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Comparative study of nephrotoxicity of potassium dichromate and chromium chloride using isolated rat renal cortical slices in vitro

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Ce mémoire intitulé:

Comparative study of nephrotoxicity of potassium dichromate and chromium chloride using isolated rat renal cortical slices *in vitro*

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Sommaire

Problématique: Il existe divers effets biologiques associés à l'exposition au chrome, ces effets dépendent de la spéciation de ce métal. Seulement l'état d'oxydation trivalent [Cr(III)] et hexavalent [Cr(VI)] présentent un intérêt pour la santé humaine. Le rein est considéré comme étant un des organes cibles de la toxicité due aux composés du chrome. La toxicité rénale, hépatique et dermique a été rapportée chez les ouvriers d'industries exposés au Cr(VI). Cependant, l'information concernant les mécanismes néphrotoxiques des composés du chrome est présentement très limitée.

Objectifs: Étudier *in vitro* le potentiel néphrotoxique (le potentiel d'induire les lésions cellulaires) du bichromate de potassium [Cr(VI)] et du chlorure de chrome [Cr(III)] en utilisant les tranches de cortex rénal isolé chez le rat. En outre, étudier la possibilité que la formation de métabolites cellulaires réducteurs, la formation d'espèces réactives oxygénées (H_2O_2, $O_2^{\cdot-}$, $^{\cdot}OH$) ou encore la perte de l'imperméabilité des pores mitochondriaux (PIPM) soient impliquées dans l'induction de ces lésions cellulaires. D'où l'étude comparative des mécanismes d'action néphrotoxique du Cr(VI) et du Cr(III) dans les tranches de cortex rénal isolé chez le rat.

Méthodologie: La viabilité cellulaire et la surproduction de la peroxydation des lipides dans les tranches de cortex rénal isolé chez le rat après exposition au bichromate de

potassium [Cr(VI)] et au chlorure de chrome [Cr(III)] ont été respectivement mesurées par le test de MTT (la mesure de la toxicité par détermination du sel de tétrazolium en formazan) et par la formation de MDA (malondialdéhyde). En outre, le rôle physiologique d'antioxydants tels la glutathion, la glutathion réductase (GSSG-R), la mélatonine, les vitamines C (acide ascorbique), E (α-tocophérol) et celui de la PIPM dans l'induction de ces lésions cellulaires corticales rénales par le bichromate de potassium et le chlorure de chrome a été étudié.

Résultats: Article 1. Le Cr(VI) engendre la cytotoxicité et la surproduction du stress oxydatif (tel que mesuré par la formation de MDA et la déplétion de la réserve des thiols: groupements sulfhydryles non liés aux protéines) à reformuler dans les tranches de cortex rénal isolé chez le rat. L'induction du stress oxydatif survient après celle de la cytotoxicité. Le prétraitement des tranches de cortex rénal avec le mannitol et le diméthyl thiourée (DMTU) avant le traitement avec le Cr(VI) a réduit de façon significative l'induction de la surproduction de la peroxydation des lipides par le Cr(VI). Cependant, ce prétraitement n'a pas réduit l'induction de la cytotoxicité. Ceci suggère que le radical hydroxyle est impliqué dans l'induction de la peroxydation des lipides par le Cr(VI) et non dans l'induction de la toxicité de ce dernier. Par contre la catalase et la superoxyde dismutase (SOD) n'ont pas réduit de façon significative l'induction de cette toxicité, suggérant que le peroxyde d'hydrogène et le radical anion superoxyde ne sont pas impliqués dans la toxicité du Cr(VI). Le traitement simultané des tranches de cortex rénal

avec le glutathion et le Cr(VI) ou leur prétraitement avec l'acide ascorbique avant d'être traitées avec le Cr(VI) ont réduit significativement les lésions cellulaires et la peroxydation des lipides. Bien que le Cr(VI) induise la PIPM, celle ci ne semble pas joué un rôle significatif dans l'induction de la cytotoxicité. La glutathion réductase non plus n'a pas joué un rôle significatif dans cette cytotoxicité. Article 2. Le Cr(III) induit la cytotoxicité à reformuler dans les tranches de cortex rénal isolé chez le rat, mais n'induit pas la surproduction du stress oxydatif. Le traitement simultané des tranches de cortex rénal avec le glutathion et le Cr(III) augmente de façon significative l'induction de la cytotoxicité et la surproduction de la peroxydation des lipides. Par contre leur prétraitement avec l'acide ascorbique suivi du traitement avec le Cr(III) diminue significativement la cytotoxicité, alors que la vitamine E n'a pas réduit de façon significative cette cytotoxicité. À reformuler, l'induction de la cytotoxicité induite par le bichromate de potassium est environ 8-fois plus forte que celle induite par le chlorure de chrome dans ce modèle expérimental.

Conclusion: Les composés du chrome [Cr(VI) et Cr(III)] induisent la cytotoxicité dans les tranches de cortex rénal isolé chez le rat. Ces résultats suggèrent que le bichromate de potassium est potentiellement plus toxique que le chlorure de chrome dans les tranches de cortex rénal isolé chez le rat. Le bichromate de potassium induit la surproduction du stress oxydatif comparativement au chlorure de chrome qui n'induit pas le stress oxydatif. L'antioxydant vitamine C peut jouer un rôle préventif dans la toxicité de ces deux

composés de chrome. D'autre part, le glutathion joue un rôle différentiel dans l'induction de la toxicité de ces composés.

Mot clés: chrome, néphrotoxicité, cytotoxicité, le mécanisme, la peroxidation des lipides, les formes d'oxygène réactives, la perte de l'imperméabilité des pores mitochondriaux

Summary

Background: The biological effects associated with chromium exposure are diverse and depend upon chromium speciation. Only the trivalent [Cr(III)] and hexavalent [Cr(VI)] forms of chromium are important for human health. The kidney is considered to be one of the target organs for toxicity of chromium compounds. Renal, hepatic and dermal toxicity have been reported in industrial workers exposed to Cr(VI). However, the information regarding the mechanisms of chromium compounds-induced nephrotoxicity is at present very limited.

Objectives: To study the nephrotoxicity potential (potential to induce cellular injury) of both potassium dichromate [Cr(VI)] and chromium chloride [Cr(III)] using isolated rat renal cortical slices *in vitro*. Furthermore, to investigate whether renal cellular injury due to these chromium compounds are mediated by the formation of cellular reductive metabolism and/or reactive oxygen species (ROS) (such as hydrogen peroxide, superoxide anion and/or hydroxyl free radicals) and/or mitochondrial permeability transition (MPT). Hence, to compare the mechanism of nephrotoxic action of Cr(VI) and Cr(III) compounds in isolated renal cortical slices.

Methods: Potassium dichromate [Cr(VI)]- and chromium chloride [Cr(III)]-induced reduction of cell viability (as measured by MTT test: measurement of the toxicity with

diphenyl-tetrazolium bromide) and increase in lipid peroxidation (LPO) [as measured by malondialdehyde (MDA) formation] were examined in isolated rat renal cortical slices. Furthermore, the effects of physiological cellular antioxidants (such as glutathione (GSH), vitamin C (ascorbic acid), vitamin E (α-tocopherol), glutathione reductase (GSSG-R), and melatonin) and/or MPT on both potassium dichromate- and/or chromium chloride-induced renal cortical cellular damage were examined.

Results: Article 1. Cr(VI) induced both concentration- and time-dependent cytotoxicity (cell viability) and oxidative stress [MDA formation and nonprotein-sulfhydryl group (NP-SH) depletion]. But Cr(VI)-induced oxidative stress occurs later than Cr(VI)-induced cytotoxicity in isolated renal cortical slices. Pre-treatment with mannitol and dimethyl thiourea (DMTU) only reduced Cr(VI)-induced LPO but not Cr(VI)-induced cytotoxicity, suggesting that hydroxyl free radicals were involved in Cr(VI)-induced LPO, but not cytotoxicity. Catalase and superoxide dismutase (SOD) failed to reduce such toxic effects, suggesting hydrogen peroxide and superoxide anion were not involved in Cr(VI)-induced toxic effects. Co-treatment with excess glutathione or pre-treatment with excess ascorbic acid reduced Cr(VI)-induced cellular damage and LPO. Although Cr(VI) induced mitochondrial permeability transition (MPT), such MPT played a non significant role in Cr(VI)-induced cytotoxicity. Glutathione reductase (GSSG-R) did not show any important role in Cr(VI)-induced nephrotoxicity. Article 2. Cr(III) induced both concentration- and time-dependent cytotoxicity in isolated rat renal

cortical slices, but no effects on oxidative stress. Co-treatment with excess GSH increased Cr(III)-induced cytotoxicity and LPO. Pre-treatment with excess vitamin C, but not vitamin E, prevented Cr(III)-induced cytotoxicity. Comparing these chromium compounds, potassium dichromate-induced cytotoxicity is found to be about 8-fold higher than that of chromium chloride in this experimental system.

Conclusion: Both chromium(VI) and chromium(III) compounds induced cytotoxicity in isolated renal cortical slices of rat. Cr(VI) possesses greater toxicity than Cr(III). Cr(VI) also induced the oxidative stress by increasing MDA formation and decreasing NP-SH content, but these occurred following the cytotoxicity occurred. Cr(III) has not shown such effects. Hydroxyl free radicals are involved in Cr(VI)-induced LPO but not cytotoxicity. GSH plays a differential role in chromium compounds-induced toxicity. For Cr(VI), it plays a protective role by decreasing Cr(VI)-induced cell viability loss and MDA formation; for Cr(III), it enhances Cr(III)-induced cytotoxicity and lipid peroxidation. Vitamin C is a powerful antioxidant to prevent both Cr(VI) and Cr(III)-induced toxicity.

Key words : chromium compounds, nephrotoxicity, cytotoxicity, mechanism, lipid peroxidation, reactive oxygen species, mitochondrial permeability transition

Table of Contents

List of Figures

List of Figures (cont)

Section 4: General discussion

List of Abbreviation

ANOVA	Analysis of variance
ATSDR	Agency for Toxic Substances and Disease Registry
BCNU	Carmustine
$CrCl_3$	Chromium chloride
Cr(III)	Trivalent chromium
Cr(VI)	Hexavalent chromium
CsA	Cyclosporine A
DFO	Deferoxamine
DMTU	Dimethyl thiourea
DTNB	5,5'-dithio-bis (2-nitrobenzoic acid)
ESR	Electron spin resonance
Fig.	Figure
g	Gram
GSH	Reduced glutathione/ glutathione
GSSG-R	Glutathione reductase
HBSS	Hanks' balanced salt solution
HPLC	High performance liquid chromatography
i.p.	Intraperitoneal
$K_2Cr_2O_7$	Potassium dichromate
kg	Kilogram
LPO	Lipid peroxidation
MDA	Malondialdehyde
mg	Milligram
min	Minute
mL	Milliliter

mM	Millimole
MPT	Mitochondrial permeability transition
MTT	Measurement of the toxicity with diphenyl-tetrazolium bromide
NP-SH	Nonprotein sulfhydryl group
PBS	Phosphate buffer solution
ROS	Reactive oxygen species
SOD	Superoxide dismutase
μg	Microgram
μM	Micromole

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SECTION 1:

GENERAL INTRODUCTION

1.1 Introduction

1.1.1 Chromium

Chromium is naturally occurring in rocks, animals, plants, soil, and in volcanic dust and gases. It is present in the environment in several different forms. The most important forms are trivalent (III) and hexavalent (VI) chromium. Trivalent chromium occurs naturally in the environment and is an essential trace metal required by the human body to promote the action of insulin in body tissues so that the body can use sugar, protein, and fat (Mertz, 1993). It is the most stable chromium compound and poorly soluble in water at pH ranging from 4-11 except for the acetate, chloride (hexahydrate), and nitrate salts. Hexavalent chromium is primarily produced by anthropogenic source. It is the second most stable chromium compound and is a strong oxidizing agent, especially in acid media. Its compounds usually exist as oxides or as oxohalides. They are soluble in water. Hexavalent chromium compounds traverse cell membranes well and are reduced to trivalent compounds intracellularly (Barceloux, 1999). The relationship between the hexavalent and trivalent states of chromium is described by the equation:

$$Cr_2O_7^{2-} + 14\ H^+ + 6\ \text{electrons} \rightarrow 2Cr^{3+} + 7\ H_2O + 1.33\ eV$$

Cr(VI) Cr(III)

The difference in electric potential between Cr(VI) and Cr(III) reflects the strong oxidizing potential of hexavalent chromium and the substantial energy (1.33 eV) required to oxidize the trivalent chromium to the hexavalent form. For practical purposes, it can be

stated that this oxidation never occurs in biological systems. But the reduction of hexavalent chromium occurs spontaneously in the organism, unless present in an insoluble form (WHO, 1988).

1.1.2 Occupational and environmental exposure

People can be exposed to chromium by breathing air, drinking water, or eating food containing chromium or through skin contact with chromium compounds. The reference dose (RfD) of US Environmental Protection Agency (EPA) for trivalent chromium is 1mg/kg/d based on a chronic animal study, in which the NOAEL is based on administering 5% chromium trioxide (Cr_2O_3) in the diet of rats. For hexavalent chromium, the RfD is 5 μg/kg/d based on a one-year drinking water study in Sprague-Dawley rats. EPA also sets the maximum level of Cr(III) and Cr(VI) allowed in drinking water at 100μg/L (ATSDR, 2000).

About 80 % of the chromium mined goes into metallurgical applications (such as ferrochromium intermediates for stainless steels, cast irons, and nonferrous alloys), 15 % is used in chromium chemicals manufacture (including chrome plating, the manufacture of dyes and pigments, leather tanning, wood preserving, textiles, and toner for copying machines) and the remainder is used in refractory application (like drilling mud, rust and corrosion inhibitors) (Barceloux, 1999). Occupational Safety and Health Administration (OSHA) regulates chromium levels in the workplace air. The occupational exposure

limits for an 8-hour workday, 40-hour workweek are 500 μg Cr/m^3 for water-soluble chromic [Cr(III)] or chromous [Cr(II)] salts and 1,000 μg/m^3 for metallic chromium [Cr(0)] and insoluble salts. The level of chromium trioxide (chromic acid) and other chromium(VI) compounds in the workplace air should not be higher than 52 μg/m^3 for any period of time (ATSDR, 2000).

1.1.3 Metabolism

Cr(VI) compounds are easier absorbed than Cr(III) compounds. Under physiological conditions, Cr(VI) exists as a chromate oxy-anion and crosses the cell membrane through non-specific phosphate/sulfate anionic transporters. Cr(VI) undergoes rapid metabolic reduction, producing the intermediate Cr(V) and Cr(IV) species and, ultimately, to Cr(III). When intracellular reductant levels are present in gross excess over Cr(VI), the initial step involves a two electron reduction to Cr(IV) followed by one electron reduction to Cr(III) (O'Brien et al, 2003). In blood, Cr(III) binds to proteins, such as transferrin, in plasma and distributes widely throughout the body. Cr(VI) entering the blood stream is taken up selectively by erythrocytes, reduced and bound to haemoglobin. It has found high-level concentration of chromium in the kidneys, lungs, and spleen (Dayan and Paine, 2001).

1.1.4 Excretion

The main routes for the excretion of chromium are via kidney/urine and bile/faeces

(Shrivastava et al, 2002). The kidney rapidly excretes most of the absorbed chromium with little retention of chromium in tissues. The urinary half-life of Cr(VI) ranges from 15 to 41 hr. Erythrocytes release chromium slowly and the possibility of limited release of chromium from a slow compartment is from several months to a few years. Urinary chromium concentrations are the highest when exposure is predominantly to Cr(VI) compounds (Barceloux, 1999). Almost all of intravenously injected chromium is excreted via the urine and only 2% is found in the faeces (WHO, 1988). Urinary excretion of total chromium has been used for decades as a biomonitoring tool to evaluate exposure to chromium compounds in occupational settings (Kerger et al, 1996). Biliary excretion accounts for about 10% of the elimination of chromium with smaller amounts of chromium appearing in hair, milk, nails and sweat (Barceloux, 1999).

1.2 Chromium-induced toxic effects

The toxic effects associated with chromium are diverse and depend upon metal speciation. Cr(VI) is generally much more toxic than Cr(III). Chromium has been extensively demonstrated to induce acute and chronic toxicity, neurotoxicity, dermatotoxicity, genotoxicity, carcinogenicity, immunotoxicity, and general environmental toxicity (Bagchi et al, 2002).

1.2.1 General effects

In adult human subjects, the lethal oral dose of soluble chromate [Cr(VI)] is considered to

be 50-70 mg/kg b.w. soluble chromates. The clinical features of acute poisoning are vomiting, diarrhoea, haemorrhage and blood loss into the gastrointestinal tract, causing cardiovascular shock. If the patient survives for more than about 8 days, the major effects resulting from oral ingestion of toxic doses of Cr(VI) are liver and kidney necrosis. The acute ingestion of large amounts of Cr(VI) compounds produces acute tubular necrosis (ATN), marked interstitial changes, and renal failure. Chronic low-dose exposure to chromium can induce low molecular weight (LMW) proteinuria and then produce chronic renal injury in chromium workers. Hepatic necrosis occurs after the acute ingestion of very large quantities of Cr(VI) compounds (Dayan and Paine, 2001; Barceloux, 1999; Wedeen and Qian, 1991).

Chromium(VI) compounds are pulmonary sensitizers, producing a generalized irritation of the conjunctiva and mucous membranes, nasal perforations, and a contact dermatitis. Inhalation of Cr(VI) compounds causes marked irritation of the respiratory tract. Thus, ulceration and perforation of the nasal septum have occurred frequently in workers employed in the chromate producing and Cr(VI)-using industries. Rhinitis, bronchospasm, pneumonia and bronchial asthma have been reported in workers exposed to Cr(VI) compounds (Dayan and Paine, 2001). Cr(VI) was associated with human lung cancer in a case report describing a chrome pigment worker with an adenocarcinoma in his nasal turbinate bone in 1890. Since the initial report, Cr(VI) compounds have been declared as a potent occupational carcinogen. IARC and the US toxicology program recognize Cr(VI)

as a known human carcinogen (Wise Sr. et al, 2002).

Both Cr(VI) and Cr(III) compounds may cause dermatitis in sensitized individuals. Chromic acid is a corrosive that produces inflammation and ulceration of the skin. Chronic ulcers of the skin and acute irritative dermatitis have been consistently reported in workers exposed to chromium containing materials. Direct skin contact with chromium compounds elicits an allergic response, characterized by eczema or dermatitis, in sensitized individuals. Contact dermatitis has been a serious problem in industries where solutions of Cr(VI) salts handled, as well as in workers exposed to stainless steel welding fumes. Chromium sensitivity is a classic delayed-type (class IV) hypersensitivity reaction. The formation of the ultimate hapten is considered to involve Cr(III) (Dayan and Paine, 2001).

1.2.2 Toxicity in experimental research

In contrast to Cr(III) compounds, Cr(VI) compounds are oxidizing agents capable of directly inducing tissue damage and cause a dose-dependent loss in cell viability. Cr(VI) is reduced to Cr(III) in cells. This reduction is assumed to be a prerequisite for chromate toxicity because only the trivalent form is able to interact with critical target molecules inside a cell (Dartsch et al, 1998; Appenroth and Kersten, 1990).

Dartsch et al (1998) investigated the nephrotoxicity and hepatotoxicity of Cr(III) and

Cr(VI) compounds in kidney epithelial cell and liver epithelial cell, respectively. The results showed that Cr(VI), but not Cr(III), had an acute cytotoxic effect and caused a dose-dependent loss in cell viability. The effective dose that caused 50% of cell death was 5 μmol/L of Cr(VI) for kidney epithelial cells and 50 μmol/L for liver epithelial cells, indicating that kidney epithelial cells are 10 times more sensitive towards Cr(VI) treatment than liver epithelial cells. In primary cultured hepatocytes, Gunaratnam and Grant (2004; 2002) found that exposure of cells to 1, 5, 10 and 50 μM Cr(VI) compounds resulted in the loss of the cell cytoskeleton, and accompanied with membrane blebbing and shrinking of the cell. Staining of the cells with annexin V and propidium iodide showed that Cr(VI) induced apoptosis at low concentrations and necrosis at higher concentrations. A significant decrease of cell viability induced by Cr(VI) also were observed in isolated hepatocytes of goldfish (Krumschnabel and Nawaz, 2004).

The cytotoxicity of both particulate (water-insoluble, $PbCrO_4$) and soluble (Na_2CrO_4) Cr(VI) in primary human bronchial fibroblasts (PHBFs) were determined. Both compounds induced concentration-dependent cytotoxicity after a 24 hr exposure in PHSFs. The relative survival was 87, 46, 26 and 2% after exposure to 0.1, 0.5, 1 and 5 $\mu g/cm^2$ $PbCrO_4$, respectively, and 74, 57, 13 and 0% after exposure to 1, 2.5, 5 and 10 μM Na_2CrO_4, respectively, indicating that Cr(VI) compounds are cytotoxic to human lung cells (Wise Sr. et al, 2002). The similar results were found in normal human lung epithelial cells at concentration ranges from 0.2 to 200 μM $K_2Cr_2O_7$. The toxic effect was

associated with increased levels of intracellular phosphoprotein and subsequent release of inflammatory cytokines IL-6 and IL-8 (Pascal and Tessier, 2004). Ning and Grant (1999) investigated the cytotoxicity of Cr(VI) in immortalized rat osteoblast cells in vitro using alkaline phosphatase (ALP) activity as an index of toxicity. Cr(VI) caused a concentration-dependent decrease in ALP activity.

Biedermann and Landolph (1990) compared the role of valence state chromium compounds on induction of cytotoxicity in diploid human fibroblasts (HFC) from foreskins and found Cr(VI) compounds ($PbCrO_4$, Na_2CrO_4 and CrO_3) were 1000-fold more cytotoxic to HFC (average 50% lethal dose 0.5 μM) than Cr(III) compounds ($CrCl_3$, Cr_2O_3, and Cr_2S_3) (average 50% lethal dose 500 μM). The similar results were observed by Flores and Perez (1999) in several human and murine cell lines. Venier et al (1982) also found that potassium dichromate [Cr(VI)] was higher cytotoxic than chromium chloride or other chromium(III) compounds in cultured hamster cells.

Cr(VI) compounds induced DNA damage, gene mutation, sister chromatid exchange (SCE), chromosomal aberrations, cell transformation and dominant lethal mutations in a number of targets, including animal in vivo, animal and human cells in vitro. The negative results were obtained for Cr(III) compounds in the large majority of tests for DNA damage and gene mutation in bacteria (Dayan and Paine, 2001). But Blasiak and Kowalik (2000) found that potassium dichromate induced DNA damage in the

lymphocytes, and $CrCl_3$ caused greater DNA migration than $K_2Cr_2O_7$.

Ovarian dysfunction in adult Swiss albino female mice was observed following Cr(VI) exposure through drinking water. Ovaries of the highest dose (750 ppm for 20 days) showed large numbers of atretic follicles and congestion in stromal tissue compared to the rest of the treated groups. The number of ova recovered from superovulated Cr(VI)-treated animals showed significant decrease in the 500 and 750 ppm dosed groups. The duration of estrus cycle increased in highest dosed (750 ppm) group (Murthy et al, 1996).

1.3 Mechanisms of chromium-induced toxic effects

A large number of studies demonstrated that chromium-induced toxicity involved in oxidative stress, DNA damage, apoptotic cell death and altered gene expression. As a general rule, Cr(VI) is much more toxic than Cr(III). Cr(VI) was transported through mammalian cell membranes by the carboxylate, sulphate and phosphate carrier systems. The intracellular reduction of Cr(VI) implies the generation of short-lived species of pentavalent [Cr(V)] and tetravalent [Cr(IV)] chromium with affinities for cellular constituents that may differ from that of Cr(III). Those chromium intermediates, radicals, and reactive oxygen species (ROS) can subsequently attack macromolecules and lead to DNA damage (Bagchi et al, 2002; Dayan and Paine, 2001; Tsou et al, 1999).

1.3.1 Apoptosis

Cr(VI)-induced apoptosis was first reported in Chinese hamster ovary (CHO) cells. Treated with sodium chromate or lead chromate particles, the CHO cells decreased in survival in colony-forming efficiency assays. And the cell growth, DNA synthesis and protein synthesis were also inhibited, respectively. Morphological features of apoptosis including nuclear fragmentation were observed in more than 90% of detached non-adherent cells and up to 22% of adherent cells. But the untreated cells remained morphologically normal (Blankenship et al, 1994; 1997).

The apoptosis caused by a set of Cr(III)-complexes in human lymphocytes was first reported by Rajaram research group in 1995. Only two of five Cr(III) complexes are found to cause apoptosis. Manygoats et al (2002) studied the cellular effects of Cr(III) and found the morphological damage caused by chromium picolinate, picolinic acid and chromic chloride in CHO AA8 cells by using transmission electron microscopy (TEM) study. The results showed 83% of analyzed cells having swollen mitochondria with degraded cristate. Apoptosis was identified by nuclear convolution and fragmentation, and cytoplasmic blebbing.

1.3.2 Oxidative stress

Chromium compounds-induced oxidative stress mainly includes enhanced production of ROS, increased lipid peroxidation and genomic DNA fragmentation. It is caused by an

imbalance between free radical generation and antioxidant defence systems. It also results in decreased antioxidant defence levels, altered redox enzyme activity, and DNA damage (Shi et al, 2004).

1.3.2.1 Reactive Oxygen Species (ROS) and lipid peroxidation (LPO)

Reactive oxygen species (ROS) refer to hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical (•OH). Both Cr(VI) and Cr(III) are biologically active oxidation states and involved in redox cycling with the production of ROS. A large number of studies have shown that chromium compounds exhibit the ability to produce reactive oxygen species, resulting in lipid peroxidation, DNA damage, and depletion of sulfhydryls (Harris and Shi, 2003; Travacio et al, 2001; Stohs and Bagchi, 1995).

In both mice and rats, acute or chronic oral administration of chromium(VI) compounds could increase production of ROS and lipid peroxidation in liver or brain, enhance excretion of urinary lipid metabolites, DNA fragmentation and apoptotic cell death. In potassium dichromate [Cr(VI)]-treated rats, the excretion of all four lipid metabolites were 1.7- to 3.0-fold greater than for chromium chloride [Cr(III)]-treated animals. In mouse brain homogenates, the oxidative stress also occurred after orally treated potassium dichromate 25 mg/kg per day. The results indicated that Cr(VI)-induced oxidative stress resulting in tissue damaging effects might contribute to the toxicity and carcinogenicity of chromium (Bagchi et al, 2002; 1997a; 1995a; 1995b and Travacio et al,

2001). In isolated rat hepatocytes, addition of $K_2Cr_2O_7$ resulted in rapid glutathione oxidation, ROS formation, lipid peroxidation (Pourahmad et al, 2003). In A549 cells, ROS levels increased in proportion to the concentration of Cr(VI). The increases were diminished by pretreatment with catalase, superoxide dismutase, or D-mannitol (Kim et al, 2003). Bagchi et al (2000) also observed approximately 3.1 to 6-fold increases in hydroxyl radical production following incubation of the K562 cells with $K_2Cr_2O_7$ at 12.5 and 25 μM concentrations. In the rat heart homogenate, hydroxyl radical formation and the lipid peroxidation levels were increased with an increase on the concentrations of Cr(VI) solution added (Coudray et al, 1992).

Cr(III) was thought to be relatively nontoxic. However, Ozawa and Hanaki (1990) demonstrated that Cr(III) can be reduced to Cr(II) by the biological reductants L-Cysteine and NADH, and in turn, the newly formed Cr(II) reacts with hydrogen peroxide to produce hydroxyl radical, which can be detected by electron spin resonance and HPLC. The resulting hydroxyl radicals are presumably responsible for tissue damaging effects. Cr(III) is capable of producing free radicals from both hydrogen peroxide and lipid hydroperoxides. Incubation of chromium chloride with hydrogen peroxide at physiological pH generated hydroxyl radical. And diethylenetriamine pentacetic acid significantly reduced hydroxyl radical yield. Incubation Cr(III) with hydroperoxide compounds resulted in the generation of lipid hydroperoxide-derived free radicals (Shi et al, 1993). Assessment of the enhanced production of reactive oxygen species for exposure

to Cr(III) compounds in cultured macrophage J774A.1 cells found that the lipid peroxidation, superoxide anion, hydroxyl radical production and DNA fragmentation are determined to increase up to 1.0 to 1.8 folds (Bagchi et al, 1997b). Balamurugan et al (2002) observed that Cr(III)-induced apoptosis involved the generation of ROS in human peripheral blood lymphocytes by examination of morphology, flow cytometry and DNA fragmentation. Pre-treatment of lymphocytes with antioxidants completely abrogate apoptosis.

1.3.2 Formation of intermediate: reactive Cr(V) and Cr(IV)

Chromium intermediates, including Cr(IV) and Cr(V), possibly generated during the metabolic reduction of Cr(VI) to Cr(III). Since Cr(V) is a putative DNA-damaging species that may be involved in the carcinogenic activity of Cr(VI) compounds. Cr(VI) does not react with isolated DNA, the reduction of Cr(VI) to lower oxidation states after entering the human system has been considered an important step in Cr(VI)-induced DNA damage and carcinogenesis. Liu and Shi's (2001) studies in vitro and in model systems indicated that Cr(V) and its related free radical formation are involved in the mechanism of Cr(VI)-induced toxicity and carcinogenesis. Electron spin resonance measurements on solutions and isolated powders provide direct evidence for the formation of long-lived Cr(V) intermediates in the reduction of Cr(VI) by glutathione reductase in the presence of NADPH and for the hydroxyl radical formation during the glutathione reductase catalyzed reduction of Cr(VI). Cr(V) intermediates catalyze

generation of hydroxyl radicals from hydrogen peroxide through a Fenton-like reaction (Shi and Dalal, 1989; 1990). Dillon et al (1998) observed that two Cr(V) complexes were genotoxic and exhibited similar potencies to that of Cr(VI) in vitro micronucleus assay in V79 Chinese hamster lung cells. On the histology of mice liver, Cr(VI) and Cr(V) produced reversible hepatic damage in a time-dependent manner. Cr(V) toxic effects had proved to be more rapid than with Cr(VI) (Pires das Neves et al, 2002). In Hojo et al's research (1999), lipid peroxidation induction by Cr(IV) and Cr(V) compounds is more effective in both liver and kidney homogenates than that by the Cr(VI) compounds. These results suggest the possible participation of Cr(IV) and Cr(V) in Cr(VI)-enhanced toxicity.

1.3.3 Role of physiological antioxidants

Two steps affect Cr(VI) reduced to Cr(III): (1) the diffusion of the Cr(VI) into cells by non-specific anion carrier and (2) the intracellular reduction of Cr(VI) to Cr(III) keeping the cytoplasmic concentration of Cr(VI) low (Arslan et al, 1987). On physiological condition, there are a great diversity of intracellular reductants or antioxidants being proposed to contribute to the reduction of Cr(VI), including both non-enzymatic and enzymatic pathways. Non-enzymatic pathway involves compounds such as reduced glutathione, cysteine, hydrogen peroxide, and the vitamins (ascorbic acid, α-tocopherol and riboflavin). Enzymatic reduction has also been demonstrated by glutathione reductase, DT-diaphorase, NADPH cytochrome c reductase and cytochrome P450 dependent

systems. The contribution of these non-enzymatic and enzymatic pathways will vary in different cells and tissues of the body and also under different pathophysiological condition (Shi et al, 2004; Gunaratnam and Grant, 2001).

1.3.3.1 Glutathione (GSH) and cysteine

In living cells, the most abundant low molecular thiol is glutathione, which is known to be present at millimolar concentrations inside cells and to act as a protective physiological antioxidant in biological systems (Sugiyama, 1992). Glutathione can reduce Cr(VI) at significant rate at physiological pH in vitro, and its occurrence in millimolar concentrations in cells makes it a primary candidate for Cr(VI) reduction in vivo (Standeven and Wetterhahn, 1991). GSH is capable of reacting with Cr(VI) to produce Cr(V), Cr(IV), GSH thiol radicals and Cr(III)-GSH complexes. Cysteine is another potentially important intracellular reductant of Cr(VI). Similar to GSH, cysteine forms coordinate amino acid-Cr(III) ternary adducts with DNA, which represent potentially mutagenic lesions (O'Brien et al, 2003). Incubation of human red blood cells with an excess of Na_2CrO_4 (10 mM) decreased the GSH content of the cells to 10% of the original amount (Wiegand et al, 1984).

The role of GSH in chromate-induced toxicity was a controversial question. Appenroth and Kersten (1990) found that an increase of renal glutathione concentration by pretreatment of rats with acetylcysteine caused an increase of proteinuria caused by

$Na_2Cr_2O_7$. Furthermore, the renal glutathione concentration of young rats was lower than those of adults, and chromate-induced nephrotoxicity was expressed less in young than that in adult rats. Glutathione depleted hepatocytes were resistant to Cr(VI) toxicity and much less dichlorofluorescin oxidation occurred (Pourahmad and O'Brien, 2001). The reduction of Cr(VI) by cysteine resulted in the formation of mutagenic Cr(III)-DNA adducts in the absence of oxidative DNA damage (Quievryn et al, 2001).

In contrast, most studies suggested that GSH may play a protective role in Cr(VI)-induced toxicity. In mice, Cr(VI)-induced nephrotoxicity was accompanied by decreased renal GSH and glutathione reductase (GSSG-R) levels. Pretreatment with buthionine sulfoximine (BSO), an inhibitor of GSH biosynthesis, enhanced Cr(VI)-induced nephrotoxicity and remarkably diminished kidney GSH and GSSG-R levels. Pretreatment with glutathione methyl ester, a GSH-supplying agent, prevented Cr(VI) from exerting a harmful effect on mouse kidney and restored kidney GSH level (Hojo and Satomi, 1991). Injection (i.p.) with a thiol compound to mice immediately after injection of chromate, mortality, ornithine carbamyl transferase activity in the serum and chromium content in the liver were diminished remarkably compared with mice injected with chromate alone. Meanwhile the urinary chromium excretion was increased. The results indicated that some thiol compounds are useful for treating chromate-induced toxicity when they are given immediately after intake of chromium (Susa et al, 1994). Standeven and Wetterhahn (1991) found the effect of GSH depletion on Cr(VI)-induced

nephrotoxicity in male Sprague-Dawley rats. Cr(VI) caused a dose-dependent decrease in cell viability and intracellular reduced glutathione levels in hepatocytes and osteoblasts (Gunaratnam and Grant, 2001; Ning and Grant, 2000).

1.3.3.2 Vitamins

Vitamin C is present normally in millimolar concentrations in humans and animals. It may have antiviral, anticancer, and antimutagenic activity. Vitamin E is located in biological membranes and plays an important antioxidant role against oxidative damage of the membrane. In rats, it was observed that vitamin C and vitamin E significantly inhibited chromate-induced nephrotoxicity by measured as the concentration of chromium in renal cortex and medulla, urinary volume, protein excretion as well as blood urea nitrogen concentration (Appenroth and Winnefeld, 1998). In animal research, it has been shown that dietary pretreatment of rats or guinea pigs with vitamin E or vitamin C protected bone marrow cells from chromium(VI)-induced cytotoxicity (Chorvatovicova et al, 1991). In experimental studies, endogenous vitamin C in rat lung, liver, kidney and human plasma effectively reduces Cr(VI) to Cr(III). The administration of exogenous ascorbic acid has been advocated in the treatment of systemic chromium poisoning and chromium dermatitis to enhance the extracellular reduction of Cr(VI) to the less bioavailable Cr(III). Parenteral ascorbic acid 0.5-5 g/kg to rats significantly reduced chromium-induced nephrotoxicity when administered 30 min before parenteral sodium dichromate and up to 1 hr after parenteral sodium chromate dosing (Bradberry and Vale,

1999). The protective role of vitamin E on Cr(VI)-induced cytotoxicity and lipid peroxidation was observed in primary cultures of rat hepatocytes or in Chinese hamster V-79 cells (Susa et al, 1996; Sugiyama et al,1989a;1989b). Pretreatment with vitamin E resulted in a decrease of DNA single-strand breaks produced by Na_2CrO_4 (Sugiyama, 1991). However, under certain conditions, vitamin C acts as a prooxidant, producing H_2O_2 and free radicals. Pretreatment of human erythrocytes with vitamin C (1 mM) and vitamin E (20 μM) significantly increased chromate-induced human erythrocytes haemoglobin oxidation in a time-dependent manner (Fernandes et al, 2000).

In animal study, it is found vitamin B_2 could decrease the nephrotoxic effect of chromate in young and adult rats (Appenroth et al, 1996). Pretreatment with vitamin B resulted in a decrease of cytotoxicity after exposure to the lethal concentration of chromate (15 μM) but did not affect the cytotoxicity at sublethal concentration of Cr(VI) in Chinese hamster V-79 cells. However, in V79 cells, treatment with vitamin B_2 enhanced an increase of chromate-induced DNA lesions (Sugiyama, 1991).

1.3.3.3 Antioxidant enzymes

Some antioxidant enzymes involved in the reduction of chromium compounds, including glutathione reductase, DT-diaphorase, NADPH cytochrome c reductase and cytochrome P450 dependent systems. Glutathione reductase appears to play an important role in the enzymatic reduction of Cr(VI). Using carmustine (BCNU), an inhibitor of glutathione

reductase, markedly protected the cells from cytotoxicity in isolated rat hepatocytes and in immortalized rat osteoblasts (FFC cells). Both DT-diaphorase and cytochrome P450 play only a minor role in detoxifying Cr(VI) and/or its metabolites (Gunaratnam and Grant, 2001; Ning and Grant, 2000). In Pourahmad and O'Brien's study (2001), DT-diaphorase and glutathione reductase did not show any significant effect on Cr(VI)-induced cytotoxic alterations. However, phenylimidazole, an inhibitor of CYP2E1, and diphenyliodonium chloride (DPI), an inhibitor of P450 reductase, protected the hepatocyte against Cr(VI)-induced cytotoxicity and lipid peroxidation. Jannetto et al (2001) found cytochrome b_5 played a key role in human microsomal chromium(VI) reduction. It acted as the mediator of electron transfer to Cr(VI) in NADPH-dependent reduction of Cr(VI) process.

1.3.3.4 Other antioxidants

Besides above mentioned, the deferoxmine (DFO) and melatonin were also found to play a protective role on Cr(VI)-induced cytotxicity and lipid peroxidation. Pretreatment of primary cultures of rat hepatocytes with DFO or melatonin reduced cells from both Cr(VI)-induced cytotoxicity and lipid peroxidation (Susa et al, 1997a, 1997b). Pretreatment of human erythrocytes with DFO (4 mM) immediately inhibited chromate-induced human erythrocytes peroxidation (Fernandes et al, 2000).

1.3.4 Mitochondrial dysfunction

Isolated rat liver mitochondria have been shown to rapidly take up chromium from the medium. Incubation of chromate with isolated rat liver mitochondria in vitro resulted in the uptake and reduction of chromium(VI), as well as the formation of the reactive intermediates (chromium (V) species). Therefore, intact mitochondria might have the ability to take up and reduce chromium(VI) and produce chromium(V) species in vitro. Incubation of chromate with isolated rat liver submitochondrial particles under anaerobic conditions in vitro resulted in reduction of Cr(VI) and increased the formation of Cr(V). Since chromium(VI) is effectively metabolized by mitochondria in vitro and chromium(V), the reactive intermediate, is formed in the process, mitochondria may play a role in Cr(VI) carcinogenesis (Rossi and Wetterhahn, 1989; Rossi et al, 1988).

Cr(VI)-induced apoptosis in Chinese hamster ovary (CHO) cells involves disruption of mitochondrial stability. A dose-dependent release of mitochondrial cytochrome c (cyt c) was observed in CHO cells exposed to sodium chromate. Cyclosporin A (CsA), an immuno-suppressant, affects the mitochondria by inhibiting the mitochondrial permeability transition (MPT), thereby preventing disruption of the trans-membrane potential. Co-treatment of CHO cells with CsA inhibited the release of cyt c and markedly abrogated Cr(VI)-induced apoptosis, suggesting that the MPT plays an important role in the regulation of mitochondrial cyt c release and that this may be a critical point in the apoptotic pathway in which cells are irreversibly committed to death

(Pritchard et al, 2000). Another research showed that the oxygen consumption of isolated rat heart mitochondria was potently depressed in presence of Na_2CrO_4, when NAD-linked substrates were oxidized (Ryberg and Alexander, 1990).

1.4 Conclusion

To sum up, chromium compounds induce toxicity in many kinds of cells, animals and humans. Cr(VI) is more toxic than Cr(III) comparing with their toxicity. Cr(VI) is easier to enter biological membrane to cells than Cr(III). In cells, Cr(VI) is metabolized through Cr(V), Cr(IV), free radicals intermediates, to Cr(III) as ultimate product. So the generation of oxidative stress, lipid peroxidation, cellular metabolism and mitochondrial dysfunction induced by chromium may be the major mechanisms which result in chromium-induced toxicity.

1.5 Objectives of the study

As a main accumulation, transport and excretion organ, kidney plays an important role in chromium metabolism and is considered as the critical excretory organ of ingested chromium. Almost all of intravenously injected chromium is excreted via the urine. Besides the main excretory organ, the kidney is also the main target organ for chromium accumulation and toxicity. However, in view of above discussions it is evident that the information regarding the mechanisms of Cr-induced nephrotoxicity is at present very limited. The present study was therefore carried out to investigate the mechanisms of

chromium [Cr(VI) and Cr(III)] induced toxicity in isolated rat renal cortical slices. And the specific objectives of the research consist of the followings:

1). To study the nephrotoxicity potential (by measuring cell viability) of potassium dichromate [Cr(VI)] and chromium chloride [Cr(III)] in isolated rat renal cortical slices and hence, to compare their renal toxicity using similar protocols.

2). To study and compare the mechanisms of nephrotoxicity induced by Cr(VI) and Cr(III) involving the following aspects:

(i) The roles of various reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anion and/or hydroxyl free radicals on Cr(VI)- and Cr(III)-induced cytotoxicity (or cell viability) in isolated rat renal cortical slices.

(ii) The role of lipid peroxidation on Cr(VI)- and Cr(III)-induced cytotoxicity (or cell viability) in isolated rat renal cortical slices.

(iii)The role of various cellular antioxidants, such as glutathione, glutathione reductase, vitamin C, vitamin E, melatonin and/or deferoxamine in Cr(VI)- and Cr(III)-induced cytotoxicity (or cell viability) in isolated rat renal cortical slices.

(iv) The role of mitochondrial permeability transition on Cr(VI)-induced cytotoxicity (or cell viability) in isolated rat renal cortical slices.

Liste des coauteurs d'Article 1

Chen J. and Chakrabarti S.K. Studies of mechanism of potassium dichromate-induced nephrotoxicity in isolated rat renal cortical slices in vitro.

Jing Chen

Saroj K. Chakrabarti

Accord des coauteurs-Article 1

Identification de l'étudiant et du programme

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Description de l'article

Chen J., Chakrabarti S.K., Studies of mechanism of potassium dichromate-induced nephrotoxicity in isolated rat renal cortical slices in vitro.

Déclaration de tous les coauteurs autres que l'étudiante

À titre de coauteur de l'article identifié ci-dessous, je suis d'accord pour que Jing Chen inclus cet article dans son mémoire de maîtrise en science qui a pour titre 'Comparative study of nephrotoxicity of potassium dichromate and chromium chloride using isolated rat renal cortical slices in vitro'.

Saroj Chakrabarti	[redacted]	29 Avril 2005
Coauteur	Signature	Date

SECTION 2:

ARTICLE 1

Studies of Mechanism of Potassium Dichromate-Induced Nephrotoxicity in Isolated Rat Renal Cortical Slices *In Vitro*

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Abstract

The kidney is considered to be one of the target organs for toxicity of Cr(VI) compounds. The information regarding the mechanisms of Cr(VI)-induced nephrotoxicity is however at present very limited. The present study was therefore carried out to investigate the mechanisms of Cr(VI)(using potassium dichromate)-induced toxicity in isolated rat renal cortical slices. The dichromate [Cr(VI)] showed both concentration (0 - 4 mM) and time (0 – 3 hr) dependent cytotoxicity, as measured by MTT assay, measurement of the toxicity with diphenyl-tetrazolium bromide. The dichromate [Cr(VI)] produced both time-dependent significant depletion of nonprotein sulfhydryl (NP-SH) contents and significant increase in lipid peroxidation (LPO). Pretreatment of renal cortical slices with either mannitol or dimethylthiourea (DMTU) failed to reduce Cr(VI)-induced toxicity but reduced LPO. Pretreatment with either catalase or superoxide dismutase (SOD) failed to reduce such toxicity and LPO. However, cotreatment with either excess glutathione (GSH) or pretreatment with excess ascorbic acid (vitamin C) reduced both the Cr(VI)-induced toxicity and LPO. Although vitamin E and melatonin significantly reduced Cr(VI)-induced LPO, they failed to prevent Cr(VI)-induced cytotoxicity. Cr(VI) induced mitochondrial permeability transition (MPT), but cyclosporine A (CsA), or carnitine, or trifluoperazine (inhibitors of MPT) failed to prevent Cr(VI)-induced cytotoxicity, suggesting a non-significant role of MPT in such toxicity. Pretreatment with carmustine (BCNU) failed to prevent Cr(VI)-induced toxicity suggesting a nonsignificant role of renal glutathione reductase activity in such toxicity. The results suggest that intracellular antioxidants vitamin C and glutathione might be the most determinant factors in Cr(VI)-induced oxidative stress and subsequent nephrotoxicity in isolated rat renal cortical slices.

Introduction

Occupational exposure to chromium involves welding, chrome-plating and manufacture of chromium pigments (Verschoor et al, 1988). Renal, hepatic and dermal toxicity have been reported in industrial workers exposed to chromium(VI) (Love, 1983; Verschoor et al, 1988). Nephrotoxicity and hepatotoxicity have also been reported in experimental animals (Appenroth and Bräunlich, 1988; Hojo and Satomi, 1991; Gunaratnam and Grant, 2001; Laborda et al, 1986; Seiken et al, 1994).

Chromium reduction intermediates [Cr(V), Cr(IV) and Cr(III)] may be toxic since they may involve the production of ROS (Stohs et al, 2000; Shi et al, 1994; O'Brien and Kortenkamp, 1994), which may be generated during physiological conditions. Various types of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot}$) and hydroxyl radicals (·OH), have been implicated in Cr(VI)-induced toxicity in different cell lines, hepatocytes and other cells (Stohs et al, 2000; O'Brien and Kortenkamp, 1994); but no such information involving these types of ROS has been clearly established in renal cells. Some intracellular antioxidants are also involved in Cr(VI) reduction, like glutathione (GSH), ascorbic acid (vitamin C), α-tocopherol (vitamin E) and glutathione reductase (GSSG-R). Furthermore, contradictory results have been reported regarding the role of physiological glutathione in chromate-induced nephtotoxicity in rats (Appenroth and Kersten, 1990; Standeven and Wetterhahn, 1991). Contradictory results have also been reported regarding the role of enzymatic glutathione reductase pathway in Cr(VI)-induced cytotoxicity in isolated rat hepatocytes (Gunaratnam and Grant, 2001; Pourahmad and O'Brien, 2001). However, the

contribution of such enzymatic pathway in Cr(VI)-induced nephrotoxicity or cytotoxicity in renal cells has not been established yet. The mammalian pineal hormone melatonin and deferoxamine (DFO) have also been reported to possess antioxidant activity in scavenging free radicals (Pieri et al, 1994; Reiter et al, 1994; Susa et al, 1997a and 1997b). But their roles in Cr(VI)-induced nephrotoxicity are still unknown.

The overall objective of this study was therefore to investigate whether Cr(VI)-induced renal cellular injury (cytotoxicity) appeared to be mediated by the formation of cellular reductive metabolism and/or ROS, such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and/or hydroxyl radicals (·OH). We have therefore examined the effects of physiological antioxidants, particularly the scavengers of ROS, as well as glutathione, glutathione reductase, vitamins C and E, melatonin on potassium dichromate-induced cellular damage in isolated renal cortical slices of rats, and the results are presented in this paper.

Materials and methods

Chemicals

Potassium dichromate ($K_2Cr_2O_7$) was purchased from BDH Inc., Toronto, Canada. Glutathione (GSH) was from Aldrich Chemical Company. All other reagents and chemicals were purchased from Sigma Chemical Company.

Animals

Adult male Sprague-Dawley rats (220 – 280 g b.w.) (Charles River Canada Inc., St. Contant, Quebec) were used. They were maintained at constant temperature (22℃) with a 12 hr light/ 12 hr dark photoperiod, housed 2-3 per cage on wood shavings and were fed Purina Laboratory chow and tap water available ad libitum. Rats were acclimated for 3 to 5 days before experiments. The experimental protocols used in this study were approved by the Ethics Committee of Université de Montréal.

Preparation of isolated rat renal cortical slices and their treatment

Rats were anaesthetized with 'Somnotal' (0.1 ml/100 g b.w. i.p.). They were decapitated and exsanguinated, then the abdomen was opened; the kidneys were removed rapidly, de-capsulated, and immediately placed into ice-cold saline solution (0.14 M NaCl and 0.02 M KCl). Following isolation of the kidneys, renal cortical slices (70 to 100 mg/per slice) were prepared using a Stadie-Riggs Microtome, weighed and placed in a flask of 3 ml ice-cold waymouth buffer medium (pH 7.4). Each group contained 4 slices. Rat renal cortical slices were treated with potassium dichromate in waymouth medium (pH7.4) at 37℃ and incubated in a rotating Dubnoff metabolic shaker at 150 rpm rotation in an atmosphere of 95% oxygen and 5% carbon dioxide. After the incubation, renal cortical slices were removed from the medium, dried with a filter paper and placed into other plastic tubes containing 3 ml phosphate buffer solution (PBS, pH7.4). Renal cortical slices were then homogenized in 3 ml PBS for 30sec. Half millilitre tissue homogenate was used to examine malondialdehyde (MDA), and 1.0ml was for nonprotein-sulfhydryl group (NP-SH). The residual tissue homogenates were centrifuged at 2000×g for 5 min

to examine cell viability; 1.0 ml of the supernatant was used (M'Bemba Meka and Chakrabarti, 2001).

Determination of cell viability (cytotoxicity)

The cell viability was evaluated by the method based on the ability of mitochondrial enzyme succinate dehydrogenase in living cells to reduce the tetrazolium compound to a soluble formazan product, which can be assayed colorimetrically. One millilitre of supernatant and 0.2 ml MTT solution (2.5 mg/ml PBS) were mixed and incubated at 37℃ in water bath for 90 minutes in darkness. Absorbance was read at 570 nm using Beckman DU-7 spectrophotometer. The result was expressed as cell viability as % of control (Mosmann, 1983).

Determination of lipid peroxidation (LPO)

This was based on the formation of malondialdehyde (MDA), which reacted with thiobarbituric acid to form red color. Half millilitre of the above tissue homogenate and 1.0 ml of reagent solution (0.188 g thiobarbituric acid, 7.5 ml 10% of trichloroacetic acid and 1.1 ml concentrated HCl mixed with distilled water to 50 ml volume) were mixed in darkness, incubated at boiling water for 15 minutes, and then cooled in ice water immediately to stop the reaction. The solution was then centrifuged at 1100×g for 15 minutes. The absorbance of the supernatant was recorded at 535 nm using Beckman DU-7 spectrophotometer. The concentration of MDA (nmol/g tissue) was calculated using a molar extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$ (Poli et al. 1979, Valentovic et al, 1999). The MDA was expressed as % of control.

Determination of nonprotein-sulfhydryl groups (NP-SH)

The method was based on colorless DTNB reduced by SH group to form yellow 2-nitro-5-thiobenzoic acid, using L-cysteine as standard. One millilitre of homogenates were mixed with 0.8 ml distilled water and 0.2 ml of 50% trichloroacetic acid (TCA). The tubes were centrifuged for 15 min at 3000×g. Half millilitre supernatant was mixed with 1.0 ml of 0.4 M Tris buffer (pH 8.9) and 0.025 ml DTNB. The absorbance was read at 412 nm using Beckman DU-7 spectrophotometer within 5 min of DTNB addition (Sedlak and Lindsay, 1968). The result was finally calculated as L-cysteine as % of control.

Determination of mitochondrial permeability transition (MPT)

The mitochondrial permeability transition (MPT) was measured as the uptake of rhodamine 123, a cationic fluorescent dye that distributed across mitochondrial membranes with respect to the trans-membrane potential. After being exposed to 1mM $K_2Cr_2O_7$, the renal cortical slices was taken out from the incubation medium (waymouth medium) and placed in Hanks' Balanced Salt Solution (HBSS) containing 1.5 μM/ml of rhodamine 123, and incubated at 37℃ for 10min in a rotating metabolic shaker (150rpm), and then change the slices to another 3ml HBSS solution, respectively. Following homogenisation and centrifugation, the uptake of rhodamine 123 in supernatant was measured fluorometrically using a Perkin-Elmer MPF 3L fluorescence spectrophotometer with excitation wavelength at 500 nm and emission wavelength at 522 nm. The capacity of mitochondria to take up the cationic dye was calculated and expressed as percentage of the total fluorescence of rhodamine 123 in the supernatant (Chakrabarti and Denniel, 1996; Wu et al, 1990).

Statistics

The data were presented as the mean±SEM for 3-4 separate experiments. Differences between groups were assessed by one-way analysis of variance (ANOVA, Tukey analysis) or t-student test. $P<0.05$ was considered as statistically significant.

Results

Cr(VI)-induced cytotoxicity (Cell viability), lipid peroxidation (MDA formation) and nonprotein sulfhydryl (NP-SH) contents in isolated rat renal cortical slices

The results of cell viability, MDA formation and NP-SH contents were summarized in Fig.2-1 and Fig.2-2. In concentration-dependent study (Fig.2-1), the isolated rat renal cortical slices were treated with 0, 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 mM $K_2Cr_2O_7$ for 120 min. With increasing concentrations of Cr(VI), cell viability was decreased significantly in a concentration-dependent manner. MDA showed significant increase from 0.5 mM of Cr(VI) compared to the control. NP-SH contents were significantly increased at low concentration (0.125 mM) followed by significant decrease from 1.0 mM when compared with control. In time-dependent study (Fig.2-2), the isolated rat renal cortical slices were incubated with 1.0mM $K_2Cr_2O_7$ for 30, 60, 90, 120 and 180min. Cr(VI) significantly reduced cell viability after 30 min of incubation and increased LPO after 90 min. NP-SH contents showed a temporary increase at the beginning and then gradually decrease. After incubation for 90 min, NP-SH was significantly lower than that of control. It is seen that

Cr(VI)-induced cytotoxicity occurred much earlier (30 min) than LPO (90 min) and NP-SH depletion (90 min) in isolated rat renal cortical slices.

Effects of ROS on Cr(VI)-induced cell viability and LPO in isolated rat renal cortical slices

The effects of ROS (such as H_2O_2, superoxide anion O_2^- and hydroxyl radicals) on dichromate-induced cytotoxicity in isolated rat renal cortical slices were studied indirectly by using scavengers of ROS. Thus, renal cortical slices were pretreated for 25 min with either catalase (scavenger of hydrogen peroxide, H_2O_2) (0, 2500 and 5000 U/ml), or superoxide dismutase (scavenger of superoxide anion, O_2^-) (0, 1350 U/ml), or mannitol (scavenger of hydroxyl radicals, ·OH) (0, 25, 50 mM), or dimethylthiourea (DMTU) (scavenger of ·OH) (0, 25, 50 mM) followed by treatment with 0.5 mM $K_2Cr_2O_7$ for 90 min and the cell viability was then measured in each case. The results are presented in Fig.2-3. It is seen that the scavengers of different ROS failed to prevent Cr(VI)-induced cytotoxicity. However, pre-treatment with either mannitol or DMTU significantly reduced Cr(VI)-induced LPO, suggesting that reactive hydroxyl groups were involved in such LPO. The antioxidants alone at concentrations used had no effects on cytotoxicity or LPO compared to control (results not shown here).

The protective roles of antioxidants on Cr(VI)-induced cytotoxicity and LPO in isolated rat renal cortical slices

To examine the roles of other cellular antioxidants on Cr(VI)-induced cytotoxicity and LPO, the isolated rat renal cortical slices were treated with 0.5 mM $K_2Cr_2O_7$ for 90 min

in the absence and presence of different antioxidants, such as reduced GSH (4 and 8 mM), ascorbic acid (vitamin C) (pretreatment: 2.5 mM and 5 mM for 45 min), (±)-α-tocopherol (vitamin E) (pretreatment: 50 mM and 100 mM for 180 min), melatonin (pretreatment: 5 mM and 10 mM for 60 min), and deferoxamine mesylate (DFO) (pretreatment: 100 μM for 15 min). As seen from Fig.2-4, excess GSH and vitamin C showed the ability to prevent Cr(VI)-induced cell viability loss and inhibit Cr(VI)-induced MDA formation. Although vitamin E and melatonin could reduce Cr(VI)-induced LPO, they failed to reduce Cr(VI)-induced cytotoxicity. DFO also could not reduce Cr(VI)-induced cytotoxicity. The antioxidants alone at concentrations used had no effects on cytotoxicity and LPO when compared to the respective control (results not shown here). These results indicated that only GSH and Vit.C have the potential to protect Cr(VI)-induced cytotoxicity and LPO.

Effects of glutathione reductase (GSSG-R) on Cr(VI)-induced cytotoxicity in isolated rat renal cortical slices

To investigate the role of glutathione reductase (GSSG-R) on Cr(VI)-induced cytotoxicity, isolated rat renal cortical slices were treated with 0.5 mM $K_2Cr_2O_7$ for 90 min in the absence and presence of either GSSG-R (pre-treatment: 80 U/ml for 15 min) or carmustine (BCNU) (an inhibitor of GSSG-R, pre-treatment: 1 and 2 mM for 30 min). The results are summarized in Fig.2-5. Pre-treatment of excess GSSG-R does not significantly change Cr(VI)-induced cytotoxicity. Inhibition of GSSG-R by BCNU also does not protect Cr(VI)-induced cytotoxicity. Carmustine and GSSG-R alone at concentrations used had no effect on cytotoxicity when compared to control (data not

shown here). The results indicate that glutathione reductase did not play an important role in Cr(VI)-induced cytotoxicity in isolate rat renal cortical slices in vitro.

The role of mitochondrial permeability transition (MPT) on Cr(VI)-induced cytotoxicity in isolated rat renal cortical slices

To evaluate the role of mitochondrial permeability transition (MPT) on Cr(VI)-induced cytotoxicity, time-dependent effect of Cr(VI)-induced MPT and the role of MPT blockers on Cr(VI)-induced cytotoxicity were studied. The results are summarized in Fig.2-6 to Fig.2-8. Fig.2-6 showed Cr(VI)-induced MPT (rhodamine 123 uptake) significantly decreased starting from 10 min to 60 min when compared to the control value. Pretreatment (Fig.2-7) or cotreatment (Fig.2-8) of renal cortical slices with MPT blockers, such as cyclosporin A, carnitine and trifluoperazin, did not reduce Cr(VI)-induced cytotoxicity, indicating Cr(VI)-induced cytotoxicity did not result from Cr(VI)-induced MPT. The MPT blockers alone at concentrations used had no effect on MPT compared to the control value (data not shown here).

Discussion

Acute exposure to Cr(VI) compounds has been reported to produce acute necrosis of renal tubules (Franchini et al, 1978) and renal lipoperoxidation in humans (Huang et al, 1999). This is in agreement with our present study. The biological effect of chromium(VI) is generally considered to be due to cellular uptake because

chromium(VI), in contrast to chromium(III), actively enters cells by the sulfate anion transport system (Jennette, 1979; Costa et al, 1984). Once inside cells, chromium(VI) is believed to be subsequently reduced through reactive intermediates to chromium(III) by cellular reductants. A great diversity of intracellular reductants have been proposed to contribute to the reduction of Cr(VI), including both non-enzymatic and enzymatic mechanisms. Non-enzymatic mechanisms involving compounds such as reduced glutathione (GSH), cysteine, hydrogen peroxide, and the vitamins (like vitamin C and vitamin E) have been proposed (Sugiyama et al, 1991 and 1989). Enzymatic reduction has been demonstrated by glutathione reductase (Ning and Grant, 2000; Shi and Dalal, 1989), DT-diaphorase (DeFlora et al, 1985) and NADPH cytochrome C reductase and cytochrome P-450-dependent system (Cupo and Wetterhahn, 1985) in cellular systems other than renal cells.

The present study has shown that various reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anion, and hydroxyl radicals, have failed to influence the potassium dichromate-induced cytotoxicity in rat renal cortical slices, as verified indirectly by the failure of scavengers of these ROS to reduce such cytotoxicity. Such a failure of Cr(VI)-induced ROS to influence other toxicities has also been reported. Thus, the cytotoxicity induced by Cr(VI) in human fibroblasts as determined by colony-forming assay was not affected by catalase treatment (Snyder, 1988). Similarly, treatments with catalase (H_2O_2 scavenger), mannitol (hydroxyl radical scavenger), superoxide dismutase and singlet oxygen scavengers such as 1,4-diaza-bicyclo[2,2,2]octane (DABCO) did not

influence chromium(VI)-induced cytotoxicity in isolated rat hepatocytes, as evaluated by the leakage of lactate dehydrogenase (Ueno et al, 1989).

On the other hand, our present study has shown that scavengers of hydroxyl radicals, such as mannitol and dimethylthiourea (DMTU) have the potential to significantly reduce potassium dichromate-induced lipid peroxidation in rat renal cortical slices, suggesting that Cr(VI)-induced generation of hydroxyl radicals are responsible for Cr(VI)-induced lipid peroxidation. Cr(VI) is thought to undergo redox cycling resulting in increased production of such reactive oxygen species (Stohs et al, 2000). The observation that hydroxyl radical scavengers reduced dichromate-induced lipid peroxidation, but not renal cytotoxicity suggests that Cr(VI)-induced lipid peroxidation is not responsible for Cr(VI)-induced cytotoxicity in rat renal cortical slices.

A number of electron spin resonance (ESR) studies have directly identified the formation of paramagnetic Cr(V) species during reduction of Cr(VI) by reduced GSH (Sugiyama, 1992). Our results of inhibition of Cr(VI)-induced cytotxicity in rat renal cortical cells by excess glutathione are in agreement with the in vivo study by Standeven and Wetterhahn (1991). Thus, depletion of GSH by buthionine sulfoximine resulted in decreased body weight gain, increased relative kidney weight, and increased blood urea nitrogen concentration without affecting the renal uptake of chromate in the metal-treated rats (Standeven and Wetterhahn, 1991). However, other study has shown an increase in Cr(VI)-induced proteinuria following pretreatment of rats with N-acetylcysteine (Appenroth and Kersten, 1990). The temporary increase of NP-SH contents in renal

cortical slices at either lower concentration of Cr(VI), or at early time period of incubation might be due to the release of protein-sulfhydryl to protein and NP-SH, due to the protein breakdown and hence, GSH biosynthesis. However, at higher concentration of dichromate [Cr(VI)], significant GSH depletion occurs, presumably due to the production of reactive intermediates, or ROS at a rate that exceeds the potential to regenerate GSH. Both Cr(VI)-induced cytotoxicity and lipid peroxidation were inhibited by cotreatment with excess GSH (4 amd 8 mM). This effect might be due to Cr(VI) reduced to Cr(III) by GSH in the medium before it entered into renal cells, because only Cr(VI) could enter cells readily and once inside the cells it is reduced by intracellular reductant to a more stable Cr(III). It is generally accepted that intracellular reduction of Cr(VI) is critical for toxicity. If Cr(VI) is reduced to Cr(III) extracellularly, it will be difficult to enter the cells and produce the toxicity in cells (Ning and Grant, 2000).

This study has shown that ascorbate pretreatment has significantly increased the cell viability reduced by dichromate, whereas such pretreatment significantly prevented dichromate-induced increase in lipid peroxidation. On the other hand, α-tocopherol pretreatment only prevented Cr(VI)-induced increase in lipid peroxidation without protecting the cell viability. Similarly, cell survival as measured by colony-forming efficiency assay showed that ascorbate, but not α-tocopherol, protected Chinese hamster ovary cells (CHO-AA8 cell line) from apoptosis induced by sodium chromate (Blankenship et al, 1997).

In vivo nephrotoxicity study has shown great differences in the protective capacity against dichromate-induced nephrotoxicity between ascorbate and GSH. Pretreatment with ascorbate completely abolished both the metabolic disturbance and nephrotoxicity induced by dichromate (Na et al, 1992). The results suggest that ascorbate-mediated Cr(VI) reduction in kinetically more favorable than that of GSH in vivo. GSH levels are about 5-fold higher than the ascorbate levels in the liver and kidney (Standeven and Wetterhahn, 1991). In vitro studies have shown that ascorbate reduces Cr(VI) at a faster rate than GSH under physiological conditions (Connett and Wetterhahn, 1985; Suzuki and Fukuda, 1990).

Ascorbate has been shown to be capable of reducing chromium(VI) to chromium(III) (Connett and Wetterhahn, 1983). The ESR signal of chromium(III) can be detected immediately following the reaction of ascorbic acid and Na_2CrO_4 (Sugiyama et al, 1991). Since pretreatment with this reductant showed an increase in cellular levels of chromium(III), the modification of the formation of those paramagnetic chromium species by ascorbic acid might be caused by cellular reduction of chromum(VI) to chromium(III) in cells. Ascorbic acid has been reported to be present normally in millimolar concentrations (2 - 5 mM) in humans and animal tissues (Horning, 1975) and to be a more potent reductant of Cr(VI) than glutathione under physiological conditions (Suzuki and Fukuda, 1990). Vitamin E appears to play a role only as a scavenger of hydroxyl radicals to reduce LPO caused by Cr(VI), but not cytotoxicity.

Enzymatic reduction of Cr(VI) involving glutathione reductase resulted in toxicity in cellular systems (Ning and Grant, 2000; Gunaratnam and Grant, 2001; Shi and Dalal, 1989). On the other hand, i.p. injection of sodium dichromate did not inhibit hepatic glutathione reductase activity even at toxic doses (Standeven and Wetterhahn, 1991). Similarly, pretreatment with carmustine (BCUN) (an inhibitor of glutathione reductase) did not protect renal cortical cells from Cr(VI)-induced cytotoxicity, suggesting that glutathione reductase does not play any role in the intracellular reduction of Cr(VI) in isolated rat renal cortical slices.

It is reported that DFO (an iron chelator) and melatonin (the pineal hormone) markedly decreased potassium dichromate-induced DNA single-strand breaks as well as Cr(VI)-induced cytotoxicity and LPO in rat hepatocytes in culture (Susa et al, 1997a and 1997b). In this study, DFO did not reduce cytotoxicity in renal cortical cells and melatonin only significantly suppressed Cr(VI)-induced lipid peroxidation, without reducing cytotoxicity in renal cortical cells. It is reported that melatonin had no effect on cellular levels of GSH, the activity of glutathione reductase, superoxide dismutase. Similarly, cellular uptake and distribution of Cr were not affected by melatonin. However, melatonin caused a 25% decrease in the levels of Cr(VI)-related hydroxyl radicals in rat hepatocytes in culture in vitro (Susa et al, 1997b). Based on their study and our present study of significant reduction of Cr(VI)-induced increase in LPO by mannitol and DMTU (scavengers of hydroxyl radicals), it is suggested that melatonin may significantly reduce such increase in LPO by scavenging Cr(VI)-induced generation of toxic hydroxyl radicals in renal cortical cells.

Cr(VI) inhibits mitochondrial respiration in vitro (Rossi and Wetterhahn, 1989). The present study has shown that dichromate induced MPT in isolated rat renal cortical slices; but failure to prevent Cr(VI)-induced toxicity following pretreatment with permeability transition pore sealing agents, such as cyclosporin A, carnitine and trifluoperazine, suggests a nonsignificant role of MPT in dichromate-induced renal toxicity. In contrast, Cr(VI) (CrO_4^{2-})-induced cytotoxicity was associated with Cr(VI)-induced MPT in isolated rat hepatocytes (Pourahmad et al, 2005).

In conclusion, mechanisms of potassium dichromate-induced cytotoxicity in isolated rat renal cortical slices are somewhat different from those in other cellular systems including isolated rat hepatocytes. The reactive oxygen species, such as H_2O_2, superoxide anion and hydroxyl radicals failed to show any significant role in potassium dichromate [Cr(VI)]-induced renal cytotoxicity, and only hydroxyl radicals played a significant role in such Cr(VI)-induced lipid peroxidation. Renal glutathione reductase and mitochondrial permeability transition appeared to play a nonsignificant role in such Cr(VI)-induced cytotoxicity. On the other hand, the present study does suggest that intracellular antioxidants such as Vitamin C and glutathione played a significant role not only in such Cr(VI)-induced oxidative stress but also in cytotoxicity in rat renal cortical slices.

Figure legend

Fig.2-1. Concentration-dependent effects of Cr(VI) on cytotoxicity, LPO and NP-SH content after exposure to $K_2Cr_2O_7$ for 120 min in isolated rat renal cortical slices. Cytotoxicity was estimated by the MTT test, lipid peroxidation by the MDA formation, and NP-SH contents by the DTNB test. The results were presented as percent of control value. The data were mean ± SEM of four separate experiments.

*: significantly different from the corresponding control, $p<0.05$.

Fig.2-2. Time-dependent effects of Cr(VI) on cytotoxicity, LPO and NP-SH content after exposure at 1 mM $K_2Cr_2O_7$ in isolated rat renal cortical slices. Cytotoxicity was estimated by the MTT test, lipid peroxidation by the MDA formation, and NP-SH contents by the DTNB test. The results were presented as percent of control value. The data were mean ± SEM of four separate experiments.

a: significantly different from the corresponding control, $P<0.05$

b: significantly different from the corresponding control, $P<0.05$

c: significantly different from the corresponding control, $P<0.05$.

Fig.2-3. Effects of scavengers of ROS on Cr(VI)-induced cytotoxicity and LPO in isolated rat renal cortical slices. All the scavengers of ROS were pre-treated with isolated rat renal cortical slices for 25 min followed by coincubation with 0.5 mM $K_2Cr_2O_7$ [Cr(VI)] for 90 min. The cell viability and MDA were measured as described

in the text. The results were presented as percent of control value. The data were mean ± SEM of four separated experiments.

a: significantly different from the corresponding control, $P<0.05$.

b: significantly different from corresponding Cr(VI) alone-treated group, $P<0.05$.

Fig.2-4. Effects of antioxidants on Cr(VI)-induced cytotoxicity and LPO in isolated rat renal cortical slices. Excess GSH (4 mM and 8 mM) were cotreated with 0.5 mM $K_2Cr_2O_7$ for 90 min, whereas other antioxidants, Vit.C (2.5 mM and 5.0 mM), or Vit.E (50 mM and 100 mM), or melatonin (5 mM and 10 mM), or deferoxamine (DFO, 100 μM), were pre-treated for 45 min, 180 min, 60 min and 15 min, respectively, before treating with 0.5 mM $K_2Cr_2O_7$ for 90 min. The results were presented as percent of control value. The data were mean ± SEM of four separate experiments.

a: significantly different from corresponding control, $p<0.05$.

b:, significantly different from corresponding Cr(VI) alone-treated group, $p<0.05$.

Fig.2-5. The role of GSSG-R and BCNU on Cr(VI)-induced cytotoxicity in isolated rat renal cortical slices. GSSG-R (80 U/ml) and BCNU (1 mM and 2 mM) are pretreated for 15 min and 30 min, respectively, before exposure to 0.5 mM $K_2Cr_2O_7$. The results were presented as percent of control value. The data were mean ± SEM of four separate experiments.

*: significantly different from control, $P<0.05$.

Fig.2-6. Time-dependent effect of Cr(VI) on MPT after exposure to 1 mM $K_2Cr_2O_7$ in isolated rat renal cortical slices. The results were presented as percentage of the total fluorescence of rhodamine 123. The data were mean ± SEM of four separate experiments.

*: significantly different from the control, $P<0.05$

Fig.2-7. Effects of cyclosporine A (CsA) pre-treatment on Cr(VI)-induced cytotoxicity after exposure at 0.5 mM $K_2Cr_2O_7$ for 90 min in isolated rat renal cortical slices. CsA (15, 30 and 60 μM) are pre-treated for 30 min before exposure to 0.5 mM $K_2Cr_2O_7$. The results were presented as percent of control value. The data were mean ± SEM of four separate experiments.

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

Fig.2-8. Effects of MPT blockers cotreatment on Cr(VI)-induced cytotoxicity after exposure at 0.5 mM $K_2Cr_2O_7$ for 90 min in isolated rat renal cortical slices Effects of cotreatment with different MPT blockers on Cr(VI)-induced cytotoxicity after exposure to 0.5 mM $K_2Cr_2O_7$ for 90 min. The results were presented as percent of control value. The data were mean ± SEM of four separate experiments.

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

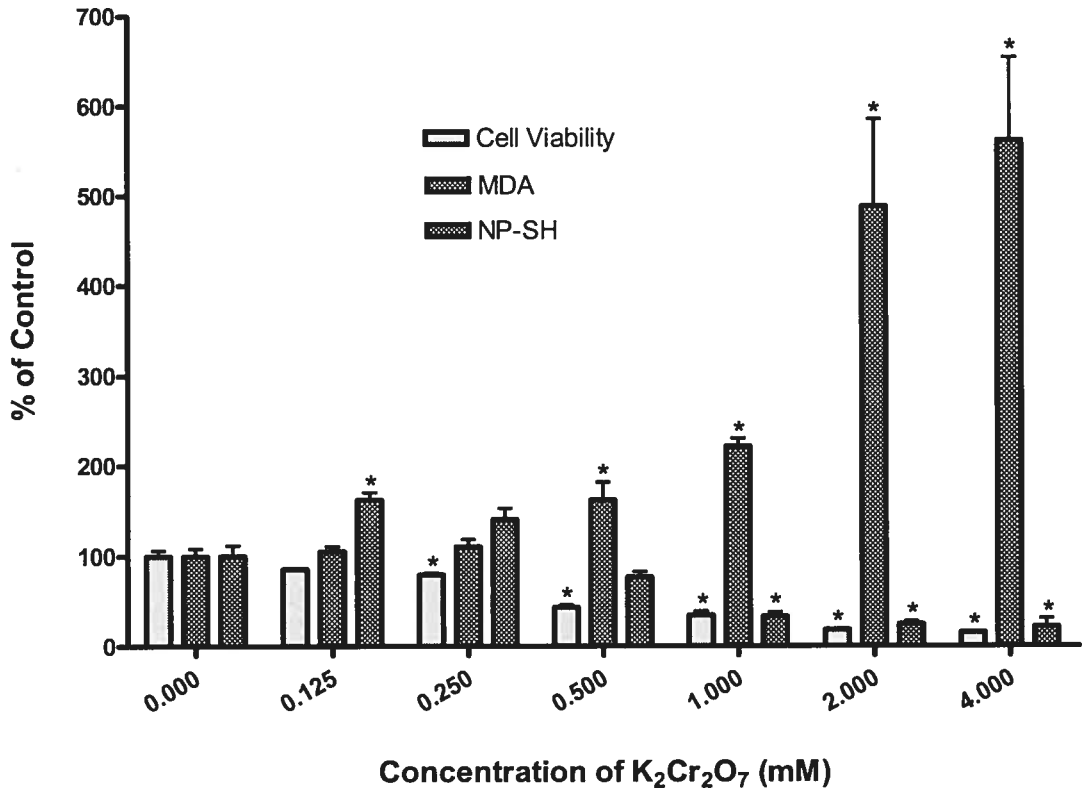
700
600
500
400
300
200
100
0
% of Control
Cell Viability
MDA
NP-SH
0.000
0.125
0.250
0.500
1.000
2.000
4.000
Concentration of $K_2Cr_2O_7$ (mM)

Fig.2-1

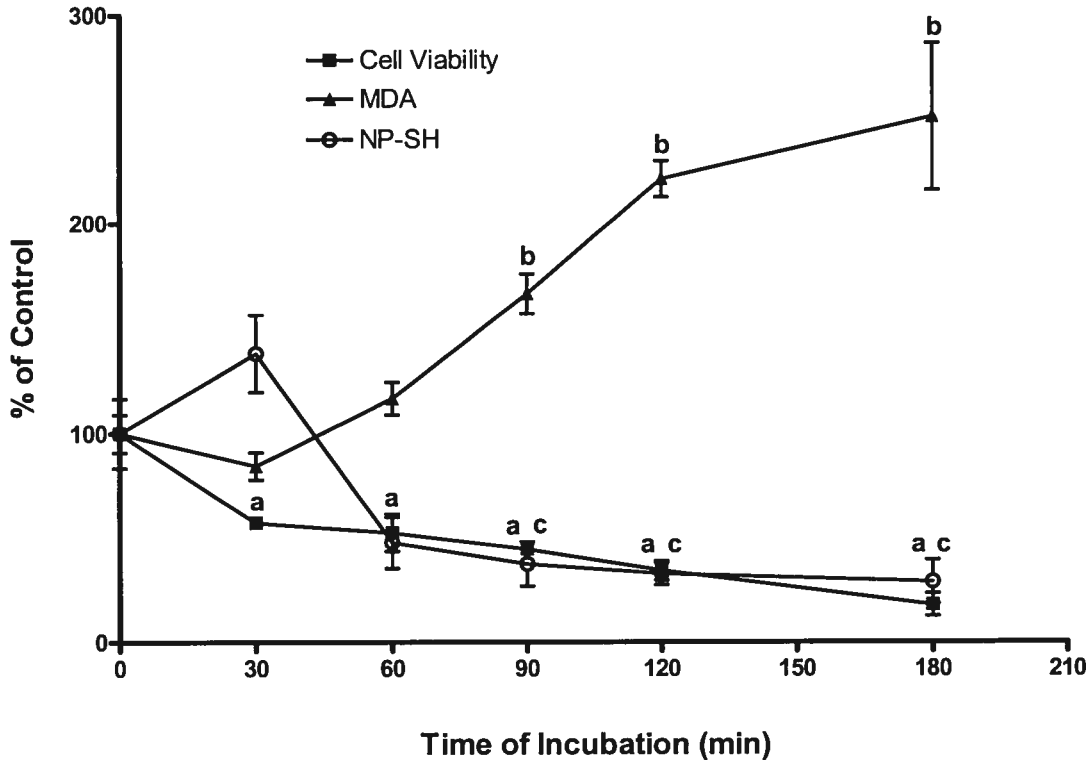
300
200
100
0
% of Control
Cell Viability
MDA
NP-SH
b
b
b
a
a c
a c
a c
0
30
60
90
120
150
180
210
Time of Incubation (min)

Fig.2-2

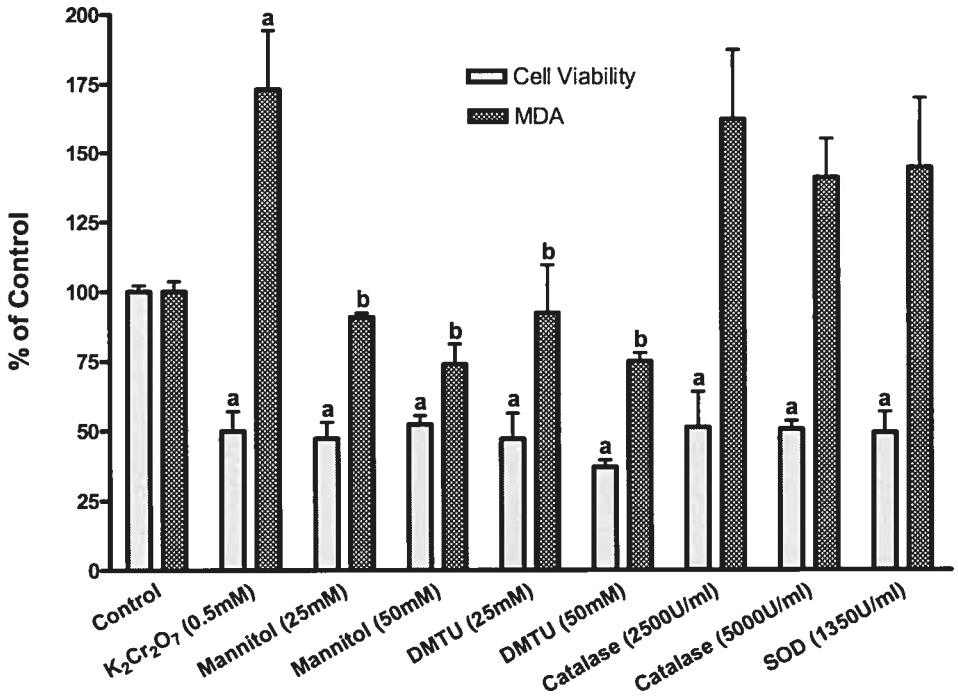
% of Control
200
175
150
125
100
75
50
25
0
Cell Viability
MDA
a
b
Control
$K_2Cr_2O_7$ (0.5mM)
Mannitol (25mM)
Mannitol (50mM)
DMTU (25mM)
DMTU (50mM)
Catalase (2500U/ml)
Catalase (5000U/ml)
SOD (1350U/ml)

Fig.2-3

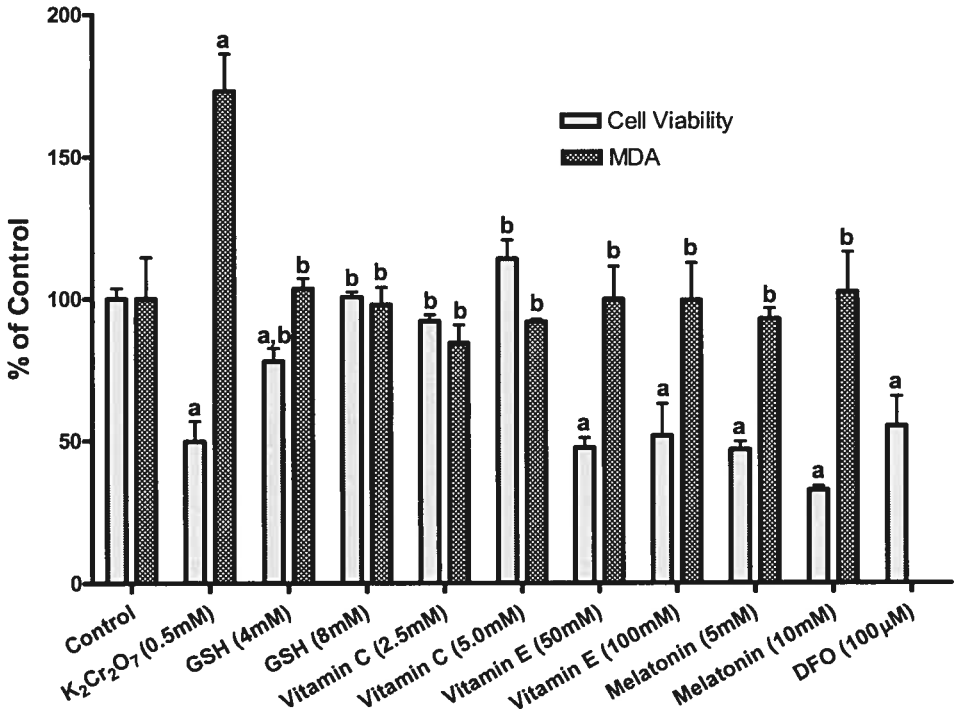
200
150
100
50
0
% of Control
Cell Viability
MDA
a
a
a,b
b
b
b
b
b
a
b
a
b
a
b
a
b
Control
K2Cr2O7 (0.5mM)
GSH (4mM)
GSH (8mM)
Vitamin C (2.5mM)
Vitamin C (5.0mM)
Vitamin E (50mM)
Vitamin E (100mM)
Melatonin (5mM)
Melatonin (10mM)
DFO (100μM)

Fig.2-4

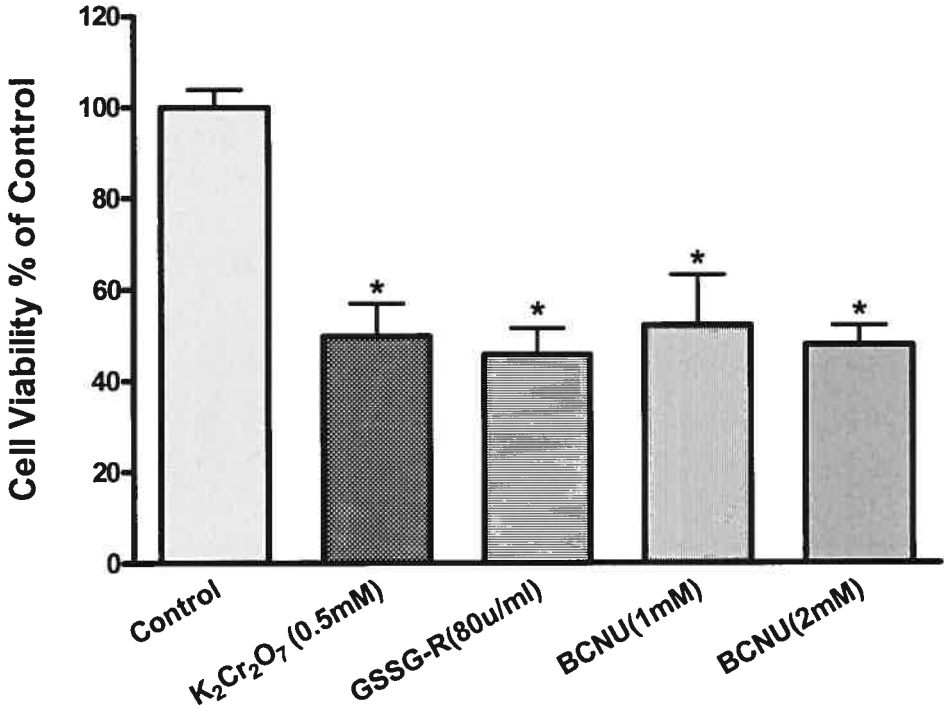
Cell Viability % of Control
120
100
80
60
40
20
0
*
*
*
*
Control
$K_2Cr_2O_7$ (0.5mM)
GSSG-R(80u/ml)
BCNU(1mM)
BCNU(2mM)

Fig.2-5

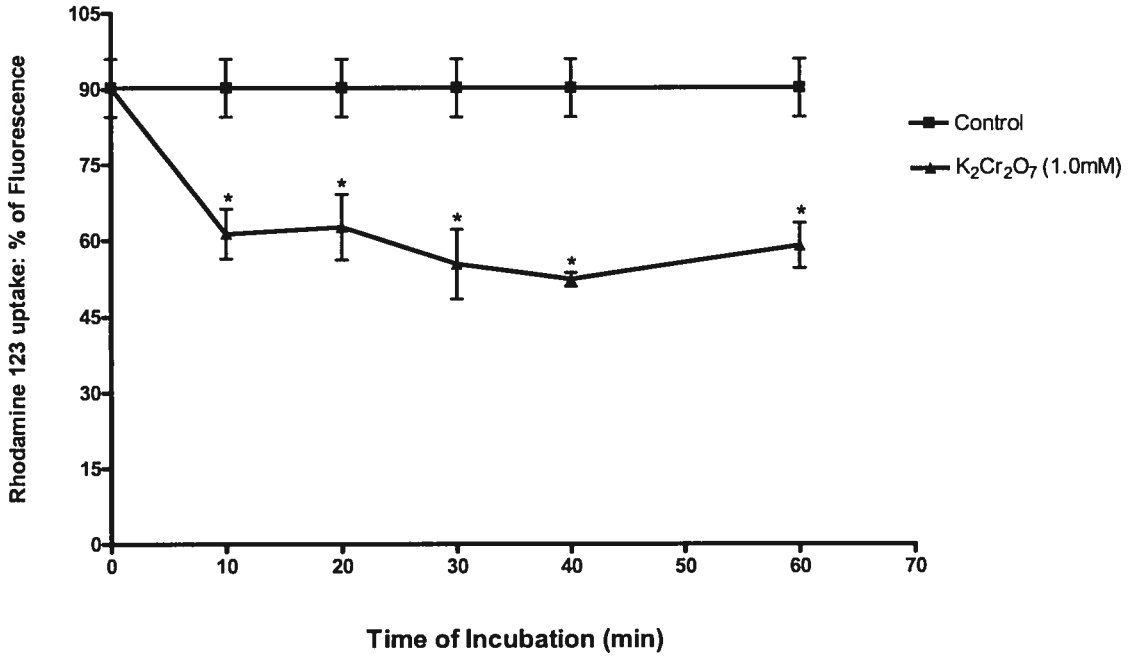
Rhodamine 123 uptake: % of Fluorescence
105
90
75
60
45
30
15
0
0
10
20
30
40
50
60
70
Time of Incubation (min)
Control
$K_2Cr_2O_7$ (1.0mM)
*

Fig.2-6

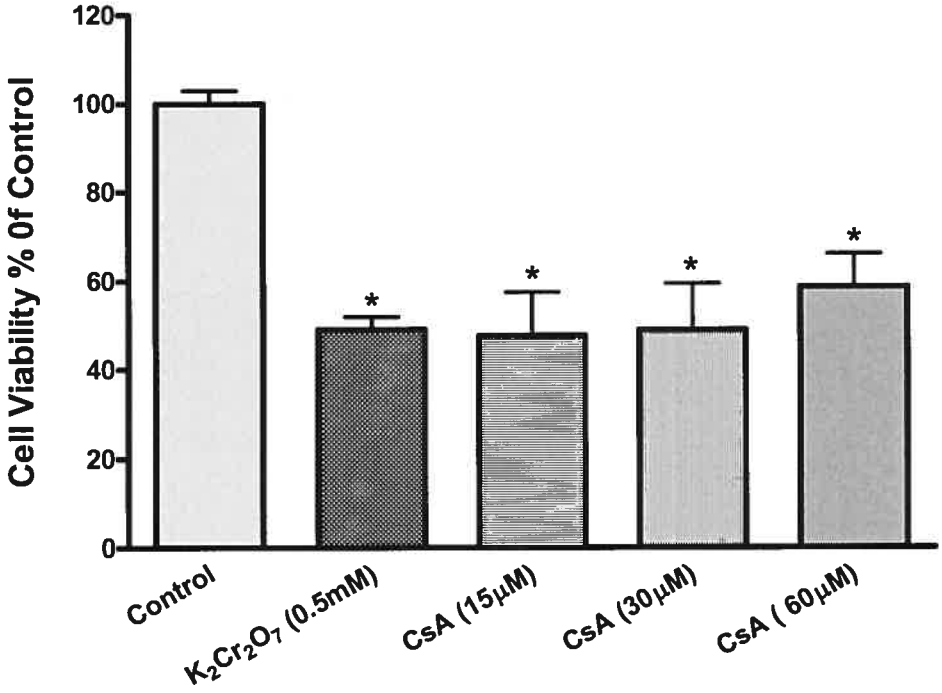
Cell Viability % 0f Control
120
100
80
60
40
20
0
*
*
*
*
Control
$K_2Cr_2O_7$ (0.5mM)
CsA (15μM)
CsA (30μM)
CsA (60μM)

Fig.2-7

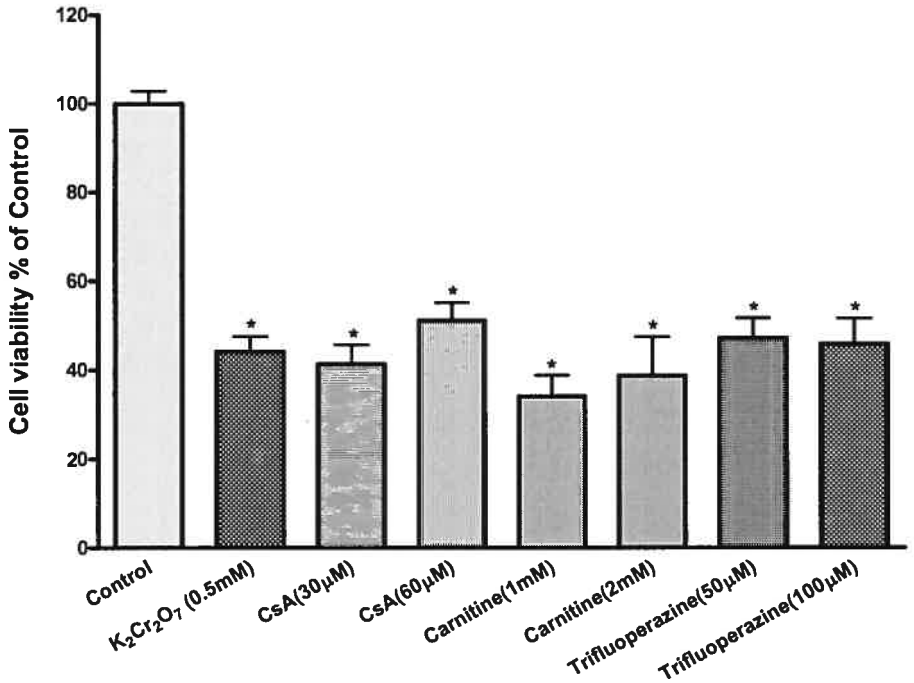
Cell viability % of Control
120
100
80
60
40
20
0
Control
$K_2Cr_2O_7$ (0.5mM)
CsA(30μM)
CsA(60μM)
Carnitine(1mM)
Carnitine(2mM)
Trifluoperazine(50μM)
Trifluoperazine(100μM)

Fig.2-8

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Liste des coauteurs d'Article 2

Chen J. and Chakrabarti S.K., Role of oxidative stress in chromium chloride-induced nephrotoxicity in isolated rat renal cortical slices.

Jing Chen

Saroj K. Chakrabarti

Accord des coauteurs-Article 2

Identification de l'étudiant et du programme

Jing Chen

Étudiante de maîtrise en santé environnementale et santé au travail

Description de l'article

Chen J., Chakrabarti S.K., Role of Reactive Oxygen Species on chromium chloride-induced nephrotoxicity in isolated rat renal cortical slices in vitro.

Déclaration de tous les coauteurs autres que l'étudiante

À titre de coauteur de l'article identifié ci-dessous, je suis d'accord pour que Jing Chen inclus cet article dans son mémoire de maîtrise en science qui a pour titre 'Comparative study of nephrotoxicity of potassium dichromate and chromium chloride using isolated rat renal cortical slices in vitro'.

Saroj Chakrabarti	[redacted]	29 Avril 2005
Coauteur	Signature	Date

SECTION 3:

ARTICLE 2

Role of Oxidative Stress in Chromium Chloride-Induced Nephrotoxicity in Isolated Rat Renal Cortical Slices

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Key words: nephrotoxicity, chromium chloride, reactive oxygen species, lipid peroxidation, glutathione, vitamins

Abstract

Trivalent chromium compounds [Cr(III)] have been found to be nephrotoxic at high concentration. However, less information is available regarding the mechanism of Cr(III)-induced nephrotoxicity. In this study, isolated rat renal cortical slices were exposed to different concentrations of chromium chloride (0-4mM) for 240min. Both concentration- and time-dependent Cr(III)-induced cytotoxicity (as measured by MTT test, the measurement of the toxicity with diphenyl-tetrazolium bromide) were observed. However, neither concentration- nor time-dependent Cr(III)-induced lipid peroxidation (LPO) [as measured by malondialdehyde (MDA) formation] was observed. Treatment of renal cortical slices with mannitol or dimethyl thiourea (scavengers of hydroxyl free radicals) failed to reduce Cr(III)-induced cytotoxicity, suggesting that reactive oxygen species, such as hydroxyl free radicals, are not involved in such toxicity. Co-treatment with excess glutathione (especially 8 mM) increased Cr(III)-induced cytotoxicity and LPO. Pre-treatment of renal cortical slices with vitamin C (5 mM) significantly reduced Cr(III)-induced cytotoxicity, whereas pre-treatment with excess vitamin E failed to reduce such toxicity. These data demonstrate that chromium chloride at very high concentration is cytotoxic to rat renal cortical slices and intracellular antioxidant vitamin C (ascorbic acid) might be an important determinant factor in such $CrCl_3$-induced cytotoxicity in isolated rat renal cortical cells.

Introduction

Trivalent chromium [Cr(III)] compounds were considered before to be non-toxic. The relationship between urinary chromium and different forms of chromium has been studied in workers exposed to chromium (Matczak et al, 1990; 1995). Cr(III) compounds may produce toxicity at relatively higher concentrations and/or depending on its chemical forms (Bagchi et al, 2002; 1997; Barcelaoux, 1999; Levis et al, 1978).

An increase in lipid peroxidation (LPO) levels in liver and kidneys was observed following chromic oral treatment of rats with 0, 2.66, 5.23 and 10 μg/kg per day of chromium picolinate for 21 days. Superoxide dismutase (SOD), glutathione peroxidase and glutathione (GSH) levels in the hepatic tissues were decreased in all the treated groups, while the hepatic catalase level decreased in the high dose group. The kidney SOD and catalase levels also decreased in all treated groups, while GSH and glutathione reductase (GSSG-R) levels were reduced in the mid- and high-dose treated groups (Mahboob et al, 2002).

Chromium picolinate caused chromic renal failure in a 33-year-old woman when she took 1200-2400 μg/day for 4-5 months to enhance weight loss. This case was presented with weight loss, anemia, hemolysis, thrombocytopenia, liver dysfunction, increase in total bilirubin, and renal failure (Cerulli et al, 1998), suggesting that chromium(III) picolinate causes serious renal impairment when ingested in excess. A second case of renal

impairment was also observed in a 49-year old female nurse who ingested 600μg/chromium picolinate/day for 6 weeks for weight reduction (Wasser et al, 1997). Increased production of superoxide anion by rat peritoneal macrophages and hepatic mitochondrial and microsomal lipid peroxidation was observed following oral administration of chromium(III) chloride hexahydrate (895 mg/kg b.w.) to female Sprague-Dawley rats. Chromium(III) picolinate and niacin-bound chromium(III) have been shown to induce cytochrome C reduction, hydroxyl radical production and DNA fragmentation in cultured J744A.1 marine macrophage cells (Bagchi et al, 2002). It has been demonstrated that Cr(III) can be reduced to Cr(II) by the biological reductants L-cysteine and NADH resulting in the formation of Cr(II) which reacts with H_2O_2 to generate hydroxyl radicals as detected by electron spin resonance (ESR) and HPLC (Ozawa and Hanaki, 1990). Similarly, free radicals were generated by reaction with H_2O_2 and lipid hydroperoxides in the presence of Cr(III) (Shi et al, 1993). These results indicate that Cr(III) has the potential to generate free radicals from both H_2O_2 and lipid hydroperoxides.

However, in this regard, the mechanism of $CrCl_3$-induced renal cellular injury is not known yet. The overall objective of this study was therefore to investigate whether Cr(III)-induced renal cellular injury or cytotoxicity appears to be mediated by the formation of cellular reductive metabolism and/or reactive oxygen species such as hydroxyl radicals. We have therefore examined the effects of physiological antioxidants such as Vitamin C (ascorbic acid), Vitamin E (α-tocopherol), and glutathione as well as the scavengers of reactive oxygen species (ROS), such as scavengers of hydroxyl free

radicals on $CrCl_3$-induced cellular damage in isolated rat renal cortical slices and the results are presented in this paper.

Materials and methods

Chemicals

Chromium chloride ($CrCl_3$), waymouth medium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, thiobarbituric acid, mannitol, dimethyl thiourea (DMTU), L-ascorbic acid (vitaminC), (±)-α-tocopherol (vitamin E), were obtained from Sigma Chemical Company. Glutathione (GSH) was from Aldrich Chemical Company.

Animals

Adult male Sprague-Dawley rats (Charles River Canada Inc., St. Contant, Quebec) were used. They were maintained at constant temperature (22℃) with a 12 hr light/ 12 hr dark photoperiod, housed 2-3 per cage on wood shavings and were fed Purina Laboratory chow and tap water available ad libitum. Rats were acclimated for 3 to 5 days and weighed 220- 280 g at the time of experiments. The experimental protocols used in this study were approved by the Ethics Committee on animal experiments, Université de Montréal.

Preparation of isolated rat renal cortical slices

Adult male Sprague-Dawley rats were anaesthetized with 'Somnotal' (0.1 ml/100 g b.w. i.p.). The rats were decapitated, exsanguinated, and then the abdomen was opened; the kidneys were removed rapidly, de-capsulated, and immediately placed into ice-cold saline solution (0.14 M NaCl and 0.02 M KCl). Following isolation of the kidneys, renal cortical slices (70 to 100 mg/per slice) were prepared using a Stadie-Riggs Microtome, weighed and placed in a flask of 3ml ice-cold waymouth buffer medium (pH 7.4). Each group contained 4 slices.

Rat renal cortical slices treatment

Rat renal cortical slices were treated with chromium chloride in waymouth medium (pH 7.4) and incubated at 37℃ in a rotating Dubnoff metabolic shaker at 150 rpm in an atmosphere of 95% oxygen and 5% carbon dioxide. After the incubation, renal cortical slices were removed from the medium, dried with a filter paper and placed into other plastic tubes containing 3 ml of phosphate buffer solution (PBS, pH 7.4).

Tissue homogenization

Renal cortical slices were homogenized in 3 ml of PBS for 30 sec. Half millilitre of the tissue homogenate was used for malondialdehyde (MDA) estimation and 1.0 ml for nonprotein sulfhydryl group (NP-SH) contents evaluation. The remaining tissue homogenate was centrifuged at 2000×g for 5 min and the supernatants were used for measurement of cell viability.

Determination of cell viability (cytotoxicity)

The cell viability was measured by MTT cleavage assay. The method is based on the ability of mitochondrial enzyme succinate dehydrogenase in living cells to reduce MTT tetrazolium salt into MTT formazan. One millilitre of supernatant from above and 0.2 ml of MTT solution (2.5 mg/ml PBS) were mixed and incubated at 37℃ in water bath for 90 min. Absorbance was read at 570 nm using Beckman DU-7 spectrophotometer. The cytotoxicity was evaluated as cell viability and expressed as percent of control value (Mosmann, 1983).

Determination of lipid peroxidation (LPO)

Evaluation of lipid peroxidation was based on the formation of malondialdehyde (MDA) which reacts with thiobarbituric acid to form red color. Half millilitre homogenate and 1.0ml reagent solution (0.188 g thiobarbituric acid, 7.5 ml 10% of trichloroacetic acid and 1.1 ml concentrated hydrochloric acid were diluted to 50 ml volume with distilled water) were mixed in darkness, incubated at boiling water for 15 minutes, and then cooled in ice water immediately to stop the reaction. The solution was centrifuged at 1100×g for 15 minutes. The absorbance of the supernatant was recorded at 535 nm using Beckman DU-7 spectrophotometer. The concentration of MDA (nmol/G tissue) was calculated using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}cm^{-1}$ (Poli et al. 1979, Valentovic et al, 1999). The result of MDA was finally expressed as MDA % of control.

Statistics

The data were presented as the mean ± SEM for 4 separate experiments. Differences between groups were assessed by one-way analysis of variance (ANOVA, Tukey analysis) or t-student test. $P<0.05$ was considered as statistically significant.

Results

Cr(III)-induced cytotoxicity and lipid peroxidation (LPO) in isolated rat renal cortical slices.

Chromium chloride ($CrCl_3$)-induced cytotoxicity and lipid peroxidation were measured and the results were summarized in Fig.3-1 and Fig.3-2. In concentration-dependent study (Fig.3-1), the isolated rat renal cortical slices were treated with 0, 1.0, 2.0 and 4.0 mM $CrCl_3$ for 240 min, respectively. With increasing concentrations of $CrCl_3$, cell viability gradually declined and be come statistically significant at 4.0 mM $CrCl_3$ when compared to control, (48.6 % of control, $p<0.01$). In time-dependent study (Fig.3-2), the renal cortical slices were exposed to 4.0 mM of $CrCl_3$ for 60, 120, 180, 240, 300 and 360 min. Cell viability and MDA were assessed. With increasing period of incubation, the percentage of cell viability significantly decreased starting at 120 min. But MDA formation appeared non significant during different period of incubation when compared to control.

Effects of ROS on Cr(III)-induced cytotoxicity and LPO in isolated rat renal cortical slices.

In this study, the effects of scavengers of ROS, namely scavengers of hydroxyl free radicals, on Cr(III)-induced cytotoxicity and LPO were measured. The results were presented in Fig.3-3. After co-treatment of renal cortical slices with either mannitol (25 and 50 mM) or dimethyl thiourea (DMTU) (10 and 20 mM) and 4.0 mM chromium chloride for 240 min, there was no change in Cr(III)-induced cytotoxicity in the presence of mannitol or DMTU. Furthermore, these scavengers failed to change the LPO compared to the groups of control and Cr(III)-treated alone.

Effects of GSH on Cr(III)-induced cytotoxicity and LPO in isolated rat renal cortical slices

Fig.3-4 presented the results of the effects of GSH cotreatment on Cr(III)-induced cytotoxicity and LPO. The results showed with increasing concentration of GSH, Cr(III)-induced cytotoxicity and LPO were gradually increased compared to control and Cr(III)-treated alone. And cotreatment with 8 mM GSH enhanced Cr(III)-induced cytotoxicity and LPO significantly compared to both control and Cr(III)-treated value ($p<0.05$).

Effects of vitamin C on Cr(III)-induced cytotoxicity and vitamin E on Cr(III)-induced cytotoxicity and LPO in isolated rat renal cortical slices

Fig.3-5 presented the results of the effects of vitamin C on Cr(III)-induced cytotoxicity and vitamin E (α-tocopherol) on Cr(III)-induced cytotoxicity and LPO. Pre-treatment of renal cortical slices with excess vitamin E for 120 min followed by incubation with 4 mM

$CrCl_3$ for 240 min did not reduce Cr(III)-induced cytotoxicity. On the other hand, pre-treatment of renal cortical slices with excess vitamin C (5 mM) for 45 min followed by treatment with 4 mM $CrCl_3$ for 240 min significantly reduced Cr(III)-induced cytotoxicity, or significantly increased cell viability compared to Cr(III)-treated group ($p<0.05$).

Discussion

The present study has shown that chromium chloride ($CrCl_3$) at very high concentrations has the potential to damage rat renal cortical cells in vitro. Furthermore, in spite of such high cytotoxic potential, $CrCl_3$ failed to induce lipid peroxidation at such high concentration. Such a renal cytotoxic potential $CrCl_3$ at much higher concentration agrees qualitatively with the results of some clinical studies (Cerulli et al, 1998; Wasser et al, 1997). Lipid peroxidation is considered to be a marker of oxidative damage to the lipids in kidney tissue. Such oxidative damage in lipids is usually caused by oxidation of lipids by reactive oxygen species (ROS), such as superoxide anion, and hydroxyl radicals. Since in our present study, chromium chloride failed to provide evidence of production of such ROS, it is not therefore surprising that Cr(III) has failed to generated such lipid peroxidation. The failure to reduce Cr(III)-induced cytotoxicity (as measured by the MTT test) in isolated rat renal cortical slices by modulators of free hydroxyl radicals such as mannitol and dimethyl thiourea suggests that the reactive oxygen species such as free hydroxyl radicals are not involved in Cr(III)-induced cytotoxicity in renal cortical cells.

Our results are in contrast to the other reported studies. Thus, Bagchi et al (2002) observed significant increase in urinary excretion of various lipid metabolites following oral administration of chromium chloride hexahydrate (895 mg/kg b.w.) to female Sprague-Dawley rats. They have also observed production of superoxide anion in peritoneal macrophage and hepatic mitochondrial and microsomal lipid peroxidation following such in vivo study (Bagchi et al, 2002). But no such information is available from this study with regard to Cr(III)-induced ROS in renal cells. Furthermore, the production of hydroxyl free radicals by chromium(III) picolinate and niacin-bound chromium(III) has been demonstrated in cultured J774A.1 macrophage cells (Bagchi et al, 2002). But our present study cannot support generation of such free hydroxyl radicals. However, similar studies carried out in our laboratory have shown that ROS such as free hydroxyl radicals played a significant role in potassium dichromate [Cr(VI)]-induced cytotoxicity in isolated rat renal cortical slices (unpublished results).

Ozawa and Hanaki (1990) pointed that Cr(III) could be reduced to Cr(II) ion by the biological reductants, such as L-cysteine (thiol group) and NADH, and thus Cr(II) could easily react with hydrogen peroxide to yield reactive oxygen species, hydroxyl radicals. Similarly, in this research, cotreatment with high concentration (8mM) of excess glutathione (thiol group also) significantly increases Cr(III)-induced cytotoxicity (cell viability loss) and the production of lipid peroxidation (Fig.3-4). The results suggest that GSH cannot protect renal cells from Cr(III)-induced cytotoxicity, whereas it enhances such toxicity and LPO production. This is in contrast to our other study where GSH

played a protective role in potassium dichromate [Cr(VI)]-induced cytotoxicity in isolated rat renal cortical slices (unpublished results).

Pretreatment with excess vitamin E (α-tocopherol) failed to increase cell viability reduced by $CrCl_3$, whereas pre-treatment of renal cortical slices with vitamin C (ascorbic acid) significantly increased the cell viability reduced by $CrCl_3$.

In conclusion, these data demonstrate that $CrCl_3$ at very high concentration is cytotoxic to rat renal cortical cells and intracellular antioxidant vitamin C (ascorbic acid) might be an important determinant factor in such $CrCl_3$-induced cytotoxicity in rat renal cortical cells, but GSH can enhance Cr(III)-induced cytotoxicity and LPO.

Figure legend

Fig.3-1 Concentration-dependent effects of Cr(III) on cytotoxicity after exposure to $CrCl_3$ for 240 min in isolated rat renal cortical slices. Cytotoxicity was estimated by the MTT test. The results were presented as cell viability as percent of control value. The data were mean ± SEM of four separate experiments.

*: significantly different from control, $p<0.05$.

Fig.3-2 Time-dependent effects of Cr(III) on cytotoxicity and LPO after exposure to 4 mM $CrCl_3$ in isolated rat renal cortical slices. Cytotoxicity (as measured by the MTT test) and lipid peroxidation (as measured by MDA formation) were measured at each time. The results were presented as percent of control value. The data were mean ± SEM of four separate experiments. *: significantly different from control, $p<0.05$.

Fig.3-3 Effects of scavengers of free hydroxyl radicals on Cr(III)-induced cytotoxicity and LPO after exposure to 4 mM $CrCl_3$ for 240min in isolated rat renal cortical slices. The scavengers of free hydroxyl radicals such as mannitol and dimethyl thiourea (DMTU) were coincubated with 4 mM $CrCl_3$ in isolated rat renal cortical slices for 240 min. The cell viability (measured by MTT test) and LPO (measured by MDA formation) were estimated as described in the text. The results were expressed as percent of control value. The values were mean ± SEM of four separated experiments. *: significantly different from control, $p<0.05$.

Fig.3-4 Effects of GSH on Cr(III)-induced cytotoxicity and LPO after exposure to 4 mM $CrCl_3$ for 240 min in isolated rat renal cortical slices. Isolated rat renal cortical slices were cotreated with 4 mM $CrCl_3$ and 4 or 8 mM GSH for 240 min. Cell viability (cytotoxicity) and LPO (MDA formation) were then measured as described in the text. The results are expressed as percent of control value. The data were mean ± SEM of four separated experiments.

a: significantly different from corresponding control, $p<0.05$.

b: significantly different from corresponding Cr(III) (4 mM) alone-treated group, $p<0.05$.

Fig.3-5 Effects of vitamin C on Cr(III)-induced cytotoxicity and vitamin E on Cr(III)-induced cytotoxicity and LPO after exposure to 4 mM $CrCl_3$ for 240 min in isolated rat renal cortical slices. Isolated rat renal cortical slices were pretreated with either vitamin C (2.5 mM and 5.0 mM) for 45 min, or vitamin E (50 mM and 100 mM) for 120 min, followed by exposure to 4 mM $CrCl_3$ for 240 min. Cell viability (cytotoxicity) and LPO were then measured as described in the text. The results are expressed as percent of the control value. The data are mean ± SEM of four separated experiments.

a: significantly different from corresponding control, $p<0.05$.

b: significantly different from corresponding Cr(III) (4 mM) alone-treated group, $p<0.05$.

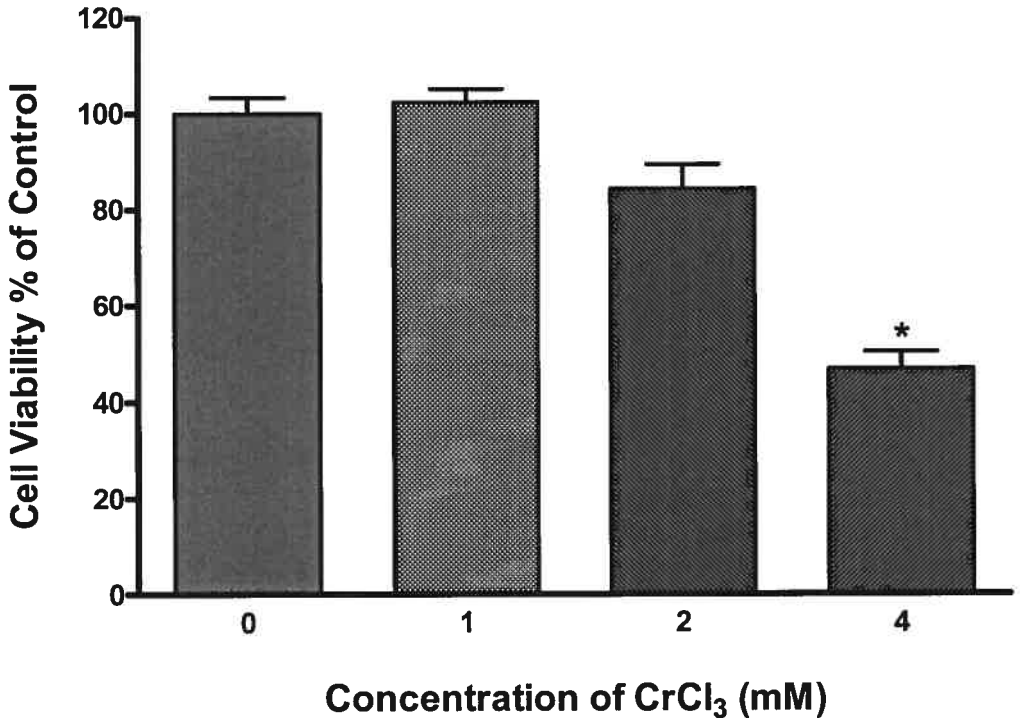
120
100
80
60
40
20
0
Cell Viability % of Control
*
0
1
2
4
Concentration of CrCl3 (mM)

Fig.3-1

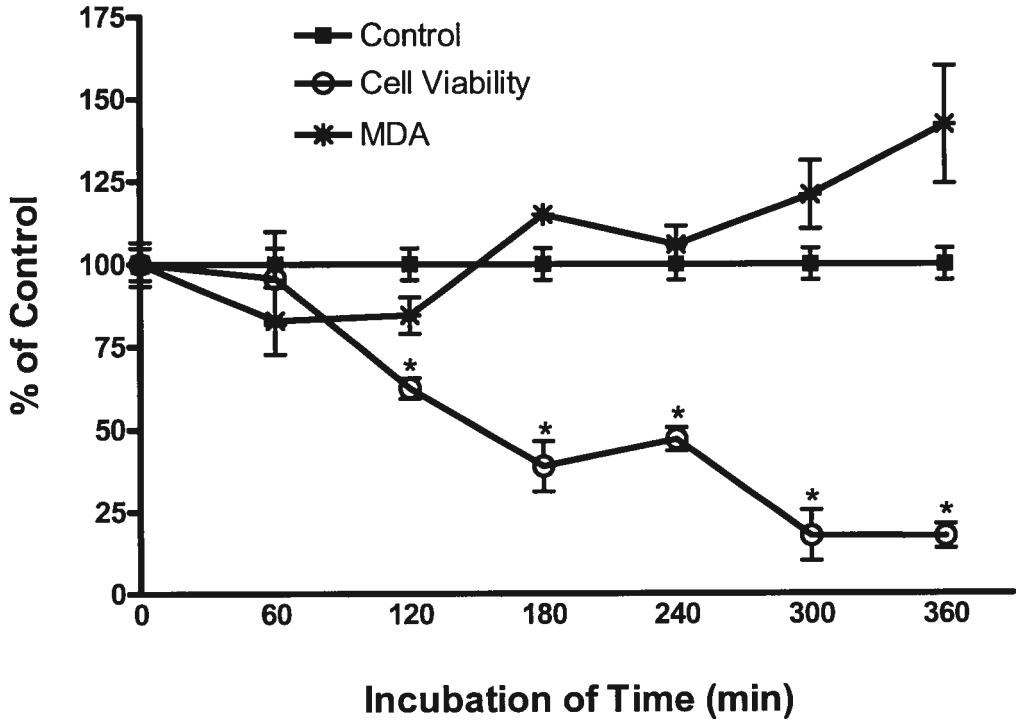
Control
Cell Viability
MDA
% of Control
175
150
125
100
75
50
25
0
0
60
120
180
240
300
360
Incubation of Time (min)

Fig.3-2

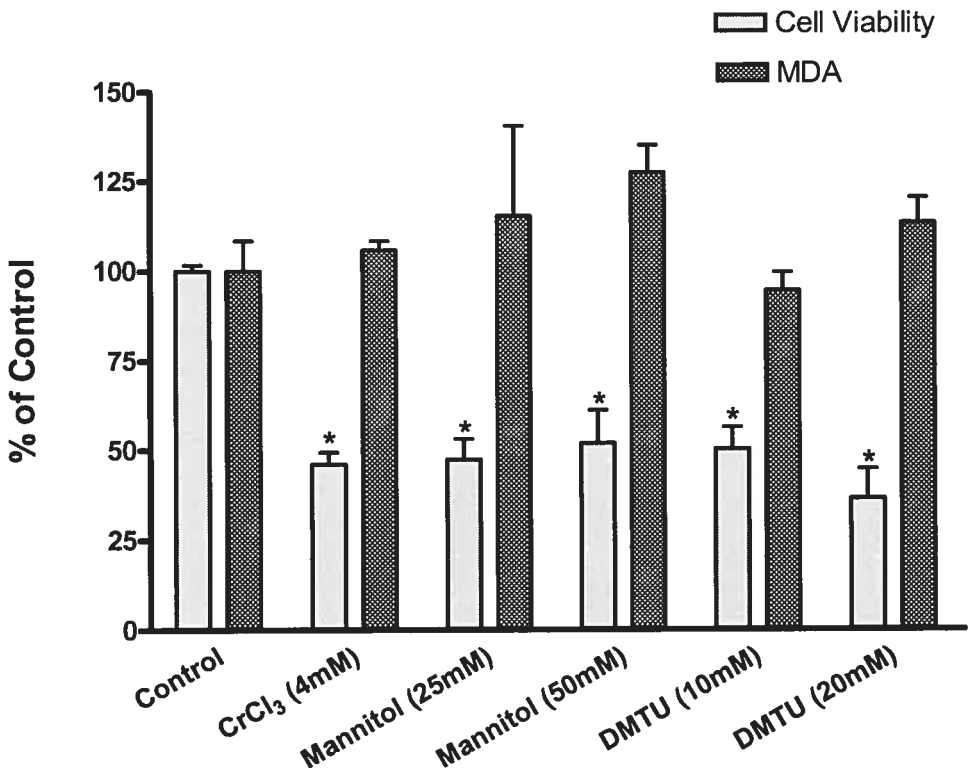
Cell Viability
MDA
% of Control
150
125
100
75
50
25
0
*
Control
CrCl3 (4mM)
Mannitol (25mM)
Mannitol (50mM)
DMTU (10mM)
DMTU (20mM)

Fig.3-3

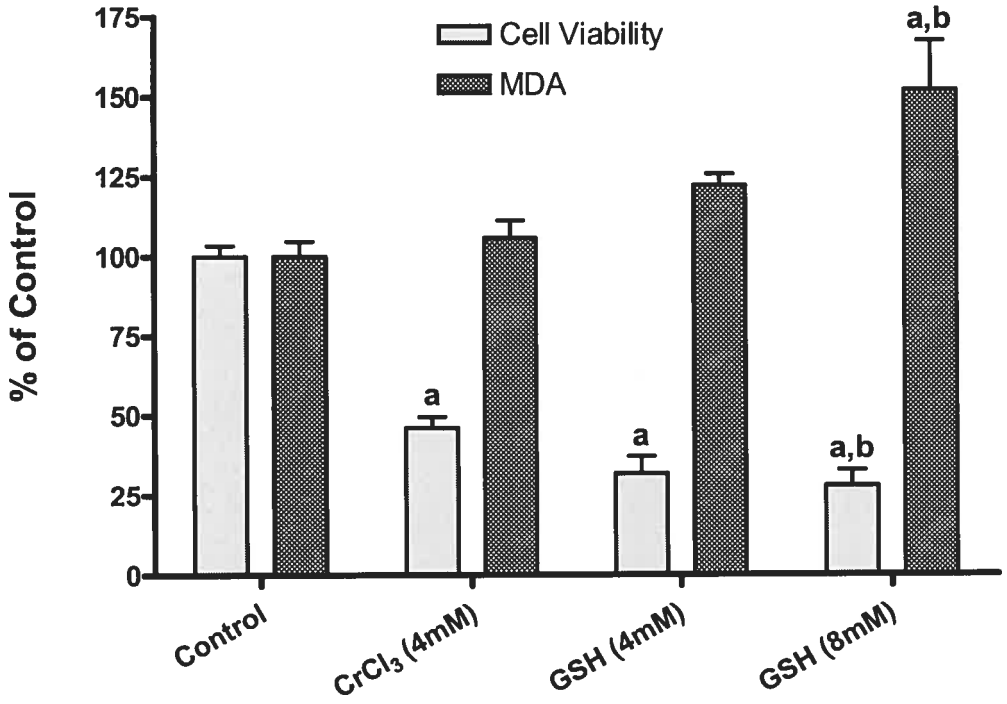
175
150
125
100
75
50
25
0
% of Control
Cell Viability
MDA
a
a
a,b
a,b
Control
CrCl3 (4mM)
GSH (4mM)
GSH (8mM)

Fig.3-4

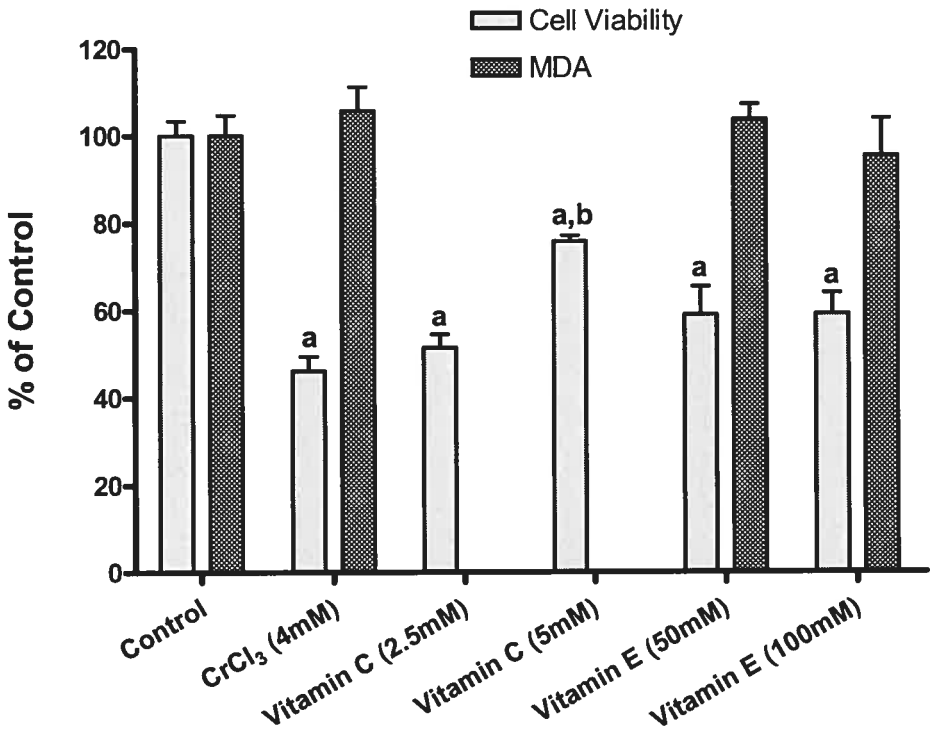
Cell Viability
MDA
% of Control
120
100
80
60
40
20
0
a
a
a,b
a
a
Control
CrCl3 (4mM)
Vitamin C (2.5mM)
Vitamin C (5mM)
Vitamin E (50mM)
Vitamin E (100mM)

Fig.3-5

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SECTION 4:

GENERAL DISCUSSION

Comparative studies of Cr(VI)- and Cr(III)-induced nephrotoxicity in isolated rat renal cortical slices

4.1. Cr(VI)- and Cr(III)-induced cytotoxicity in isolated rat renal cortical slices in vitro

Chromium is known to have cytotoxic effects on cells. In general, Cr(VI) demonstrated significantly higher toxicity than Cr(III). Our study has shown that both Cr(VI) and Cr(III) induced concentration- and time-dependent effects on loss of cell viability in isolated rat renal cortical slices (Fig.4-1 and Fig.4-2A). Cell viability was gradually reduced and produced significant great loss of cell viability starting at exposure to 1 mM $K_2Cr_2O_7$ [Cr(VI)] for 120 min (34.2% of control) compared to exposure to 4 mM of $CrCl_3$ [Cr(III)] for 120 min (62.4% of control) in Fig.4-1. Thus, Cr(III) presents a relatively lower toxicity than that of Cr(VI) in isolated rat renal cortical slices. Potassium dichromate-induced cytotoxicity is about 8-fold higher than that induced by chromium chloride in isolated rat renal cortical slices in vitro. These results are similar to those in several human and murine cell lines (Flores and Perez, 1999). In diploid human fibroblastic cells (HFC), Cr(VI) compounds are 1000-fold more cytotoxic (LC_{50} is 0.5 μM) than Cr(III) compounds (LC_{50} is 500 μM) (Biedermenn and Landolph, 1990). In rats, LD_{50} of sodium dichromate is only 50 mg/kg, but that of chromium chloride is up to 1790 mg/kg (Bagchi et al, 2002). Such a difference in cytotoxicity between Cr(VI) and Cr(III) can be ascribed to a differential permeability of the plasma membranes due to two different oxidation states of chromium. Cell membranes have been found to be practically impermeable to Cr(III), whereas Cr(VI) has been able to cross such biological membranes easily (Appenroth and Kersten, 1990) and hence, exerts more toxic effects.

4.2 The effects of reactive oxygen species (ROS) on Cr(VI)- and Cr(III)-induced cytotoxicity

Reactive oxygen species are believed to be involved in the toxicity of chromium compounds. Various types of ROS, such as H_2O_2, superoxide anion and hydroxyl free radicals have been implicated in both Cr(VI)- and Cr(III)-induced toxicity in different cell lines, hepatocytes and other cells (Stohs et al, 2000; O'Brien and Kortenkamp, 1994; Bagchi et al, 2002). But in our study, pretreatment with either catalase (scavengers of H_2O_2), superoxide dismutase (scavenger of superoxide anion, O_2^-) failed to reduce both Cr(VI)-induced cytotoxicity and lipid peroxidation (LPO). On the other hand, pretreatment of renal cortical slices with either mannitol (scavenger of extracellular hydroxyl free radicals), or dimethyl thiourea (DMTU) (scavenger of intracellular hydroxyl free radicals) failed to reduce Cr(VI)-induced toxicity (Fig.4-3A), but significantly reduce Cr(VI)-induced LPO (Fig.4-3B). Similarly, both mannitol and DMTU failed to inhibit $CrCl_3$ [Cr(III)]-induced cytotoxicity or, failed to increase the cell viability reduced by $CrCl_3$ (Fig.4-3A). Thus, the reactive oxygen species do not play any significant role in both Cr(VI)- and Cr(III)-induced cytotoxicity in rat renal cortical cells. In contrast to Cr(VI), $CrCl_3$ [Cr(III)] does not produce ROS and does not induce LPO in our study.

4.3 The role of glutathione (GSH) on chromium-induced cytotoxicity and LPO

Glutathione is the most important antioxidant in cells or tissues. Hojo and Satomi (1991) found that administration of Cr(VI) caused systemic and kidney injuries in

mice, accompanying a lowering of renal GSH. In our study, we have observed that potassium dichromate [Cr(VI)] caused GSH level (measured as NP-SH content) to increase temporarily in Cr(VI) treatment at 0.125 mM for 120 min or 1 mM for 30 min, but this level decreased significantly when the concentration or treatment time was increased in isolated rat renal cortical slices (Fig.2-1 and Fig.2-2). Furthermore, from Fig.4-4A and Fig.4-4B, we observed that cotreatment with excess reduced GSH (4 mM and 8 mM) resulted in decreasing Cr(VI)-induced cytotoxicity and LPO, whereas such cotreatment enhanced Cr(III)-induced cytotoxicity and produced significant increase of LPO in isolated rat renal cortical slices, indicating that GSH played a differential role in chromium-induced toxicity in isolated rat renal cortical slices. One side, GSH plays a protective role on Cr(VI)-induced cytotoxicity and LPO. On the other side, it enhances Cr(III)-induced cytotoxicity and LPO in isolated rat renal cortical slices.

4.4 The role of vitamin C and vitamin E in chromium-induced cytotoxicity or LPO

To further compare the role of cellular antioxidants on Cr(VI)- and Cr(III)-induced renal toxicity, we examined the effects of ascorbic acid (vitamin C) and α-tocopherol (vitamin E) on Cr(VI)- and Cr(III)-induced toxicity. Pretreatment of vitamin C with isolated rat renal cortical slices can reduce both Cr(VI)- and Cr(III)-induced cell viability loss and Cr(VI)-induced lipid peroxidation. However, pretreatment of vitamin E failed to reduce both Cr(VI)- and Cr(III)-induced toxicity, but only reduce Cr(VI)-induced LPO (Fig.4-5). Thus, intracellular antioxidants vitamin C (ascorbic

acid) might be the most determinant factors in potassium dichromate [Cr(VI)]-induced oxidative stress and subsequent nephtotoxicity in isolated rat renal cortical slices, whereas intracellular vitamin C seems to be the most important determining factor in chromium chloride ($CrCl_3$)-induced nephrotoxicity in renal cortical slices.

4.5 Conclusion

In our research, both Cr(VI) and Cr(III) have the potential to induce cytotoxicity in isolated rat renal cortical slices, but with different degree. Cr(VI)-induced cytotoxicity seems to be 8-fold higher than that of Cr(III) in isolated rat renal cortical slices in vitro. Moreover, Cr(VI) [but not Cr(III)] produced lipid peroxidation following the occurrence of cytotoxicity. Reactive oxygen species (ROS) involving hydroxyl free radicals are involved in Cr(VI)-induced LPO but not cytotoxicity. Glutathione plays a differential role in chromium compounds-induced cytotoxicity in isolated rat renal cortical slices. One side, it plays a protective role in Cr(VI)-induced cytotoxicity and LPO by decreasing cell viability loss and MDA formation. On the other side, it enhances Cr(III)-induced cytotoxicity and lipid peroxidation. Vitamin C is a powerful cellular antioxidant to prevent both Cr(VI) and Cr(III)-induced renal toxicity, but not vitamin E.

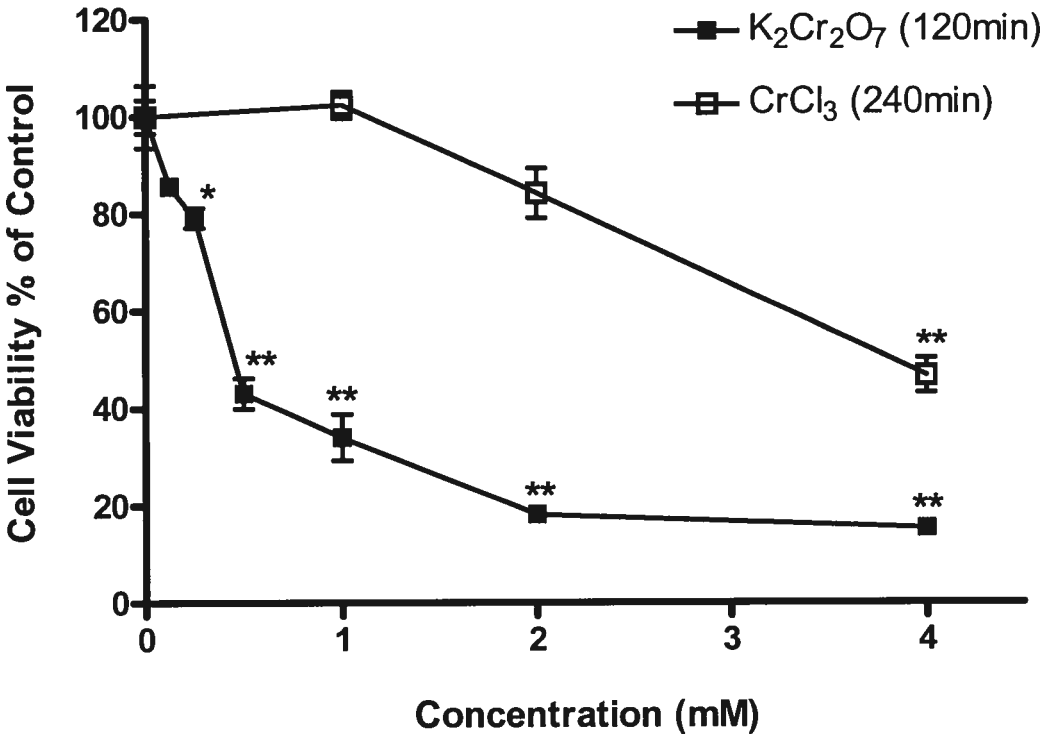

Fig.4-1 Concentration-dependent effects of Cr(VI) and Cr(III) on cytotoxicity after exposure to different concentrations of $K_2Cr_2O_7$ for 120 min and those of $CrCl_3$ for 240 min in isolated rat renal cortical slices. Cytotoxicity is estimated by the MTT test. The results are expressed as % of control value. The values are mean ± SEM of four separate experiments.

*: $p<0.05$, significantly different from control;

**: $p<0.01$, more significantly different from control.

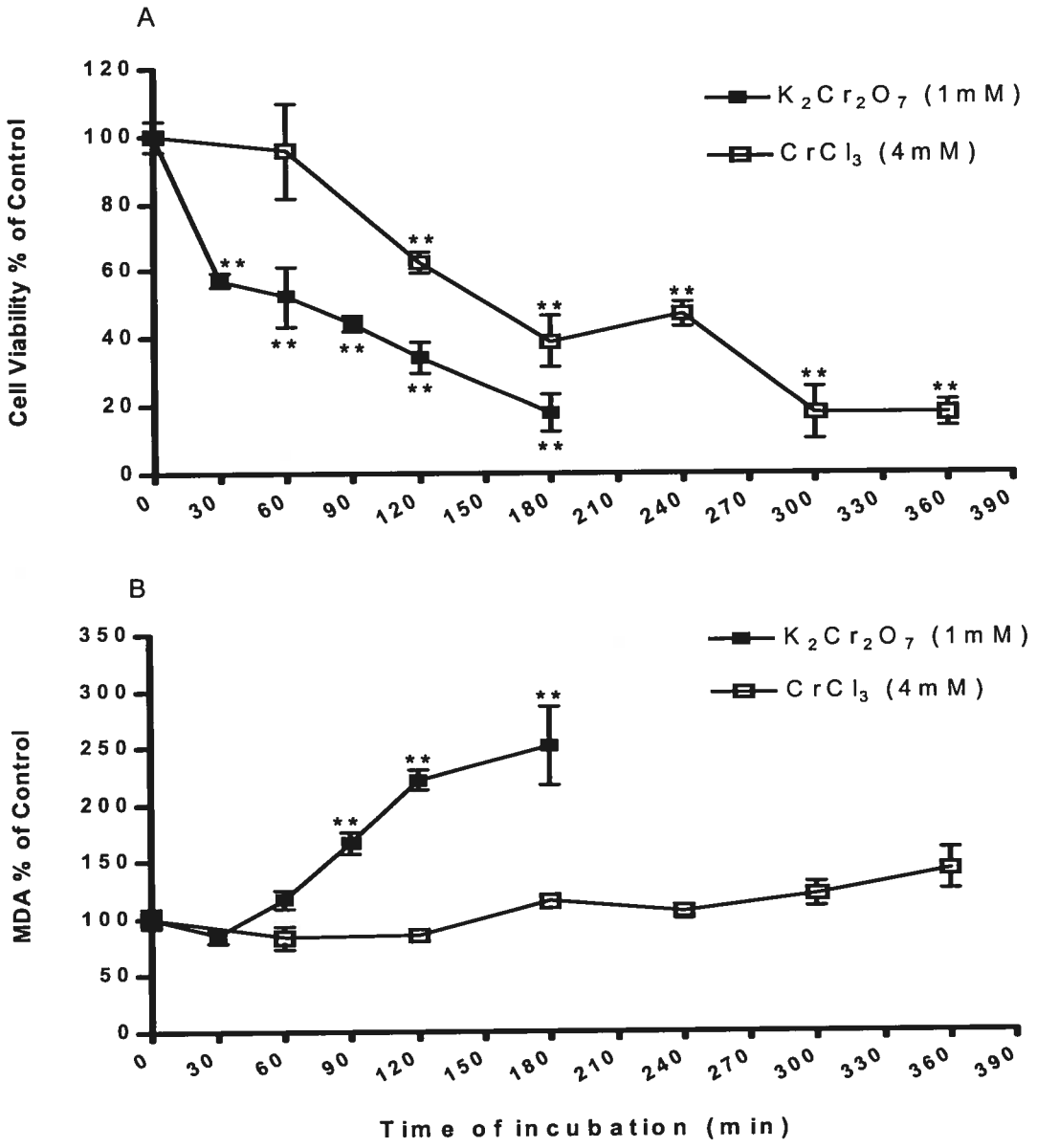

Fig.4-2 Time-dependent effects of Cr(VI) and Cr(III) on cytotoxicity (A) and lipid peroxidation (B) after exposure to 1 mM $K_2Cr_2O_7$ and 4 mM $CrCl_3$ in isolated rat renal cortical slices. Cytotoxicity was estimated by the MTT test. LPO was estimated by MDA formation. The results are presented as % of control value. The values are mean ± SEM of 4 separate experiments.

**: $p<0.01$, more significantly different from control.

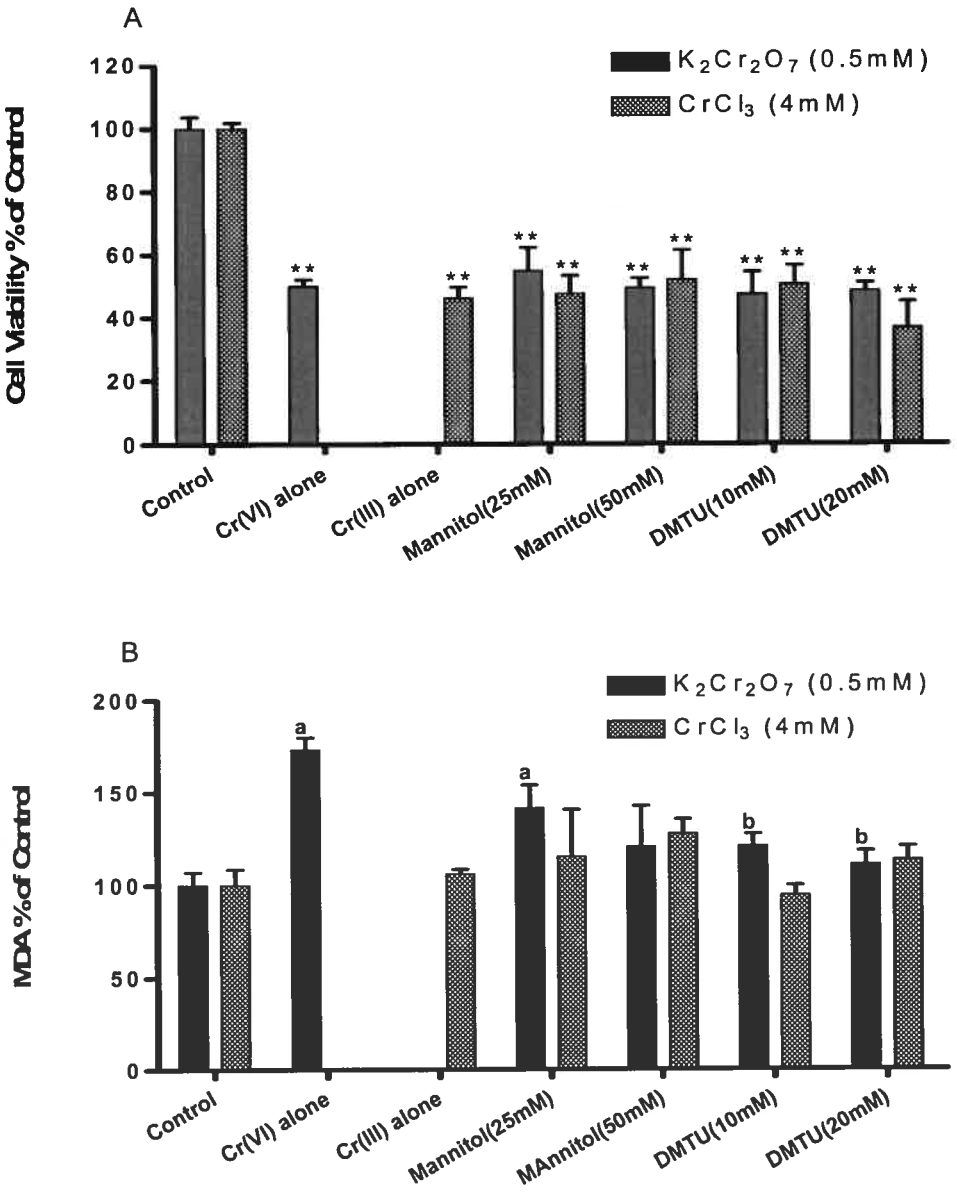

Fig.4-3 Effects of scavengers of free hydroxyl radicals (ROS) on Cr(VI)- and Cr(III)-induced cytotoxicity (A) and LPO (B) in isolated rat renal cortical slices. The scavengers were cotreated with 0.5 mM $K_2Cr_2O_7$ for 90 min or 4 mM $CrCl_3$ 240 min in isolated rat renal cortical slices. Cytotoxicity was estimated by the MTT test. LPO was estimated by MDA formation. The results are presented as % of control value. The values are mean ± SEM of 4 separate experiments.

a: significantly different from control, $p<0.05$;

b: significantly different from corresponding chromium compounds, $p<0.05$.

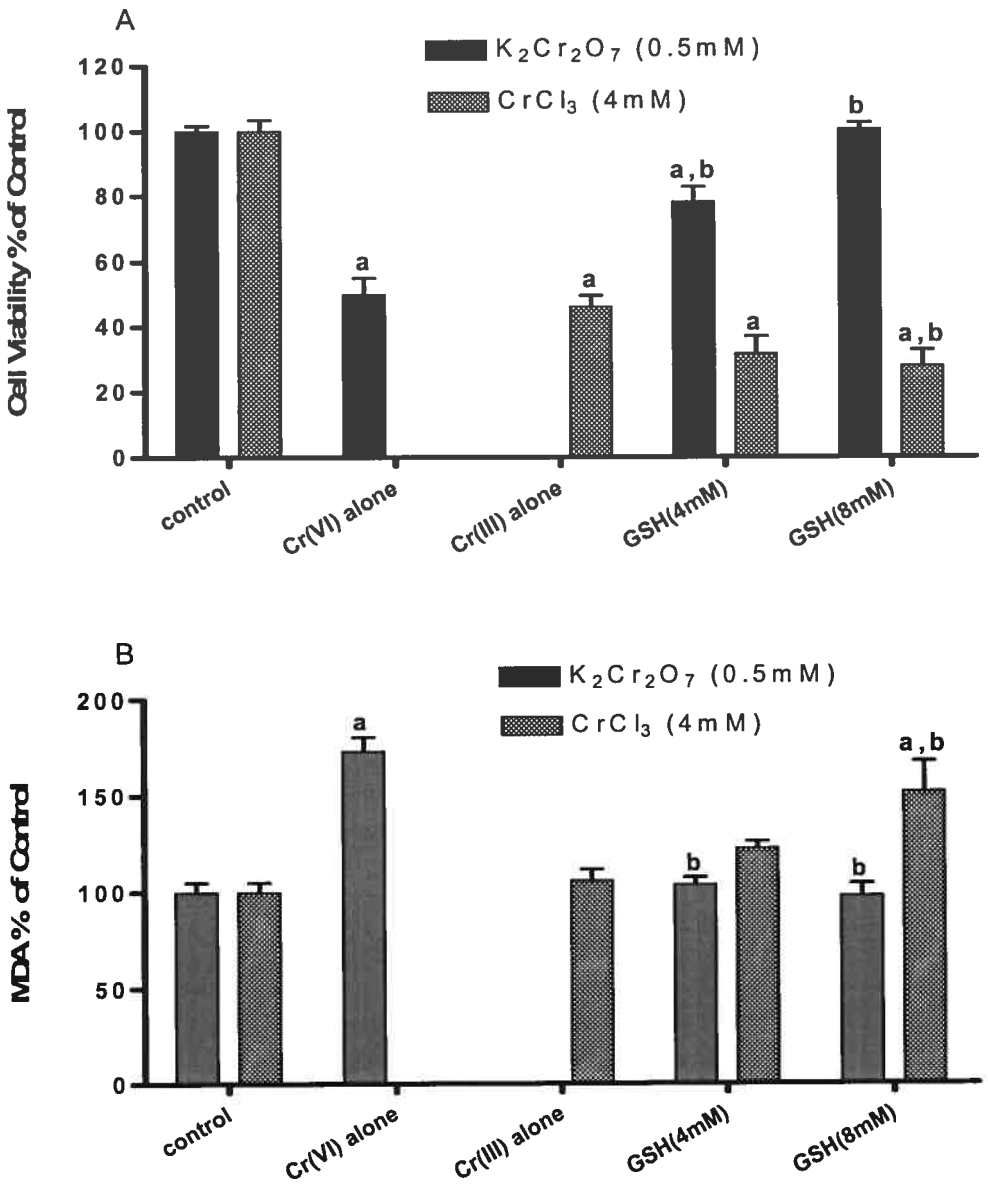

Fig.4-4 Effects of glutathione (GSH) on Cr(VI)- and Cr(III)-induced cytotoxicity (A) and LPO (B) in isolated rat renal cortical slices. GSH was cotreated with 0.5 mM $K_2Cr_2O_7$ for 90 min or 4 mM $CrCl_3$ for 240 min in isolated rat renal cortical slices. Cytotoxicity was estimated by the MTT test. LPO was estimated by MDA formation. The results are presented as % of control value. The values are mean ± SEM of 4 separate experiments.

a: significantly different from control, $p<0.05$;

b: significantly different from Cr(VI) (0.5 mM) or Cr(III) (4 mM)-treated group, $p<0.05$.

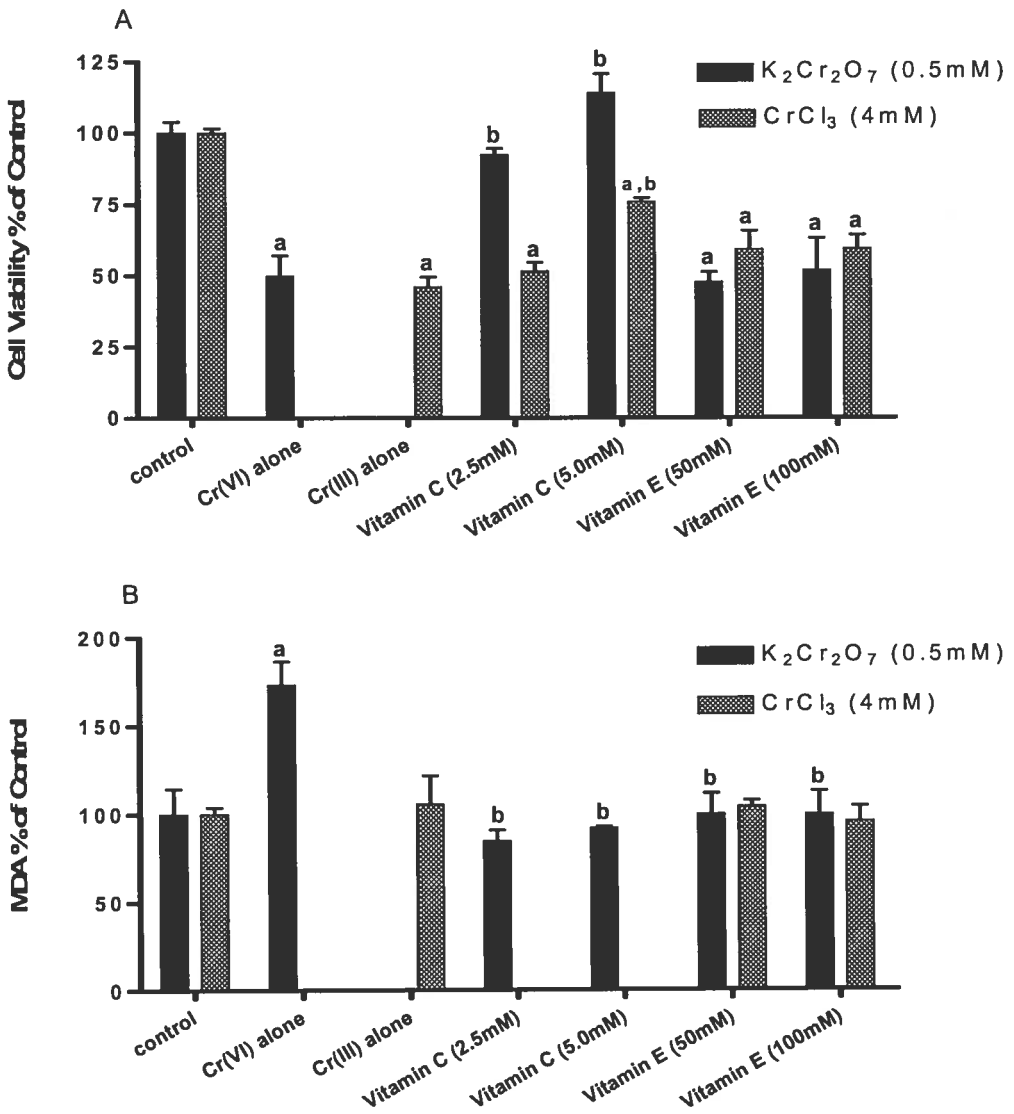

Fig.4-5 Effects of vitamin C on Cr(VI)- and Cr(III)-induced cytotoxicity (A) and Cr(VI)-induced LPO (B) and those of vitamin E on Cr(VI)- and Cr(III)-induced cytotoxicity (A) and LPO(B) in isolated rat renal cortical slices. Vitamin C was pretreated for 45 min before treatment with 0.5 mM $K_2Cr_2O_7$ or 4 mM $CrCl_3$. Vitamin E was pretreated for 180 min before treatment with 0.5 mM $K_2Cr_2O_7$ and for 120 min before treatment with 4 mM $CrCl_3$. Cytotoxicity was estimated as MTT test. LPO was estimated as MDA formation. The results are presented as % of control value. The values are mean ± SEM of 4 separate experiments.

a: significantly different from control, $p<0.05$;

b: significantly different from Cr(VI) (0.5mM) or Cr(III) (4mM)-treated group, $p<0.05$.

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