Vitamin-E Supplementation Ameliorates Chromium-and/or Nickel Induced Oxidative Stress *in Vivo*

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(Received October 31, 2005; Accepted December 5, 2005)

The present study was designed to investigate the *in vivo* effects of nickel chloride [$NiCl_2$; 8, and 16 mg/kg body weight (b wt)] and/or Potassium dichromate ($K_2Cr_2O_7$; 5 and 10 mg/kg b wt) in the liver of adult mice. The beneficial effects of vitamin E (2 mg/kg) along with their combination were also studied. The antioxidative indices including oxidative lipid peroxidation (LPO) and antioxidative enzymes such as glutathione (GSH), total sulfhydryl (–SH) groups, total ascorbic acid (TAA), superoxide dismutase (SOD) and catalase (CAT) were evaluated in the hepatic tissue using well established techniques. Nickel and/or chromium treatments to mice revealed a significant decline in the levels of these antioxidant parameters as compared to control. Concomitantly a significant increase in lipid peroxidation was obtained. These data indicated that the treatment induced oxidative stress in the hepatic tissue of treated mice, which was more pronounced by combination of metal salts. But supplementation of vitamin E with $NiCl_2$ + $K_2Cr_2O_7$ to mice exerted no significant alterations in the liver antioxidant system as compared to control, thus indicating its ameliorative role. These findings suggest that vitamin E prevents LPO and protects the antioxidant system in the mouse liver.

Key words —— nickel, chromium (VI), oxidative stress, vitamin E, antioxidant cytoprotection, mouse liver

INTRODUCTION

Chromium and nickel individually are considered potential health hazards. These are major components of various steels and their salts are used extensively in plating. Thus both these metals are important materials in many industries and hence it is not possible to stop exposure to them. Oral exposure of human to levels much greater than background has resulted in death, gastrointestinal, haematological, hepatic, renal and neurological effects.[1] Studies by Knight *et al.*[2] suggest that the acute Ni^{+2} toxicity in rats is associated with lipid peroxidation in target organs. Studies by Misra *et al.*[3] demonstrated that the amount of lipid peroxidation (LPO) increased while the hepatic glutathione (GSH) levels and catalase (CAT) activity significantly decreased by nickel. Das *et al.*[4] demonstrated that nickel sulfate administration to male albino rats significantly increased the levels of LPO and simultaneously decreased glutathione level and glutathione peroxidase activity in the liver.

Chromium (VI) and chromium (0) are generally produced by industrial processes.[5] Cr(VI) compounds are oxidizing agents capable of directly inducing tissue damage.[6,7] Accidental or intentional swallowing of large amount of Cr(VI) causes stomach upset, ulcers, convulsions, liver and kidney damage and even death. It has also been reported to cause severe liver effects in workers exposed to CrO_3 in chrome plating industry.[5] Hexavalent chromium results in enhanced formation of reactive oxygen species (ROS), including superoxide anion, hydroxyl radical and nitric oxide, decreased cell viability, increased cellular and genomic hepatic DNA fragmentation, enhanced intracellular oxidized states, membrane damage apoptic and necrotic cell death.[8]

Thus much work has been done on the toxic effects of chromium and nickel alone but there is still a need to study their combined toxicity and amelioration by Vitamin E on Ni/Cr poisoning. Hence the present study was undertaken to evaluate the effects of chromium alone and in combination in the liver with respect to lipid peroxidation, glutathione, total ascorbic acid, total sulfhydryl groups, superoxide

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dismutase and catalase levels of mice. The effect of vitamin E supplementation along with nickel/and or chromium has also been investigated, as its role on heavy metal alone toxicity was studied earlier in our laboratory.[9)]

MATERIALS AND METHODS

Animals —— Healthy adult male albino mice (*Mus musculus*) of Swiss strain, weighing between 35–40 g were obtained from Alembic Pharmaceuticals, Vadodara, India, under the Animal Maintenance and Registration No. 167/1999/CPCSEA from the ministry of Social Justice and Empowerment, Government of India. All the animals were acclimatized seven days prior to the commencement of the treatment. These were housed in an air-conditioned animal house at 26 ± 2°C and exposed to 10–12 hr of daylight and a relative humidity of 30–70%. The animals were maintained on a standard chow [National Institute of Occupational Health (NIOH), Ahmedabad, India]. It contains wheat (70%), gram (20%), fish meal (5%), and yeast powder (5%) and water *ad libitum*.

Dose Selection —— Potassium dichromate ($K_2Cr_2O_7$; SD Fine Chemicals, Mumbai, India) and nickel chloride ($NiCl_2$; Himedia Laboratories Ltd., Mumbai, India) were suitably diluted in distilled water at different dose levels based on LD_{50} values. The dose for vitamin E (α Tocopherol acetate E, Merck India Ltd., Mumbai, India) was selected on the basis of earlier work in our laboratory.[10)] Route of administration was oral.

Experimental Design —— The animals were divided into nine groups. Group I served as control, Group II animals were orally administered with vitamin E. Group III and IV animals were treated with $NiCl_2$ at doses of 8 mg and 16 mg/kg weight (wt). Group V and VI animals were treated with $K_2Cr_2O_7$ at doses of 5 mg and 10 mg/kg wt respectively. Group VII and VIII were given combined treatment of nickel and chromium at both dosage levels respectively. Group IX animals were supplemented with vitamin E (2 mg/kg) along with 16 and 10 mg of $NiCl_2 + K_2Cr_2O_7$ respectively. The treatments were given orally to the experimental animals for 30 days. The control animals were provided with distilled water.

Biochemical Analysis —— At the end of each treatment animals were weighed and sacrificed by cervical dislocation. The liver of all the animals were dissected out, blotted free of blood and weighed.

LPO: LPO was determined by the method of Ohkawa *et al.*[11)] To the sample tube containing 0.1 ml distilled water, 0.75 ml of 20% acetic acid, 0.2 ml sodium dodecyl sulphate (SDS), 0.75 ml of 1% thiobarbituric acid (TBA), and 0.1 ml of homogenate was added. The blank tube contained 0.1 ml distilled water instead of homogenate. The solutions were mixed and heated in a water bath for 60 min at 95°C. The tubes were cooled, mixed, and centrifuged at 1000 *g* for 15 min. The optical density (OD) of the supernatant was read at 532 nm and was expressed as n moles malonyldialdehyde (MDA) formed/100 mg tissue wt.

CAT: The activity of CAT was assayed by the modified method of Luck.[12)] The assay mixture consisted of 0.5 ml 50 mM phosphate buffer, pH 7.0 and 1 ml aliquot. The blank readings were subtracted from the test readings for obtaining correction factor for the spontaneous reaction. The decrease in absorbance was noted every 5 for 30 sec at 240 nm. The enzyme activity was expressed as μmol of H_2O_2/mg protein.

Super oxide dismutase (SOD) was assayed by the method of Kakkar *et al.*[13)] In the assay system, control consisted of 2.4 ml phosphate buffer (0.052 M), 0.1 ml phenazine methosulphate (186 μmol), 0.3 ml of nitroblue tetrazolium chloride (30 μmol), 0.2 ml fresh NADH (780 μmol). To the sample system 0.2 ml of enzyme was added prior to the addition of NADH. The reaction was stopped by addition of 1 ml of acetic acid. Four ml of n-butanol was then added and shaken vigorously. The tubes were centrifuged for 10 min at 2000 rpm. The optical density was measured at 560 nm. Its activity was measured as units SOD/mg protein.

GSH: GSH was estimated by the method of Grunert and Philips.[14)] A known amount of tissue was homogenized in 3 ml 3% metaphosphoric acid (HPO_3), 1 ml distilled water and saturated with salt solution (1.5 g NaCl). The tubes were centrifuged at 800 *g* for 5 min. Two ml aliquot of supernatant was added to the sample tube containing 6 ml saturated NaCl solution and allowed to stand for 10 min at 20°C. The blank tube was run with 2 ml of 2% HPO_3 instead of the supernatant aliquot. Sodium nitroprusside and sodium cyanide, 1 ml each was added to the blank and sample tubes. The optical density was measured at 520 nm and the levels were expressed as μg/100 mg tissue wt.

Total Sulfhydryl (–SH) Groups: Total –SH groups were estimated by modified method of Sedlak and

Table 1. Antioxidative Levels in Liver of Control and Experimental Groups

PARAMETER	CONTROL	$NiCl_2$		$K_2Cr_2O_7$	
		8 mg	16 mg	5 mg	10 mg
Lipid peroxidation (MDA/60 min/100 mg tissue weight)	29.66 ± 0.94 (100)	33.20 ± 1.01* (112)	$38.47 \pm 0.68^{\dagger}$ (130)	$40.05 \pm 0.47^{\dagger}$ (135)	$43.08 \pm 1.05^{\dagger}$ (145)
Glutathione (mg/100 mg tissue weight)	176.21 ± 3.56 (100)	166.45 ± 1.85* (95)	$151.37 \pm 1.17^{\dagger}$ (86)	164.44 ± 1.81* (93)	$149.48 \pm 0.77^{\dagger}$ (84)
Total −SH groups (mg/g tissue weight)	14.46 ± 0.27 (100)	13.37 ± 0.25** (92)	$12.25 \pm 0.13^{\dagger}$ (85)	13.28 ± 0.25** (92)	$12.11 \pm 0.10^{\dagger}$ (84)

PARAMETER	$NiCl_2 + K_2Cr_2O_7$		Vit. E	$NiCl_2 + K_2Cr_2O_7$ + Vit E
	16 mg	10 mg		
Lipid peroxidation (MDA/60 min/100 mg tissue weight)	$46.18 \pm 0.61^{\dagger}$ (156)	$50.25 \pm 0.64^{\dagger}$ (169)	29.32 ± 0.77^{NS} (99)	30.17 ± 0.56^{NS} (102)
Glutathione (mg/100 mg tissue weight)	$138.00 \pm 1.42^{\dagger}$ (78)	$118.80 \pm 0.89^{\dagger}$ (67)	183.05 ± 4.32^{NS} (104)	172.31 ± 1.60^{NS} (98)
Total −SH groups (mg/g tissue weight)	$10.59 \pm 0.10^{\dagger}$ (73)	$9.07 \pm 0.13^{\dagger}$ (63)	13.90 ± 0.22^{NS} (96)	13.77 ± 0.35^{NS} (95)

Values are mean ± S.E.; $NiCl_2$ = nickel chloride; $K_2Cr_2O_7$ = potassium dichromate; MDA = Malonyldialdehyde formed; NS = non significant. *$p < 0.05$; **p, 0.01; †$p < 0.001$; Figures in parentheses indicate percent (%) values.

Lindsey[15] 0.5 ml homogenate (made in 0.02 M Tris EDTA) was mixed with 1.5 ml of 0.2 M Tris EDTA buffer and 0.01 M 5′,5′-dithiobis-2-nitrobenzoic acid (DTNB). The mixture was made up to 10 with 7.9 ml of absolute methanol. A reagent blank (without sample) and a sample blank (without DTNB) were prepared in a similar manner. The tubes were allowed to stand for 15 min and then centrifuged for 15 min at 3000 *g* and the respective supernatants were read at 412 nm. Total sulfhydryl groups were expressed as mg/g tissue wt.

Total Ascorbic Acid (TAA): TAA was determined by the method of Roe and Kuether.[16] To 4 ml of homogenate, 1 ml of 2,4-dinitrophenyl-hydrazine reagent was added and then a drop of thiourea was added in order to activate the reaction. Blank tube was run with 4 ml of 6% tricarboxylic acid (TCA) instead of homogenate and the standard tube with 4 ml ascorbic acid solution (10 μg/ml). The tubes were kept in boiling water bath for 15 min and cooled. 5 ml of sulphuric acid was added to all the tubes and the tubes were allowed to stand for 30 min. The optical density of the color was measured at 540 nm. Its levels were expressed in mg/g tissue wt.

Statistics ——— Data were statistically analyzed using Student's *t*-test along with percent values and analysis of variance (ANOVA).

RESULTS

Biochemical Studies

GSH and Total –SH Groups: The levels of glutathione and total –SH groups had a significant ($p < 0.05$, 0.01, and 0.001) depletion following $NiCl_2$ and/or $K_2Cr_2O_7$ exposure in a dose dependent manner. The depletion was much more pronounced ($p < 0.001$) by the combined treatment (Table 1).

TAA: A significant decline was observed in the levels of total ascorbic acid after $NiCl_2$, and/or $K_2Cr_2O_7$ feeding. Its levels were further depleted ($p < 0.001$) by combination (Table 2).

Lipid Peroxidation: A significant ($p < 0.05$, 0.001) elevation was noted in lipid peroxidation after $NiCl_2$ and/or $K_2Cr_2O_7$ treatments gradually whereas the combined treatment had more impact ($p < 0.001$) (Table 1).

SOD and CAT: SOD showed a significant ($p < 0.05$, 0.001) reduction in its activity after the exposure to the toxicants. Similarly a significant ($p < 0.01$, 0.001) decrease was noted in catalase activity after $NiCl_2$ and/or $K_2Cr_2O_7$ treatments (Table 2).

All the above mentioned parameters exhibited no significant changes as compared to that of control by vitamin E alone and co-administration of vi-

Table 2. Antioxidative Parameters in Liver of Control and Experimental Groups

PARAMETER	CONTROL	$NiCl_2$		$K_2Cr_2O_7$	
		8 mg	16 mg	5 mg	10 mg
Total ascorbic acid (mg/g tissue weight)	4.51 ± 0.17 (100)	4.04 ± 0.05* (89)	$3.67 \pm 0.05^{\dagger}$ (81)	4.01 ± 0.7* (88)	$3.74 \pm 0.08^{\dagger}$ (83)
Super oxide dismutase (Units SOD/mg protein)	0.78 ± 0.06 (100)	0.66 ± 0.03^{NS} (85)	0.60 ± 0.03* (77)	0.62 ± 0.04* (79)	0.56 ± 0.03** (72)
Catalase (μmol of H_2O_2/mg protein)	34.79 ± 0.44 (100)	33.59 ± 0.22** (97)	$29.80 \pm 0.15^{\dagger}$ (86)	30.18 ± 0.47** (87)	$29.22 \pm 0.32^{\dagger}$ (84)

PARAMETER	$NiCl_2 + K_2Cr_2O_7$		Vit. E	$NiCl_2 + K_2Cr_2O_7$ + Vit. E
	16 mg	10 mg		
Total ascorbic acid (mg/g tissue weight)	$3.27 \pm 0.07^{\dagger}$ (72)	$3.00 \pm 0.03^{\dagger}$ (66)	4.37 ± 0.19^{NS} (97)	4.28 ± 0.08^{NS} (95)
Super oxide dismutase (Units SOD/mg protein)	$0.50 \pm 0.02^{\dagger}$ (64)	$0.47 \pm 0.02^{\dagger}$ (60)	0.78 ± 0.01^{NS} (100)	0.73 ± 0.02^{NS} (94)
Catalase (μmol of H_2O_2/mg protein)	$24.15 \pm 0.56^{\dagger}$ (69)	$20.53 \pm 0.44^{\dagger}$ (59)	35.07 ± 0.26^{NS} (101)	35.00 ± 0.03^{NS} (101)

Values are mean ± S.E.; $NiCl_2$ = nickel chloride; $K_2Cr_2O_7$ = potassium dichromate. *$p < 0.05$; **p, 0.01; †$p < 0.001$. Figures in parentheses indicate percent (%) values.

tamin E along with nickel and chromium treatments (Tables 1 and 2).

DISCUSSION

Numerous studies have reported toxic and carcinogenic effects induced when animals and humans are exposed to certain metals. Detailed studies in the past two decades have shown that metals like iron, copper, cadmium, chromium mercury, nickel vanadium possess the ability to produce reactive free radicals resulting in DNA damage, lipid peroxidation, depletion of protein, sulfhydryl and other effects. The toxic effect of metals involves hepatotoxicity, neurotoxicity and nephrotoxicity.[17] Nickel and chromium are major components of various steel industries. Toxic and carcinogenic effects of nickel have been well documented in occupationally exposed workers.[1] Similarly chromium (VI) compounds are widely recognized as human carcinogens. From the epidemiological studies, it is suggestive that hexavalent chromium causes increased risk of bone, prostate lymphomas, Hodgkins *etc*. reflecting the ability of hexavalent chromium to penetrate all tissues in the body.[18] Due to their extensive use in industry, there is also a need to investigate their combined toxicity in organ system and mitigative role of vitamin on their toxicity. Hence the present study was undertaken in mice as an animal model. Liver with its metabolic detoxifying function is extremely vulnerable to harmful substances. Certain metals are known to act as catalyst for the production of free radicals in biological systems.[19] The most important consequences of free radical production are lipid peroxidation increase and change in permeability of cell membrane.[20] Lipid peroxidation is often discussed as a cause of metal induced toxicity.[21] In the present study there was a significant increase in the LPO levels after the treatments. Corroborating with present data Misra *et al*.[3] noted high level of lipid peroxidation in the liver with an increased concentration of H_2O_2 followed by a reduction in the activity of enzyme catalase which is an H_2O_2 scavenging enzyme by nickel. These two effects could augment the potential of oxidative cell damage in this study. Chromium is also found to affect LPO at both concentrations thus leading to cell injury. Studies by Bagchi *et al*.[8] corroborate with our findings. Moreover recent studies in our laboratory indicated increased levels of LPO in the hepatic tissue of male mice treated with chromium or nickel salts.[9]

One of the mechanisms by which heavy metals produce effects is through their interaction with cellular sulfhydryl groups in proteins. Sulfhydryl groups thus serve as a source of electrons for reduction and also mediate the methylation process. When the availability of free thiol group is low, enhanced expression of toxicity in the form of oxidative stress could occur. Thus the reduction in total –SH group in our study indicates toxicity status of the tissue by these toxicants. Several physiological mechanisms

(antioxidative defense mechanisms) dispose of free radicals/ROS by directly scavenging them or by interrupting the already occurring lipid peroxidation chain reaction to limit their tissue damage. Glutathione performs a pivotal role in maintaining the metabolic and transport functions of cells. Its conjugation helps in detoxification by binding electrophiles that could otherwise bind to proteins or nucleic acids, resulting in cellular damage and genetic mutation.[22] The decreased glutathione levels in the present study could be due to its involvement in the mechanisms of detoxification of various xenobiotics,[23] inhibition of lipid peroxidation by scavenging free radicals[24,25] as well as reducing dehydroascorbic acid to reduced form. Its depletion could also be due to its participation in the formation of nickel and chromium complexes, which are excreted during protection over liver by metal poisoning. Studies by Das *et al*.[4] and Bagchi *et al*.[8] showed decreased levels of GSH by nickel and chromium intoxication in support of our data.

Ascorbic acid is known to be a powerful reducing agent, which helps in activating several enzymes, and acts as an antioxidant for detoxifying toxic substances.[26] A decrease in levels of total ascorbic acid under chromium and/or nickel induced stress suggests that the stored ascorbic acid is rapidly oxidized in the liver. In support to our data, studies by Das *et al*.[4] also reported altered ascorbate metabolism in the liver by nickel treatment. The protective action of ascorbic acid on heavy metal toxicity is well documented *via* its free radical scavenging mechanism and detoxification effect.[27–30]

The enzyme SOD catalyses dismutation of superoxide radical, leading to formation of hydrogen peroxide, which in turn is detoxified by the enzyme catalase.[31] Studies by Das *et al*.[4] also reported reduced activity of these enzymes by chromium in the hepatic tissue in agreement with our data. Supplementation of vitamin E with Nickel + chromium to mice exhibited mitigating effect in the liver, as all the above-mentioned biochemical tests were not altered. Vitamin E alone supplementation also produced no significant changes. Lebovitz *et al*.[32] documented that vitamin exhibited greatest protection against metal toxicity. Experimental studies by Sokol[33] and Appenroth *et al*.[34] have reported that antioxidants of vitamin E family have protective effects against metal induced adverse effects in man and laboratory animals. Susa *et al*.[35] also reported that pre-treatment with vitamin E normalized the level of non-enzymatic antioxidants such as glutathione and vitamin suppressed by dichromate. Thus these studies corroborate with the present investigation and confirm the role of vitamin E as a scavenger of free radicals, probably preserving structural and functional integrity of sub cellular organelles thereby protecting the hepatic tissue.

The present findings hence revealed that nickel and/or chromium caused formation of free radicals in the liver by reducing the antioxidant indices. These adverse effects were more pronounced by the combination leading to liver dysfunction. However vitamin E supplementation to nickel and chromium combination fed mice exhibited no effects in liver antioxidant system indicating its antioxidative property. This vitamin thus ameliorates metal exerted oxidative liver damage and might have a therapeutic use in heavy metal induced oxidative stress in human.

Acknowledgements One of the authors M. V. R. is thankful to University Grants Commission (UGC), New Delhi for Financial assistance.

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