relates to cellular toxicity/cell death due to $NiCl_2$ is not known.

Nickel has been shown to inhibit mitochondrial succinate dehydrogenase activity in rat lung epithelial cells (Riley et al, 2003). Nickel subsulfide (Ni_3S_2) derived particles were found to be bound preferentially, though less markedly than lymphocytes, to mitochondria, Golgi apparatus, and peroxisomes from lung cells (Shirali et al, 1992). Similarly, the binding of nickel sulfides to both

rat and human lymphocyte subcellular structures, such as the cell membranes, euchromatin, mitochondria, and Golgi system has been reported (Hildebrand et al, 1987). However, the information how such Ni-induced disturbances in mitochondria could affect Ni-induced cellular toxicity or cell death is presently unknown. Very few studies have been reported relating to the toxic effect of nickel with disturbance in calcium homeostasis. For example, an increase in calcium concentrations in mice pancreas has been observed following nickel administration (Funakoshi et al, 1996). Ni^{2+}-treated renal epithelial A6 cells have been shown to release significantly $[Ca^{2+}]_i$ (Faurskov and Bjerregaard, 2002). Levels of free intracellular calcium $[Ca^{2+}]_i$ were increased in nickel-treated cells (Salnikow et al, 1999). However, how such increase in $[Ca^{2+}]_i$ relates to Ni-induced toxicity or cell death is not known. Furthermore, the mechanism leading to the elevation of $[Ca^{2+}]_i$ in nickel-treated cells is also presently not known. Besides, the possibility whether different calcium channels could influence nickel-induced toxicity or cell death is yet to be verified. Therefore, the present investigation was carried out to study the *in vitro* cytotoxic action of nickel sulfate ($NiSO_4$) and its ability to induce different types of oxidative stress, mitochondrial permeability transition and changes in intracellular Ca^{2+} homeostasis in isolated human lymphocytes. Hence, the roles of various oxidative stress, mitochondrial permeability transition and intracellular Ca^{2+}

homeostasis in $NiSO_4$-induced lymphocyte death have been examined.

Materials and methods

Materials

Nickel sulfate hexahydrate ($NiSO_4.6H_2O$, green) was obtained from Sigma–Aldrich Canada Ltd, Ont., Canada. All other chemicals and reagents were obtained from Sigma, unless otherwise mentioned. $NiSO_4.6H_2O$ is highly soluble in water, saline or incubation buffer. The concentration of nickel was determined by electrothermal atomic absorption spectrometry.

The protocols described below were approved by the Ethics Committee on Human Research, Université de Montréal.

Measurement of cell viability in isolated human lymphocytes

Human whole blood was obtained from 4 healthy donors provided by Sainte-Justine Hospital, Montreal. Peripheral blood samples from 4 healthy non-smoking donors between ages 27 and 42 and who were not previously exposed to any radiation or heavy metal compounds or drug therapy and who did not take any alcohol at least two days before this experiment were used in the present experiments. Questionnaires were obtained from each blood donor to

evaluate exposure history. Methodology for the handling of human blood was followed according to the strict guidelines as devised for clinical workers at Sainte-Justine Hospital, Montreal. Human peripheral blood was collected in sodium-heparinized vacutainers. Separation of peripheral blood lymphocytes was carried out under sterile conditions on Ficoll-Paque (Pharmacia) gradients by the method of Boyum (1976). Aliquots of the heparinized whole blood diluted with an equal volume of ice-cold physiological saline (1:1) were gently applied on an equal volume of Ficoll-Paque in centrifuge tubes. The samples were then centrifuged at 400 g for 30 min. The resultant interface (buffy coat) was then carefully aspirated from the gradient, washed twice in ice-cold Dulbecco's phosphate-buffered saline by centrifugation at 200 g for 10 min. The subsequent pellet of purified lymphocytes was finally resuspended in RPMI 1640 medium and adjusted to the desired lymphocyte concentration (1 to $5x10^6$ cells per ml) in RPMI 1640 medium (pH 7.4). This range of cell concentrations was necessary depending on the type of study protocol used. The approximate yield of lymphocytes was determined by counting the cells on a hemocytometer. The viability of the cells was determined by trypan blue dye exclusion.

Determination of $NiSO_4$-induced human lymphocyte death

For developing cell death in a short duration of exposure (3-4 h)

using human lymphocytes in suspension, we had to use higher concentrations (0-4 mM) of $NiSO_4$. The stock solution of $NiSO_4$ was diluted to the appropriate concentrations using RPMI 1640 medium. Isolated human lymphocytes were exposed for 0-4 h at 37°C to either control, or $NiSO_4$ (0-4 mM) in a humidified atmosphere of 95% oxygen and 5% carbon dioxide. At the end of such exposure, lymphocytes from each group were washed twice with RPMI 1640 medium. An equal volume of 0.4% trypan blue dye reagent was then added to the lymphocyte suspension and the percentage of dead lymphocytes was determined under a field microscope, using trypan blue exclusion. To determine the time-dependent effect of $NiSO_4$-induced lymphocyte death, isolated human lymphocytes were exposed at 37°C with $NiSO_4$ (1.5 mM), or control at different times, e.g. 30, 60, 90, 120, 180 and 240 min followed by measurement of lymphocyte death by trypan blue dye exclusion as described above.

Measurements of $NiSO_4$-induced generation of reactive oxygen species/oxidative stress

The production of hydrogen peroxide (H_2O_2) in control and Ni-treated lymphocytes was assayed following a colorimetric method developed by Graf and Penniston (1980). The method is based on the oxidation of iodide in the presence of ammonium molybdate and photometry of the resulting blue starch-iodine complex (which is

stable for several hours) was carried out at 570 nm. The concentration of H_2O_2 was finally estimated from a standard curve. The formation of cellular superoxide anion (O_2^-) was determined by the nitroblue tetrazolium (NBT) reduction assay as described by Rauen et al., (2000) with minor modifications. NBT was added to RPMI 1640 medium (pH 7.4) in a final concentration of 1 mg/ml and lymphocytes were incubated in this medium for 0-3 h at 37°C without and with 1.5 mM $NiSO_4$. At the end of the incubation period, lymphocytes were separated and carefully washed with Hanks' Balanced Salt Solution and then lysed at 37°C with 5% sodium dodecyl sulfate in phosphate buffer (80 mM, pH 7.8) containing 0.45% gelatin. The samples were centrifuged for 5 min at 13000 g. The absorbance due to formazan at 540 nm was determined against a lyses buffer blank immediately and within 2 min after such formation. The concentration of O_2^- was evaluated following the method of Rauen et al., (2000). Lipid peroxidation (LPO) was determined indirectly by measuring the production of malondialdehyde (MDA) followed by its reaction with thiobarbituric acid as described by Schnellmann (1988). Briefly, aliquots of the cell suspension (control and Ni-treated) corresponding to 1 x 10^6 lymphocytes (0.5 ml) were deproteinized with 0.5 ml TCA (10%) and centrifuged. The supernatant (0.9 ml) was then mixed with 0.5 ml 2-thiobarbituric acid reagent (0.76%), heated in a boiling waterbath for 10 min, and

allowed to cool. Sample absorbance was then measured at 535 nm. Protein content was estimated by the method of Bradford, using bovine serum albumin as a standard (Bradford, 1976).

Effects of scavengers of reactive oxygen species on $NiSO_4$-induced lymphocyte death

In order to study the involvement of reactive oxygen species in the induction of $NiSO_4$-induced lymphocyte death, scavengers of H_2O_2 such as catalase (0, 1000-2000 U/ml) and scavengers of hydroxyl radical such as dimethyl thiourea (DMTU) (0-20 mM) and mannitol (0-50 mM), glutathione (GSH) (0-8 mM) and N-acetylcysteine (NAC) (0-8 mM) (scavengers of ROS) were first separately incubated for 15 min before treatment human lymphocytes with 1.5 mM $NiSO_4$ for 3 h at 37°C. The lymphocyte death was estimated as described above. To determine the iron-mediated oxidative damage in Ni-compound-induced lymphocyte death, deferoxamine (DFO) (0-100 μM) was incubated either alone or pretreated for 15 min before incubation with nickel compound for 3 h at 37°C and the lymphocyte death was determined as mentioned above.

Effect of $NiSO_4$ on total, nonprotein, and protein-bound sulfhydryl contents in human lymphocytes

Both protein-bound (P-SH) and nonprotein sulfhydryl group

(NP-SH) contents in lymphocytes were determined using the method of Sedlak and Lindsay (1968). Determination of total sulfhydryl (T-SH) contents: Briefly, aliquots of 0.25 ml of the cell suspension (Ni-treated) were mixed with 0.75 ml of 0.2 M Tris buffer, pH 8.2, and 0.05 ml of 0.01 M 5,5'-dithiobis-(2-nitrobenzoic acid (DTNB). A reagent blank (without sample) and a sample blank (without DTNB) were prepared in a similar manner. The test tubes after stoppered with rubber caps were immediately agitated and the reaction mixtures centrifuged at approximately 3000 g at room temperature for 15 min. The absorbance of the supernatants was read in a Beckman model DU-7 Spectrophotometer at 412 nm. Determination of NP-SH groups: Aliquots of 0.5 ml of the lymphocyte suspension were mixed in 15 ml test tubes with 0.4 ml of distilled water and 0.1 ml of 50% trichloroacetic acid (TCA). The tubes were immediately agitated and centrifuged at 3000 g at room temperature for 15 min. 0.5 ml of the supernatant was mixed with 1 ml of 0.4 M Tris buffer, pH 8.9, and 0.025 ml DTNB and the sample was shaken. The absorbance was read within 5 min of the addition of DTNB at 412 nm against a reagent blank. The contents of different sulfhydryl groups were quantified by comparison of the results from a standard curve. The content of PB-SH is calculated by subtracting the content of NP-SH from that of T-SH.

Determination of the mitochondrial permeability transition (MPT)

Rhodamine 123, a cationic fluorescent dye whose mitochondrial fluorescence intensity decreases quantitatively in response to dissipation of the mitochondrial membrane potential, was used to evaluate perturbations in mitochondrial membrane potential (Wu et al. (1990). Both control and Ni-treated lymphocytes as described above were incubated at 37°C with 1.5 μM rhodamine 123 in an incubator for 10 min with gentle shaking, followed by washing the lymphocytes with the culture medium. Thereafter, lymphocytes were suspended in culture media prior to fluorescence measurement with excitation at 493 nm and emission at 522 nm using a Perkin-Elmer MPF 3 L spectrofluorimeter.

To determine the effect of cyclosporin A on MPT, lymphocytes were cotreated at 37°C for 3 h with 2 and 5 μM cyclosporin A (specific inhibitor of MPT, and calcineurin and Ca^{2+} release from mitochondria) and 1.5 mM $NiSO_4$, or without Ni-compound (control). First, lymphocyte death was estimated as described above, and immediately thereafter incubated at 37°C with 1.5 μM rhodamine 123 as described above. The uptake of rhodamine in both control and Ni-treated lymphocytes was measured fluorometrically as described above.

Measurement of intracellular Ca^{2+}, $[Ca^{2+}]_i$

At first isolated human lymphocytes were treated at 37°C for 0-2 h with 1.5 mM $NiSO_4$, as described above. The lymphocytes were then washed from their original medium with Hanks' Balanced Salts Solution (HBSS) (137 mM NaCl, 5.4 mM KCl, 8 mM $MgSO_4$, 0.4 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$ and 15 mM glucose) and resuspended in oxygenated (95% O_2, 5% CO_2) HBSS supplemented with 1% of FCS (loading medium) and incubated with 5 μM Fura-2 AM at 37°C for 30 min. Then the cells were centrifuged for 5 min at 1800 rpm, washed twice with HBSS, and resuspended in the HBSS at a concentration of 1.5 x 10^6 cells/ml. The cells were transferred to a cuvette in a Perkin-Elmer MPF 3 L spectrofluorometer. Changes in $[Ca^{2+}]_i$ were evaluated at 25°C by measuring the intensity of the fluorescence emission at 500 nm with the excitation at 336 nm and 366 nm as described by Grynkiewicz et al. (1985). The Fura-2 AM fluorescence ratio signal was calibrated in terms of $[Ca^{2+}]_i$ as described by Grynkiewicz et al. (1985) and according to the following equation: $[Ca^{2+}]_i = K_d(R-R_{min})/(R_{max}-R)(F_o/F_s)$ where K_d is 184 nM (a value for the Fura-2 AM-Ca^{2+} complex), R the ratio 336/366 nm of fluorescence of the indicator, R_{min} the ratio 336/366 nm of Fura-2 AM in the Ca^{2+}-free HBSS, R_{max} the ratio of Fura-2 AM in the presence of saturating Ca^{2+} concentration (1 mM $CaCl_2$) and F_o/F_s the ratio of 366 nm excitation fluorescence at zero and saturating Ca^{2+} levels.

To determine the role of intracellular calcium, $[Ca^{2+}]_i$ in $NiSO_4$-induced human lymphocyte death, isolated lymphocytes were treated at 37°C for 3 h with (1.5 mM) $NiSO_4$ alone, or simultaneously with each of the following calcium channel blockers/antagonist such as verapamil (12 and 25 μM), nifedipine (12 and 25 μM), diltiazem (50 μM), ruthenium red (inhibitor of Ca^{2+} uptake by mitochondria) (2 and 5 μM), dantrolene (inhibitor of Ca^{2+} release from sacroplasmic reticulum) (25 and 50 μM), cyclosporin A (inhibitor of Ca^{2+} release from mitochondria) (2 μM) and BAPTA (Ca^{2+} chelator) (3 and 6 mM). At the end of the incubation period, the lymphocyte death was estimated in control and Ni-treated groups as described above. Such concentrations of different modulators of $[Ca^{2+}]_i$ used in this study were found to be non-toxic.

Data analysis

The data are presented as the mean ± SEM for four separate experiments. Data were analysed by analysis of variance. Difference between treatment means was tested by Tukey-Kramer multiple comparisons test. For time-dependent effects, the treatment means were compared with corresponding control including a zero-time control value. The level of significance was set at $p < 0.05$.

Results

Concentration- and time-dependent effects of nickel sulfate ($NiSO_4$) on human lymphocyte death *in vitro*.

Freshly isolated human lymphocytes were treated *in vitro* with various concentrations of $NiSO_4$ (0-4 mM) at 37°C for 4 hours. The results of such concentration-dependent effects on human lymphocyte death (as measured by the trypan blue exclusion) are presented in Fig. 1A. The respective minimum and maximum concentrations required for significant minimum and maximum (100%) lymphocyte death due to exposure to $NiSO_4$ are 1 and 4 mM $NiSO_4$ respectively. $NiSO_4$ has shown also the time-dependent effects on human lymphocyte death (Fig. 1B). The earliest time at which a significant lymphocyte death occurred due to 4 mM $NiSO_4$ was after 120 min of such exposure.

Effects of $NiSO_4$ on the generation of reactive oxygen species, or oxidative stress.

The excess generation of H_2O_2 due to exposure of human lymphocytes to 1.5 mM $NiSO_4$ at different time periods (0-3 h) has been presented in Fig. 2A. Significant generation of H_2O_2 due to $NiSO_4$ started after 15 min of such exposure and attained a maximum value

after 60 min of such exposure followed by gradual diminution but still significantly high during later time periods. Time-dependent effect of $NiSO_4$ on the production of lipid peroxidation (LPO) (as measured by malondialdehyde (MDA) formation) in human lymphocytes is shown in Fig. 2B. Significant increase of LPO due to $NiSO_4$ started to occur following 60 min of such exposure, and remained significantly high till 180 min of such exposure. Since the generation of ROS is often accompanied by a change in the thiol status, we also determined the effects of $NiSO_4$ on lymphocyte nonprotein (NP)- and protein-sulfhydryl (P-SH) contents. Significant time-dependent diminution of both nonprotein- and protein sulfhydryl contents has been observed starting after 15 min of exposure to 4 mM $NiSO_4$. Such diminution of NP-SH reached a maximum value of about 10-12% of the control value following 60-240 min of exposure of human lymphocytes to this Ni-compound (Fig. 3). Similarly, a maximum decrease of about 10-12% of the control value for P-SH contents was observed following 60-240 min of exposure of lymphocytes to $NiSO_4$ (Fig. 3). $NiSO_4$ failed to produce any such increase of superoxide anion compared to control during any time period of exposure used in this study (results no shown here).

Effects of reactive oxygen species (ROS) scavengers and antioxidants on $NiSO_4$-induced human lymphocyte death

To examine the role of ROS on $NiSO_4$-induced human lymphocyte death, we have evaluated the effects of various ROS antagonists on such lymphocyte death. Since significant depletion of nonprotein sulfhydryl content (the reductive reserve) occurred much earlier than the lymphocyte death due to $NiSO_4$ we have therefore examined whether this was associated with Ni-induced lymphocyte death. It is seen that pre-treatment of lymphocytes for 15 min with excess GSH significantly reduced such cell death induced by nickel sulfate (Fig. 4). Pretreatment of lymphocytes with excess *N*-acetylcysteine significantly reduced Ni-compound-induced lymphocyte death (Fig. 4). Similarly, pretreatment of human lymphocytes with catalase (a H_2O_2 scavenger) for 15 min significantly reduced lymphocyte death induced by $NiSO_4$ (Fig. 4). Pretreatment of lymphocytes with either DMTU, or mannitol (hydroxyl radical scavenger) for 15 min significantly prevented $NiSO_4$-induced cell death (Fig. 4). The effect of DFO, a potent iron-chelator, on Ni-induced lymphocyte death was also studied. When lymphocytes were treated with $NiSO_4$ in the presence of DFO the percentage of cell death induced by $NiSO_4$ was significantly reduced (Fig. 4). The values of lymphocyte death for the different ROS scavengers- and antioxidants-alone-treated group were of 5.3±1.5 to 6.1±1.3 and not significantly different from control (results no shown here).

Role of mitochondrial permeability transition (MPT) on $NiSO_4$-induced lymphocyte death.

When isolated human lymphocytes were exposed to 1.5 mM $NiSO_4$ for different periods of time, e.g. 15, 30, 60, 120, 180 min, significant induction of MPT compared to the control value was observed starting after 60 min exposure to $NiSO_4$ (Fig. 5). Furthermore, the MPT continued to be significantly induced due to the $NiSO_4$ until 180 min of exposure used in this study (Fig. 5). We have also utilized a specific MPT pore inhibitor, cyclosporin A (CsA) in order to examine whether the MPT pore is directly involved in the lymphocyte death induced by $NiSO_4$. Thus when lymphocytes were treated for 3h with 1.5 mM $NiSO_4$ in the presence of 2 μM CsA, the reduced Rhodamine 123 uptake caused by the Ni-compound was significantly ameliorated, and reached a nearly control value due to CsA cotreatment (Table 1). At the same time, such cotreatment with CsA significantly prevented the lymphocyte death induced by $NiSO_4$ (Table 1).

Effects of intracellular calcium homeostasis on nickel sulfate-induced lymphocyte death.

This study was designed to seek evidence of any impaired calcium regulation whether occurring early during Ni^{2+}-induced lymphocyte death and signs of a close temporal relationship between

the onset of lymphocyte death and any impaired calcium ion (Ca^{2+}) regulation due to $NiSO_4$. At first changes in the free intracellular Ca^{2+} influx and release from intracellular stores were investigated with $NiSO_4$ and the results are presented (Fig. 6A). Significant increase in free intracellular Ca^{2+} level, $[Ca^{2+}]_i$ started after 30 min of exposure to $NiSO_4$ and continued to increase with time and reached a maximum increase of about three times the control value after 120 min of such treatment (Fig. 6A). Therefore, the effects of Ni^{2+}-induced lymphocyte death were investigated using different calcium channel blockers involving $[Ca^{2+}]_i$ influx and release as well as Ca^{2+} uptake. As pharmacological tools, we used primarily compounds that were known to act on cell membranes. However, since elevated $[Ca^{2+}]_i$ can also originate from intracellular stores in mitochondria, or smooth endoplasmic reticulum (Trump et al, 1989), and since mitochondria were shown to be affected by exposure to $NiSO_4$ in this study, we therefore tested the possibility that modulating intracellular calcium fluxes using both Ca^{2+} channel blockers/antagonist, inhibitor of Ca^{2+} release/uptake, and Ca^{2+} chelator might prevent the $NiSO_4$-induced lymphocyte death and the results are presented in Fig. 6B. The values of lymphocyte death for diltiazem-, nifedipine-, verapamil-, dantrolene-, CsA-, ruthenium red-, and BAPTA-alone-treated group were of 2.7±1.2 to 5.1±1.1 and not significantly different from control (results no shown here). It is seen that cotreatments of lymphocytes

with $NiSO_4$ and different Ca^{2+} channel blockers, such as diltiazem, nifedipine and verapamil considerably and significantly prevented in concentration-dependent manner the lymphocyte death induced by $NiSO_4$. Similarly cotreatments of lymphocytes with $NiSO_4$ and different inhibitor of Ca^{2+} release, such as dantrolene, CsA, and inhibitor of Ca^{2+} uptake such as ruthenium red significantly and considerably protected in concentration-dependent manner against lymphocyte death induced by Ni-compound (Fig. 6). Furthermore, cotreatment of human lymphocytes with $NiSO_4$ and 1,2-bis (2-Aminophenoxy) ethane-*N*,*N*,N´,*N*´-tetraacetic acid tetrapotassium salt (BAPTA) (an intracellular Ca^{2+} chelator) significantly protected in concentration-dependent manner against Ni-compound-induced lymphocyte death (Fig. 6).

Discussion

Evidence has been presented in this study to suggest that oxidative damage-inducing free radicals may play an important role in the underlying biochemical mechanisms involved in human lymphocyte death due to $NiSO_4$. Increased generation of various types of ROS (e.g. H_2O_2, hydroxyl radical) as well as depletions of NP-SH and P-SH, and activation of LPO due to $NiSO_4$ are all believed to be the initiators of pathogenesis of lymphocyte death, as they appeared

before any occurrence of $NiSO_4$-induced lymphocyte death. These are further confirmed as follows. The percentage of $NiSO_4$-induced dead cells is significantly reduced by treatment with excess GSH, or excess NAC (a precursor of GSH). Excess GSH may be involved in the metabolism of free radicals and hydroperoxides. The inhibition of $NiSO_4$-induced human lymphocyte death by catalase suggests that H_2O_2 participates in such lymphocyte death. Similarly, the significant prevention of $NiSO_4$-induced lymphocyte death by mannitol and DMTU indicates that $NiSO_4$-induced cell death also occurs via generation of excess reactive hydroxyl radical species in isolated human lymphocytes. Significant prevention of lymphocyte death induced by this $NiSO_4$ has also been observed following pretreatment with DFO. Iron acts as a Fenton catalyst to produce hydroxyl radical from H_2O_2. DFO being an iron chelator has the potential to inhibit the process of Ni^{2+}-induced ROS, resulting in prevention against $NiSO_4$-induced lymphocyte death. Thus this Ni-compound may express its cytotoxic (cell death) potential by way of iron-mediated oxidative damage as well. Furthermore, these findings provide support for iron chelator therapy in protection against $NiSO_4$ induced oxidative damage.

The mechanism by which $NiSO_4$ increases generation of ROS in human lymphocytes is not clear. It has been shown that peptides

containing the glycyl-glycyl-L-histidyl sequence trigger nickel-dependent production of oxygen radicals through reaction with H_2O_2. When chelated with peptides containing the glycyl-glycyl-L-histidyl sequence, Ni^{2+} could also peroxidize lipids either through H_2O_2 disproportionation and hydroxyl radical production, or directly by reaction with the lipid peroxides (Torreilles and Guérin, 1990). However, Ni^{2+} ions themselves have been shown to induce the formation of oxidized DNA bases (Kawanishi et al, 2002; Nackerdien et al, 1991). Both Ni_3S_2 (insoluble) and $NiCl_2$ (soluble) have been shown to induce increased oxidants in intact cultured mammalian cells as detected by dichlorofluorescein fluorescence (Huang et al, 1993), and by nickel chloride in isolated human lymphocytes (Chen et al, 2003). In view of above considerations, whether $NiSO_4$ is capable of only directly inducing increased generation of H_2O_2, or indirectly through some biological chelators cannot be determined from this study and warrants further investigation.

CsA is often considered as a specific blocker of the MPT as well as a specific inhibitor of mitochondrial membrane potential (Lemasters et al, 1998a and 1998b). Since CsA cotreatment markedly reduced the lymphocyte death induced by $NiSO_4$ (Table 1), it indicates that (a) $NiSO_4$ has the potential to induce mitochondrial dysfunction in human lymphocytes and (b) the MPT plays equally an important

role in $NiSO_4$-induced lymphocyte death signalling process. CsA affects the mitochondria by inhibiting the induction of MPT, thereby may prevent disruption of the transmembrane potential caused by $NiSO_4$. Studies with isolated mitochondria show that MPT pore favors a closed state, but some physiological and pathological signals trigger pore opening (Bernardi and Petronilli, 1996). The fully opened state creates a channel for ≤ 1.5 kDa molecules, resulting in dissipation of the H^+ gradient across the membrane and uncoupling of the respiratory chain (Green and Amarante-Mendes, 1998). Thus opening of high conductance pores in the mitochondrial inner membrane triggered by $NiSO_4$ precipitates the onset of the MPT, which could lead to membrane depolarization and release of ions and other physiological effects. The MPT has been linked to significant depletion of cellular ATP (Duchen, 2000; Qian et al, 1999). It has been postulated that the MPT uncouples mitochondria and causes consumption of ATP by mitochondrial ATPases. Thus, studies by Arsalane et al (1994) on the cytotoxicity of nickel carbonate hydroxide using guinea pig alveolar macrophages in culture have shown significant generation of free radicals and a depletion of cellular energy reserve particularly ATP. The mechanism of pore blockage by CsA has not yet been resolved, but may involve binding with the mitochondrial matrix protein, cyclophilin family protein associated with MPT pore (also called peptidylprolyl-cis-trans isomerase (PPIase)

rather than direct interaction with the pore itself (Nicolli et al, 1996; Connern et al, 1992).

Ni-compound-induced initial ROS or oxidative stress is responsible for Ni-compound-induced MPT was obtained from our previous study that pretreatment of human lymphocytes with either catalase (a H_2O_2 scavenger), or excess GSH significantly and effectively inhibited the nickel carbonate hydroxide-induced MPT (unpublished results). Our previous study has shown that nickel carbonate hydroxide-induced destabilization of cellular calcium homeostasis is caused not only by Ni-compound-induced oxidative stress but also by Ni-compound-induced MPT. This is substantiated by the observation that not only excess catalase and GSH (scavengers of ROS) but also CsA (a specific blocker of the MPT and a specific inhibitor of mitochondrial membrane potential) significantly reduced nickel carbonate hydroxide-induced elevation of $[Ca^{2+}]_i$. Consequently, both ROS and mitochondria were causally involved in nickel carbonate hydroxide-induced destabilization of cellular calcium homeostasis (manuscript submitted). The present study has identified several calcium sites as additional targets by which Ni^{2+} may perturb cellular Ca^{2+} homeostasis which may therefore result in human lymphocyte death. Thus, analyses of the different mechanisms involving the effects of different modulators of calcium channel blockers governing

the increase of $[Ca^{2+}]_i$ suggest that the protective effects of nifedipine, verapamil and diltiazem against $NiSO_4$-induced increase of $[Ca^{2+}]_i$ and the consequent lymphocyte death are due to inhibition of calcium movements through voltage-operated calcium channel protein involving plasma membranes. Furthermore, the present study has also indicated that other calcium channel blockers are also able to inhibit calcium movements through other mechanisms as well. Thus, $NiSO_4$-induced lymphocyte death can also be significantly prevented by inhibiting Ca^{2+} release from sacroplasmic reticulum by dantrolene cotreatment as well as inhibiting Ca^{2+} release from mitochondria by CsA treatment and inhibiting Ca^{2+} uptake by mitochondria by ruthenium red treatment. Thus, $NiSO_4$ may increase $[Ca^{2+}]_i$ via release of Ca^{2+} from voltage-operated calcium channel sensitive protein, as well as from mitochondria, and sarcoplasmic reticulum. Soluble nickel has been shown to compete with calcium for channels and to be taken up through calcium channels (Refvik and Andreassen, 1995; Funakoshi et al, 1997). It has been shown that Ni uptake by rat hepatocytes occurs, at least in part, through the Ca channel transport processes. Pretreatment with nicardipine or verapamil, potent inhibitors of Ca channels, decreased Ni uptake by 20% (Funakoshi et al, 1997). It is possible that apparently competes with the essential metals like calcium to use the same pathways, thus disrupting the intracellular balance of Ca^{2+} and resulting in cell death. However,

other mechanisms of Ni uptake might also be involved which requires further study. Since exogenous addition of BAPTA, a specific chelator of free intracellular calcium, $[Ca^{2+}]_i$ also attenuated Ni^{2+}-induced lymphocyte death (Fig. 6), it indicates that elevation of intracellular calcium was essential for such death. The present data may provide some new insights into the mechanisms of Ni-compound-induced human lymphocyte death, as well as contribute important information for the treatment and prevention of adverse health effects in workers of nickel-related industries using the modulators of oxidative stress, mitochondrial damage, and calcium dehomeostasis. In conclusion the mechanism of $NiSO_4$-induced activation of death signalling pathways involves not only the excess generation of various types of ROS and oxidative stress, and MPT, but also destabilization of cellular calcium homeostasis.

ACKNOWLEDGMENTS

The authors sincerely thank Sainte-Justine Hospital, for obtaining blood samples from donors and from the laboratory of vascular immunology of Dr. Jean-Claude Mamputu of CHUM, Research Center, Notre-Dame Hospital, Montreal.

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FIGURE CAPTIONS

FIG. 1. (A) Concentration-dependent effects of nickel sulfate ($NiSO_4$) on human lymphocyte death. Freshly isolated human lymphocytes were treated *in vitro* with various concentrations of $NiSO_4$ at 37°C for 4 hours. (B) Time-dependent effects of $NiSO_4$ on human lymphocyte death. Isolated lymphocytes were exposed *in vitro* to 1.5 mM of $NiSO_4$ for different time periods at 37°C. Cell death was determined by trypan blue dye exclusion. Values represent mean ± SEM from four separate experiments involving four separate blood donors. *Significantly different from control, $p < 0.05$. [a]Significantly different between $NiSO_4$-treated group, $p < 0.05$.

FIG. 2. (A) The generation of hydrogen peroxide (H_2O_2) following exposure of isolated human lymphocytes to 1.5 mM of nickel sulfate ($NiSO_4$) at various time periods (10-180 min). For details, see Materials and Methods. (B) The production of lipid peroxidation (as measured by MDA formation) following exposure of human lymphocytes to $NiSO_4$ for various time periods. The results are mean ± SEM for 4 separate experiments. *Significantly different from control,

$p < 0.05$.

FIG. 3. Time-dependent effects of nickel sulfate ($NiSO_4$) on (A) nonprotein sulfhydryl (NP-SH) and (B) protein sulfhydryl (P-SH) contents following exposure of human lymphocytes to $NiSO_4$ for various times. For details, see Materials and Methods. The values represent mean ± SEM from four different experiments. *Significantly different from control, $p < 0.05$.

FIG. 4. Effects of various scavengers of ROS on nickel sulfate ($NiSO_4$)-induced human lymphocyte death. Human lymphocytes were either first pretreated for 15 min, or not followed by exposure to 4 mM $NiSO_4$ for 3 h at 37°C. Lymphocyte death was measured by trypan blue dye exclusion. Results are mean ± SEM for four separate experiments.Control (Ctl), A= $NiSO_4$ (1.5 mM), B= Catalase pretreated (1000 U/ml), C= Catalase pretreated (2000 U/ml), D= GSH pretreated (4 mM), E= GSH pretreated (8 mM), F= NAC pretreated (4 mM), G= NAC pretreated (8 mM), H= Mannitol pretreated (25 mM), I= Mannitol pretreated (50 mM), J= DMTU pretreated (10 mM), K= DMTU pretreated (20 mM), L= DFO pretreated (100 μM). *Significantly different from $NiSO_4$-alone treated group, $p < 0.01$.

FIG. 5. Time-dependent effects of nickel sulfate on the mitochondrial

permeability transition (MPT) (or, the mitochondrial membrane potential). The MPT was evaluated by the capacity of lymphocytes to take up the fluorescent cationic dye rhodamine 123. The results expressed as percentage of the total fluorescence of rhodamine 123 in the incubation medium and are means ± SEM of four separate experiments. *Significantly different from control, $p < 0.001$.

FIG. 6. (A) Time-dependent effect of nickel sulfate ($NiSO_4$)-induced increase in intracellular calcium ion concentration, $[Ca^{2+}]_i$. For details, see Materials and Methods. (B) Effects of various Ca^{2+} antagonists on $NiSO_4$ (1.5 mM)-induced human lymphocyte death. For details, see Materials and Methods. Control (Ctl), A= $NiSO_4$ alone, B= $NiSO_4$ plus 50 μM diltiazem, C= $NiSO_4$ plus 12 μM nifedipine, D= $NiSO_4$ plus 25 μM nifedipine, E= $NiSO_4$ plus 12 μM verapamil, F= $NiSO_4$ plus 25 μM verapamil, G= $NiSO_4$ plus 3 mM BAPTA, H= $NiSO_4$ plus 6 mM BAPTA, I= $NiSO_4$ plus 2 μM ruthenium red, J= $NiSO_4$ plus 5 μM ruthenium red, K= $NiSO_4$ plus 25 μM dantrolene, L= $NiSO_4$ plus 50 μM dantrolene, M= $NiSO_4$ plus 2 μM cyclosporin A. Results are mean ± SEM of four separate experiments. *Significantly different from Ni-compound-alone treated group, $p < 0.001$.

Table 1. Effects of cyclosporin A (CsA) (2 μM) on nickel sulfate ($NiSO_4$) (1.5 mM)-induced mitochondrial permeability transition (MPT) and lymphocyte death in isolated human lymphocytes cotreated at 37°C for 3 h.

Treatment	MPT % of Rhodamine 123 uptake (Mean ± SEM)[1]	% of Lymphocyte death (Mean ± SEM)[1]
Control	96 ± 1.58	5.2 ± 1.33
Rotenone (2 μM) (positif control)	69 ± 2.30	19.3 ± 1.47
CsA	94 ± 1.78	6.5 ± 1.65
$NiSO_4$	85 ± 1.78	35.8 ± 1.63
$NiSO_4$ + CsA	93 ± 1.95*	8.3 ± 1.80*

[1] Results are mean ± SEM of four separate experiments.

* Significantly different from $NiSO_4$-alone-treated group, $p < 0.05$.

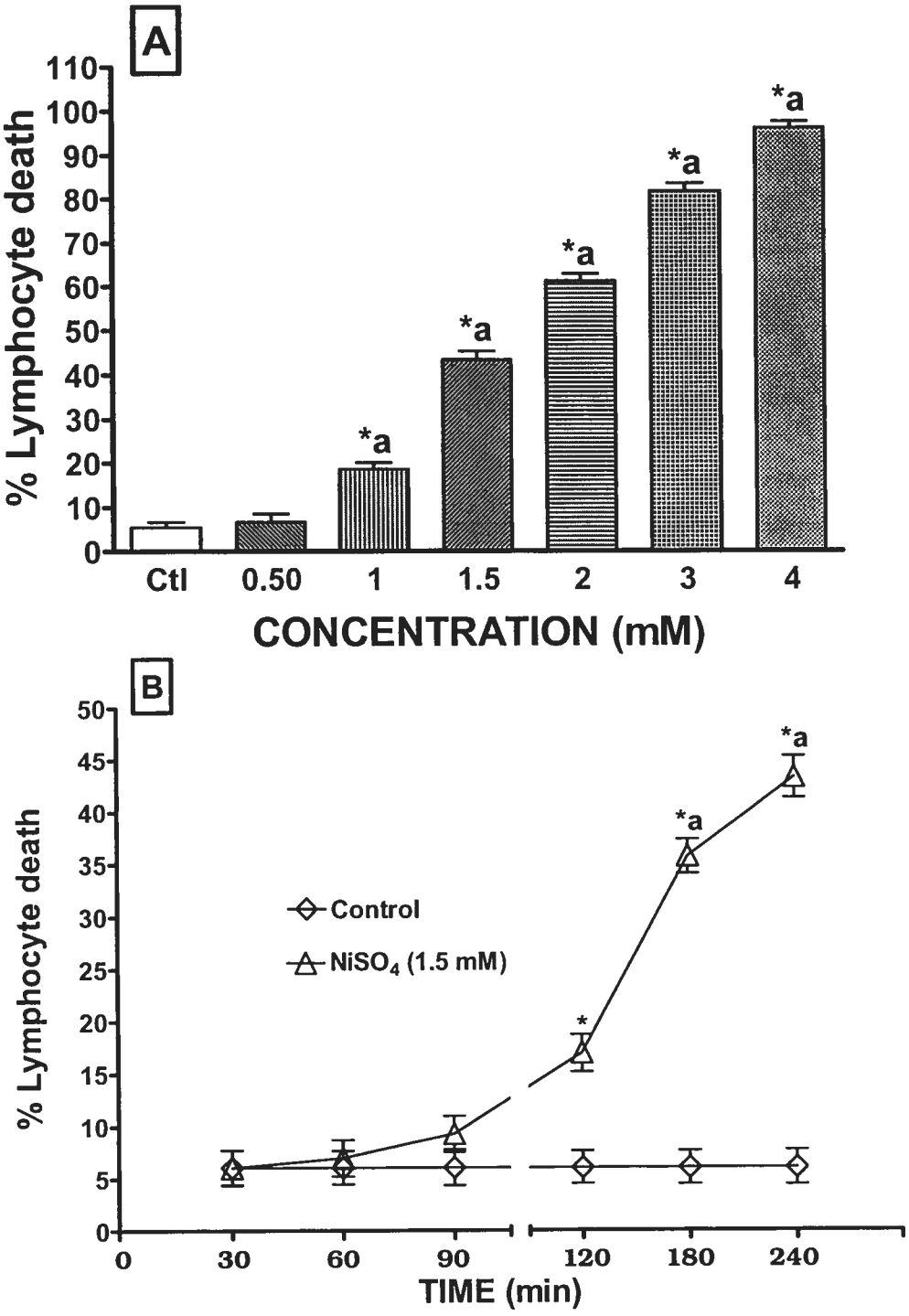
A
% Lymphocyte death
110
100
90
80
70
60
50
40
30
20
10
0
*a
Ctl
0.50
1
1.5
2
3
4
CONCENTRATION (mM)
B
% Lymphocyte death
50
45
40
35
30
25
20
15
10
5
0
Control
NiSO4 (1.5 mM)
*
*a
0
30
60
90
120
180
240
TIME (min)

Figure 1

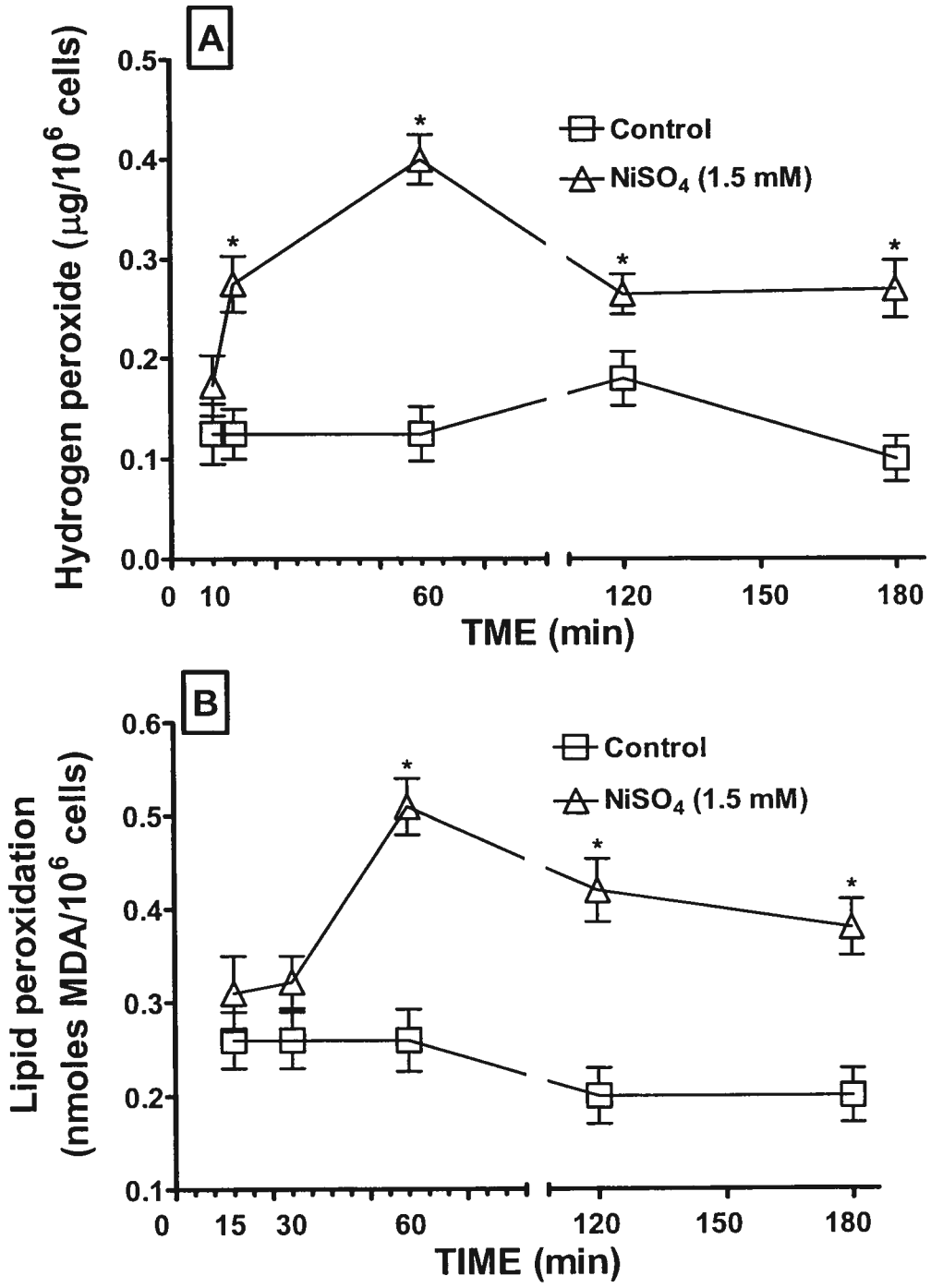
A
Hydrogen peroxide (μg/10^6 cells)
0.5
0.4
0.3
0.2
0.1
0.0
0
10
60
120
150
180
TME (min)
Control
NiSO4 (1.5 mM)
B
Lipid peroxidation (nmoles MDA/10^6 cells)
0.6
0.5
0.4
0.3
0.2
0.1
0
15
30
60
120
150
180
TIME (min)
Control
NiSO4 (1.5 mM)

Figure 2

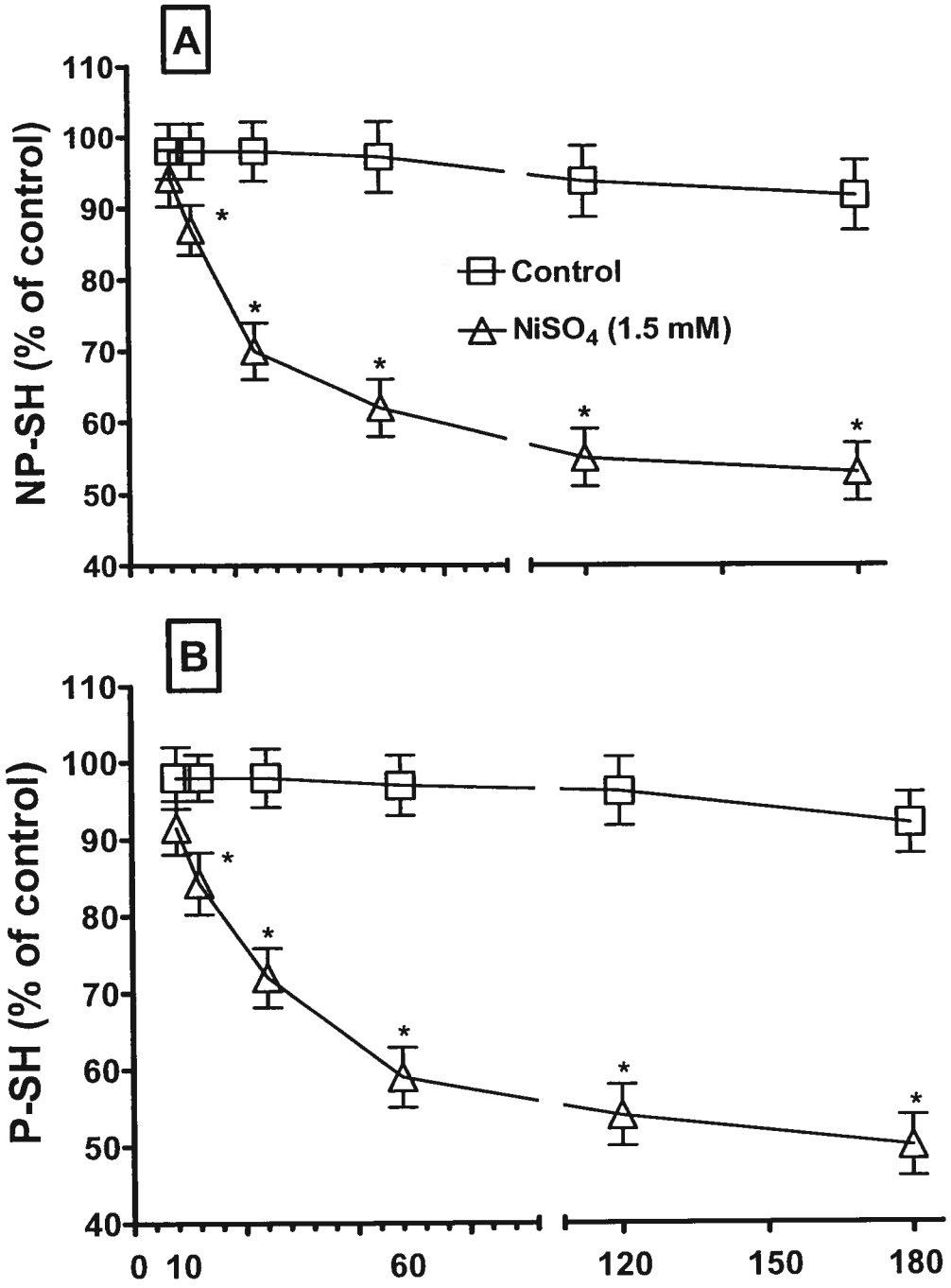
A
NP-SH (% of control)
110
100
90
80
70
60
50
40
Control
NiSO4 (1.5 mM)
*
B
P-SH (% of control)
0 10
60
120
150
180

Figure 3

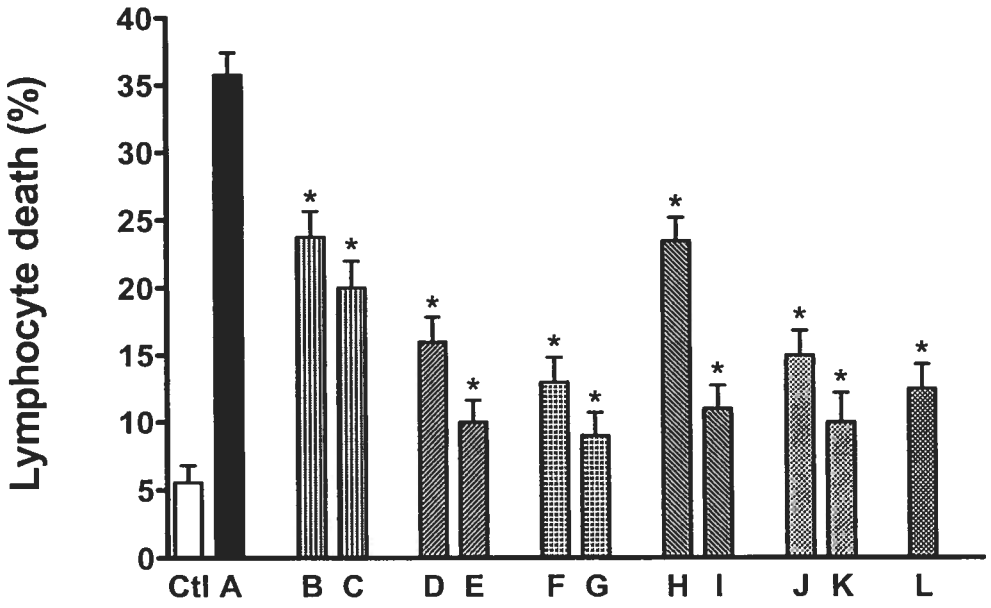

Figure 4

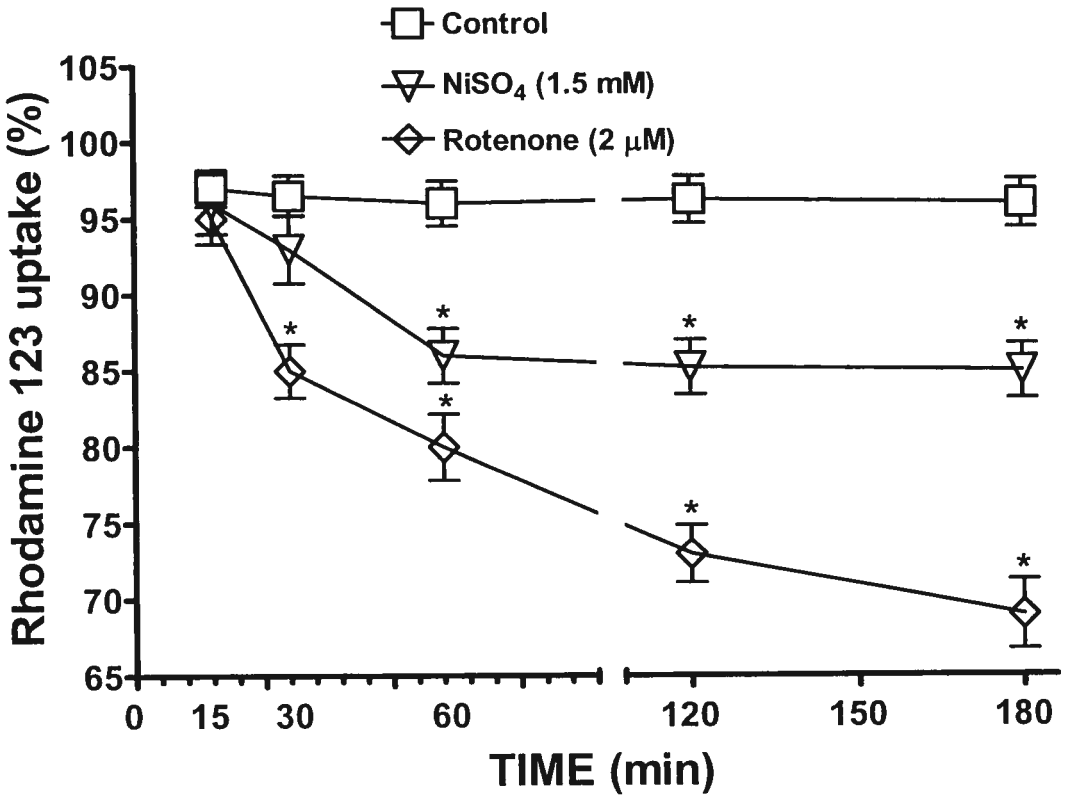
Control
NiSO4 (1.5 mM)
Rotenone (2 μM)
Rhodamine 123 uptake (%)
105
100
95
90
85
80
75
70
65
0
15
30
60
120
150
180
TIME (min)
*

Figure 5

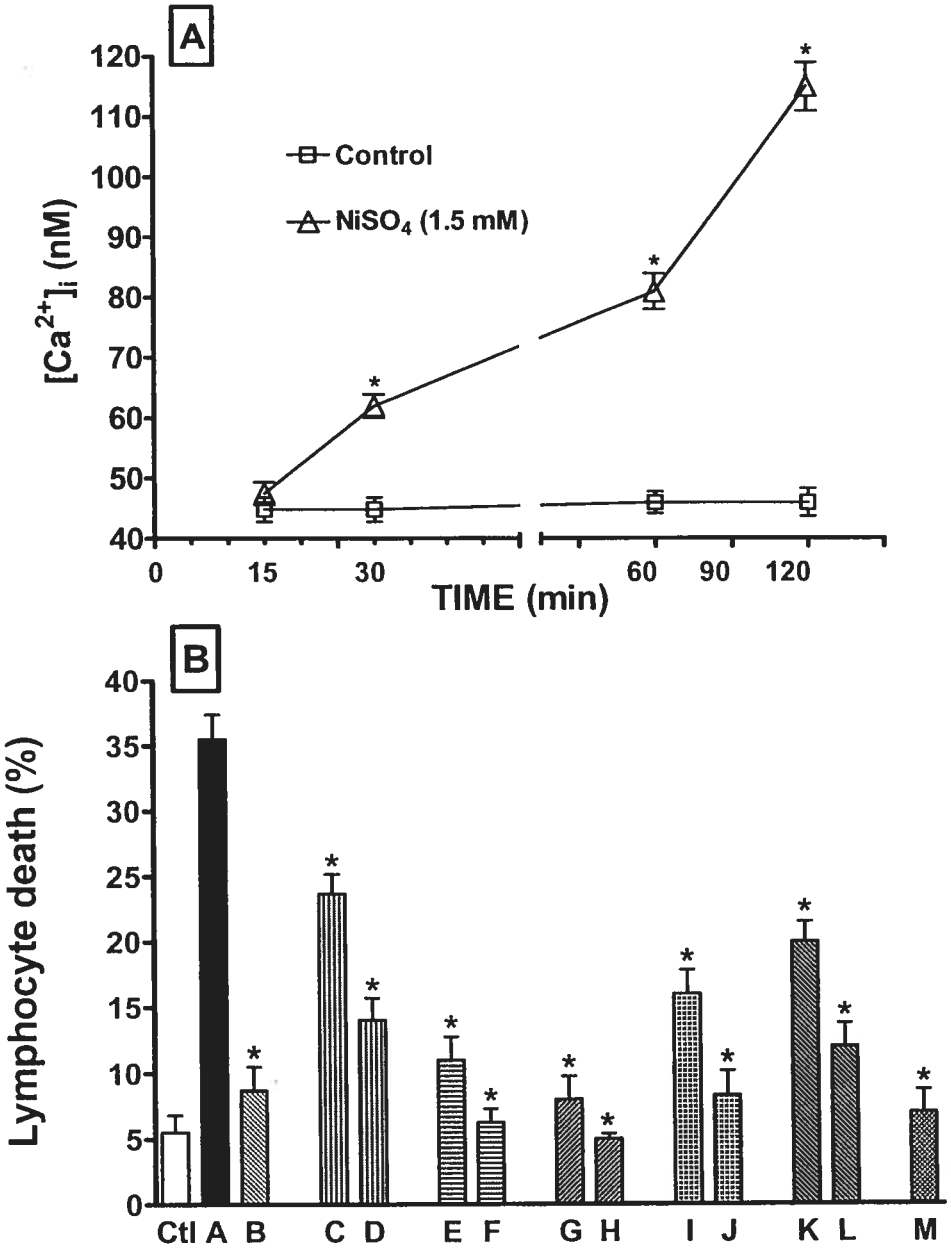
A
120
110
100
90
80
70
60
50
40
[Ca2+]i (nM)
Control
NiSO4 (1.5 mM)
0
15
30
60
90
120
TIME (min)
B
40
35
30
25
20
15
10
5
0
Lymphocyte death (%)
Ctl A B C D E F G H I J K L M

Figure 6

III. CHAPITRE II: Mécanismes moléculaires de la génotoxicité des composés de nickel dans les lymphocytes humains.

Role of oxidative stress and intracellular calcium in nickel compound-induced DNA single-strand breaks in chromosomal and nuclear chromatin in human blood lymphocytes *in vitro* using electron microscopy *in situ* end-labeling.[1]

Role of oxidative stress and intracellular calcium in nickel compound-induced sister-chromatid exchanges, replication index and mitotic index in human peripheral blood lymphocytes in culture.[1]

[1]Articles à soumettre pour publication.

III.1. ARTICLE IV

Role of oxidative stress and intracellular calcium in nickel compound-induced DNA single-strand breaks in chromosomal and nuclear chromatin in human blood lymphocytes *in vitro* using electron microscopy *in situ* end-labeling.

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Abstract

The effects of nickel sulfate, and soluble forms of nickel carbonate hydroxide, nickel subsulfide and nickel oxide on the induction of DNA single-strand breaks in chromosomal and nuclear chromatin in human blood lymphocytes in culture were studied. The induction of DNA single-strand breaks (SSBs) was measured using the method of electron microscopy *in situ* end-labeling. The data were quantified by the percentage increase in immunogold particles (IGPs) per μm^2 chromatin (labeling density) involving both metaphase chromosomal chromatin and interphase nuclear chromatin following exposure of human blood lymphocytes in culture to a very low concentration of either nickel sulfate, or soluble forms of either nickel carbonate hydroxide, or nickel oxide, or nickel subsulfide for 2 h. The metaphase chromosomal chromatin showed significantly higher DNA SSBs resulting in strong colloidal gold labeling of chromosomes due to all four Ni-compounds at 15 μM concentration as compared to the control value. However, exposure to only nickel carbonate hydroxide and nickel oxide showed significantly higher DNA SSBs in nuclear chromatin when compared to the control value. Both nickel carbonate hydroxide and nickel oxide produced significantly higher induction of SSBs than those of nickel subsulfide and nickel sulfate in chromosomal chromatin. Nickel carbonate hydroxide-induced SSBs

were found to be significantly higher than those due to nickel oxide in nuclear chromatin. Overall, the genotoxic potency seems to be decreased as follows: nickel carbonate hydroxide > nickel oxide ≥ nickel subsulfide > nickel sulfate. Ni-compound-induced DNA SSBs were found to be significantly higher in chromosomal chromatin than those in nuclear chromatin. Pretreatment of human blood lymphocytes with either catalase (a H_2O_2 scavenger), or superoxide dismutase (a scavenger of superoxide anion radical) or dimethylthiourea (a hydroxyl radical scavenger), or N-acetylcysteine (GSH precursor), significantly reduced DNA SSBs in both chromosomal and nuclear chromatin induced by nickel carbonate hydroxide, suggesting that various types of oxidative stress are involved in such genotoxicity. Deferoxamine (a highly specific iron chelator) pretreatment prevented nickel carbonate hydroxide-induced SSBs in both chromosomal and nuclear chromatin suggesting a role of iron-mediated oxidative stress generating hydroxyl radical in such Ni-compound-induced genotoxicity. Simultaneous treatment with either verapamil (an inhibitor of Ca^{2+} through plasma membranes), or dantrolene (an inhibitor of mobilization of $[Ca^{2+}]_i$ from endoplasmic reticulum), or BAPTA (a Ca^{2+} chelator), significantly reduced SSBs in both chromosomal and nuclear chromatin induced by this Ni-compound. These results indicate that Ni-compound-induced destabilization of calcium homeostasis is also involved in the

induction of DNA SSBs in both chromosomal and nuclear chromatin from human blood lymphocytes.

KEY WORDS: Human blood lymphocytes; nickel compounds; DNA single-strand breaks; chromosomal and nuclear chromatin; oxidative stress; intracellular calcium.

Introduction

Occupational exposure to nickel compounds occurs principally through mining, smelting, and refining operations, alloy production, electroplating and welding operations during manufacture of steel, other alloys and batteries. In 1990 the International Committee on Nickel Carcinogenesis in Man suggested that respiratory cancer risks are primarily related to chronic exposure to soluble nickel compound at concentrations above 1mg/m^3 and to less soluble forms at concentrations above 10 mg/m^3 (IARC, 1990). Nickel matte refining has been associated with high exposure to dusts of nickel subsulfide and nickel oxide, whereas in electrolytic refining operations workers are exposed to aerosols of nickel sulfate and nickel chloride. Nickel carbonate hydroxide is used in nickel plating, in colours and glazes for ceramics and in high pure form it is used in electronic components. Thus, these nickel compounds possess potentials risks

to occupational and environmental health.

The increased frequencies of chromosomal aberrations and sister-chromatid exchanges (SCEs) (compared to the control group) in the peripheral lymphocytes of workers occupationally exposed to nickel have been reported in many studies (Perminova et al., 2001; Werfel et al., 1998; Myslak and Kosmider, 1997; Senft et al., 1992; Popp et al., 1991; Deng et al., 1988; Decheng et al., 1987; Waksviks et al., 1984; Waksviks and Boyen, 1982). Thus, these studies have established that human blood lymphocytes are also the target cells for genotoxicity of nickel compounds. However, in most cases the workers in nickel industries are generally exposed to a number of other metals as well (e.g. Chromium in electroplating refinery, copper and other metals in a nickel refinery, and iron, manganese and chromium in welding operations) in addition to nickel. Since the exposure in nickel industrial environments is always mixed, it is difficult to clearly estimate the genotoxic risks associated with individual nickel compounds alone whether water-soluble, or water-insoluble forms (Oller et al., 1997). Chen et al. (2003) reported single-strand DNA breakage due to water-soluble nickel chloride as measured by Comet assay in isolated human lymphocytes *in vitro*. Ni-compounds have been shown to produce DNA single-strand breaks in Chinese hamster ovary cells using the method of molecular weight of DNA (Robison et

al., 1982) and in the rat kidney (Misra et al., 1993). However, the information of such DNA strand breaks due to water-insoluble Ni-compounds in human blood lymphocytes is not available at present. Furthermore, studies of Ni-compound-induced DNA single-strand breaks in both chromosomal and nuclear chromatin are at present very limited and are hardly available in human blood lymphocytes.

Ni compounds are known to produce a variety of genetic effects on chromatin (Kasprzak et al., 2003; Costa et al., 2002). Ni-compounds have the ability to selectively damage heterochromatin which appears to be the site of chromosomal damage by Ni-compounds (Costa et al., 2002). In this regard, the technique for *in situ* detection of induced DNA single-strand breaks under electron microscopy (Gosálvez et al., 1993) enabling to visualize the location and quantification of the induced DNA single-strand breaks along both chromosomal and nuclear chromatin fibres has been used very limited for the evaluation of genotoxicity. This technique was used by Assad et al. (1997) for evaluating DNA damage in human blood lymphocytes due to a commercial pure titanium as biomaterial, and Fernandez et al. (1993) for detecting DNA strand breaks by hydroxyl radicals using Chinese hamster Don cells.

Various studies have shown the involvement of oxidative stress

in Ni-compound-induced genotoxicity (see review by Kasprzak et al., 2003; reviews by Denkhaus and Salnikow, 2002; Cavallo et al., 2003; Kawanishi et al., 2002; Costa et al., 2002; Cangul et al., 2002; Chakrabarti et al., 2001; Wozniak and Blasiak, 2002; Chakrabarti et al., 1999). However, the detailed mechanistic studies underlying such toxicity involving blood lymphocytes are still very limited (Chen et al., 2003; Wozniak and Blasiak, 2002; Chakrabarti et al., 2001). In this regard, the role of various types of oxidative stress in the development of Ni(II)-compound-induced genotoxicity in human lymphocytes is still not fully known, and hence, needs further evaluation.

Furthermore, studies related to the genotoxic effects of nickel with disturbance in calcium metabolism are at present also very limited. Levels of free intracellular calcium $[Ca^{2+}]_i$ were increased in nickel-treated cells (Faurskov and Bjerregaard, 2002; Salnikow et al., 1999). Nickel-transformed cells displayed ability to rapidly proliferate *in vitro* in a low-calcium media suggesting alteration of intracellular calcium metabolism in nickel-transformed cells (Swierenga et al., 1976). *In vivo* administration of Ca^{2+} prevented the formation of lung adenomas caused by nickel or lead (Poirier et al., 1984). In this regard, the role of intracellular Ca^{2+} in Ni(II)-compound-induced DNA single-strand breaks in human blood lymphocytes is unknown and therefore, needs evaluation.

DNA in fixed chromosomes is a very stable molecule that basically retains its native structure in the proteinaceous chromosomal matrix. Briefly, the main features of the technique for *in situ* detection and quantification of induced DNA single-strand breaks under electron microscopy involve the following: Exonuclease III (Exo III) incubation enabled to amplify induced DNA breaks by releasing nucleotides at free 3´ hydroxyl ends from nickel double-stranded DNA. The resulting single-strand areas hybridize with short oligonucleotides of random sequences which behave as primers for Escherichia coli DNA polymérase I. This polymérase fills the amplified single-strand gaps with nucleotides including biotinylated-16-dUTP which is finally detected by immunogold binding to chromatin. Therefore, the present investigation was carried out to examine the genotoxic potential of different nickel compounds using *in situ* evaluation of Ni(II)-induced DNA single-strand breaks in human blood lymphocytes along both chromosomal and nuclear chromatin fibres under electron microscopy. Furthermore, the effects of various types of oxidative stress and intracellular Ca^{2+} on such Ni(II)-compound-induced DNA single-strand breaks were examined and the results are presented in this paper.

Materials and methods

Materials

Nickel sulfate hexahydrate ($NiSO_4.6H_2O$, green), nickel subsulfide (Ni_3S_2, 150 mesh, black) nickel oxide (NiO, black) and nickel carbonate hydroxide tetrahydrate ($2NiCO_3.3Ni(OH)_2.4H_2O$, green) were obtained from Sigma–Aldrich Canada Ltd, Ontario, Canada. All other chemicals and reagents were obtained from Sigma, unless otherwise mentioned.

$NiSO_4.6H_2O$ is highly soluble in water, saline or incubation buffer. Ni_3S_2, NiO, and $2NiCO_3.3Ni(OH)_2.4H_2O$ are insoluble in water or physiological saline. Therefore, they were dissolved in 0.05 M Tris-HCl buffer, pH 7.4, by incubation at 37°C for 12h with shaking using the method of Lee *et al.* (1982). Substantial amounts of all these nickel compounds were dissolved, leading to soluble nickel concentrations of 8-16 mM depending on the nickel compound. The concentration of nickel was determined by electrothermal atomic absorption spectrometry.

The protocols described below were approved by the Ethics Committee on Human Research, Université de Montréal.

Measurement of cell viability in isolated human lymphocytes

Human whole blood was obtained from 4 healthy donors provided by Hôpital Sainte-Justine, Montréal. Peripheral blood samples from 4 healthy non-smoking donors between ages 27 and 42 and who were not previously exposed to any radiation or heavy metal compounds or drug therapy and who did not take any alcohol at least two days before this experiment were used in the present experiments. Questionnaires were obtained from each blood donor to evaluate exposure history. Methodology for the handling of human blood was followed according to the strict guidelines as devised for clinical workers at Hôpital Sainte-Justine, Montréal. The peripheral lymphocytes were isolated under sterile conditions by the method of Boyum (1980). Blood was diluted 1:1 with Ca^{2t} and Mg^{2t} free PBS solution at room temperature [(KCl (0.2 g/L), KH_2PO_4 (0.2 g/L), NaCl (8 g/L), $Na_2HPO_42H_2O$ (1.4 g/L)]. Diluted blood (30 ml) was carefully layered over 10 ml portions of a Ficoll-Paque (Pharmacia) solution (density 1.077 g/ml) in 50 ml plastic centrifuge tubes. Gradients were then centrifuged at 400 x g for 20 min at room temperature. After centrifugation, the upper layer of clear plasma (18 ml) was discarded. Cells of the interface between plasma and Ficoll-Paque layer (buffy coat) werecarefully collected in ice-cold 50 ml plastic centrifuge tubes, sedimented at 500 x g for 10 min at 4°C, washed in PBS, resedimented by centrifugation at 500 x g, and finally resuspended in

PBS. A portion of the cell suspension was diluted with 0.13% trypan blue in PBS, and viable cells were counted in a hemocytometer, taking into account of the integrity of the cell membrane. About 95% of the cells isolated were mononuclear and excluded trypan blue. Aliquots of the lymphocyte cells were then added to a total volume of 3 ml of supplemented RPMI 1640 medium. The supplemented RPMI 1640 media contained 10% fetal calf serum, 0.1% gentamycin, 1% glutamine (from Invitrogen, Burlington, Canada) and 1% phytohaemagglutinin (Murex Diagnostics, Dartford, England). After 46 h of the initiation of culture, the cells were treated in the absence or presence of different concentrations (0-30 μM) of either nickel carbonate hydroxide, or nickel subsulfide, or nickel sulfate, or nickel oxide for 2h at 37°C in the dark in a humidified atmosphere of 95% oxygen and 5% carbon dioxide. Following the end of such exposure, cells were washed twice with RPMI-1640 and incubated in fresh complete RPMI-1640 culture medium for another 24 hr. An equal volume of 0.4% trypan blue reagent was then added to the cell suspension and the percentage of the viable cells was counted in a hemocytometer under a light microscope (Leitz Wetzlar), using trypan blue exclusion.

Cell treatment and slide preparation

Human whole blood lymphocyte in culture were prepared by

incubating 0.3 ml of fresh heparinized whole blood in 5 ml of supplemented RPMI-1640 media for a total of 72 h at 37°C as already described by Lemieux *et al.* [1990]. For treatments appropriate aliquots of a freshly made stock solution of each nickel compound mentioned above were added to 5.3 ml for 2 hr after 46 hr of initial culture to give a final concentration of 15 μM, whereas an equal volume of the aliquot containing only RPMI-1640 medium was added to another culture to serve as a control. Both control and Ni-treated cells were then washed twice with RPMI-1640 and incubated finally in fresh complete RPMI-1640 culture medium for another 24 hr. After a total of 70 h of incubation, cells were then arrested at metaphase using colcemid (0.1 μg/ml) (Invitrogen, Burlington, Canada). Two hours later, cells were centrifuged at 2000 rpm for 10 min, resuspended in 0.075 M KCI (Sigma Chemical Co., Mississauga, Canada), and incubated at 37°c for 15 min. The cells in culture were then centrifuged for another 5 min and fixed 3 times in methanol: acetic acid (3: 1) (Fisher Scientific, Ottawa, Canada). Finally, chromosome and nuclei spreads were prepared by letting one drop of the suspension fall onto cold and condensation-covered slides which were then air-dried.

In situ end-labeling (ISEL)

For quantification of *in vitro* chromatin DNA breakage, *in situ*

end-labeling (ISEL) technique was then performed following the method of Assad *et al.* (1997) with some minor modifications. Spread chromosomes and nuclei were incubated at 37°C for 30 min in a moist chamber in the presence of 100 units of *E. coli* Exonuclease III (New England Biolabs, Mississauga, Ontario, Canada) in 100 μL of incubation buffer. Slides were then thoroughly washed with 2x standard saline citrate (2xSSC, pH 7.0) for 15 min and with phosphate-buffered saline (PBS) for another 15 min. Labeling of chromatin was performed using a multiprimer DNA labeling system Kit (Amersham Life Science/Pharmacia Biotech, Baie D'Urfé, Canada) in which dTTP had been replaced by biotinylated-11-dUTP (Enzo Diagnostics, Farmingdale, NY) at a concentration of 15 μM. The reaction was carried out with 25 μL of the labeling mix in a moist chamber at 37°C for 75 min and was terminated with 100 μL of 0.5 M EDTA (pH 8.0) (BDH Inc, Toronto, Canada). The slides were then washed with 2xSSC for 5 min, rinsed twice in PBS containing 0,8% human serum albumin (HSA) (Bayer Inc., Etobicoke, Canada) for 5 min each, and then incubated with rabbit anti-biotin antibodies (Enzo Diagnostics, Farmingdale, NY) (diluted 1:100 PBS with 0.8% HSA) for 45 min in a moist chamber at 37°C. They were rinsed again twice in PBS containing 0.8% HSA and incubated with biotinylated goat anti-rabbit antibodies (Invitrogen, Burlington, Canada) (1:100 PBS with 0.8% HSA) for 45 min at 37°C. This step was followed by another 45-

min incubation at 37°C with rabbit anti-biotin antibodies followed by a 45 min incubation with anti-rabbit IgG-colloidal gold complex of 10 nm in diameter per particle (Sigma Chemical Co., Mississauga, Canada) (1:10 PBS with 0.8% HSA). After two washes, slides were immersed in 3% glutaraldehyde at 4°C for 30 min. Chromatin was dehydrated in an ethanol series (30, 50, 70, 90 and 100%) and a dichloroethane series (25, 50, 70, 90 and 100%) for 3 min each. Finally, the slides were coated by a quick dip in 0.5% formvar (J.B.EM Services, Montreal, Canada) dissolved in dichloroethane and allowed to dry vertically. Chromatin materials were then transferred from the slide to the electron microscopy nickel grids (J.B.EM Services, Montreal, Canada) following the method of Messier *et al.* (1986).

Electron microscopy (EM)

Chromosomes and nuclei on nickel grids were observed using a Philips EM208 transmission electron microscopy (Philips Electron Optics, Eindhoven, the Netherlands) operated at 90 kV with a magnification of x 28 000. Chromosomes were selected on the basis of their representative labeling among the metaphase, their ease of identification of the labeling density and their small size for purposes of the photography. ISEL assay was analysed by detection of DNA single-strand breakage by identifying the immunogold particles (IGPs) in each Ni-treated chromatin as well as in untreated chromatin, and

was followed by quantification of DNA single-strand breakage by counting the number of IGPs labelled in each chromatin.

Immunogold counting

The labeling density was evaluated by counting the number of IGPs per μm^2 in 10 chromosomes and 10 nuclei per sample. Analysis of gold particle counts were performed on a PC computer using the public domain NIH Image program developed at the U.S National Institutes of Health (Rasband and Bright, 1995) and available on the Internet at http: //rsb.info.nih.gov/nih-image/. The mean values of the IGPs or % increases in IGPs per μm^2 in metaphase chromosomal and interphase nuclear chromatin were estimated in each sample and were taken as indices of DNA single-strand breaks in each sample.

To investigate the role of reactive oxygen species (ROS) in the induction of DNA single-strand breaks in both chromosomal and nuclear chromatin by nickel carbonate hydroxide, human blood lymphocytes after 46 h of initial culture (as described above), were pretreated for 15 min with either such as superoxide dismutase (SOD) (a scavenger of superoxide anion (O_2^-) radical) (2 500 U/ml), or catalase (a H_2O_2 scavenger) (1 000 U/ml), or dimethyl thiourea (DMTU) (a hydroxyl radical scavenger) (20 mM), or *N*-acetylcysteine (NAC) (a antioxidant) (8 mM) followed by treatment with 15 μM nickel

carbonate hydroxide for 2 h at 37°C. The induction of DNA single-strand breaks in both chromosomal and nuclear chromatin was measured following the rest of the protocol as described above. To determine the role of iron in nickel carbonate hydroxide-induced chromatin damage, deferoxamine (DFO) (100 μM) was added alone or 15 min before nickel carbonate hydroxide (15 μM), and DNA single-strand breaks in both chromosomal and nuclear chromatin was estimated following the rest of the protocol as described above.

To determine the role of intracellular Ca^{2+}, human blood lymphocytes, after 46 hr of initial culture, were treated for 2 hr at 37°C with either 15 μM nickel carbonate hydroxide alone, or in the presence of modulator of intracellular calcium fluxes, such as either verapamil (25 μM), or dantrolene (50 μM), or 1,2-bis(*O*-aminophenoxy)ethane-*N,N,N'* ,*N'*-tetraacetic acid tetra-(acetoxymethy)ester (BAPTA) (3 mM). The induction of DNA single-strand breaks in both chromosomal and nuclear chromatin was then measured following the rest of the protocol as described above.

Statistical analysis

The standard error of the mean for the data was determined by the use of binominal variances. The data are expressed as the mean ± SEM for four separate experiments. The data were analysed by one-

way analysis of variance (ANOVA). The difference between treatments means was also tested by Tukey-Kramer multiple comparison test. The level of significance was set at $p < 0.05$.

Results

Effects of different nickel compounds on DNA single-strand breaks in human blood lymphocytes *in vitro*.

The effect of exposure of human lymphocytes to low concentrations (0-120 μM) of nickel sulfate and soluble forms of nickel carbonate hydroxide, nickel subsulfide, and nickel oxide for 2 h on cellular viability, as estimated by the trypan blue exclusion test is shown in Figure 1. It is seen that the viability of Ni-treated cells was not significantly different from that of untreated cells.

Induction of DNA single-stranded breaks (SSBs) as measured by the percentage increase in immunogold particles (IGPs) per μm^2 chromatin (labeling density) involving both metaphase chromosomal chromatin and interphase nuclear chromatin following exposure of human blood lymphocytes in culture to soluble forms of four different nickel compounds for 2 h are presented in Table 1 and Figures 1-3. Both nickel carbonate hydroxide- and nickel oxide-treated chromosomes showed much strong DNA breakage-associated

immunogold tagging demonstrating strong uniform staining over the entire chromatin except for centrometric regions which remained almost unlabeled (Fig. 2), whereas both nickel subsulfide and nickel sulfate showed some intermediate DNA breakage as verified by some medium but uniform staining over the entire chromatin, whereas the control showed very low immunogold tagging (Fig. 2). As compared with metaphase chromosomes, both nickel carbonate hydroxide- and nickel oxide-exposed interphase nuclei demonstrated somewhat less strong DNA breakage-associated immunogold tagging which was homogeneously dispersed over the entire nuclear chromatin (Fig. 3). On the other hand, both nickel subsulfide- and nickel sulfate-exposed nuclei showed immunogold tagging, although dispersed over the entire chromatin, much lower than those due to nickel carbonate hydroxide and nickel oxide, and almost comparable to the labeling found in the control (Fig. 3). It is seen that chromosomal chromatin showed significantly higher DNA single-strand breaks which resulted in strong colloidal gold labeling of metaphase chromosomes due to the soluble forms of all four different nickel compounds when compared to the control value (Fig. 2). On the other hand, nuclear chromatin showed significantly higher DNA single-strand breaks due to exposure to only nickel carbonate hydroxide and nickel oxide as measured by strong colloidal gold labeling of nuclear specimens when compared to the control value (Fig.3).

Furthermore, both nickel carbonate hydroxide and nickel oxide showed significant higher induction of DNA single-strand breaks than those of nickel sulfate and nickel subsulfide in chromosomal chromatin (Fig. 2). Furthermore, nickel carbonate hydroxide-induced DNA single-strand breaks were found to be significantly higher than those due to nickel oxide in interphase nuclear chromatin (Fig.3). Based on the results obtained in Fig. 2 and 3 with regard to DNA single-strand breaks in chromosomal chromatin, and nuclear chromatin, the genotoxic potency seems to be increased in the following order: nickel carbonate hydroxide > nickel oxide > nickel subsulfide ≥ nickel sulfate.

A significant difference in DNA single-strand breaks between chromosomal chromatin and nuclear chromatin was also observed. Thus, the number of immunogold particles per μm^2 chromatin was found to be significantly greater in chromosomal chromatin than those in nuclear chromatin. Nonspecific labeling was found to be nearly absent regardless of treatment. The percentage increases in DNA single-strand breaks over the control value involving chromosomal chromatin due to low equimicromolar amounts (15 μM) of different nickel compounds were found to be as follows: 393%, 127%, 140% and 107% IGPs/μm^2 chromosomal chromatin for nickel carbonate hydroxide, nickel oxide, nickel subsulfide and nickel sulfate

respectively, whereas such percentage increases were found to be respectively 271, 110, 74, and 24% IGPs/μm^2 nuclear chromatin (Figs. 2 and 3) (Table 1)

Role of various oxidative stress in Ni-compound-induced DNA single-strand breaks in human blood lymphocytes

Since previous studies from our laboratory have shown that soluble forms of nickel carbonate hydroxide and nickel subsulfide as well as nickel sulfate shared a similar biochemical mechanism of Ni-compound-induced human lymphocyte death *in vitro* involving oxidative stress and calcium dehomeostasis (M'Bemba-Meka et al., manuscript submitted), our present study was therefore designed to study the similar mechanism using only nickel carbonate hydroxide as a model. We have therefore examined the effects of various scavengers of such ROS on soluble form of nickel carbonate hydroxide-induced chromatin damage in human blood lymphocytes and the results are presented in Figs. 4, 5 and Table 2. Pretreatment of human blood lymphocytes with superoxide dismutase (SOD) (a scavenger of O_2^-) (2500 U/ml) for 15 min followed by treatment with 15 μM nickel carbonate hydroxide for 2h significantly reduced DNA single-strand breaks induced by nickel carbonate hydroxide alone involving both chromosomal and nuclear chromatin, as verified by the observation that the labeling density (as measured by number of

immunogold particles per μm^2) was significantly reduced by 45.4% and 51% in chromosomal and nuclear chromatin respectively when compared to the corresponding Ni-compound-alone-treated groups (Table 2, Figs.4 and 5). Pretreatment of human blood lymphocytes with catalase (a H_2O_2 scavenger) (1000 U/ml) for 15 min followed by treatment with 15 μM nickel carbonate hydroxide for 2h significantly reduced Ni-compound-induced DNA single-strand breaks, as verified by the observation that the number of immunogold particles per μm^2 chromatin was significantly reduced by 39.7% and 41% in chromosomal chromatin and nuclear chromatin respectively when compared to the respective values of such labeling density in chromosomal and nuclear chromatin in corresponding Ni-compound-alone-treated groups (Table 2, Figs. 4 and 5). Pretreatment of human blood lymphocytes with dimethylthiourea (DMTU) (a hydroxyl radical scavenger) (20 mM) for 15 min followed by treatment with 15 μM nickel carbonate hydroxide for 2h significantly reduced Ni-compound-induced DNA single-strand breaks as evidenced by the observation that the number of immunogold particles per μm^2 chromatin was significantly reduced by 46% and 56% in chromosomal and nuclear chromatin respectively when compared to the respective values of such labeling densities in corresponding Ni-compound-alone-treated groups (Table 2, Figs. 4 and 5). In order to assess the role of iron-mediated oxidative stress, deferoxamine (DFO) was used to block the

iron-mediated oxidative stress. Thus, pretreatment of human blood lymphocytes with 150 μM DFO for 15 min followed by treatment with 15 μM of the same Ni-compound for 2 h resulted in significant reduction of such DNA damage induced by this Ni-compound as evident from the result that the number of immunogold particles per μm^2 chromatin was significantly reduced by 42% and 44.9% in chromosomal and nuclear chromatin respectively when compared to the respective values of such labeling densities in corresponding Ni-compound-alone-treated groups (Table 2, Figs. 4 and 5). Ni(II) compounds are known to deplete glutathione (GSH) contents in human lymphocytes (M'Bemba-Meka et al., manuscript submitted), and in rat liver and kidney (Cartana et al., 1992; Chakrabarti and Bai, 1999). Besides, oxidative stress also involves oxidation of GSH. We have therefore examined the effect of pretreatment with excess N-acetylcysteine (NAC) (a precursor of GSH) on the soluble form of Ni-compound-induced DNA single-strand breaks in human blood lymphocytes. The results are presented in Table 2, and Figs. 4 and 5. Thus, pretreatment of human blood lymphocytes with excess NAC (8 mM) for 15 min followed by treatment with 15 μM same Ni-compound for 2 h significantly reduced Ni-compound-induced DNA single-strand breaks as evident from the result that the number of immunogold particles per μm^2 chromatin was significantly reduced by 60.5% and 41% in chromosomal and nuclear chromatin respectively when

compared to the respective values of such labeling densities in corresponding Ni-compound-alone-treated groups (Table 2, Figs. 4 and 5).

Effects of intracellular calcium ion, $[Ca^{2+}]_i$ on Ni-compound-induced DNA single-strand breaks in human blood lymphocytes

Previous studies from our laboratory have demonstrated that Ni-compound-induced destabilization of calcium homeostasis could play a significant role in Ni-compound-induced human lymphocyte death (M'Bemba-Meka et al., manuscript submitted). We have therefore investigated whether Ni-compound-induced destabilization of calcium homeostasis could influence Ni-compound-induced DNA single-strand breaks in human blood lymphocytes. We therefore tested the possibility that modulating intracellular calcium fluxes using both Ca^{2+} channel blocker and intracellular calcium ion (Ca^{2+}) antagonist might prevent the Ni-compound-induced DNA single-strand breaks in human blood lymphocytes and the results are presented in Table 3, Figs. 6 and 7. Simultaneous treatment of human blood lymphocytes in culture with 15 μM nickel carbonate hydroxide (soluble form) and 25 μM verapamil (which is able to inhibit movements of Ca^{2+} through plasma membranes) for 2 h significantly reduced Ni-compound-induced DNA single-strand breaks as verified by the observation that the number of immunogold particles per μm^2

chromatin was significantly reduced by 46.9% and 50.8% in chromosomal and nuclear chromatin respectively when compared to the respective value of such labeling densities in corresponding Ni-compound-alone-treated groups (Table 3, Figs. 6 and 7). Similarly, cotreatment of human blood lymphocytes in culture with the same Ni-compound (15 μM) and 50 μM dantrolene (which is known to inhibit mobilization of $[Ca^{2+}]_i$ from endoplasmic reticulum) for 2 h significantly reduced Ni-compound-induced DNA single-strand breaks as evident from the result that the number of immunogold particles per μm^2 chromatin was significantly reduced by 35% and 40.5% in chromosomal and nuclear chromatin respectively when compared to the respective value of such labeling densities in corresponding Ni-compound-alone-treated group (Table 3, Figs. 6 and 7). Finally, cotreatment of human blood lymphocytes in culture with 15 μM of the same Ni-compound and 3 mM of 1,2-bis (2-Aminophenoxy) ethane-*N*,*N*,N´,*N*´-tetraacetic acid tetra-(acetoxymethyl)ester (BAPTA), a specific Ca^{2+} chelator (Salnikow et al., 2000) for 2 h significantly reduced Ni-compound-induced DNA damage as evident from the result that the number of immunogold particles per μm^2 chromatin was somewhat but significantly reduced by 13.8% and 33% in chromosomal and nuclear chromatin respectively when compared to the respective values of such labeling densities in corresponding Ni-compound-alone-treated groups (Table 3, Figs. 6 and 7).

Discussion

Based on our present study of the interactions of $NiSO_4$ and soluble forms of nickel carbonate hydroxide, nickel oxide and nickel subsulfide with both chromosomal and nuclear chromatin from human blood lymphocytes, the genotoxic potential (as measured by DNA single-strand breaks) seems to be decreased in the following order: nickel carbonate hydroxide > nickel oxide ≥ nickel subsulfide > nickel sulfate. Significantly higher rate of DNA single-strand breakages and significantly elevated SCE values have been observed in lymphocytes of welders exposed to nickel and chromium (Werfel et al., 1998). DNA single-strand breaks were observed in lungs and kidneys of rats following parenteral administration of $NiCl_2$ (Saplakoglu et al., 1997). Previous studies from our laboratory have shown that the extent of double-strand DNA breaks in liver, kidney, and lymphocytes of mice and rats following *in vivo* administration of Ni-compounds depends not only on the nickel speciation but also on the exposure route and animal species (Chakrabarti and Bai, 1995). Both single- and double-strand breaks were observed in Ni(II)-treated blood lymphocytes *in vitro* (Cai and Zhuang, 1999). *In vitro* exposure of human lymphocytes to $NiCl_2$ produced DNA damage as measured by comet assay (Chen et al., 2003).

Exposure of workers in nickel industries to atmospheric nickel varies from 5-15 mg/m^3 in Falconbridge nickel refinery, Norway (Doll et al., 1990) to 70-699 mg/m^3 in Sherritt Gordon hydrometallurgical nickel refinery, Fort Saskatchewan, Alberta (Egedahl et al., 1991). Based on a scatter diagram showing the linear relationship between atmospheric nickel levels and plasma nickel concentrations for seven individuals working in a Ni_3S_2 matte crushing department (Nieboer et al., 1992), the mean plasma concentrations of nickel are estimated to be 60 μg/L and 400 μg/L for atmospheric nickel levels of 15 mg/m^3 and 100 mg/m^3 respectively. When rat lymphocytes were exposed to 2 mM of Ni_3S_2 (soluble form) for 2 hr at 37°C, the measured nickel uptake by lymphocytes was found to be 4.38 μg/ml (Chakrabarti et al., 2001). Based on this data , the approximate nickel uptake by human lymphocytes is estimated to be 33 and 264 μg per liter following exposure to 15 and 120 μM Ni_3S_2 respectively for 2 hr. These values are well within the range of plasma nickel concentrations found in some nickel workers mentioned above.

The present results have shown that both chromosomes and nuclei from human blood lymphocytes exposed *in vitro* to very low concentration (15 μM) of four different Ni-compounds demonstrated significantly higher DNA single-strand breaks (as compared to the respective control), as verified by the higher immunogold labeling of

both Ni-treated chromosomal and nuclear chromatin fibres. Control cultures processed by ISEL showed some immunogold labeling on both chromatin fibres. This may be due to some spontaneous DNA single-strand breaks which are habitually present in living cells, and/or the result of physical and chemical manipulations (Fertil et al., 1984). The degree and distribution of chromatin immunogold-labeling (hence the DNA single-strand breaks) in all four Ni-compound-treated human lymphocytes seem to be significantly higher in chromosomal chromatin than those in nuclear chromatin. This indicates that the chromosomal chromatin is found to be more sensitive to Ni-compound-induced DNA single-strand breaks than the nuclear chromatin. Thus the chromosome damage induced by nickel compounds seemed to be a more sensitive parameter of the genotoxicity of nickel, the extent of which was found to be correlated better with the carcinogenic potency of nickel compounds (Sen and Costa, 1985). It is possible that the level of nickel bound to chromosomal chromatin from human lymphocytes may be higher than that from nuclear chromatin, and such difference could be related to the difference in nickel ion uptake. Nickel has been shown to produce chromosome damage selectively in heterochromatic regions, including damage to the heterochromatic long arm of the X chromosome of Chinese hamster cells, as well as gaps, breaks, and exchanges in a heterochromatin predominantly (Sen and Costa, 1986;

Conway et al., 1987; Costa et al., 2002). However, Ni^{2+} actually induced an increase in chromatin condensation, and a metaphase chromosome is maximally condensed (Borochov et al., 1984). The major difference between the heterochromatic and nuclear matrix proteins is that heterochromatic proteins contain significantly much higher amount of histone H1 than nuclear matrix proteins. Thus histone H1 may be the specific protein in heterochromatin that binds Ni^{2+} and may catalyze the formation of reactive oxygen species (ROS) (Huang et al., 1995). Since the binding of Ni^{2+} to DNA is relatively weak, the principal target for nickel binding in nuclear chromatin appears to be the proteins; especially the histones and protamines (see review by Bal et al., 2000). Briefly, at physiological pH, strong Ni(II)-binding motifs have been observed in protamine P2 and in core histones H3 and H2A, and a weak one in histone H4. It has been shown that heterochromatic proteins further significantly enhanced the formation of 8-oxo-dG (7,8-dihydro-8-oxo-2´-deoxyguanosine) induced by $NiCl_2$ plus H_2O_2. In contrast to heterochromatic proteins, nuclear matrix proteins inhibited the production of 8-oxo-dG by $NiCl_2$ plus H_2O_2 (Huang et al., 1995). In nuclear chromatin, the DNA molecule, having phosphate anions and nitrogen and oxygen donor groups, is capable of binding to Ni^{2+} like other metal cations. However, the chromatin proteins even have stronger potential than DNA to bind to Ni^{2+} (Bal et al., 1997; Kasprzak and Buzard, 2000; Zoroddu et al.,

2000). This may explain why nickel like other heavy metals is found in cell nuclei following *in vivo* administration (Berg, 1986; Peskin and Shlyahova, 1986). The generation of H_2O_2 and O_2^- was also detected in cell nuclei (Peskin and Shlyahova, 1986). Therefore, the bound nickel may catalyze the generation of excess ROS in the cell nucleus that may lead to oxidative damage to DNA and other nuclear components as well (Kasprzak and Buzard, 2000; Coogan et al., 1989). Besides chromosomal damage, the major oxidative effects in DNA due to exposure to nickel include strand breaks, cross-linking, depurination and base modifications (Coogan et al., 1989; Chakrabarti et al., 1999, 2001; Kasprzak and Buzard, 2000; Costa et al., 2002).

In this study following the method of Lee et al (1982) significant concentrations (≈10 mM) of Ni(II) were found in solution following incubation of either insoluble nickel subsulfide, or nickel carbonate hydroxide, or nickel oxide up to 12 hr in 0.05M Tris-HCl buffer, pH 7.4. However, the nature of the chemical forms of these Ni-compounds after solubilizing in Tris-HCl buffer, pH 7.4 was not determined in our present study. On the other hand, this increase in solubility for Ni_3S_2 for example, can be accounted for by the ability of Tris to coordinate nickel (II) ion and form soluble charged complexes (Dotson, 1972). The solubilized nickel exhibited electronic absorption

spectra and magnetic moments characteristic of a octahedral nickel (II). So, it is assumed that insoluble nickel oxide and insoluble nickel carbonate hydroxide may be changed to a soluble form with Tris involving some type of Ni^{2+}-coordinated complex. On the other hand, a variety of reactive intermediates can also be formed during the process of oxidative cellular solubilization of Ni_3S_2 and NiS, for example. Their sensitivity to oxidation by ambient oxygen facilitates their dissolution in biological fluids. Such dissolution may eventually result in intracellular formation of soluble Ni(II) complexes with natural ligands, such as amino acids and proteins (Kasprzak et al., 2003).

Evidence presented in this study to suggest that oxidative damage-inducing free radicals may play an important role in the underlying biochemical mechanisms involved in soluble form of nickel carbonate hydroxide-induced DNA single-strand breaks. Since pretreatment of human blood lymphocytes with catalase (a H_2O_2 scavenger) significantly reduced nickel carbonate hydroxide (soluble form)-induced increase in DNA single-strand breaks in both metaphase chromosomal and interphase nuclear chromatin, it suggests that excess reactive oxygen species such as H_2O_2 is implicated in this Ni-compound-induced DNA single-strand breaks in both chromosomal and nuclear chromatin of human blood

lymphocytes. Significant prevention of soluble form of nickel carbonate hydroxide-induced DNA single-strand breaks in both metaphase chromosomal chromatin and interphase nuclear chromatin by superoxide dismutase (SOD) suggests the participation of excess superoxide anion radical (O_2^-) as well in such DNA single-strand breaks. Similarly, the significant prevention of soluble form of nickel carbonate hydroxide-induced DNA single-strand breaks in both chromosomal and nuclear chromatin by dimethylthiourea (DMTU) (a hydroxyl radical scavenger) indicates that Ni-compound-induced such DNA single-strand breaks also occur via generation of excess reactive hydroxyl radical species in human blood lymphocytes. Iron acts as a Fenton catalyst to produce hydroxyl radical from H_2O_2. Studies from our laboratory involving Ni-compound-induced human lymphocyte death have directly demonstrated time-dependent generation of excess H_2O_2 and superoxide anion radical by soluble form of nickel carbonate hydroxide (M'Bemba-Meka et al., manuscript submitted). Deferoxamine (DFO) being an iron chelator has the potential to inhibit the process of Ni-compound-induced ROS, thereby can prevent against Ni-compound-induced DNA single-strand breaks. Since DFO pretreatment significantly reduced nickel carbonate hydroxide-induced DNA single-strand breaks in both chromosomal and nuclear chromatin, it suggests that this nickel compound may express its genotoxic potential by way of iron-mediated oxidative damage as well.

Furthermore, these findings provide support for iron chelator therapy in protection against Ni-compound-induced oxidative DNA damage. The percentage of Ni-compound-induced DNA single-strand breaks is significantly reduced by pretreatment of human blood lymphocytes in culture with excess N-acetylcysteine (a precursor of GSH), suggesting that intracellular GSH level is one of the determining factors in the susceptibility of human lymphocytes to the genotoxic effects of Ni-compound. Excess GSH may be involved in the metabolism of free radicals and hydroperoxides. Ni-compound (for example, nickel carbonate hydroxide)-induced generation of excess H_2O_2 in isolated human lymphocytes is significantly reduced not only by catalase and DFO, but also by excess GSH (M'Bemba-Meka et al., manuscript submitted). This suggests that the loss of cellular thiol reserve also resulted in significant generation of H_2O_2 and hydroxyl radical. However, since catalase and/or DFO also significantly prevented the depletion of nonprotein-sulfhydryl (NP-SH) induced by Ni-compounds, it indicates that generation of ROS such as H_2O_2 and $^{\cdot}OH$ are also responsible for the loss of cellular thiol. This is not unusual as both the generation of H_2O_2 and depletion of cellular NP-SH contents started at the same time, e.g. after 15 min of exposure to Ni-compounds in isolated human lymphocytes (M'Bemba-Meka et al., manuscript submitted). Similarly, increased generation of various types of ROS (e.g. H_2O_2, superoxide anion, hydroxyl radical) as well as

depletions of NP-SH and protein-bound sulfhydryl (P-SH), and activation of lipid peroxidation due to Ni-compounds including nickel carbonate hydroxide at non cytotoxic concentration are all believed to be the initiators of pathogenesis of lymphocyte death, as they appeared before any occurrence of Ni-compound-induced human lymphocyte death (M'Bemba-Meka et al., manuscript submitted). Reactive oxygen species (ROS) such as superoxide radical ion (O_2^-), H_2O_2 and $^{\cdot}OH$ have been implicated in the etiology of many human diseases including cancer (Halliwell and Gutheridge, 1989). An increased generation of ROS within cells could lead to DNA damage by various mechanisms (Halliwell and Aruoma, 1991).

The mechanism by which Ni(II)-compounds increase generation of ROS in human lymphocytes is not clear. Ni_3S_2 is believed to activate neutrophils and cause substantial production of H_2O_2 levels (Zhong et al., 1990). It has been shown that peptides containing the glycyl-glycyl-L-histidyl sequence trigger nickel-dependent production of oxygen radicals through reaction with H_2O_2. When chelated with peptides containing the glycyl-glycyl-L-histidyl sequence, Ni^{2+} could also peroxidize lipids either through H_2O_2 disproportionation and hydroxyl radical production, or directly by reaction with the lipid peroxides (Torreilles and Guérin, 1990). Both soluble Ni(II) and insoluble Ni_3S_2 increased the formation of intracellular ROS even at

the nucleus when cells were exposed to Ni_3S_2 (Huang et al., 1994). Ni^{2+} and DNA are unlikely to be responsible for the genotoxic effects observed in cells exposed to Ni^{2+}. Therefore, it has been proposed that Ni^{2+} reacts with endogenous H_2O_2 in cells to form hydroxyl radicals ($^{\bullet}OH$) which may then cause DNA damage. Ni^{2+} is known to induce increased oxidative stress in both the cytoplasm and nuclei of living Chinese hamster ovary cells (Huang et al., 1993, 1994). Furthermore, Ni^{2+} is known to enhance oxidative damage to DNA bases following incubation with human chromatin *in vitro* (Nackerdien et al., 1991). *In vivo* studies have shown that Ni^{2+} induces oxidative DNA base damage in chromatin isolated from renal and hepatic cells of Ni-treated pregnant rats and fetuses (Kasprzak et al., 1992). The oxidative effects of nickel depend on its ability to form the Ni(III)/Ni(II) redox couple at pH 7.4. This is favored only when Ni(II) is complexed by some natural ligands, including peptides and proteins (Bal et al., 2000; Margerum and Anliker, 1988).

However, Ni^{2+} ions themselves have been shown to induce the formation of oxidized DNA bases (Kawanishi et al., 1989; 2002; Nackerdien et al., 1991). Similarly, nickel subsulfide (soluble form)-induced formation of reactive oxygen species has been detected by dichlorofluorescein fluorescence in isolated rat lymphocytes *in vitro* (Chakrabarti et al., 2001). Both nickel subsulfide (insoluble) and

nickel chloride (soluble) have been shown to induce increased oxidants in intact cultured mammalian cells as detected by dichlorofluorescein fluorescence (Huang et al., 1993), and by nickel chloride in isolated human lymphocytes (Chen et al., 2003). Studies from our laboratory have also shown excess generation of hydrogen peroxide and superoxide anion radical from soluble form of nickel carbonate hydroxide-treated human lymphocytes *in vitro* (M'Bemba-Meka et al., manuscript submitted). Similarly, the effects of nickel carbonate hydroxide ($NiCO_3.Ni(OH)_2.2H_2O$) on alveolar macrophage functions were characterized by large generation of free radicals and a depletion of cellular energy reserves (Arsalane et al., 1992).

Our present study has provided evidences both directly and indirectly that the mechanism of nickel carbonate hydroxide-induced DNA single-strand breaks involves not only the excess generation of various types of ROS and oxidative stress, but also destabilization of cellular calcium homeostasis. Similar mechanisms involving both oxidative stress and calcium dehomeostasis have also been implicated in Ni-compound-induced human lymphocyte death (M'Bemba-Meka et al., manuscript submitted). However, our previous study has shown that Ni-compound-induced destabilization of cellular calcium homeostasis in isolated human lymphocytes is caused by Ni-compound-induced ROS/oxidative stress (M'Bemba-Meka et al.,

manuscript submitted). The present study has identified some calcium sites as additional targets by which Ni-compound may perturb cellular Ca^{2+} homeostasis which may therefore result in increased DNA single-strand breaks in both chromosomal and nuclear chromatin from human blood lymphocytes. Thus, analysis of the mechanism involving the effect of modulator of calcium channel blocker governing the increase of $[Ca^{2+}]_i$ suggest that the protective effect of verapamil against Ni-compound-induced DNA single-strand breaks in chromosomal and nuclear chromatin is due to inhibition of calcium movement through voltage-operated calcium channel protein involving plasma membranes. Furthermore, the present study has also indicated that other calcium channel blocker is also able to inhibit calcium movement through other mechanism as well. Thus, Ni-compound-induced DNA single-strand breaks in both chromosomal and nuclear chromatin can also be significantly prevented by inhibiting Ca^{2+} release from sacroplasmic reticulum by dantrolene cotreatment. However, it warrants further study whether there are more sensitive targets for Ni^{2+}. It is possible that soluble form of nickel (Ni^{2+}) from nickel carbonate hydroxide apparently compete with the essential metals like calcium to use the same pathways, thus disrupting the intracellular balance of Ca^{2+} and resulting in DNA single-strand breaks. Soluble nickel has been shown to compete with calcium for channels and to be taken up through

calcium channels (Refvik and Andreassen, 1995; Funakoshi et al., 1997). It has been shown that Ni uptake by rat hepatocytes occurs, at least in part, through the Ca channel transport processes. Pretreatment with nicardipine or verapamil, potent inhibitors of Ca^{2+} channels, decreased Ni uptake by 20% (Funakoshi et al., 1997). However, other mechanisms of Ni uptake might also be involved which requires further study. Ca^{2+} is considered as one of the most important intracellular second messengers and is maintained at a very steep gradient between the outside and the inside of all mammalian cells (Pozzan et al., 1994; Rosen et al., 1995). Very few studies have related the carcinogenic and/or toxic effects of nickel with disturbances in calcium metabolism (see review by Denkhaus and Salnikow, 2002). Thus, an increase in calcium concentration has been observed in mice pancreas after nickel administration (Funakoshi et al., 1996). Nickel-transformed cells could rapidly proliferate in media deficient in calcium suggesting an alteration of intracellular calcium metabolism in nickel-transformed cells (Swierenga et al., 1976). Cytoplasmic Ca^{2+} pulses signal gene expression associated with cell growth, differentiation, and apoptosis of many different types of cells in the body (Rosen et al., 1995; Nicotera and Orrenius, 1988). Since exogenous addition of BAPTA, a specific chelator of free intracellular calcium, $[Ca^{2+}]_i$ also attenuated Ni^{2+}-induced DNA single-strand breaks in both chromosomal and

nuclear chromatin, it indicates that elevation of intracellular calcium was essential for such DNA damage. Similarly, other studies from our laboratory have also shown that either intracellular Ca^{2+} channel blocker, or intracellular Ca^{2+} antagonist, or Ca^{2+} chelator is capable of preventing human lymphocyte death induced by soluble form of nickel carbonate hydroxide.

In conclusion, it is unlikely that any single biochemical event is only responsible for nickel carbonate hydroxide (soluble form)-induced DNA single-strand breaks in both chromosomal and nuclear chromatin from human blood lymphocytes. Ni^{2+}-induced disruptions in cellular biochemical processes including various types of oxidative stress, and calcium dehomeostasis are equally critical events in Ni-carbonate hydroxide-induced DNA single-strand breaks in both chromosomal and nuclear chromatin derived from human blood lymphocytes *in vitro*. Further studies are required to verify whether similar mechanisms exist for the three other Ni-compounds producing DNA single-strand breaks in both chromosomal and nuclear chromatin from human blood lymphocytes. Thus the present data may provide some new insights into the mechanisms of Ni-compound-induced DNA single-strand breaks in human blood lymphocytes as well as contribute some important information for the treatment and prevention of potential adverse health effects among workers in

nickel-related industrial environments.

ACKNOWLEDGMENTS

The authors sincerely thank the Sainte-Justine Hospital, Montréal for obtaining blood samples from donors, the Department of Pathology (Sainte-Justine Hospital, Montréal) for electron microscopy facility. They also thank Mrs. Fléchère for expert technical assistance and Mr. Jean Léveillé for expert photographical assistance.

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FIGURE CAPTIONS

FIG. 1. Effect of exposure of human blood lymphocytes in culture to different nickel compounds on cellular viability. 0-30 μM of soluble forms of nickel carbonate hydroxide, or nickel subsulfide, or nickel sulfate, or nickel oxide was injected into the cultures at 46 h after initiation of culture for a 2 h exposure during a total 72 h culture period. The cellular viability was estimated by the trypan blue

exclusion test. Values represent mean ± SEM from four separate experiments involving four separate blood donors.

FIG. 2. Electron microscopy *in situ* end-labeling images (x 28 000) of (a) untreated lymphocyte metaphase chromatin, (b) nickel carbonate hydroxide-treated lymphocyte metaphase chromatin, (c) nickel oxide-treated lymphocyte metaphase chromatin, (d) nickel subsulfide-treated lymphocyte metaphase chromatin, and (e) nickel sulfate-treated lymphocyte metaphase chromatin. For details, see Materials and Methods.

FIG. 3. Electron microscopy *in situ* end-labeling images (x 28 000) of (a) untreated lymphocyte interphase chromatin, (b) nickel carbonate hydroxide-treated lymphocyte interphase chromatin, (c) nickel oxide-treated lymphocyte interphase chromatin, (d) nickel subsulfide-treated lymphocyte interphase chromatin, and (e) nickel sulfate-treated lymphocyte interphase chromatin. For details, see Materials and Methods.

FIG. 4. Effects of various scavengers of reactive oxygen species (ROS) on electron microscopy *in situ* end-labeling images (x 28 000) of (a) untreated lymphocyte metaphase chromatin, (b) nickel carbonate hydroxide (NiCH)-treated lymphocyte metaphase chromatin, (c) NiCH

plus catalase-treated lymphocyte metaphase chromatin, (d) NiCH plus N-acetylcysteine (NAC)-treated lymphocyte metaphase chromatin, (e) NiCH plus DMTU-treated lymphocyte metaphase chromatin, (f) NiCH plus superoxide dismutase-treated lymphocyte metaphase chromatin, (g) NiCH plus deferoxamine-treated lymphocyte metaphase chromatin. For details, see Materials and Methods.

FIG. 5. Effects of various scavengers of reactive oxygen species (ROS) on electron microscopy *in situ* end-labeling images (x 28 000) of (a) untreated lymphocyte interphase chromatin, (b) NiCH-treated lymphocyte interphase chromatin, (c) NiCH plus catalase-treated lymphocyte interphase chromatin, (d) NiCH plus NAC-treated lymphocyte interphase chromatin, (e) NiCH plus DMTU-treated lymphocyte interphase chromatin, (f) NiCH plus superoxide dismutase-treated lymphocyte interphase chromatin, (g) NiCH plus deferoxamine-treated lymphocyte interphase chromatin. For details, see Materials and Methods.

FIG. 6. Effects of various intracellular calcium ion $[Ca^{2+}]_i$ modulators on electron microscopy *in situ* end-labeling images (x 28 000) of (a) untreated lymphocyte metaphase chromatin, (b) NiCH-treated lymphocyte metaphase chromatin, (c) NiCH plus 25 μM verapamil-treated lymphocyte metaphase chromatin, (d) NiCH plus 50 μM

dantrolene-treated lymphocyte metaphase chromatin, (e) NiCH plus 3 mM BAPTA-treated lymphocyte metaphase chromatin. For details, see Materials and Methods.

FIG. 7. Effects of various $[Ca^{2+}]_i$ modulators on electron microscopy *in situ* end-labeling images (x 28 000) of (a) untreated lymphocyte interphase chromatin, (b) NiCH-treated lymphocyte interphase chromatin, (c) NiCH plus 25 μM verapamil-treated lymphocyte interphase chromatin, (d) NiCH plus 50 μM dantrolene-treated lymphocyte interphase chromatin, (e) NiCH plus 3 mM BAPTA-treated lymphocyte interphase chromatin. For details, see Materials and Methods.

TABLE I

Effects of soluble forms of different nickel compounds on DNA single-strand breaks in human blood lymphocytes

Treatment	Labeling density (Immunogold particles/μm^2)	
	Metaphase chromosomal chromatin (Mean ± SEM)	Interphase nuclear chromatin (Mean ± SEM)
Control	55 ± 3.5	49 ± 1.4
$NiSO_4$ (5 μM)	61 ± 3.4	49 ± 4.2
$NiSO_4$ (15 μM)	114 ± 9.4*[b]	61 ± 4.5
Ni_3S_2 (5 μM)	63 ± 3.6	49 ± 3.0
Ni_3S_2 (15 μM)	132 ± 6.0*[b]	85 ± 2.6*
NiO (5 μM)	65 ± 2.9	50 ± 2.7
NiO (15 μM)	125 ± 5.6*[ab]	103 ± 3.9*
2 $NiCO_3$.3Ni$(OH)_2$ (5 μM)	67 ± 4.3	53 ± 3.9
2 $NiCO_3$.3Ni$(OH)_2$ (15 μM)	271 ± 1.7*[ab]	182 ± 7.1*

* Significantly different from control, $p < 0.01$.
[a] Significantly different from all other Ni-treated group, $p < 0.001$.
[b] Significantly different from corresponding Ni-treated interphase nuclear chromatin group, $p < 0.01$.

TABLE II

Role of various oxidative stress in $2NiCO_3.3Ni(OH)_2$-induced DNA strand breaks in human blood lymphocytes

Treatment	Labeling density (Immunogold particles/μm^2)	
	Metaphase chromosomal chromatin (Mean ± SEM)	Interphase nuclear chromatin (Mean ± SEM)
Control	55 ± 3.5	49 ± 1.4
2 $NiCO_3.3Ni(OH)_2$ (15 μM)	271 ± 3.3	185 ± 2.9
Catalase (1 000 U/ml) + $2NiCO_3.3Ni(OH)_2$ (15 μM)	161 ± 21.1*	109 ± 3.0*
NAC (8 mM) + $2NiCO_3.3Ni(OH)_2$ (15 μM)	107 ± 11.9*	107 ± 5.5*
DMTU (20 mM) + $2NiCO_3.3Ni(OH)_2$ (15 μM)	147 ± 18.6*	81 ± 5.8*
SOD (2 500 U/ml) + $2NiCO_3.3Ni(OH)_2$ (15 μM)	148 ± 18.5*	90 ± 6.4*
DFO (100 µM) + $2NiCO_3.3Ni(OH)_2$ (15 μM)	157 ± 15.8*	102 ± 5.0*

* Significantly different from Ni-compound-alone-treated group, $p < 0.001$.

TABLE III

Role of intracellular calcium ion in $2NiCO_3.3Ni(OH)_2$-induced DNA strand breaks in human blood lymphocytes

Treatment	Labeling density (Immunogold particles/μm^2)	
	Metaphase chromosomal chromatin (Mean ± SEM)	Interphase nuclear chromatin (Mean ± SEM)
Control	55 ± 3.5	49 ± 1.4
2 $NiCO_3.3Ni(OH)_2$ (15 μM)	275 ± 2.1	185 ± 2.9
Verapamil (25 μM) + $2NiCO_3.3Ni(OH)_2$ (15 μM)	146 ± 5.1*	91 ± 6.8*
Dantrolene (50 μM) + $2NiCO_3.3Ni(OH)_2$ (15 μM)	176 ± 6.1*	110 ± 5.7*
BAPTA (3 mM) + $2NiCO_3.3Ni(OH)_2$ (15 μM)	236 ± 8.4*	124 ± 4.2*

* Significantly different from Ni-compound-alone-treated group, $p < 0.01$.

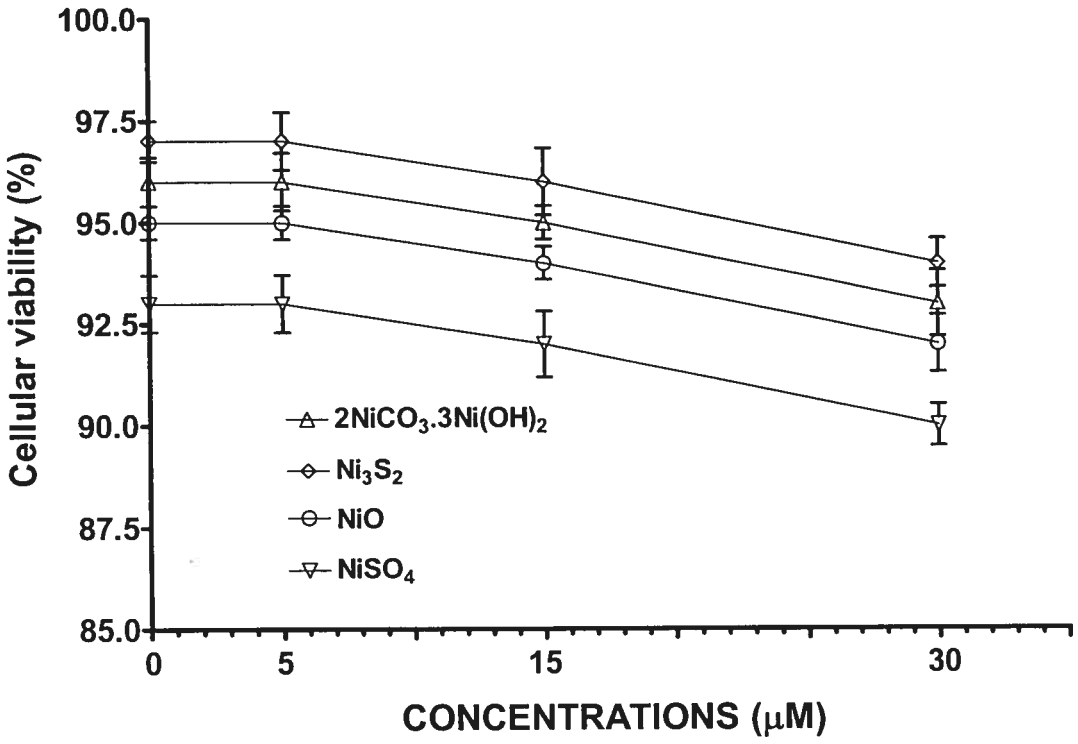
100.0
97.5
95.0
92.5
90.0
87.5
85.0
Cellular viability (%)
0
5
15
30
CONCENTRATIONS (μM)
2NiCO3.3Ni(OH)2
Ni3S2
NiO
NiSO4

FIG. 1

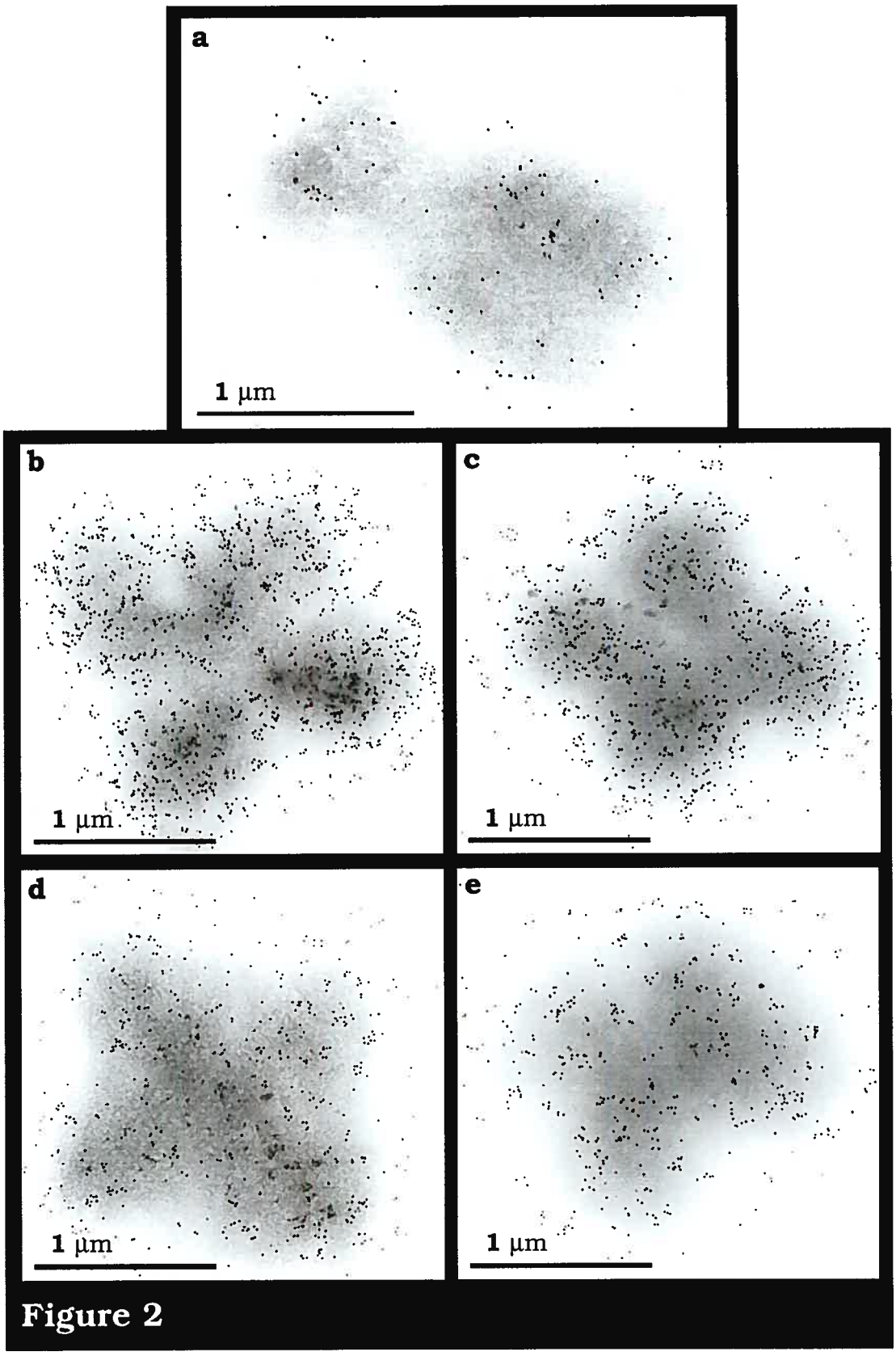

Figure 2

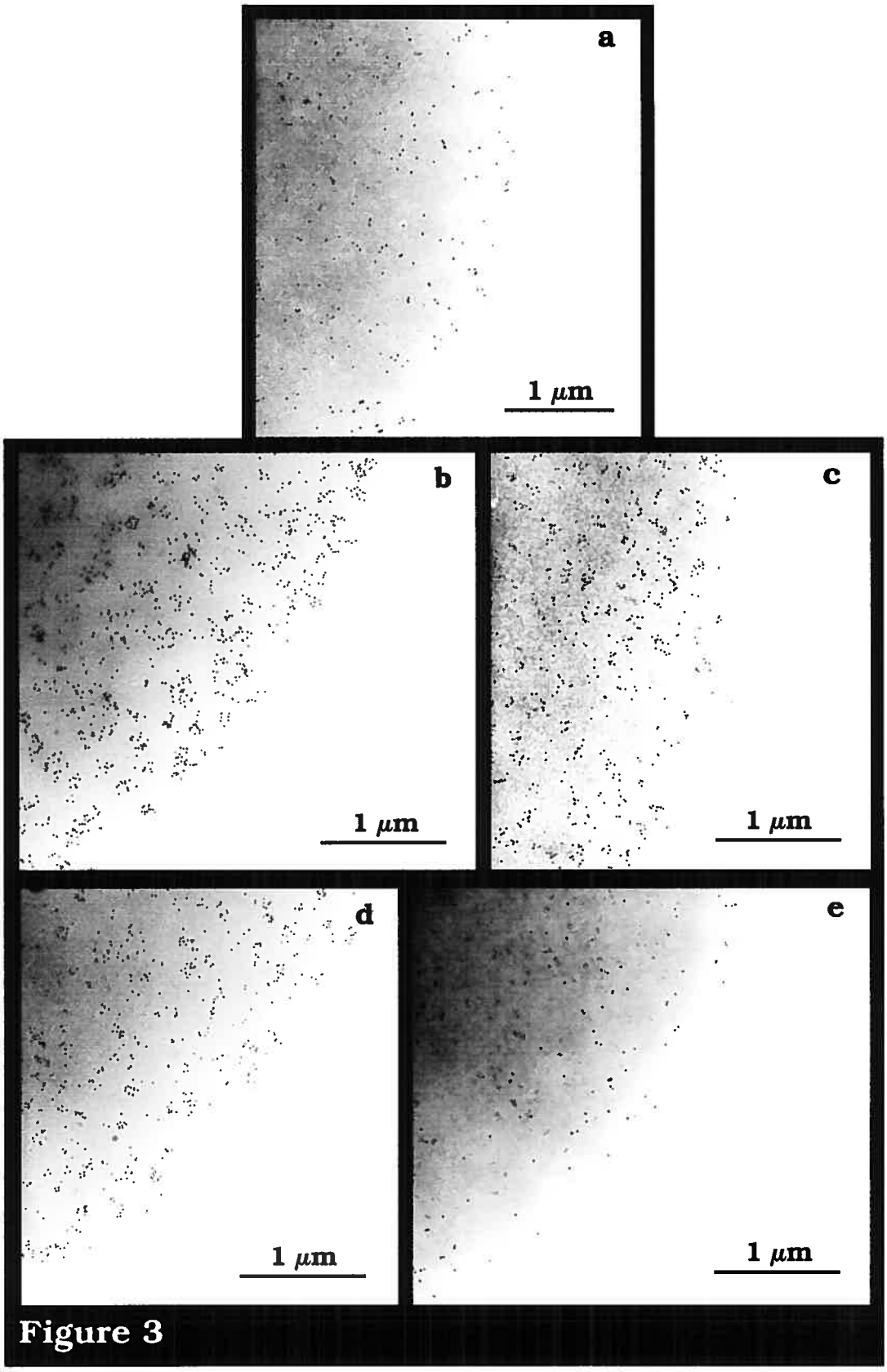

Figure 3

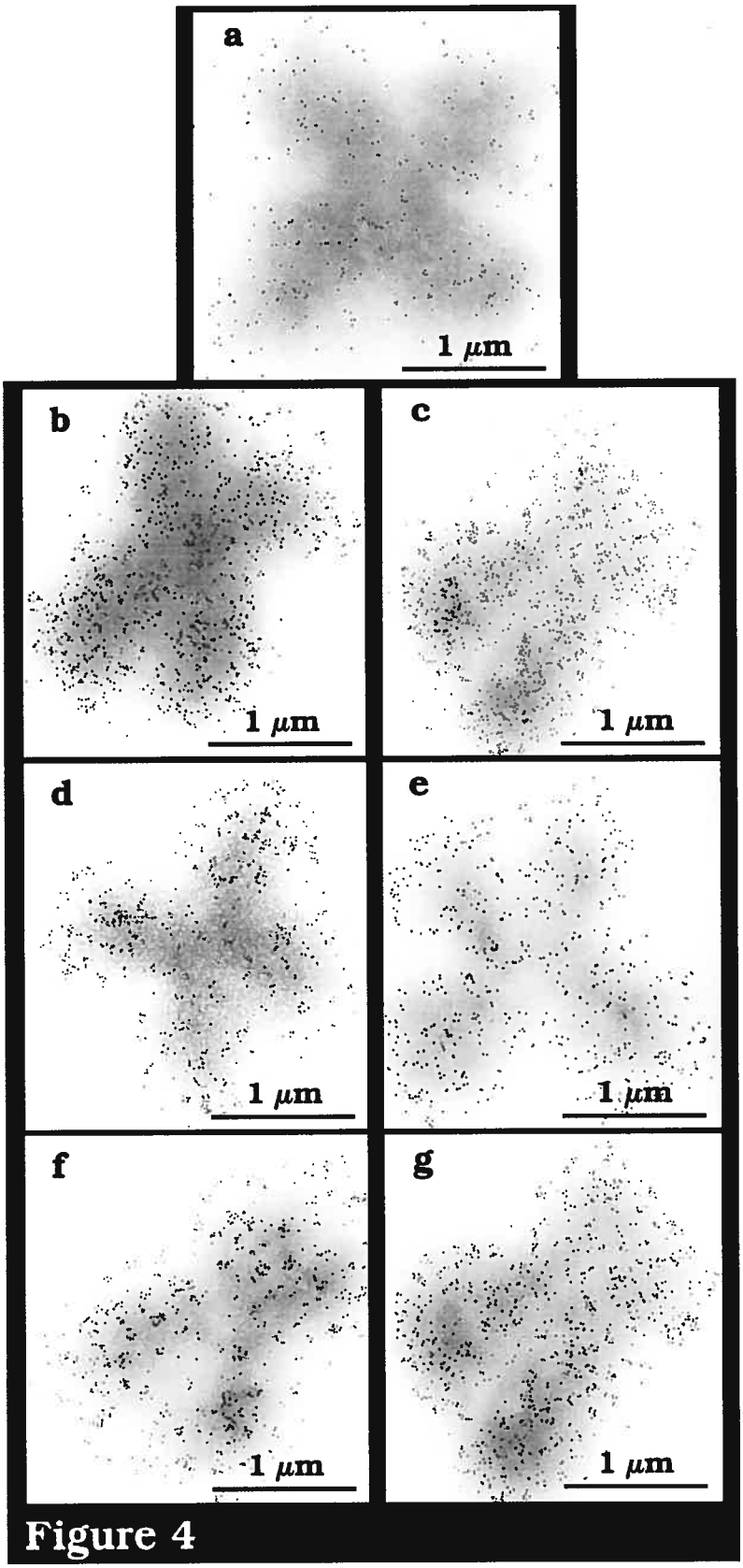

Figure 4

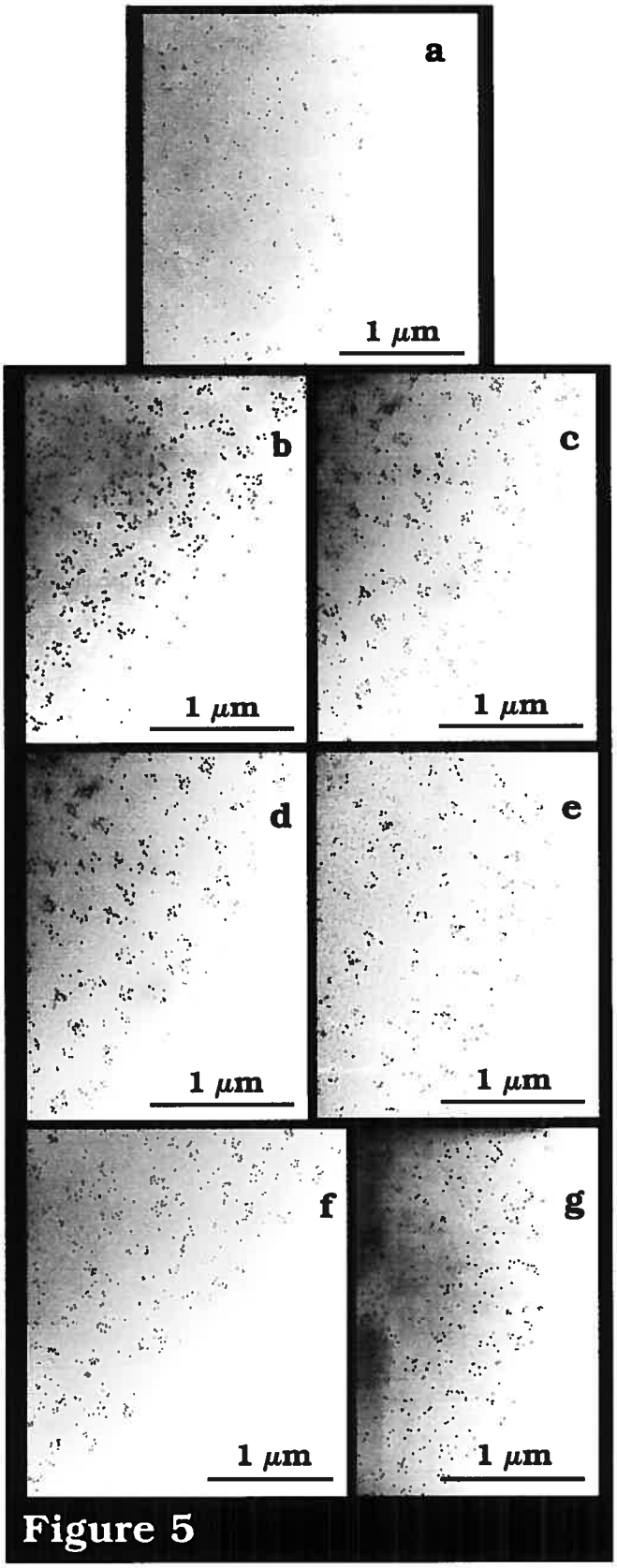

Figure 5

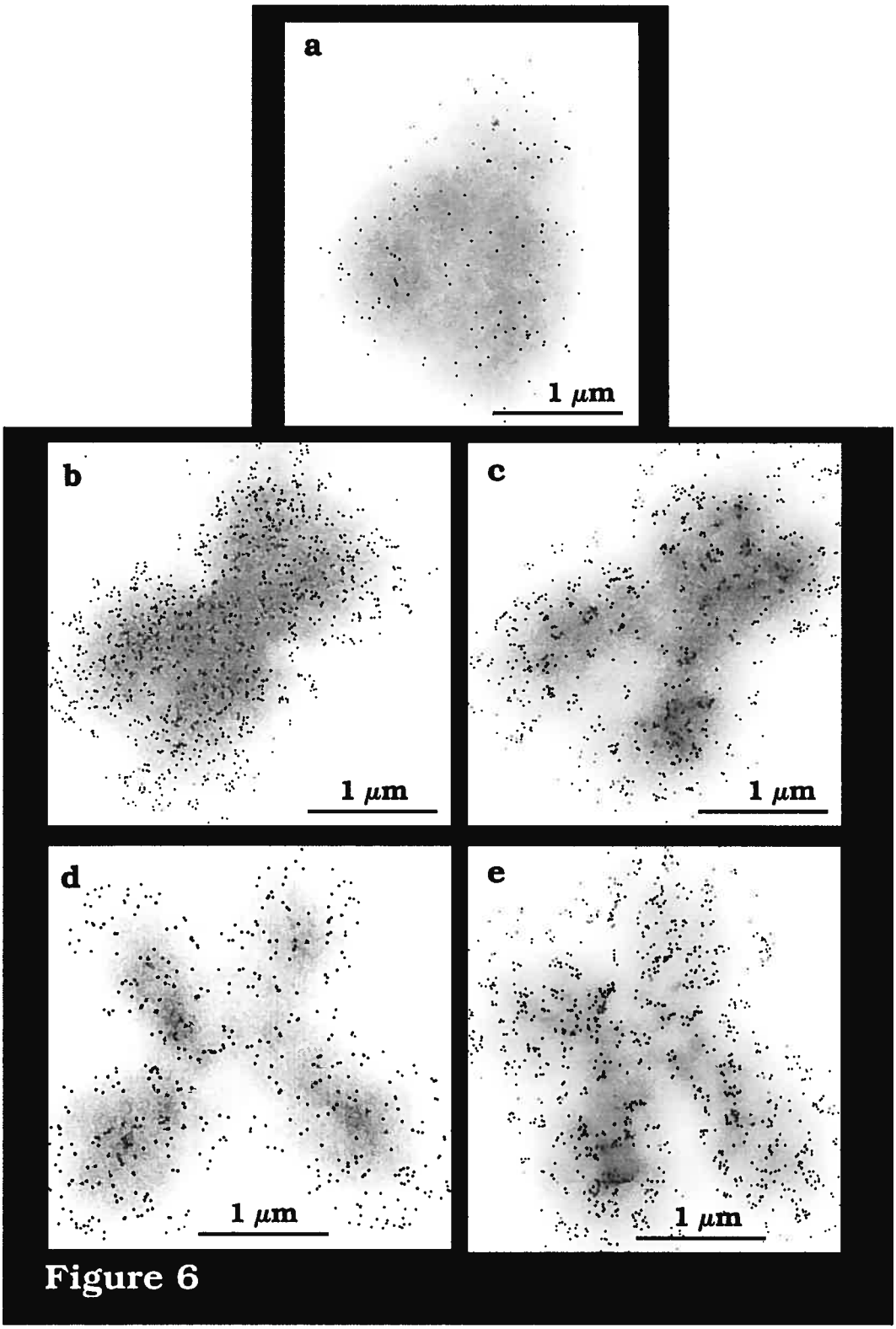

Figure 6

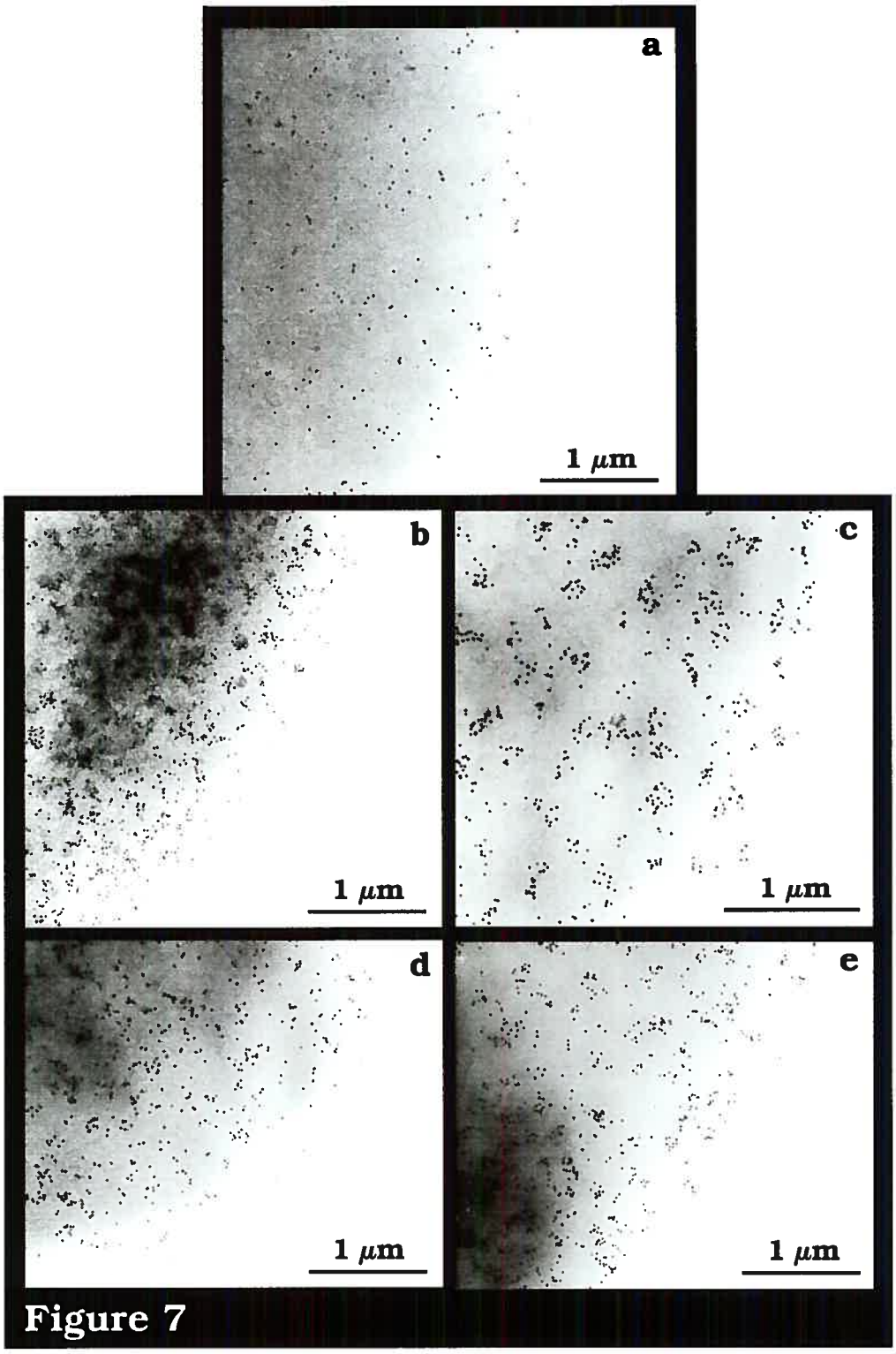

Figure 7

III.2. ARTICLE V

Role of oxidative stress and intracellular calcium in nickel compound-induced sister-chromatid exchanges, replication index and mitotic index in human peripheral blood lymphocytes in culture.

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Abstract

Human peripheral lymphocytes from whole blood cultures were exposed to either soluble form of nickel carbonate hydroxide (0-60 μM), or of nickel subsulfide (Ni_3S_2) (0-120 μM), or of nickel oxide (NiO) (0-120 μM), or of nickel sulfate ($NiSO_4$) (0-120 μM) for a short duration of 2 h during a total of 72 h culture period. After the end of 72 h, the sister-chromatid exchanges (SCEs), replication index (RI), and mitotic index (MI) were measured for each nickel compound. Nickel carbonate hydroxide (NiCH) at 30 μM but Ni_3S_2 and NiO at 120 μM produced moderate but significant increase in the SCE frequency per cell compared to the control value, whereas $NiSO_4$ failed to produce any such significant increase. Except $NiSO_4$, the soluble forms of NiCH, Ni_3S_2 and NiO produced significant cell-cycle delay (as measured by inhibition of RI) as well as significant inhibition of MI at respective similar concentrations mentioned above. Pretreatment of human blood lymphocytes with catalase, or superoxide dismutase, or dimethylthiourea, or *N*-acetylcysteine, or deferoxamine significantly prevented NiCH-induced changes in SCEs, RI and MI. This suggests the participation of H_2O_2, superoxide anion radical, hydroxyl radical and iron chelator in such cytogenotoxicity. Cotreatment of NiCH with either verapamil (inhibitor of $[Ca^{2+}]_i$ movement through plasma membranes), or dantrolene (inhibitor of $[Ca^{2+}]_i$ release from

sarcoplasmic reticulum), or BAPTA (Ca^{2+} chelator) significantly prevented NiCH-induced changes in cytogenotoxicity. It suggests that $[Ca^{2+}]_i$ is also implicated in such genotoxicity. Overall the data indicate that both iron-mediated oxidative stress involving Fenton-Haber/Weiss reaction and calcium dehomeostasis are involved in such genetic damage.

KEY WORDS: Human blood lymphocytes; nickel compounds; sister-chromatid exchanges; replication index; mitotic index; oxidative stress/calcium.

Introduction

Occupational exposure to nickel compounds occurs principally through mining, smelting, and refining operations, alloy production, electroplating and welding operations during manufacture of steel, other alloys and batteries. Nickel matte refining has been associated with high exposure to dusts of nickel subsulfide and nickel oxide, whereas in electrolytic refining operations workers are exposed to aerosols of nickel sulfate and nickel chloride. Nickel carbonate hydroxide is used in nickel plating, in colours and glazes for ceramics and in high pure form it is used in electronic components.

Epidemiological and experimental studies have established that nickel is a human carcinogen (Oller et al., 1997; Kasprzak et al., 2003; Kawanishi et al., 2002; Denkhaus and Salnikow, 2002; ICNCM Report, 1990). Nickel is known to be both mitostatic and clastogenic. The increased frequencies of chromosomal aberrations and sister-chromatid exchanges (SCEs) (compared to the control group) in the peripheral lymphocytes of workers occupationally exposed to nickel have been reported in many studies (Werfel et al., 1998; Myslak and Kosmider, 1997; Elias et al., 1989; Popp et al., 1991; Senft et al., 1992; Deng et al., 1988; Decheng et al., 1987; Waksviks and Boysen, 1982; Waksviks et al., 1984; Perminova et al., 2001). These results have shown that the lymphocytes are also the target cells for nickel compounds. Although the majority of these studies have shown an increased incidence of chromosomal aberrations, or of SCEs associated with occupational exposure to nickel, these reports did not provide enough details about exposure history of workers, experimental protocols and blood concentrations of nickel. Furthermore, in most cases the workers in nickel industries are generally exposed to a number of other metals as well (e.g. chromium in electroplating refinery, copper and other metals in a nickel refinery, and iron, manganese and chromium in welding operations) in addition to nickel. Since the exposure in nickel industrial environments is always mixed, it is difficult to clearly estimate the

genotoxic risks associated with individual nickel compounds whether water-soluble or water-insoluble forms (Oller et al., 1997). Besides, the studies of cytogenotoxic potential of various insoluble forms of individual nickel compounds involving SCEs, replication index and mitotic index in human blood lymphocytes are hardly available at present. Most of such studies are even limited to water-soluble form of nickel compounds such as nickel chloride, and nickel sulfate (Sahu et al., 1995; Conway et al., 1986; Christie et al., 1991; Larramendy et al., 1981; Newman et al., 1982; Wolff, 1980). Therefore, in this study the cytogenotoxicity of different Ni-compounds was evaluated in human blood lymphocytes in culture using the SCEs, replication index and mitotic index assay for chromosomal damage.

Although oxidative mechanism(s) may be involved in nickel compound-induced genotoxicity (see reviews by Kasprzak et al., 2003; Costa et al., 2002; Kawanishi et al., 2002; Chakrabarti et al., 2001; Chakrabarti et al., 1999), studies involving the roles of various types of oxidative stress in the development of Ni(II)-induced cytogenotoxicity involving SCEs, replication index and mitotic index in human peripheral blood lymphocytes (as target cells) are at present unknown. Previous studies from our laboratory have demonstrated the role of various types of oxidative stress in affecting nickel compound-induced DNA single-strand breaks in both chromosomal

and nuclear chromatin from human blood lymphocytes *in vitro* using electron microscopy *in situ* end-labeling (M'Bemba-Meka et al., manuscript submitted). Nickel-transformed cell displayed the ability to rapidly proliferate *in vitro* in a low-calcium media suggesting alteration of intracellular calcium metabolism in nickel-transformed cells (Swierenga et al., 1976). *In vivo* administration of Ca^{2+} prevented the formation of lung adenomas caused by nickel or lead (Poirier et al., 1984). Studies from our laboratory have also demonstrated the role of intracellular calcium dehomeostasis in both Ni(II)-compound-induced human lymphocyte death (cytotoxicity) and DNA single-strand breaks *in vitro* (M'Bemba-Meka et al., manuscripts submitted). But the role of intracellular calcium in the development of Ni(II)-induced SCEs, replication index and mitotic index in human blood lymphocytes is unknown at present. The present study was therefore carried out to investigate whether various types of oxidative stress as well as intracellular calcium ion are involved in nickel compound-induced SCEs, replication and mitotic indices in human peripheral blood lymphocytes in culture.

Materials and methods

Materials

Nickel sulfate hexahydrate ($NiSO_4.6H_2O$, green), nickel

subsulfide (Ni_3S_2, 150 mesh, black) nickel oxide (NiO, black) and nickel carbonate hydroxide tetrahydrate ($2NiCO_3.3Ni(OH)_2.4H_2O$, green) were obtained from Sigma–Aldrich Canada Ltd, Ontario, Canada. All other chemicals and reagents were obtained from Sigma, unless otherwise mentioned.

$NiSO_4.6H_2O$ is highly soluble in water, saline or incubation buffer. Ni_3S_2, NiO, and $2NiCO_3.3Ni(OH)_2.4H_2O$ are insoluble in water or physiological saline. Therefore, they were dissolved in 0.05 M Tris-HCl buffer, pH 7.4, by incubation at 37°C for 12h with shaking using the method of Lee et al. (1982). Substantial amounts of all these nickel compounds were dissolved, leading to soluble nickel concentrations of 8-16 mM depending on the nickel compound. The concentration of nickel was determined by electrothermal atomic absorption spectrometry.

The protocols described below were approved by the Ethics Committee on Human Research, Université de Montréal.

Measurement of cell viability in isolated human lymphocytes

Fresh heparinized peripheral blood samples from 4 healthy non-smoking donors between ages 27 and 42 and who were not previously exposed to any radiation or heavy metal compounds or drug therapy

and who did not take any alcohol at least two days before this experiment were used in the present experiments. Questionnaires were obtained from each blood donor to evaluate exposure history. Methodology for the handling of human blood was followed according to the strict guidelines as devised for clinical workers at Hôpital Sainte-Justine, Montréal. The peripheral lymphocytes from human blood were isolated under sterile conditions by the method of Boyum (1980). Aliquots of heparinized blood was diluted 1:1 with Ca^{2t} and Mg^{2t} free PBS solution at room temperature [(KCl (0.2 g/L), KH_2PO_4 (0.2 g/L), NaCl (8 g/L), $Na_2HPO_42H_2O$ (1.4 g/L)]. Diluted blood (30 ml) was carefully layered over 10 ml portions of a Ficoll-Paque (Pharmacia) solution (density 1.077 g/ml) in 50 ml plastic centrifuge tubes. Gradients were then centrifuged at 400 x g for 20 min at room temperature. After centrifugation, the upper layer of clear plasma (18 ml) was discarded. Cells of the interface between plasma and Ficoll-Paque layer (buffy coat) were carefully collected in ice-cold 50 ml plastic centrifuge tubes, sedimented at 500 x g for 10 min at 4°C, washed in PBS, resedimented by centrifugation at 500 x g, and finally resuspended in PBS. A portion of the cell suspension was diluted with 0.13% trypan blue in PBS, and viable cells were counted in a hemocytometer, taking into account of the integrity of the cell membrane. About 95% of the cells isolated were mononuclear and excluded trypan blue. Aliquots of the lymphocyte cells were then

added to a total volume of 3 ml of supplemented RPMI 1640 medium. The supplemented RPMI 1640 media contained 10% fetal calf serum, 0.1% gentamycin, 1% glutamine (from Invitrogen, Burlington, Canada) and 1% phytohaemagglutinin (Murex Diagnostics, Dartford, England). After 46 h of the initiation of culture, the cells were treated in the absence or presence of different concentrations (0-120 μM) of either nickel carbonate hydroxide, or nickel subsulfide, or nickel sulfate, or nickel oxide for 2h at 37°C in the dark in a humidified atmosphere of 95% oxygen and 5% carbon dioxide. Following the end of such exposure, cells were washed twice with RPMI-1640 and incubated in fresh complete RPMI-1640 culture medium for another 24 hr. An equal volume of 0.4% trypan blue reagent was then added to the cell suspension and the viability was estimated by measuring the fraction of cells that excluded trypan blue taking into account of the integrity of the cell membrane. The percentage of the viable cells was then counted in a hemocytometer under a light microscope (Leitz Wetzlar).

Measurement of Cytogenetic Parameters

Human whole blood lymphocyte cultures were prepared by incubating 0.3 ml of fresh heparinized peripheral whole blood in 5 ml of RPMI-1640 medium containing 10% fetal calf serum, 0.1% gentamycin, 1% glutamine (all purchased from Invitrogen, Burlington,

Canada) and 1% phytohaemagglutinin (PHA) (Murex Diagnostics, Dartford, England). For treatments aliquots of a freshly made stock solution of nickel compounds were added after 46 hr of initiation of culture to 5.3 ml cultures to give the appropriate final concentrations 15-60 μM for $2NiCO_3.3Ni(OH)_2$, 15-120 μM for Ni_3S_2, $NiSO_4$ and NiO, whereas an equal volume of the aliquot containing only RPMI-1640 medium was added to another culture to serve as a control. After a 2 hr treatment with each Ni-compound, cells were washed twice with RPMI-1640 and incubated in fresh complete culture RPMI-1640 medium for the last 24 hr. Thus, human whole blood lymphocytes were cultured for 72 hr in 5% CO_2 at 37°C in the dark. Four cultures per individual were studied. Thus, after 48 hr of initial culture, 5-bromo-2-deoxyuridine (BrdU) was added (final concentration: 10 μg/ml) and the lymphocytes were harvested 24 hr later. Two hours before harvesting (after 70 hr), colcemid (0.1 μg/ml) was added.

Chromosome preparation

At the end of 72 hr, the cells were centrifuged at 2000 rpm for 10 min, resuspended in a hypotonic solution (0.056 M KCl), and incubated again at 37°C for 15 min. The cells were then centrifuged for another 6 min and fixed in absolute methanol and glacial acetic acid (3:1 v/v). Spreads were prepared, air-dried, and the fluorescence-photolysis-Giemsa method (Perry and Wolff, 1974), was used for

differential chromatid staining and coded for analysis following the standard protocol.

SCE Analysis

For the SCE assay a total of 50 well-spread diploid metaphases were scored in M_2 cells per experiment, giving a total of 200 cells per experiment for SCE analysis. For each mean SCE determination, only mitoses with 42-46 chromosomes were examined. The data were expressed as mean number of SCE per cell.

Cell-cycle kinetics

In addition to SCE examination, the BrdU differential staining technique was used to evaluate the effect of Ni-compounds on cell replication. The proportion of cells in the first (M_1), second (M_2) and third (M_3) metaphases of 100 consecutive mitosis for each treatment allowed to determine the cell proportion kinetics. The replication index (RI), an indirect measure of studying cell-cycle progression was calculated as follows:

$$RI = (\%1M_1) + 2(\%M_2) + 3(\%M_3)/100$$

(Lazutka, 1991) which indicate the average number of times the cells have divided in the medium from the time of addition of BrdU until

harvesting (Lamberti et al., 1983).

Mitotic index

Mitotic index (MI) (the fraction of cells in a cell population that are undergoing mitosis at a given time) was determined by randomly selecting metaphase cells, scoring 1000 cells from each experiment for a total of 4000 cells from four separate experiments and expressed as the number of mitosis among 1000 nuclei.

Role of oxidative stress

For studies with free radical scavengers, the cells were separately incubated for 15 min with either catalase (1000 U/ml) (H_2O_2 scavenger), or dimethylthioruea (DMTU) (20 mM) (hydroxyl radical scavenger), or superoxide dismutase (SOD) (5000 U/ml) (O_2^- scavenger), or *N*-acetylcysteine (NAC) (8 mM) (general antioxidant), or deferoxamine (100 μM) (iron chelator), before incubating with 30 μM soluble form of nickel carbonate hydroxide (NiCH) for 2 hr at 37°C. The SCE frequency, RI and MI were then measured following the rest of the protocols as mentioned above.

Role of intracellular calcium

To study the effects of intracellular calcium modulator on NiCH-induced SCE frequency, replication index and mitotic index,

verapamil (which inhibits $[Ca^{2+}]_i$ influx by binding to the inside of voltage-operated calcium channel (VOC) protein) (25 μM)), dantrolene (which inhibits $[Ca^{2+}]_i$ release from sarcoplasmic reticulum) (50 μM), and BAPTA (3 mM) (calcium chelator) were added simultaneously with 30 μM NiCH to the cells. The potential effects of verapamil, dantrolene, and BAPTA were then evaluated by the measurement of the SCE frequency, RI and MI, as described above.

Statistical analysis

The standard error of the mean for the data was determined by the use of binominal variances. The data are expressed as the mean ± SEM for four separate experiments. The data were analysed by one-way analysis of variance (ANOVA). The difference between treatments means was also tested by Tukey-Kramer multiple comparison test. The level of significance was set at $p < 0.05$.

Results

Effects of different nickel compounds on sister-chromatid exchanges (SCEs), replication index (RI) and mitotic index (MI) in human blood lymphocytes in culture.

Human peripheral lymphocytes from whole-blood cultures were exposed to either nickel sulfate (0-120 μM), or soluble form of nickel

subsulfide (0-120 μM), or soluble form of nickel oxide (0-120 μM), or soluble form of nickel carbonate hydroxide (0-60 μM) for only 2 h during a total of 72 h culture period. After the end of 72 h of culture, the SCEs, RI and MI were measured for each nickel compound. The results of Ni-compound-induced SCEs are shown in Fig. 2. It is seen that nickel carbonate hydroxide at 30 μM showed a mild but significant increase in SCEs per cell compared to the control value, whereas both nickel subsulfide and nickel oxide showed similar significant mid increase of SCEs only at 120 μM concentration (maximum concentration used in this study). On the other hand nickel sulfate (which is highly soluble) failed to show any such significant increase in SCE frequency under the experimental conditions used in this study (Fig. 2). The significant increase in frequency of SCEs/cell due to nickel carbonate hydroxide was found to be about 1.53 times higher than that of the control, whereas in nickel subsulfide- and nickel oxide-treated lymphocytes, such significant increase was found to be about 1.42 times higher than that of the control. There was no significant impairment of the viability of the lymphocytes (as measured by the trypan blue exclusion) observed with different nickel compounds at the concentrations up to 120 μM used in this study (Fig. 1).

The results of cell-cycle delay as measured by replication index

(RI) are shown in Fig. 3A. The data indicate that there was a significant cell-cycle delay due to exposure of lymphocytes to soluble forms of nickel carbonate hydroxide, nickel subsulfide and nickel oxide. But no such significant cell-cycle delay (RI) was observed with nickel sulfate (Fig. 3A). The minimum concentration level that resulted in significant cell-cycle delay (as measured by RI) was 30 μM due to exposure to nickel carbonate hydroxide, whereas it was found to be 60 μM due to both nickel subsulfide and nickel oxide for such RI (Fig. 3A). The replication index (RI) was been found to be significantly decreased compared to the control value due to nickel carbonate hydroxide at 30 μM, and nickel subsulfide and nickel oxide at concentration up to 120 μM.

The numbers of first (M_1), second (M_2), and third cycle cells (M_3) scored in control and Ni-treated human blood lymphocytes as well as the values of replication indices due to different nickel compounds are presented in Table 1. It is seen that with increasing concentrations of nickel subsulfide and nickel oxide, fewer cells were able to reach the second generation, whereas an increasing number of cells remained at the first generation during the time of the experiment. Treatment with nickel carbonate hydroxide (30 μM) resulted only a very few cells to reach the second generation, and no second generation cells could be seen at concentration higher than 30 μM. The same is true for

nickel subsulfide and nickel oxide at some higher concentrations. However, with nickel sulfate treatment the replication index observed was not statistically significantly different from that of control at any concentration of nickel sulfate used (Table 1, Fig. 3A).

The effects of treatments of human blood lymphocytes in culture with different nickel compounds on mitotic index are presented in Fig. 3B. Exposure of human peripheral lymphocytes from whole-blood cultures to either nickel carbonate hydroxide, or nickel subsulfide, or nickel oxide resulted in a concentration-dependent inhibition of mitotic index compared to the control value. The minimum concentration levels that resulted in significant inhibition of mitotic index (compared to the control value) due to nickel carbonate hydroxide, nickel subsulfide and nickel oxide were 30, 60, and 60 μM respectively (Fig. 3B). On the other hand, nickel sulfate again failed show any such significant inhibition at any concentration used in this study.

Effects of various types of oxidative stress in Ni-compound-induced sister-chromatid exchanges, replication index and mitotic index in human blood lymphocytes

To examine the role of various types of reactive oxygen species/oxidative stress in Ni-compound-induced cytogenotoxicity,

such as sister-chromatid exchanges (SCEs), replication index (RI), and mitotic index (MI), we have evaluated the effects of various scavengers of reactive oxygen species (ROS) on such cytogenetic parameters using human blood lymphocytes in culture. Since previous studies from our laboratory have shown that soluble forms of nickel carbonate hydroxide and nickel subsulfide as well as nickel sulfate shared a similar biochemical mechanism of Ni-compound-induced human lymphocyte death *in vitro* involving oxidative stress (M'Bemba-Meka et al., manuscript submitted), our present study was therefore designed to study the similar mechanism using only nickel carbonate hydroxide as a model. Pretreatment of human peripheral lymphocytes from whole-blood cultures with catalase (H_2O_2 scavenger) (1000 U/ml) for 15 min followed by treatment with 30 μM nickel carbonate hydroxide for 2 h significantly reduced the Ni-compound-induced increase of SCEs to nearly the control value (Fig. 4). Pretreatment of human blood lymphocytes in culture with superoxide dismutase (a scavenger of superoxide anion radical, O_2^-) (5000 U/ml) for 15 min followed by treatment with 30 μM Ni-compound for 2 h significantly reduced the Ni-compound-induced increase of SCEs to its control value (Fig. 4). Similarly, pretreatment of human blood lymphocytes in culture with either 20 mM dimethylthiourea (a hydroxyl radical ($^{\bullet}OH$) scavenger), or 100 μM deferoxamine (a blocker for iron-mediated oxidative stress), or pretreatment with 8 mM *N*-acetylcysteine (a

precursor of glutathione, an antioxidant) for 15 min followed by treatment with 30 μM Ni-compound for 2 h significantly reduced the Ni-compound-induced increase of SCEs to its control value (Fig. 4). Significant decrease in replication index and mitotic index (compared to the control values) caused by 2 h treatment with 30 μM nickel carbonate hydroxide was finally significantly increased to nearly their control values following similar pretreatments with scavengers, or modulators of different types of oxidative stress as mentioned above (Table 2).

Effects of modulators of intracellular calcium ion on nickel carbonate hydroxide-induced SCEs, replication index and mitotic index in human blood lymphocytes

Previous studies from our laboratory have demonstrated that Ni-compound-induced destabilization of calcium homeostasis could play a significant role in Ni-compound-induced human lymphocyte death (cytotoxicity) *in vitro* (M'Bemba-Meka et al., manuscript submitted). We have therefore tested the possibility that modulating intracellular calcium fluxes using both Ca^{2+} channel blocker and intracellular calcium ion (Ca^{2+}) antagonist might prevent the Ni-compound-induced changes in various cytogenetic parameters such as sister-chromatid exchanges (SCEs), replication index and mitotic index using human blood lymphocytes in culture, and the results are

presented in Fig. 5 and Table 3. Simultaneous treatment of human blood lymphocytes in culture with 30 μM nickel carbonate hydroxide (soluble form) and 25 μM verapamil (which is able to inhibit movements of Ca^{2+} through plasma membranes) for 2 h significantly reduced Ni-compound-induced increase in SCEs close to the control value (Fig. 5). Similarly, cotreatment of human blood lymphocytes in culture with 30 μM of the same Ni-compound and 50 μM dantrolene (which is known to inhibit mobilization of intracellular calcium ion, $[Ca^{2+}]_i$ from endoplasmic reticulum) for 2 h significantly reduced Ni-compound-induced increase in SCEs close to the control value (Fig. 5). Cotreatment of human blood lymphocytes in culture with 30 μM Ni-compound and 3 mM of 1,2-bis (2-Aminophenoxy) ethane-*N*,*N*,N´,*N*´-tetraacetic acid tetra-(acetoxymethyl)ester (BAPTA), a specific Ca^{2+} chelator (Salnikow et al., 2000) for 2 h significantly reduced Ni-compound-induced increase in SCE frequency close to the control value (Fig. 5). Significant decrease in replication index and mitotic index caused by 2 h treatment with 30 μM nickel carbonate hydroxide was finally significantly increased to nearly their control values following similar cotreatments with verapamil, dantrolene and BABTA (Table 3) as mentioned above.

Discussion

Our study has demonstrated that *in vitro* exposure of human blood lymphocytes in culture for 2 h to reasonably low concentrations of soluble forms of nickel carbonate hydroxide (NiCH), nickel subsulfide and nickel oxide produced significant increases in SCEs and decreases in both RI and MI. Both low concentrations of nickel compounds and short duration (2h) of exposure were used to avoid any possible Ni-induced cytotoxicity in human blood lymphocytes. Exposure of workers in nickel industries to atmospheric nickel varies from 5-15 mg/m^3 in Falconbridge nickel refinery, Norway (Doll et al., 1990) to 70-699 mg/m^3 in Sherritt Gordon hydrometallurgical nickel refinery, Fort Saskatchewan, Alberta (Egedahl et al., 1991). Based on a scatter diagram showing the linear relationship between atmospheric nickel levels and plasma nickel concentrations for seven individuals working in a Ni_3S_2 matte crushing department (Nieboer et al., 1992), the mean plasma concentrations of nickel are estimated to be 60 μg/L and 400 μg/L for atmospheric nickel levels of 15 mg/m^3 and 100 mg/m^3 respectively. When rat lymphocytes were exposed to 2 mM of Ni_3S_2 (soluble form) for 2 hr at 37°C, the measured nickel uptake by lymphocytes was found to be 4.38 μg/ml (Chakrabarti et al., 2001). Based on this data, the approximate nickel uptake by human lymphocytes is estimated to be 33 and 264 μg per liter

following exposure to 15 and 120 μM Ni_3S_2 respectively for 2 hr. These values are well within the range of plasma nickel concentrations found in some nickel workers mentioned above.

Based on there cytogenetic parameters, the soluble form of NiCH was found to be more potent and active as a cytogenotoxic agent than those of the other nickel compounds studied. The cytogenotoxic potential as measured by SCEs, RI and MI decreases as: NiCH > Ni_3S_2 ≈ NiO > $NiSO_4$. Significantly higher rate of DNA single-strand breaks and elevated SCEs have been observed in lymphocytes of welders exposed to nickel and chromium (Werfel et al., 1998). Soluble forms of insoluble nickel compounds at low μM concentrations and 2 h of exposure are shown to be weakly clastogenic, yielding about 1.55 fold increase in the frequency of SCE from that of control. In Chinese hamster ovary cells $NiCl_2$ and NiS were also found to be weak inducers of SCE frequency. Ni_3S_2 (1 and 10 μg/ml) produced similar marginal increase in SCE frequency but not dose-dependent way (Saxholm et al., 1981). A significant increase in SCE frequency compared to the control has been observed following long duration (36h) of such exposure to 25 μM $NiSO_4$ (Sahu et al., 1995). Similarly, a significant increase in SCE frequency has been observed following treatment of human blood lymphocytes with 119 μM $NiCl_2$ for 64 hr (Newman et al., 1982).

SCEs represent a relevant first level of response to DNA damage, being related to specific locus mutation (Carrano et al., 1978) and to in vitro transformation (Popescu et al., 1981). SCE formation results from reciprocal exchanges between sister chromatids during DNA replication. The induction of SCEs proved to be a very sensitive indicator of DNA damage from a diverse class of chemical agents (Perry and Thomas, 1984). Since increase in SCEs is considered to be a reflection of errors in DNA replication (Kato, 1974; Perry, 1980), and genotoxic damage due to inhibition of DNA replication usually affects cell kinetics (Khalil and Maslat, 1990), therefore both SCEs and RI involving cell-cycle delay could be used as cytogenotoxic marker for Ni-compounds.

Regulation of the cell-cycle is a very important and fundamental process which makes growing cells to follow an orderly progression of DNA replication and mitosis. Therefore, any disturbance in cell-cycle will have some important implication in carcinogenesis, since cancer cells grow relatively independently, by overcoming certain cell-cycle restraint thereby allowing proliferation of neoplastic cells. Overall our results showed that Ni-compounds except $NiSO_4$ strongly inhibited proliferation of the lymphocytes and arrested growth. It may suggest that inhibited growth of cells after Ni-treatment was observed due to an extended G_2 phase. Lymphocytes that enter the G_1 phase of the

cell-cycle proliferate at a lower rate in the presence of nickel than in control (lower percentage of third generation of lymphocytes). Alterations in SCE frequencies induced by various mutagens and carcinogens have been related to cell-cycle effects (Galloway et al., 1987; Loveday et al., 1990). In this study soluble forms of Ni-compounds that produce a delay in the cell-cycle cause a moderate but significant increase in SCE frequency. Nickel compounds have been shown to influence cell growth by selectively blocking the S-phase of the cell-cycle (Costa et al., 1982). The reduction in MI at low micromolar concentrations of soluble forms of Ni-compounds in this study may be attributed to inhibition of DNA synthesis, preventive effect of Ni-compounds on protein binding in the cells and induction of genetic damage (Preston, 1999).

Following the method of Lee et al (1982) significant concentrations (≈10 mM) of Ni(II) were found in solution following incubation of either insoluble Ni_3S_2, or NiCH, or NiO up to 12 hr in 0.05M Tris-HCl buffer, pH 7.4. However, the nature of the chemical forms of insoluble Ni-compounds after solubilizing in Tris-HCl buffer, pH 7.4 was not determined in our study. However, this increase in solubility for Ni_3S_2 was due to the ability of Tris to coordinate Ni^{2+} and form soluble charged complexes (Dotson, 1972). The solubilized nickel exhibited electronic absorption spectra and magnetic moments

characteristic of a octahedral nickel (II). So, it is assumed that insoluble nickel oxide and insoluble NiCH may be changed to a soluble form with Tris involving some type of Ni^{2+}-coordinated complex.

Significant prevention of NiCH-induced changes in SCE frequency, RI and MI by catalase, superoxide dismutase and DMTU suggests the participation of H_2O_2, superoxide anion radical (O_2^-) and hydroxyl radical respectively in such cytogenotoxicity. Significant prevention of NiCH-induced changes in SCE frequency, RI and MI by deferoxamine, an iron chelator suggests an iron-mediated oxidative damage is involved in NiCH-induced cytogenotoxicity. Thus, NiCH-induced cytogenotoxicity in human lymphocytes may be caused by Fenton/Haber-Weiss reaction generating hydroxyl radicals:

$$Fe^{2+} + H_2O_2 \rightarrow OH^{\bullet} + OH^- + Fe^{3+}$$

$$O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2$$

so that the sum of these two reactions, e.g. $H_2O_2 + O_2^- \rightarrow OH^- + OH^{\bullet} + O_2$. Thus, the $OH^{\bullet}$ is the ultimate agent causing NiCH-induced cytogenotoxicity. Similar prevention by excess N-acetylcysteine (a precursor of GSH) suggests that excess GSH may be involved in the

metabolism of free radicals generated.

The mechanism of Ni(II)-compound-induced increased generation of ROS in human lymphocytes is not clear. Ni_3S_2 is believed to activate neutrophils and cause substantial production of H_2O_2 levels (Zhong et al., 1990). The peptides containing the glycyl-glycyl-L-histidyl sequence trigger nickel-dependent production of oxygen radicals through reaction with H_2O_2 (Torreilles and Guérin, 1990). The oxidative effects of nickel depend on its ability to form the Ni(III)/Ni(II) redox couple at pH 7.4. This is favored only when Ni(II) is complexed by some natural ligands, including peptides and proteins (Bal et al., 2000; Margerum and Anliker, 1988). However, Ni^{2+} ions themselves induced the formation of oxidized DNA bases (Kawanishi et al., 1989, 2002; Nackerdien et al., 1991). Similarly, Ni_3S_2-induced formation of reactive oxygen species has been detected by dichlorofluorescein fluorescence in isolated rat lymphocytes *in vitro* (Chakrabarti et al., 2001). Both Ni_3S_2- and $NiCl_2$-induced increased oxidants in intact cultured mammalian cells as detected by dichlorofluorescein fluorescence (Huang et al., 1993), and by $NiCl_2$ in isolated human lymphocytes (Chen et al., 2003). Studies from our laboratory have also shown excess generation of hydrogen peroxide and superoxide anion radical from soluble form of NiCH-treated human lymphocytes *in vitro* (manuscript submitted).

The present study has shown that Ni-compound-induced perturbation of cellular Ca^{2+} homeostasis may also result in Ni-compound-induced changes in SCE frequency, RI and MI. Thus, the protective effect of verapamil against Ni-compound-induced cytogenetic damage is due in part, to inhibition of calcium movement through voltage-operated calcium channel protein involving plasma membranes. The preventive effect of dantrolene against NiCH-induced cytogenetic damage indicates that intracellular Ca^{2+} release from sarcoplasmic reticulum is also involved in such cytogenotoxicity. However, it warrants further study whether there are other calcium-sensitive targets for Ni^{2+}. It is possible that Ni^{2+} from NiCH apparently competes with the essential metals like calcium to use the same pathways, thus disrupting the intracellular balance of Ca^{2+} and resulting in cytogenetic damage. Soluble nickel has been shown to compete with calcium for channels and to be taken up through calcium channels (Refvik and Andreassen, 1995; Funakoshi et al., 1997). Very few studies have related the carcinogenic and/or toxic potential of nickel with disturbances in calcium metabolism (see review by Denkhaus and Salnikow, 2002). Since exogenous addition of BAPTA, a specific chelator of free intracellular calcium, $[Ca^{2+}]_i$ also attenuated Ni^{2+}-induced changes in SCE frequency, RI and MI, it indicates that elevation of intracellular calcium was essential for such cytogenetic damage.

In conclusion, Ni^{2+}-induced disruptions in cellular biochemical processes including various types of oxidative stress, and calcium dehomeostasis are equally critical events in NiCH-induced cytogenetic damage in human blood lymphocytes *in vitro*. The present data may provide some new insights into the mechanisms of Ni-compound-induced cytogenetic damage in human blood lymphocytes as well as contribute some important information for the treatment and prevention of potential adverse health effects among workers in nickel-related industrial environments. Besides, the SCEs, RI and MI are recognized biomarkers which can be used for early biomonitoring of workers health using their blood lymphocytes as well as those of population living near such environments.

ACKNOWLEDGMENTS

The authors sincerely thank Mrs. Fléchère Fortin for expert technical assistance and the Hôpital Sainte-Justine, Montréal for obtaining blood samples from donors.

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FIGURE CAPTIONS

FIG. 1. Effect of *in vitro* exposure of human blood lymphocytes to different nickel compounds for 2 h on cell survival or cellular viability,

as estimated by trypan blue exclusion test. The results are averages ± SEM of four separate experiments.

FIG. 2. Effects of different concentrations of various nickel compounds (2 h exposure) on the induction of sister-chromatid exchanges (SCEs) in cultured human blood lymphocytes. The results are averages ± SEM of four separate experiments. *Significantly different from control, $p < 0.05$.

FIG. 3. (A) Effects of different concentrations of various nickel compounds (2 h exposure) on replication index (cell-cycle) in cultured human blood lymphocytes. The results are averages ± SEM of four separate experiments. *Significantly different from control, $p < 0.05$. (B) Effects of different concentrations of various nickel compounds (2 h exposure) on mitotic index in cultured human blood lymphocytes. The results are averages ± SEM of four separate experiments. *Significantly different from control, $p < 0.05$.

FIG. 4. Effects of different modulators of oxidative stress on nickel carbonate hydroxide-induced SCEs in cultured human blood lymphocytes. For details, see Materials and Methods. The results are averages ± SEM of four separate experiments. *Significantly different from control, $p < 0.05$.

FIG. 5. Effects of different modulators of calcium homeostasis on nickel carbonate hydroxide-induced SCEs in cultured human blood lymphocytes. For details, see Materials and Methods. The results are averages ± SEM of four separate experiments. *Significantly different from control, $p < 0.05$.

Table I

Analysis of cell growth kinetics after nickel compounds exposure *in vitro*.

Treatment	Percent cells in			Replication index
	M_1	M_2	M_3	
Control	26	56	18	1.92
$2NiCO_3.3Ni(OH)_2$ (30 μM)	91	09	-	1.09*
$NiSO_4$ (60 μM)	25	60	15	1.90
$NiSO_4$ (120 μM)	25	60	15	1.90
Ni_3S_2 (60 μM)	69	30	1	1.32*
Ni_3S_2 (120 μM)	81	19	-	1.19*
NiO (60 μM)	69	31	-	1.31*
NiO (120 μM)	89	11	-	1.11*

* Significantly different from control, $p < 0.05$.

Table II

Effects of ROS scavengers and antioxidants on $2NiCO_3.3Ni(OH)_2$-induced mitotic frequency and human blood lymphocyte replication.

Treatment	Mitotic index (Mean ± SEM)	Replication index
Control	7.10 0.67	1.92
$2NiCO_3.3Ni(OH)_2$ (30 μM)	3.60 ± 0.22	1.00
$2NiCO_3.3Ni(OH)_2$ (30 μM) + DMTU (20 mM)	6.40 ± 0.42*	1.80*
$2NiCO_3.3Ni(OH)_2$ (30 μM) + Catalase (1 000 U/ml)	6.30 ± 0.82*	1.70*
$2NiCO_3.3Ni(OH)_2$ (30 μM) + SOD (5 000 U/ml)	6.60 ± 0.58*	1.80*
$2NiCO_3.3Ni(OH)_2$ (30 μM) + NAC (8 mM)	6.20 ± 0.72*	1.80*
$2NiCO_3.3Ni(OH)_2$ (30 μM) + DFO (100 μM)	7.60 ± 0.85*	1.90*

* Significantly different from $2NiCO_3.3Ni(OH)_2$-alone-treated group, $p < 0.05$.

Table III

Effects of modulators of intracellular calcium fluxes on $2NiCO_3.3Ni(OH)_2$-induced mitotic frequency and human blood lymphocyte replication.

Treatment	Mitotic index (Mean ± SEM)	Replication index
Control	7.10 0.67	1.92
$2NiCO_3.3Ni(OH)_2$ (30 μM)	3.60 ± 0.22	1.00
$2NiCO_3.3Ni(OH)_2$ (30 μM) + Verapamil (25 μM)	6.70 ± 0.88*	1.80*
$2NiCO_3.3Ni(OH)_2$ (30 μM) + Dantrolene (50 μM)	6.55 ± 0.41*	1.70*
$2NiCO_3.3Ni(OH)_2$ (30 μM) + BAPTA (3 mM)	6.20 ± 0.57*	1.70*

* Significantly different from $2NiCO_3.3Ni(OH)_2$-alone-treated group, $p < 0.05$.

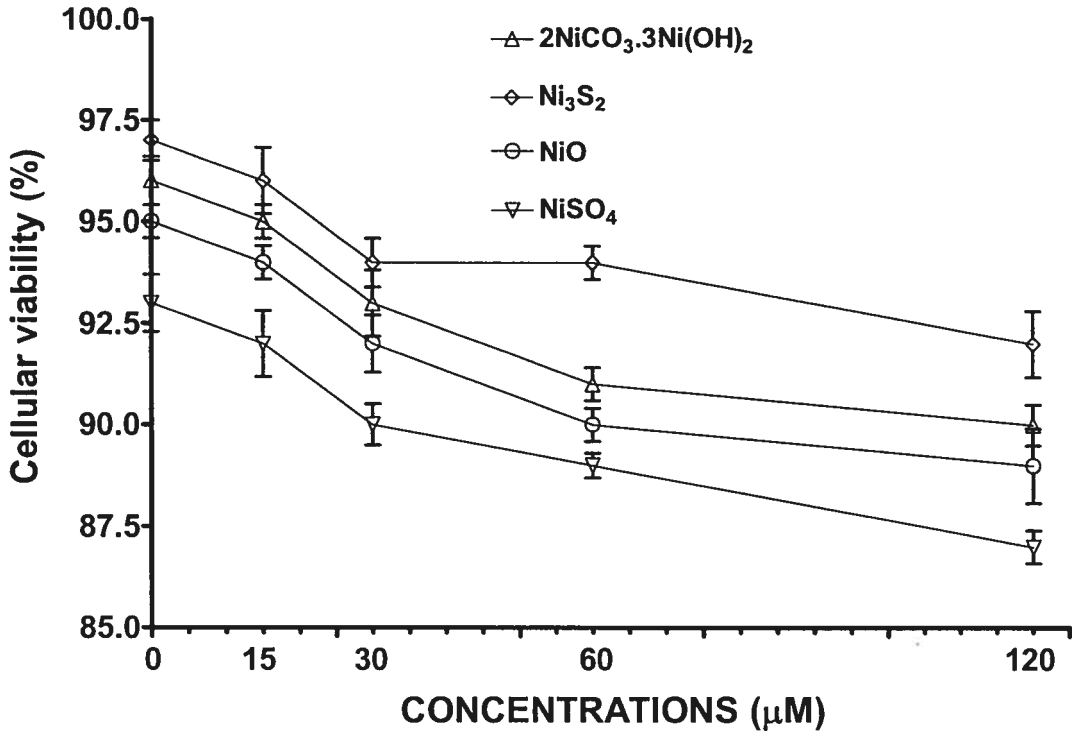
100.0
97.5
95.0
92.5
90.0
87.5
85.0
Cellular viability (%)
0
15
30
60
120
CONCENTRATIONS (μM)
2NiCO3.3Ni(OH)2
Ni3S2
NiO
NiSO4

FIG. 1

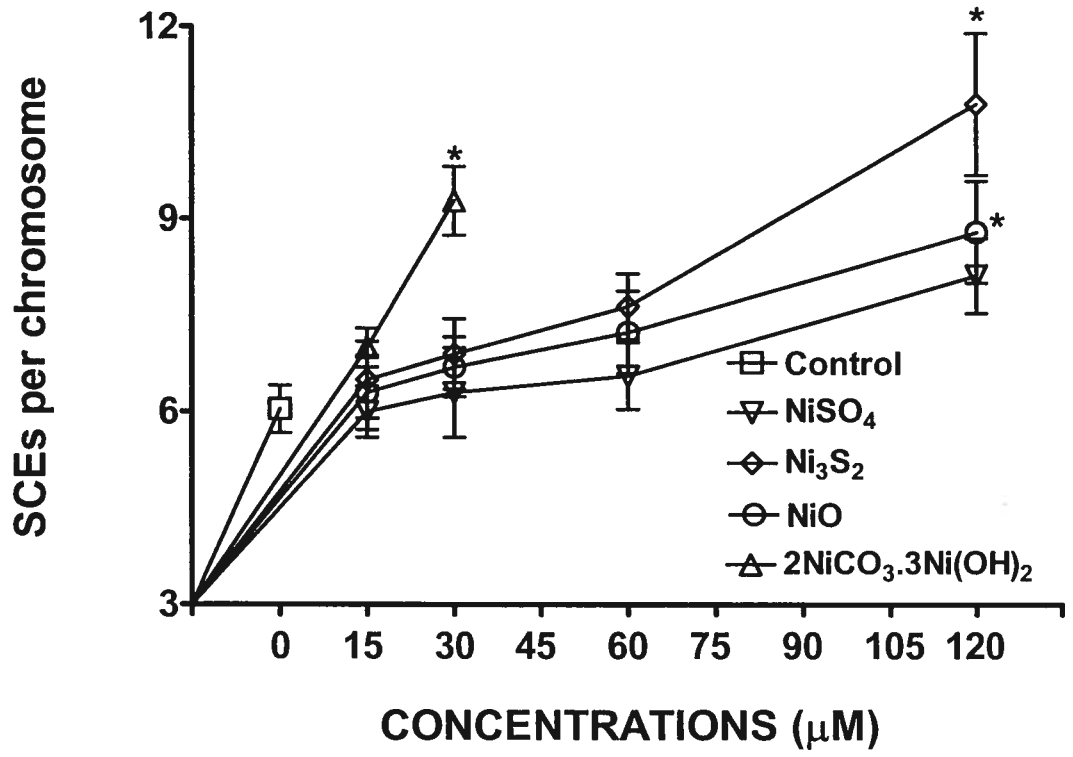
12
9
6
3
SCEs per chromosome
0
15
30
45
60
75
90
105
120
CONCENTRATIONS (μM)
Control
NiSO4
Ni3S2
NiO
2NiCO3.3Ni(OH)2
*
*
*

FIG. 2

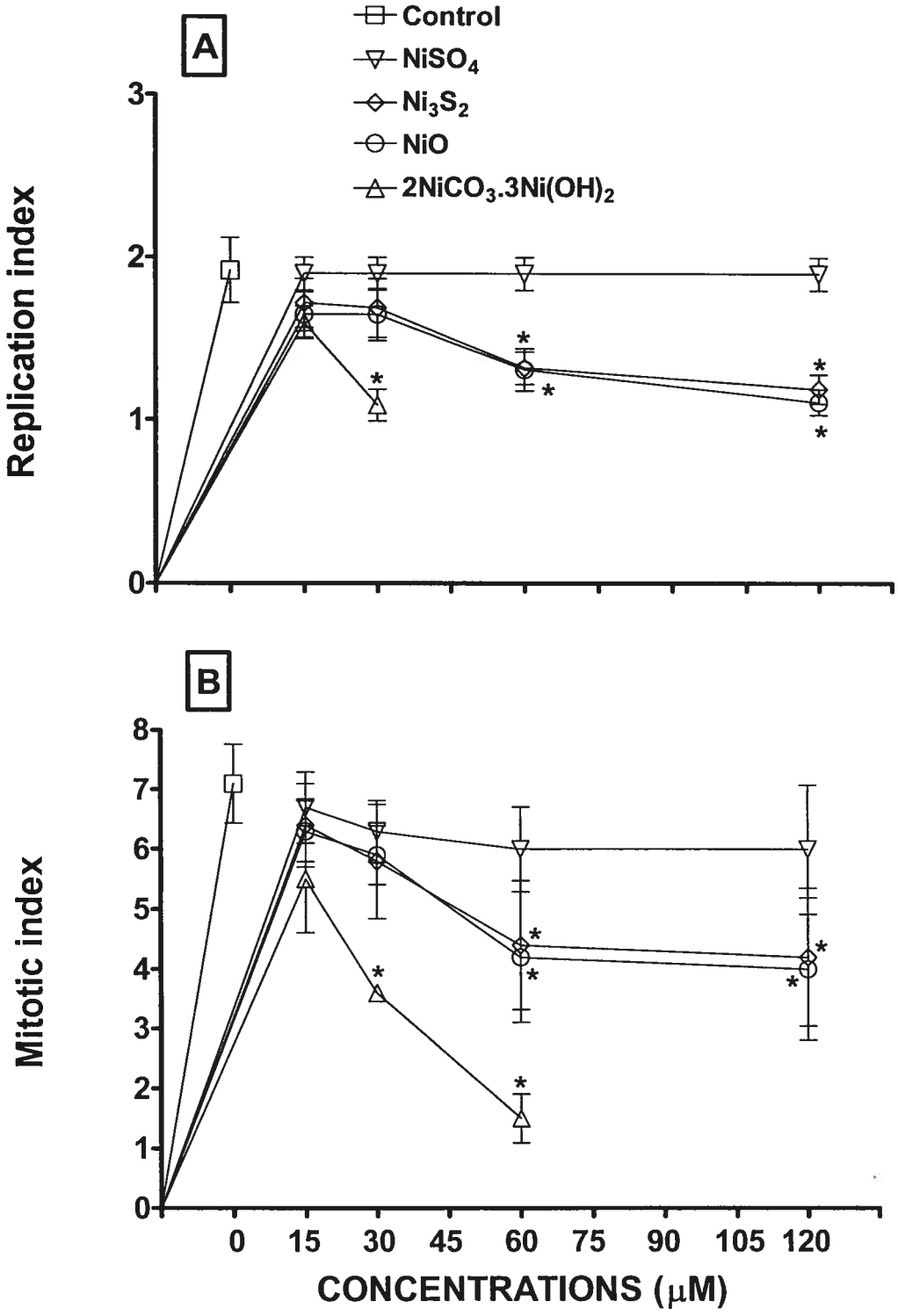
A
Control
NiSO4
Ni3S2
NiO
2NiCO3.3Ni(OH)2
Replication index
3
2
1
0
B
Mitotic index
8
7
6
5
4
3
2
1
0
0 15 30 45 60 75 90 105 120
CONCENTRATIONS (μM)

FIG. 3

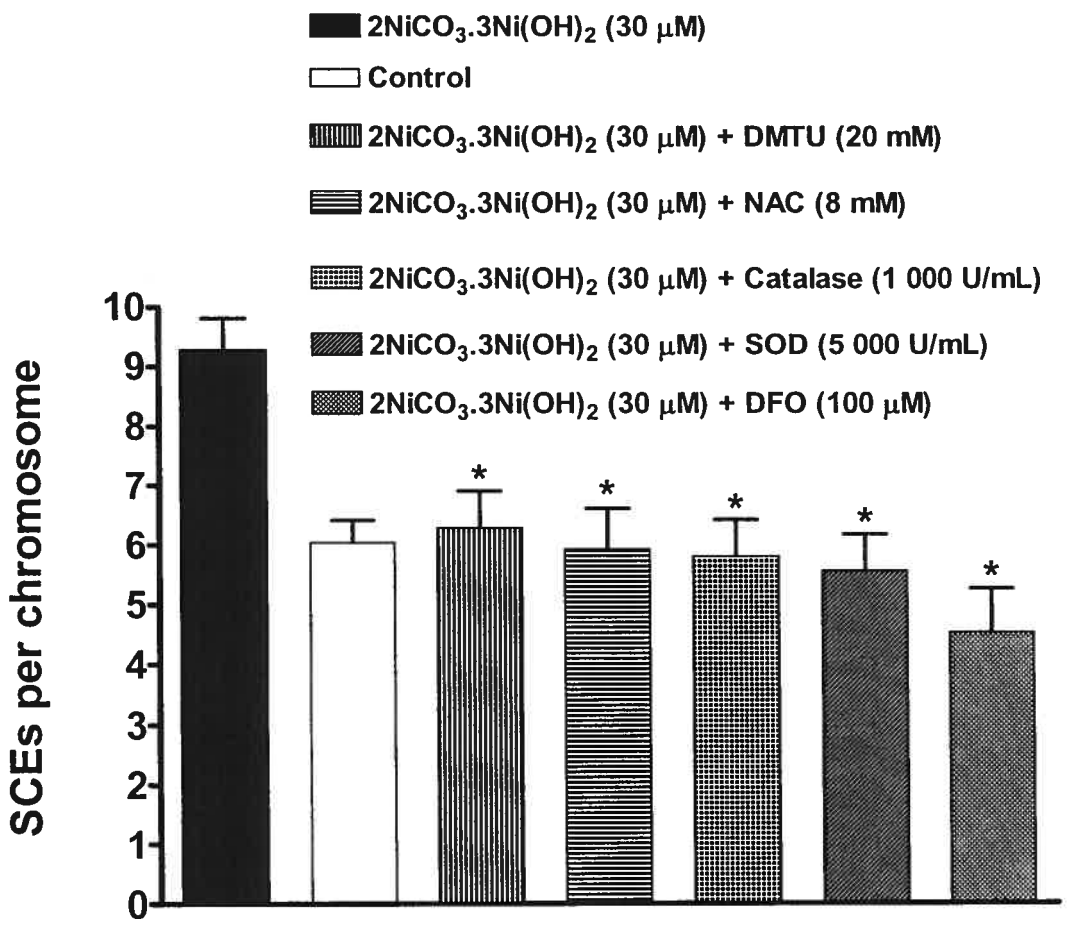
2NiCO3.3Ni(OH)2 (30 μM)
Control
2NiCO3.3Ni(OH)2 (30 μM) + DMTU (20 mM)
2NiCO3.3Ni(OH)2 (30 μM) + NAC (8 mM)
2NiCO3.3Ni(OH)2 (30 μM) + Catalase (1 000 U/mL)
2NiCO3.3Ni(OH)2 (30 μM) + SOD (5 000 U/mL)
2NiCO3.3Ni(OH)2 (30 μM) + DFO (100 μM)
SCEs per chromosome
0
1
2
3
4
5
6
7
8
9
10
*
*
*
*
*

FIG. 4

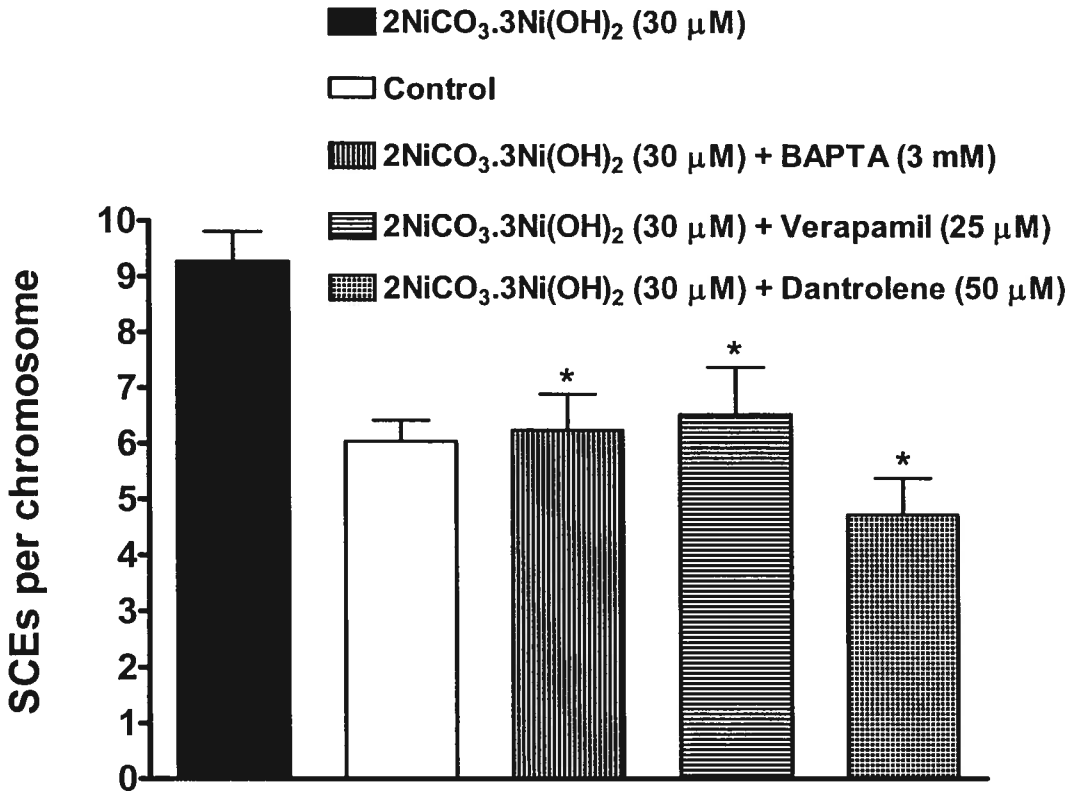
2NiCO3.3Ni(OH)2 (30 μM)
Control
2NiCO3.3Ni(OH)2 (30 μM) + BAPTA (3 mM)
2NiCO3.3Ni(OH)2 (30 μM) + Verapamil (25 μM)
2NiCO3.3Ni(OH)2 (30 μM) + Dantrolene (50 μM)
SCEs per chromosome
0
1
2
3
4
5
6
7
8
9
10
*
*
*

FIG. 5

IV. DISCUSSION ET CONCLUSION GÉNÉRALE

IV.1. Discussion générale

Les différentes formes chimiques de nickel auxquels les lymphocytes humains du sang périphérique sont exposés *in vitro* causent de façon différentielle, en plus de la mortalité des lymphocytes, des dommages génotoxiques.

IV.1.1. Potentiel cytotoxique des composés de nickel sur les lymphocytes sanguins humains isolés

Notre étude montre que l'ampleur de l'induction de la mortalité des lymphocytes dépend fortement de la nature chimique des composés de nickel. Ainsi, la forme soluble de l'hydroxyde carbonate de nickel est potentiellement le plus cytotoxique comparativement aux autres composés de nickel étudiés. À des concentrations identiques, les résultats de cette étude montrent que le taux de mortalité des lymphocytes exposés aux différents composés de nickel diminue dans l'ordre suivant: l'hydroxyde carbonate de nickel > le sous-sulfure de nickel > le sulfate de nickel > l'oxyde de nickel. Cet ordre du potentiel toxique est en accord avec les conclusions des autres études (Dunnick et coll., 1988; Benson et coll., 1988, 1992; Haratake et coll., 1992; M'Bemba-Meka et Chakrabarti, 2001). Dunnick et coll., (1988), Benson et coll., (1988) et Haratake et coll., (1992) ont étudié les effets

toxiques respiratoires des composés de nickel chez des rats exposés par inhalation au sulfate, à l'oxyde et au sous-sulfure de nickel sous forme d'aérosol, pendant 12 jours, 6 h/j, 5 jours/semaine. L'ordre du potentiel toxique a été établi sur la base de la plus faible concentration induisant un effet toxique pour chacun des composés de nickel. Ainsi, Dunnick et coll., (1988) et Benson et coll., (1988) suggèrent une diminution du potentiel toxique dans l'ordre suivant: le sous-sulfure de nickel (0.4 mg/m^3) > le sulfate de nickel (0.8 mg/m^3) > l'oxyde de nickel (0.9 mg/m^3). D'autre part, Haratake et coll., (1992) ont montré que le sous-sulfure de nickel cause plus de lésions inflammatoires dans les poumons des rats que l'oxyde de nickel. Pour leur part, Benson et coll., (1992) ont réalisé une étude comparative de cytotoxicité pulmonaire en exposant *in vitro* les macrophages alvéolaires et les cellules épithéliales pulmonaires des rats aux composés de nickel. Les auteurs ont établi le potentiel cytotoxique des composés de nickel dans l'ordre suivant: le sous-sulfure de nickel > le sulfate de nickel > l'oxyde de nickel. De plus, dans une étude de néphrotoxicité effectuée antérieurement dans notre laboratoire (M'Bemba-Meka et Chakrabarti, 2001), des tranches du cortex rénal de rats ont été exposées aux composés de nickel. Le potentiel néphrotoxique de ces composés a été établi dans l'ordre suivant: l'hydroxyde carbonate de nickel > le sous-sulfure de nickel > le sulfate de nickel > l'oxyde de nickel (M'Bemba-Meka et Chakrabarti, 2001).

Malheureusement dans la litterature il y a très peu d'études sur l'évaluation du potentiel cytotoxique des différents composés de nickel. Celle existantes sont réalisées sur des cellules animales mais aucune d'entre-elles portent sur des lymphocytes sanguins humains.

IV.1.2. Potentiel génotoxique des composés de nickel sur les lymphocytes sanguins humains en culture

Notre étude montre que le potentiel génotoxique des composés de nickel sur la chromatine chromosomique et nucléaire tel que mesuré par la quantification des bris simple-brin à l'ADN diminue dans l'ordre suivant: l'hydroxyde carbonate de nickel > l'oxyde de nickel ≥ le sous-sulfure de nickel > le sulfate de nickel. Par contre l'induction de la fréquence des échanges entre chromatides-sœurs diminue dans l'ordre suivant: l'hydroxyde carbonate de nickel > le sous-sulfure de nickel > l'oxyde de nickel > le sulfate de nickel. Dans une étude *in vivo*, Werfel et coll., (1998) ont observé un taux significativement élevé des bris simple-brin à l'ADN et des valeurs nettement plus élevées pour les échanges entre chromatides-sœurs dans les lymphocytes sanguins des soudeurs exposés au nickel et au chrome. Pour leur part, Saplakoglu et coll., (1997) ont mis en évidence une augmentation des bris simple-brin à l'ADN dans les poumons et les reins de rats après l'administration du chlorure de

nickel par la voie parentale. De plus, les bris simple-brin et double-brin d'ADN ont aussi été observés *in vitro* dans les lymphocytes sanguins humains exposés au chlorure de nickel à des concentrations trois fois plus élevées que celles utilisées dans notre étude (Cai and Zhuang, 1999).

Nos résultats montrent que les lymphocytes sanguins humains en culture de 72 h, traités pendant 2 h (24 h avant la fin de la culture) à une très faible concentration (15 μM) (comparativement à 25 μM utilisés dans les études existantes) avec les quatre différents composés de nickel présentent des taux de bris simple-brin à l'ADN significativement élevés comparés au contrôle. De plus, la densité du marquage des bris simple-brin à l'ADN dans la chromatine des chromosomes est significativement plus élevée que la densité observée dans la chromatine des noyaux. Il est possible que le degré de liaison entre le nickel et la chromatine chromosomique des lymphocytes humains soit plus élevé que celui entre le nickel et la chromatine nucléaire. Une telle différence peut être un facteur déterminant de la captation de l'ion nickel par la chromatine. Le premier niveau d'organisation de la chromatine dans le noyau interphasique et dans les chromosomes métaphasiques, est la fibre nucléosomique, stabilisée par l'histone H1. Dans les chromosomes métaphasiques les boucles d'ADN sont attachées par leurs bases à deux protéines Sc1 et

Sc2 (respectivement 170 et 135 KDa) (Lewis et Laemmli, 1982). La protéine Sc1 est la protéine non-histone la plus abondante des chromosomes métaphasiques. Elle se lie à l'ADN et trois molécules sont détectées en moyenne par boucle d'ADN dans les chromosomes métaphasiques (Gasser et Laemmli, 1987). Earnshaw et Heck (1885) ont montré que la protéine Sc1 est identique à la topoisomérase II. La cohésion entre les boucles d'ADN adjacentes semble être assurée par des interactions protéine-protéine ou protéine-ADN pour former un réseau interne ou «échafaudage» le long de l'axe du chromosome (Gasser et Laemmli, 1987). Dans le noyau interphasique, la structure équivalente de l'échafaudage chromosomique est plus complexe en morphologie et composition. Elle est composée de la lamina périphérique, d'un réseau interne qui pourrait éventuellement intégrer de l'ARN (Benyajati et Worcel, 1976) en plus de protéines non-histones, et d'un nucléole résiduel (Gasser et Laemmli, 1987). Cette structure est appelée matrice nucléaire (Berezney et Coffey, 1977), lamina (Aaronson et Blobel, 1975), membrane nucléaire (Comings, 1980) ou cage (Cook et Brazell, 1980). Lebkowski et Laemmli (1982) ont suggéré l'existence de deux types (I et II) d'échafaudages nucléaires correspondant à deux niveaux de condensation de la fibre de chromatine dans le noyau. Dans une autre étude, Lewis et Lebkowski (1984) ont établi la composition protéique des deux types d'échafaudages: les échafaudages de type I

contiennent en plus des lamines, la protéine Sc1 (topoisomerase II), composant majoritaire des échafaudages métaphasiques, et un nombre indéterminé d'autres protéines. Les lamines A, B et C sont les principaux constituants des structures de type II. On note des différences d'ordre morphologique entre les types I et II. Les échafaudages de type I présentent, outre la lamina périphérique, une structure filamemteuse à l'intérieur du noyau résiduel, alors que les échafaudages de type II montrent simplement une couche laminaire périphérique. Donc, dans l'hétérochromatine, l'histone H1 peut être l'une des protéines qui se lie à l'ion Ni^{2+} et peut catalyser la formation d'espèces radicalaires de l'oxygène (Huang et coll., 1995). L'effet majeur de nickel sur le genome passe par l'interméaiaire de son interaction avec les protéines de la chromatine parce que l'ion nickel a une grande affinité avec les protéines qu'avec l'ADN (Zoroddu et coll., 2002; Bal et coll., 2000). La structure différentielle entre les chromosomes métaphasiques et les noyaux interphasiques pourrait expliquer la différence de densité du marquage des bris à l'ADN dans les chromosomes et les noyaux. Huang et collaborateurs (1995) ont aussi démontré que les protéines de l'hétérochromatine augmentent considérablement la formation de 8-oxo-dg (7,8-dihydro-8-oxo-2´-deoxyguanosine) induit par le chlorure de nickel en présence du peroxyde d'hydrogène. Cependant, on ne peut negliger de mentionner qu'il existe une deuxième hypothèse pour expliquer cette différence

dans la densité du marquage des bris simple brin en interphase et en métaphase. Le dégré de compaction de la chromatine métaphasique est plus élevé que celui de la chromatine interphasique; il pourait alors être normal de retrouver une plus grande densité de bris par μm^2 dans les chromosomes. Par ailleurs, nos résultats collaborent bien avec ceux de l'étude de Borochov et collaborateurs (1984) qui ont montré que l'ion Ni^{2+} augmentent la condensation de la chromatine.

Plusieurs études ont démontré que les composés de nickel ne sont pas mutagènes puisqu'ils ciblent préférentiellement la chromatine inactive (Sen et Costa, 1986b; Conway et coll., 1987; Costa et coll., 2002). L'effet sélectif du Ni sur l'hétérochromatine mène à une autre découverte intéressante qui a un impact sur les mécanismes moléculaires de la carcinogenèse du Ni. Cet effet implique la capacité du Ni à rendre sélectivement silencieux des gènes qui sont autour de la région de l'hétérochromatine (Costa et coll., 2002). L'ion Ni^{2+} condense les gènes qui sont autour de l'hétérochromatine et change leur structure et celle de la chromatine, ceci entraîne l'hyperméthylation de ces gènes (donc l'inactivation des gènes) (Costa et coll., 2002). S'il s'agit des gènes suppresseurs de tumeurs, en ce moment là il pourrait y avoir un développement de tumeurs. Malheureusement, les études d'évaluation du potentiel cytogénotoxique des différentes formes chimiques de nickel sur les

lymphocytes sanguins humains sont très limitées présentement.

IV.1.3. Mécanismes d'action toxique des composés de nickel

IV.1.3.1. Rôle du stress oxydatif dans l'induction de la mortalité des lymphocytes

Notre étude a mis en évidence différents dommages oxydatifs induits par les espèces radicalaires de l'oxygène produites par les différents composés de nickel. Les résultats de notre étude suggèrent que ces dommages peuvent jouer un rôle important dans les mécanismes biochimiques impliqués dans la mortalité des cellules. La surproduction de divers types d'espèces radicalaires de l'oxygène (H_2O_2, O_2^-, $^{\cdot}OH$), la déplétion au niveau cellulaire des groupements sulfhydryles liés (SH-P) et non liés (SH-NP) aux protéines, et la peroxydation des lipides (POL) causées par les composés de nickel seraient des initiateurs de la mortalité des lymphocytes. Cette hypothèse est appuyée par nos résultats démontrant que le pourcentage de cellules mortes suite à l'exposition aux composés de nickel est significativement réduit par le traitement avec le glutathion ou la *N*-acétylcystéïne (un précurseur de GSH). Le glutathion est prescrit pour soigner l'insuffisance hépatique, l'inflammation des

voies respiratoires et l'asthénie. Il agit en se liant au groupement thiol pour constituer la deuxième ligne de défense contre les radicaux libres. La *N*-acétylcystéïne est indiquée dans le traitement symptomatique des syndromes d'hypersécrétion bronchiques. Elle a aussi d'autres propriétés pharmacologiques telles l'activité antioxydant liée à sa capacité de restaurer les stocks intracellulaires de cystéïne et de glutathion (Aruoma et coll., 1989), l'activité antidote comme traitement contre l'empoisonnement à l'acétaminophène (analgésique) et aux métaux lourds (Smilkstein et coll., 1988; Martin et coll., 1990). La *N*-acétylcystéïne agit par réduction en entraînant l'ouverture des ponts disulfures des macromolécules (mucoprotéines) responsables de la viscosité du mucus bronchique. Ceci suggère que le niveau intracellulaire en GSH est un facteur déterminant dans la susceptibilité des lymphocytes humains aux effets cytotoxiques de sulfate, de sous-sulfure et d'hydroxyde carbonate de nickel. La surproduction de H_2O_2 induite par les composés de nickel est considérablement réduite non seulement par la catalase (un piégeur de peroxyde d'hydrogène) et la deferoxamine (DFO) (un chélateur du fer), mais aussi par le GSH (antioxydant). La catalase est une enzyme constituant la deuxième ligne de défense de l'organisme contre les radicaux libres. Elle agit spécifiquement contre le peroxyde d'hydrogène en le transformant en eau. La deferoxamine est un médicament utilisé en prévention de l'hémochromatose secondaire

aux transfusions chez les sujets atteints de thalassémie majeure (Olivieri et coll., 1994). Elle est donc employée pour éliminer l'excès de fer du corps. Elle est également indiquée comme antidote dans le traitement d'empoisonnement aigu au fer particulièrement chez les jeunes enfants. La deferoxamine capte les ions ferriques de la sidérophiline sans capter ceux de l'hémoglobine pour former des chélates solubles. Elle a une importante spécificité pour le fer car la constante de stabilité vis-à-vis des autres métaux est beaucoup plus faible. Cela suggère que la déplétion de la réserve cellulaire en thiol résulte aussi de la surproduction de H_2O_2 et du radical hydroxyle. Cependant, puisque la catalase et la DFO ont aussi empêché la déplétion de SH-NP induite par les composés de nickel, ceci confirme que la surproduction d'ERO tels H_2O_2 et $^{\bullet}OH$ est aussi responsable de cette déplétion. Nos résultats suggèrent aussi que le peroxyde d'hydrogène participe à cette mortalité puisque la mortalité des lymphocytes causée par les composés de nickel est inhibée par la catalase. Aussi, l'inhibition de cette mortalité par le mannitol et le diméthylthiourea (DMTU) (des piégeurs de radical hydroxyle) indique qu'elle est induite par les composés de nickel via la surproduction d'espèces radicalaires hydroxyles. Nos résultats démontrent également que l'action préventive de la superoxyde dismutase (SOD) (un piégeur de l'anion superoxyde) sur la mortalité des lymphocytes causée par l'hydroxyde carbonate et le sous-sulfure de nickel est une

indication de la participation de l'anion superoxyde dans ce processus mortel. La SOD est une enzyme qui agit comme seconde ligne de défense de l'organisme spécifiquement contre l'anion superoxyde en le transformant en eau et oxygène. Comparable à nos résultats, la POL induite dans le rein et le foie des rats suite à une injection intrapéritonéale d'acétate de nickel, un composé soluble de nickel (107 μM), a été considérablement réduite par la SOD (Misra et coll., 1990). Chen et collaborateurs (2003) ont démontré que la surproduction du radical hydroxyle induite par le chlorure de nickel (un composé soluble de nickel) dans les lymphocytes sanguin humains est inhibée par la catalase, le glutathion et le mannitol, mais pas par la SOD. Nos résultats montrent que le sulfate de nickel, également un composé soluble de nickel, ne semble pas induire l'anion superoxyde dans les lymphocytes humains. D'autre part, une diminution importante de la mortalité des lymphocytes exposés à l'hydroxyde carbonate, au sous-sulfure et au sulfate de nickel a été aussi observée dans notre étude suite au pré-traitement des cellules avec la DFO. Le fer agit comme catalyseur de la réaction Fenton pour produire le radical hydroxyle à partir du peroxyde d'hydrogène (Ryan et Aust, 1992). Comme la deferoxamine est un chélateur du fer, il a le potentiel d'inhiber le processus d'induction d'ERO par les ions nickel. Ceci a pour résultat de prévenir la mortalité des lymphocytes. Ainsi, chacun des composés de nickel peut actionner son potentiel

cytotoxique (la mortalité cellulaire) à travers la voie des dommages oxydatifs via la médiation du fer. Ces résultats fournissent des évidences montrant que la chélation du fer par la DFO est un apport thérapeutique protégeant les lymphocytes contre l'induction des dommages oxydatifs par les composés de nickel.

Plusieurs études ont démontré l'implication du stress oxydatif dans le mécanisme d'action toxique des composés de nickel. Chakrabarti et coll., (1999) ont démontré que le sous-sulfure de nickel induit la formation d'ERO dans les cellules corticales rénales isolées chez des rats. Huang et coll., (1993) et Chen et coll., (2003) ont aussi mis en évidence la surproduction d'oxydants par le sous-sulfure et le chlorure de nickel dans les cellules de mammifères en culture. Contrairement aux études citées ci-haut, notre étude a montré que les composés de nickel induisent soit directement la surproduction de divers types d'ERO tels le radical hydroxyle, l'anion superoxyde, le peroxyde d'hydrogène; ou soit indirectement à travers quelques modulateurs biologiques (catalase, SOD, mannitol, etc.) piégeant les ERO dans les lymphocytes humains. Nous avons aussi démontré que le sulfate de nickel, composé soluble de nickel n'induit pas de façon significative, comparativement au contrôle, la surproduction de l'anion superoxyde dans les lymphocytes humains.

IV.1.3.2. Rôle des mitochondries dans l'induction de la mortalité des lymphocytes

La perte de l'imperméabilité des pores mitochondriaux (PIPM) peut être définie comme étant un potentiel-dépendant de la conductivité du canal de la membrane interne mitochondriale. Cette dépendance du potentiel paraît être une propriété intrinsèque des pores. L'induction de cette perte d'imperméabilité est caractérisée par le gonflement des mitochondries, la fuite des petits ions tels le K^+, le Mg^+ et le Ca^{2+}, et la perte du potentiel de la membrane mitochondriale. Dans un état complètement ouvert, le diamètre du pore apparent est d'environ 3 nm, cette mesure est basée sur la libération du soluté à travers les pores (Haworth et. Hunter, 1979) et sur les mesures successives de la conductivité (Szabó et. Zoratti, 1991).

La cyclosporine A est un inhibiteur spécifique de la perte de l'imperméabilité des pores mitochondriaux (PIPM), et est aussi un modulateur spécifique de l'intégrité de la membrane mitochondriale (Lemasters et coll., 1998a, 1998b; Quian et coll., 1997). Nous avons traité simultanément les lymphocytes avec la cyclosporine A à chacun des trois composés de nickel. La cyclosporine A a réduit remarquablement la mortalité des lymphocytes. Ceci montre que les

trois composés de nickel ont le potentiel d'induire le dysfonctionnement mitochondrial dans les lymphocytes humains en suspension et que la PIPM joue également un rôle important dans l'induction du processus de la transmission des signaux entraînant leur mortalité. L'étude sur des mitochondries isolées montre que certains signaux physiologiques et pathologiques sont des facteurs déclenchants de l'ouverture des pores mitochondriaux (Bernardi et Petronilli, 1996). L'ouverture complète crée un canal de conductivité pour des molécules inférieures ou égales à 1.5 kDa ce qui a pour résultat la dissipation du gradient H^+ à travers la membrane et le découplage de la chaîne respiratoire (Green et Amarante-Mendes, 1998). Ainsi, l'ouverture des pores mitochondriaux déclenchée par les composés de nickel favorise fortement le passage des particules à travers la membrane interne mitochondriale. C'est le début du passage d'un état imperméable à un état perméable des pores mitochondriaux; ceci pourrait mener à la dépolarisation de la membrane et à la libération d'ions et d'autres effets physiologiques. Duchen (2000) et Qian et coll., (1999) ont démontré que ce changement de l'intégrité de la membrane mitochondriale est lié à la déplétion de l'ATP cellulaire. Il a été postulé que la PIPM entraîne le découplage des mitochondries et la consommation de l'ATP par les enzymes ATPases mitochondriaux. Ainsi, les études d'Arsalane et coll., (1992, 1994) sur la cytotoxicité de l'hydroxyde carbonate de

nickel dans les macrophages alvéolaires du cobaye en culture ont montré la production des radicaux libres et la déplétion de la réserve de l'énergie cellulaire particulièrement de l'ATP. Cependant, le mécanisme de blocage des pores mitochondriaux par la cyclosporine A n'a pas encore été démontré. La cyclosporine A pourrait agir en se liant à la protéine de la matrice mitochondriale, protéine de la famille de la cyclophiline (appelée aussi PPIase) associée à la PIPM plutôt qu'en interagissant directement avec les pores mitochondriaux eux-mêmes (Nicolli et coll., 1996; Conneru et coll., 1992). Nous avons montré que le pré-traitement des lymphocytes humains avec le catalase ou avec le glutathion inhibe significativement la PIPM induite par les composés de nickel. Ceci suggère que les espèces radicalaires de l'oxygène ou le stress oxydatif surproduits par les composés de nickel sont responsables de la PIPM. Notre étude a donc établi un lien temporel entre l'induction d'ERO, la déplétion de la réserve des thiols et les événements biochimiques survenus par la suite, telle la PIPM. Notre étude a aussi mis en évidence directement et indirectement les mécanismes par lesquels les différents composés de nickel induisent une activation de la transmission des signaux amenant à la mortalité des lymphocytes. Ces mécanismes impliquent, non seulement la surproduction de divers types d'ERO et du stress oxydatif et le déclenchement de la PIPM, mais aussi la déstabilisation de l'homéostasie intracellulaire du calcium. En outre, notre étude a

démontré également que la déstabilisation de l'homéostasie intracellulaire du calcium induite par les composés de nickel est causée non seulement par les ERO/stress oxydatif mais aussi par la PIPM. Par conséquent, les ERO/stress oxydatif et les mitochondries sont impliqués de façon causale dans l'induction de la déstabilisation de l'homéostasie intracellulaire du calcium.

IV.1.3.3. Rôle du calcium intracellulaire dans l'induction de la mortalité des lymphocytes

Notre étude a identifié plusieurs sites du calcium comme cibles supplémentaires par lesquels les ions Ni^{2+} peuvent perturber l'homéostasie intracellulaire du calcium et causer ainsi la mortalité des lymphocytes humains. En effet, l'analyse du rôle des différents inhibiteurs du flux de calcium à travers les membranes plasmiques tels que le nifedipine, le verapamil et le diltiazem suggère que l'effet protecteur empêchant l'augmentation du calcium intracellulaire est dû à l'inhibition des déplacements du Ca^{2+} à travers le canal calcique contrôlés par la calmoduline impliquant une modification de la différence de potentiel de la membrane plasmique (Catterall et Striessnig, 1992). Le nifedipine inhibe le calcium extracellulaire par blocage des canaux calciques potentiel-dépendant en se liant aux protéines de ces canaux à l'extérieur des membranes plasmiques

(Catterall et Striessnig, 1992). C'est un agent vasodilatateur efficace utilisé pour traiter l'angine de poitrine, l'hypertension et la migraine. Le verapamil est un inhibiteur du calcium extérieur qui bloque les canaux calciques potentiel-dépendant en se liant aux protéines de ces canaux à l'intérieur des membranes plasmiques (Catterall et Striessnig, 1992). Il est utilisé en clinique pour traiter les personnes souffrant d'hypertension essentielle. Le diltiazem est un médicament connu comme étant un bloqueur des canaux calciques. Il inhibe le calcium à travers les canaux calciques potentiel-dépendant (Catterall et Striessnig, 1992). Nous avons aussi démontré que d'autres types d'inhibiteurs des canaux calciques qui agissent au niveau du stockage intracellulaire procurent cet effet protecteur. C'est ainsi le dantrolene, la cyclosporine A et le ruthénium rouge ont réduit la mortalité des lymphocytes. Le dantrolene empêche la libération du calcium contenu dans le réticulum sarcoplasmique (une variété de réticulum endoplasmique lisse, complexe et permettant le stockage des ions calcium) (Ohta et coll., 1990). Il est utilisé pour détendre certains muscles du corps et pour soulager les spasmes. La cyclosporine A est un inhibiteur de la calcineurine (une protéine-kinase libérée par les mitochondries) et de la libération du calcium de mitochondries (Packer et Murphy, 1994). C'est aussi un médicament ayant une activité immunosuppressive puissante utilisé dans les transplantations d'organes pour empêcher le rejet de la greffe (Manez

et coll., 1995). Le ruthénium rouge inhibe le transport du calcium par les mitochondries (Gunter et Pfeiffer, 1990). Finalement, nous avons aussi observé cet effet avec le BAPTA, un chélateur spécifique du calcium dans le cytosol (Guidarelli et coll., 1996). Il agit en complexant sélectivement les ions calcium. À la lumière de ces résultats, étant donné que le Ni^{2+} se lie fortement aux protéines (Huang et coll., 1995), nous pensons que le Ni^{2+} (à partir de sulfate de nickel, de sous-sulfure et d'hydroxyde carbonate de nickel) se lie à la calmoduline en la modifiant et en contrôlant le couplage des phénomènes de membrane (combinaison du « premier messager» avec le récepteur de membrane); ceci aboutit à l'ouverture des canaux de calcium de la membrane et des organites cellulaires, ce qui entraîne l'afflux et la diffusion massive du calcium dans la cellule. Il est possible aussi que ces trois composés de nickel soient en compétition avec les ions métal essentiel comme le calcium pour les mêmes voies. La perturbation de l'équilibre intracellulaire d'ions Ca^{2+} pourrait ainsi provoquer la mortalité cellulaire. Refvik et Andreassen (1995) et Funakoshi et collaborateurs (1997) ont démontré que le composé soluble de nickel est en compétition avec le calcium pour les canaux calciques, et que le nickel arrive à contrôler et passer à travers ces canaux. Funakoshi et collaborateurs (1997) ont observé une diminution significative de 20 % de la captation du nickel suite au pré-traitement des hépatocytes de rat avec le nicardipine ou le

verapamil.

IV.1.3.4. Rôle du stress oxydatif dans l'induction des dommages à l'ADN

La surproduction de H_2O_2 et O_2^- a été détectée dans le noyau cellulaire suite à l'exposition des cellules au nickel (Peskin et Shlyahova, 1986). La liaison du nickel avec l'ADN peut catalyser la surproduction d'ERO dans le noyau cellulaire, ce qui pourrait entraîner des dommages oxydatifs à l'ADN et aux autres composants nucléaires (Kasprzak et Buzard, 2000; Coogan et coll., 1989). Outre les dommages chromosomiques, les effets oxydatifs majeurs à l'ADN dus à l'exposition au nickel tels les bris simple-brin d'ADN, les liaisons croisées protéine-ADN, l'épuration de purine et la modification des bases azotées ont été tous observés dans plusieurs études (Coogan et coll., 1989; Chakrabarti et coll., 1999, 2001; Kasprzak et Buzard, 2000; Costa et coll., 2002).

Les évidences présentées dans notre étude suggèrent que les dommages oxydatifs induits par les radicaux libres peuvent jouer un rôle important dans les mécanismes biochimiques impliqués dans l'induction des bris simple-brin à l'ADN par l'hydroxyde carbonate de nickel. Le pré-traitement des lymphocytes sanguins humains avec,

entre autres la catalase, la superoxyde dismutase et le DMTU a, dans tous les cas, réduit significativement les bris simple-brin à l'ADN dans la chromatine chromosomique et nucléaire. De la même façon, ces modulateurs biochimiques du stress oxydatif ont réduit de façon significative les échanges entre chromatides-sœurs. Ces données suggèrent que l'excès des espèces radicalaires de l'oxygène telles le peroxyde d'hydrogène, l'anion superoxyde et le radical hydroxyle est impliqué dans l'induction des bris simple-brin dans la chromatine par l'hydroxyde carbonate de nickel.

Puisque la deferoxamine est un chélateur du fer, il a le potentiel d'inhiber le processus d'induction d'ERO par les composés de nickel (Chakrabarti et Bai, 1999). De cette façon, la deferoxamine peut prévenir les bris simple-brin à l'ADN. Nos résultats démontrent que le pré-traitement des lymphocytes avec la deferoxamine réduit significativement les bris simple-brin causés par l'hydroxyde carbonate de nickel dans la chromatine chromosomique et nucléaire. Ceci suggère que ce composé de nickel peut exercer son potentiel génotoxique par la voie des dommages oxydatifs à médiation ferreux. Ces résultats montrent que la chélation du fer par la deferoxamine est un apport thérapeutique de protection des lymphocytes humains contre l'induction des dommages oxydatifs à l'ADN par le nickel. Par ailleurs, le pourcentage des bris simple-brin à l'ADN causés par les

composés de nickel est aussi significativement réduit par le pré-traitement des lymphocytes sanguins humains en culture avec la *N*-acétylcystéïne (un précurseur de GSH). Cela suggère aussi que le niveau intracellulaire en GSH est un des facteurs déterminants de la susceptibilité des lymphocytes humains aux effets génotoxiques des composés de nickel.

Par ailleurs, plusieurs études supportent les résultats de notre étude sur l'implication du stress oxydatif dans les mécanismes génotoxiques du nickel. Par exemple, Huang et collaborateurs (1994) ont démontré que l'ion Ni^{2+} induit un stress oxydatif dans le cytoplasme et le noyau des cellules ovariennes de l'hamster chinois. Chen et coll., (2003) ont montré également que le chlorure de nickel induit une surproduction d'oxydants dans les lymphocytes humains isolés. Nackerdien et coll., (1991) et Kawanishi et coll., (2002) ont observé que les ions Ni^{2+} induisent eux-mêmes la formation des bases oxydées d'ADN. De la même façon, le sous-sulfure de nickel produit des espèces radicalaires de l'oxygène dans les lymphocytes isolés de rat (Chakrabarti et coll., 2001).

IV.1.3.5. Rôle du calcium intracellulaire dans l'induction des dommages à l'ADN

Notre étude a identifié des cibles supplémentaires par lesquelles les composés de nickel peuvent perturber l'homéostasie cellulaire du calcium entrainant comme conséquence l'augmentation des bris simple-brin à l'ADN dans la chromatine chromosomique et nucléaire des lymphocytes sanguins humains en culture. Ainsi nous avons analysé l'effet protecteur de différents inhibiteurs des canaux calciques qui empêchent l'augmentation intracellulaire du calcium. Nos données suggèrent que l'effet protecteur du verapamil contre l'induction des bris simple-brin à l'ADN dans la chromatine est dû à l'inhibition des déplacements des ions calcium à travers le canal calcique potentiel-dépendant de la protéine (calmoduline) de la membrane plasmique. D'autre part, les bris simple-brin causés par l'hydroxyde carbonate de nickel ont été aussi significativement inhibés par le dantrolene par réduction de la libération des ions Ca^{2+} contenus dans le réticulum sarcoplasmique. Il est possible que l'hydroxyde carbonate de nickel soit en compétition avec les ions métal essentiel comme le calcium pour les mêmes voies entraînant la perturbation de l'équilibre intracellulaire du calcium et provoque les bris simple-brin à l'ADN. Il y a très peu d'études sur les effets carcinogènes et/ou toxiques du nickel liés à la perturbation du

métabolisme de calcium (Denkhaus et Salnikow, 2002).

IV.2. Conclusion générale

Notre étude montre que l'induction de la mortalité des lymphocytes et l'induction des dommages génotoxiques à la chromatine dépendent fortement de la nature chimique des composés de nickel. Dans nos conditions expérimentales, l'hydroxyde carbonate de nickel s'avère le composé de nickel le plus cytotoxique et génotoxique. Nos résultats révèlent également que le sulfate, le sous-sulfure et l'hydroxyde carbonate de nickel agissent à travers un même mécanisme. En effet, ces composés de nickel causent un dysfonctionnement dans le processus biochimique cellulaire. Ce mécanisme implique la production de divers types d'espèces radicalaires oxygénées/stress oxydatif, la déplétion de la réserve des thiols, le déclenchement de la perte de l'imperméabilité des pores mitochondriaux et la déstabilisation de l'homéostasie intracellulaire du calcium. Ces événements critiques entraînant non seulement des dommages à l'ADN, mais activent aussi la transmission des signaux conduisant à la mortalité des lymphocytes humains.

Étant donné que les lymphocytes circulent continuellement dans le sang, dans la lymphe et dans tous les organes de l'organisme,

nos résultats suggèrent que l'exposition humaine aux composés de nickel, particulièrement à l'hydroxyde carbonate de nickel, pourrait être un grand risque pour la santé humaine. Nos résultats de recherche fournissent des nouvelles informations pour la compréhension des mécanismes cellulaires et moléculaires de toxicité des composés de nickel. En effet, nos résultats montrent que l'usage des antioxydants et des certains médicaments inhibiteurs de l'afflux du calcium intracellulaire utilisés en clinique pour traiter l'hypertension essentielle réduisent considérablement les dommages causés à l'ADN et protègent également les lymphocytes contre la mortalité induite par les composés de nickel.

Les résultats de cette recherche fournissent également des informations importantes sur la prévention des dommages causés à l'ADN des personnes travaillant dans l'industrie du nickel. Ces dommages peuvent, potentiellement, à long terme être à l'origine d'une panoplie des effets néfastes sur la santé incluant les effets héréditaires (cassures de chromosome et réparation irrégulière du matériel génétique), les anomalies congénitales et le développement du cancer. La surveillance biologique précoce est présentement le seul moyen utilisé pour le suivi de l'état de santé des travailleurs de l'industrie du nickel. Malheureusement, elle ne semble pas être un moyen efficace pour l'évaluation des risques sur la santé des

travailleurs, mais simplement un indicateur d'exposition. Étant donné l'ampleur des dommages causés à l'ADN par les différents composés de nickel, nous suggérons que des études humaines *in vivo* dans des conditions d'exposition environnementale et/ou occupationnelle auprès des travailleurs de l'industrie du nickel aussi bien qu'auprès de la population qui vit à proximité de tels environnements, devraient être menées afin de valider les dommages causés à l'ADN comme bioindicateurs de l'action cancérogène des composés de nickel. Nous suggérons aussi que la technique EM-ISEL puisse être utilisé comme un test à court terme pour prédire le potentiel des effets néfastes sur la santé à long terme.

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