EFFECT OF L-ASCORBIC ACID ON NICKEL-INDUCED ALTERATIONS IN SERUM LIPID PROFILES AND LIVER HISTOPATHOLOGY IN RATS

Kusal K. Das,[1] Amrita Das Gupta,[1] Salim A.Dhundasi,[1] Ashok M.Patil,[2] Swastika N. Das[3] and Jeevan G.Ambekar[4]

[1]Department of Physiology and [2]Department of Pathology, Al-Ameen Medical College, Bijapur 586108 Karnataka, India; [3]Department of Engineering Chemistry, B.L.D.E.A's. Dr. P.G. Halakatti College of Engineering and Technology and [4]Department of Biochemistry, Sri B.M. Patil Medical College, Bijapur, 586103, Karnataka, India

ABSTRACT

Nickel exposure greatly depletes intracellular ascorbate and alters ascorbate-cholesterol metabolism. We studied the effect of the simultaneous oral treatment with L-ascorbic acid (50 mg/100 g body weight (BW) and nickel sulfate (2.0 mg/100 g BW, i.p) on nickel-induced changes in serum lipid profiles and liver histopathology. Nickel-treated rats showed a significant increase in serum low-density lipoprotein-cholesterol, total cholesterol, triglycerides, and a significant decrease in serum high-density lipoprotein-cholesterol. In the liver, nickel sulfate caused a loss of normal architecture, fatty changes, extensive vacuolization in hepatocytes, eccentric nuclei, and Kupffer cell hypertrophy. Simultaneous administration of L-ascorbic acid with nickel sulfate improved both the lipid profile and liver impairments when compared with rats receiving nickel sulfate only. The results indicate that L-ascorbic acid is beneficial in preventing nickel-induced lipid alterations and hepatocellular damage.

Reprint requests to: Kusal K.Das, Professor, Department of Physiology, Al-Ameen Medical College, Bijapur-586108;Karnataka, India e-mail: kusaldas@yahoo.com

INTRODUCTION

Heavy or toxic metals are trace metals with a density at least five times that of water. As they cannot be metabolized by the body, these metals are bioaccumulative. Nickel salts are considered an industrial health hazard /1/. Human occupational exposure to nickel occurs primarily via the inhalation or ingestion of metal containing diets, which is particularly high among stainless steel welders, miners and metallurgy workers /2–3/. After entering the body, nickel penetrates all organs and accumulates primarily in bone, liver and kidney /3–4/, being excreted in bile and urine /5/. Nickel concentrations in the urine of normal human subjects vary from 0.1 to 13.3 μg/L, whereas in the urine of welders, nickel has been found at a concentration of > 18.8 μg/L /6/. If nickel enters and accumulates in the body tissues faster than the body's detoxification pathways can dispose of it, a gradual build-up of these toxins will occur. Exposure to a high concentration of nickel is not necessary to produce a state of toxicity in the body because the metal accumulates in body tissues and over time can reach toxic levels /3/. Systems in the body where nickel can induce impairment and dysfunction include blood and cardiovascular systems, detoxification pathways (colon, liver, kidney, skin), endocrine (hormonal), energy production pathways, enzymatic, gastrointestinal, immune, nervous (central and peripheral), reproductive, and the urinary tract /7–10/.

Nickel induces severe liver and kidney damage by altering several marker enzymes and ascorbate-cholesterol metabolism /4/. Evidence in humans is sufficient for the carcinogenicity of nickel but the precise molecular mechanism of nickel induced carcinogenesis is undefined /11/. The most plausible mechanism that may be operative in vivo is the generation of reactive oxygen species (ROS), which initiate lipid peroxidation (LPO), thereby causing oxidative damage to critical macromolecules like proteins or DNA, as well as cell damage or death /12–13/. Liver, the major site of detoxification is the primary target of environmental and occupational toxicity. Protection of hepatic cell from nickel-induced alteration of physiological chemistry becomes a necessity /14/.

Ascorbate is a well-known antioxidant required by all mammalian cells for proper functioning. The effect of nickel compounds on cell is complex and most likely involves modulation of many metabolic pathways. Recently it was found that the nickel-induced activation of HIF-1 transcription factor and up-regulation of hypoxia inducible genes reported in /15/ is due to greatly depleted intracellular ascorbate levels /16/. The supplementation of the media with ascorbate increased intracellular ascorbate and reversed both metal-induced stabilization of HIF-1 and HIF-1α-dependent gene expression /16/. Apart from these, the suppression of HIF-1α protein levels by ascorbate at physiological concentrations was demonstrated in tumor cell lines /17/. Deficiency of ascorbic acid has a direct association with increased atherosclerosis in the guinea pig, and its intake was shown to have a relation to increased atherosclerosis in quails, rabbits, rats, and humans /18/. Low density lipoproteins (LDL) deposited under the endothelial cell of the arterial walls can undergo oxidation by oxygen free radicals released from arterial endothelium and smooth muscle cells /19/. Ascorbate administration exerts an antioxidant effect in hypercholesterolemic rats and also exerts a protective role against the peroxidative damage of lipids /20/. Several studies are underway to determine the effect of antioxidant supplementation following heavy metal exposure. The data suggest that antioxidants may play an important role in abating certain hazards of heavy metals /21–22/.

The present study was designed to ascertain the effect of L-ascorbic acid on nickel sulfate ($NiSO_4$, $6H_2O$) induced alteration of serum lipid profile and to evaluate histological findings of liver in male albino rats.

EXPERIMENTAL

Adult (aged 60 to 70 days) laboratory-bred male Wister rats weighing 160 ± 5 g each were fed with laboratory stock diet and water *ad libitum* for 7 days. The acclimatized animals were divided into four groups of six animals each. The chances of nickel toxicity

occurring by the oral route are remote because a large amount of nickel is required to produce a toxic effect by ingestion. Nickel salts administered intravenously, intraperitoneally (i.p.), or subcutaneously are highly toxic /2/. Hence, nickel sulfate was administered i.p. in this study. Group I served as an untreated control. Group II rats were administered nickel sulfate (Sigma Chemicals, St. Louis, Missouri, USA) in double-distilled water at a dose of 2.0 mg/100g BW. i.p. on alternate days until the tenth dose /23/. Group III rats were treated orally with ascorbic acid at a dose of 50 mg/100g BW /24 , and group IV rats received both nickel sulfate (2.0 mg/100g BW, i.p) and ascorbic acid (50 mg/100g BW, orally) simultaneously on alternate days until the tenth dose.

The entire experimental protocol was approved by institutional ethical committee and utmost care was taken during the experimental procedure, as well as at the time of sacrifice, according to the Helsinki Declaration of 1964 /25 . The rats were sacrificed at the end of the last dose after an overnight fast. Blood samples were collected in a centrifuge tube and allowed to form serum. Serum total cholesterol, LDL-cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides were assayed using an enzymatic estimation kit (ERBA-diagnostics Mannheim, GmbH, Germany) /26–27/.

Mean ± SEM values were calculated for each group. To determine the significance of inter-group differences, we analyzed each parameter separately. A one way analysis of variance (ANOVA) was carried out at 1% Fisher's distribution to determine which of the groups differed among themselves. Duncan's multiple range test was applied with the level of significance fixed at $P < 0.05$ /28/.

For the evaluation of histopathology, liver from each rat was stored in 10% neutral buffered formalin solution. Three lobes of each rat liver were processed and sectioned. By following routine histological techniques, the samples were put into paraffin, and serial sections of 5 μm were taken from tissue blocks. For each rat, three slides were made and stained with hematoxylin and eosin. The preparations were evaluated under a photomicroscope and photographed (Olympus BH-2 with Samsung Digital Color Camera, Model No. SDC-242).

TABLE 1

Effect of nickel sulfate on serum lipid profile in rats

Group	LDL-C (mg/dL)	TC (mg/dL)	HDL-C (mg/dL)	TG (mg/dL)
I	46.05 ± 4.84^a	68.13 ± 8.33^a	18.33 ± 1.24^a	80.16 ± 6.48^a
II	62.55 ± 4.66^b	86.8 ± 9.48^b	11.36 ± 2.48^b	135.6 ± 10.48^b
III	43.38 ± 4.74^a	69.66 ± 6.64^a	15.53 ± 4.64^c	84.5 ± 9.46^a
IV	58.89 ± 4.84^b	75.33 ± 7.51^c	15.00 ± 2.48^c	119.3 ± 18.42^c

Treatment groups: I- untreated control; II- nickel sulfate; III- L-ascorbic acid; IV- nickel sulfate + L-ascorbic acid. LDL-C, low density lipoprotein-cholesterol; TC, total cholesterol; HDL-C, high density lipoprotein-cholesterol; TG, triglyceride; each value is mean + SEM of six observations in each group. In each column, values with different superscripts (a, b, c) were significantly different from each other ($p < 0.05$).

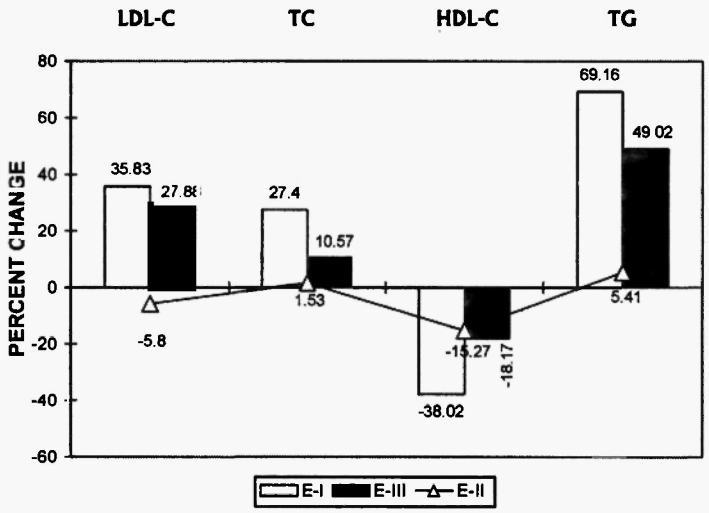

Fig. 1: Percent change chart of serum lipid profile of nickel sulfate treated rats. Legend as in Table 1. E I = Group I vs. Group II; E II = Group I vs. Group III; E III = Group I vs. Group IV

RESULTS

Biochemical analysis

Table 1 shows that in Group II, nickel induced a significant increase in serum LDL-cholesterol, total cholesterol, and triglyceride levels and a significant decrease in the serum HDL-cholesterol level in comparison with the control Group I. Group IV (nickel sulfate + L-ascorbic acid) also showed a significantly elevated level of serum LDL-cholesterol level, total cholesterol, and triglycerides and lower level of HDL-cholesterol in comparison with Group I, but the total cholesterol and triglyceride levels were significantly lower and HDL-cholesterol significantly higher that those in Group II.

Figure 1 shows the percent change increase of LDL-cholesterol, total cholesterol, and triglyceride and the percent change decrease of serum HDL-cholesterol in Group IV in comparison with the control Group I. But when compared with Group II receiving nickel sulfate only, the rise in LDL-cholesterol, total cholesterol, triglyceride, and fall in serum HDL-cholesterol were remarkably less (E I vs. E III). Rats receiving L-Ascorbic acid alone (Group III) did not show any significant variation in the parameters studied above when compared with control (Group I) [E I vs. E II]

Histopathological evaluation

The histological structure of normal untreated rat liver (Group I) showed a normal architecture (Figs. 2A, 2B). In Group II (+ nickel sulfate), all six rats showed loss of normal architecture (Figs. 3A, 3B). Apart from that, large cells with vacuolated cytoplasm, eccentric nuclei, and Kupffer cell hypertrophy were also seen. In Group III (L-ascorbic acid), all six rats showed an intact hepatic cellular architecture but a moderate cellular hypertrophy and enlarged nuclei (Figs. 4A, 4B). In Group IV (nickel + ascorbic acid), all showed near to normal histological results except for some sinus congestion (Figs. 5A, 5B).

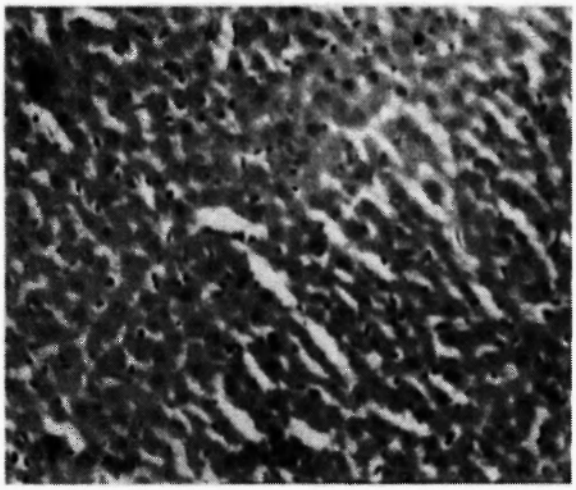

Fig. 2: **(A)**Paraffin section of the liver of an untreated control rat showing central vein (cv) with radiating hepatocytes and sinusoidal spaces (s). 10 x 10 X

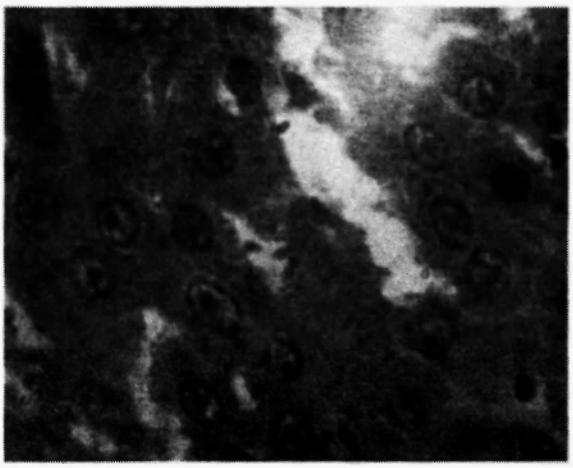

Fig. 2: **(B)** Paraffin section of an untreated control rat liver. × 45 x 10

Fig. 3: (A) Section of the liver of a rat treated with nickel sulfate Loss of normal architecture and fatty changes (?) are seen. 10x10 magnification

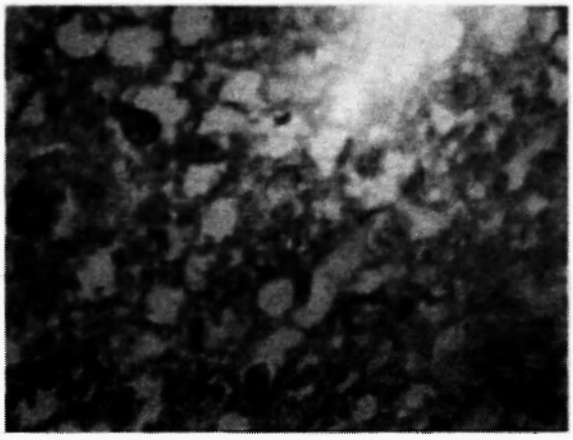

Fig. 3: (B) Section of liver from a rat treated with nickel sulfate. Large cell with vacuolated cytoplasm, eccentric nuclei; Kupffer cell hypertrophy is seen. 45x10 magnification

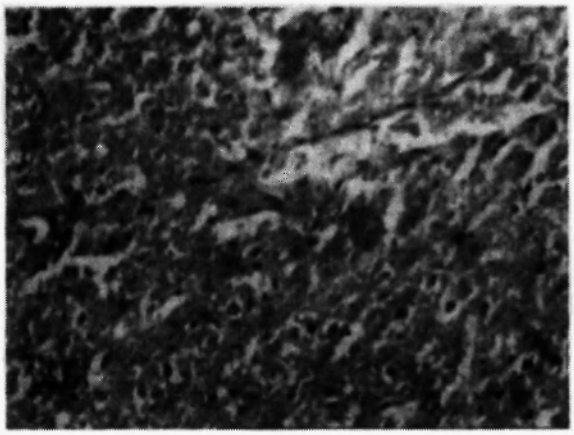

Fig.4: **(A)** Paraffin section of the liver of a rat treated with L-ascorbic acid. Congested vessels are seen. Hepatocellular architecture is intact. 10x10 magnification

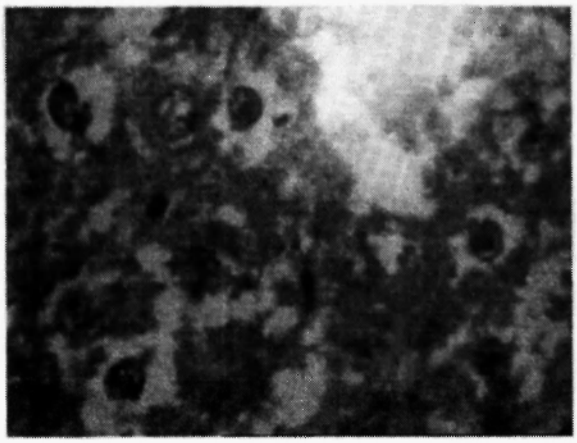

Fig. 4: **(B)** Paraffin section of the liver of a rat treated with L-ascorbic acid. Moderate cellular hypertrophy and enlarged nuclei are seen. 45x10 magnification

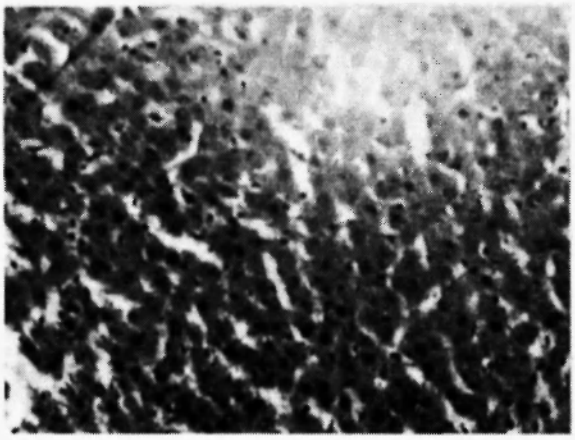

Fig. 5: (A) Paraffin section of the liver of a rat treated with nickel sulfate + L-ascorbic acid. Near normal hepatic architecture is seen. 10 x 10 magnification

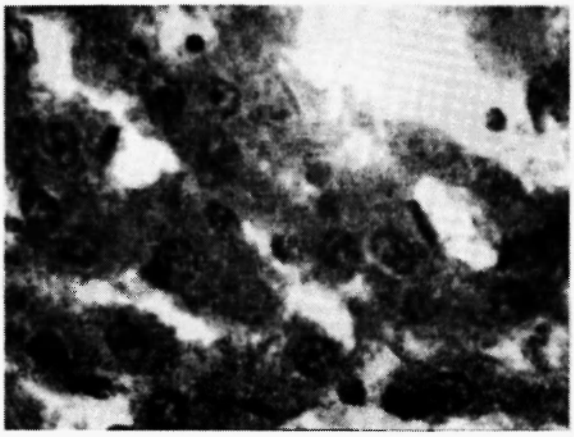

Fig. 5: (B) Paraffin section of liver from a rat treated with nickel sulfate + L-ascorbic acid. Hepatocytes show prominent nuclei. Sinus congestion is also visible. 45 x 10 magnification

DISCUSSION

Cholesterol is a soft, waxy substance formed among lipids in the blood stream and in body cells. The compound is an important part of a healthy body because it is used to form cell membranes and several hormones and is necessary for other functions. Cholesterol and other lipids cannot dissolve in the blood but rather must be transported to and from the cells by low-density lipoproteins (LDL) and high-density lipoproteins (HDL). High-density lipoprotein-cholesterol tends to carry cholesterol away from the arteries and back to the liver. Therefore, a high level of LDL-cholesterol and a low level of HDL-cholesterol is a high risk factor for heart disease /29/. High liver cholesterol levels can be due to hepatic dysfunctioning.

The nickel-induced rise of serum LDL-cholesterol, total cholesterol, and triglycerides and fall of serum HDL-cholesterol observed here in Group II could be due to changes in the gene expression of hepatic enzymes like HMG-CoA reductase (hydroxy-methylglut-aryl-CoA reductase), which in turn depresses LDL-receptor gene expression. Defects in the LDL-receptor interfere with cholesterol uptake from the blood stream, which in turn causes excess cholesterol synthesis in the liver and high levels of serum total cholesterol and LDL-cholesterol /30/. Heavy-metal-induced change in the gene expression of HMG-CoA reductase in rats has already been reported /31/.

In the present study, the nickel-induced rise in serum triglycerides (Group II) is possibly due to a lack of activity in blood vessels of lipoprotein lipase, which breaks up the triglyceride inside the chylomicrons, releasing fatty acids in the process. Fatty acids can either be used by the muscles as energy or be absorbed by fat cells, in which they are incorporated again into triglycerides that can be stored for further energy needs. Although proving conclusively that elevated triglycerides alone can cause atherosclerosis is difficult, hyper-lipidemia is increasingly recognized as being associated with other conditions that increase the risk of atherosclerosis, including a low level of HDL-cholesterol, insulin resistance, poorly controlled diabetes mellitus, and small, dense LDL-cholesterol particles /19/.

In the present study, when compared with Group II, the significant improvement in lipid profiles in Group IV rats treated concomitantly with L-ascorbic acid and nickel sulfate agrees with reports that ascorbic acid can help decrease the levels of total and LDL-cholesterol and triglycerides and increase the level of HDL-cholesterol /32 . L-ascorbic-acid-induced increase in LDL-receptors and facilitation of lipoprotein-cholesterol clearance has also been reported /33/. In Group IV, ascorbic acid could counteract nickel-induced changes in HMG-CoA reductase activities and thereby improve the serum lipid profile/34/. When ascorbic acid levels are low, the body compensates and manufactures more cholesterol; conversely, high levels of vitamin C inhibit HMG-CoA reductase and lower the total cholesterol level. As nickel decreases the cellular transportation of ascorbic acid and depletes intracellular ascorbic acid, resulting in intracellular hypoxia and metabolic dysfunction /16 , supplementation with L-ascorbic acid could reverse the nickel-induced alteration of hepatic enzymes activities that regulate cholesterol biosynthesis.

The liver is the primary organ for the detoxification, bio-transformation, and excretion of xenobiotics. The results of our histo-pathological studies show that in nickel sulfate-treated rats, liver tissue lost its normal characteristics and architectural organization (Figs. 3A, 3B), and injurious signs were observed in individual hepatocytes, some of which were swollen with granular cytoplasm and others showed cytoplasmic vacuolization with giant nuclei or eccentric nuclei (Fig. 3B). Such histopathological observations clearly indicate that nickel is hepatotoxic. The change in cellular integrity could be due to the oxidative stress developed by the nickel-induced generation of reactive oxygen species (ROS), such as hydroxyl ion (OH^-), superoxide radical (O^{2-}), or hydrogen peroxide (H_2O_2). The significant improvement in the histopathology of the liver in rats treated simultaneously with nickel sulfate and L-ascorbic acid (Figs. 5A , 5B) points to a beneficial effect of L-ascorbic acid as an effective antioxidant to combat free-radical-induced hepatocellular damage.

The moderate cellular hypertrophy with enlarged nuclei and congested blood vessels observed in the liver of rats treated with L-ascorbic acid only (Figs. 4A, 4B) was a surprising finding.

Possibly, an additional supplementation of L-ascorbic acid in normal rats (who can synthesize ascorbic acid) might be counterproductive. Antioxidants like vitamin C can have an opposite pro-oxidant effect under certain conditions, particularly when consumed in large doses /3ƃ .

Thus, nickel sulfate, a toxic heavy metal, adversely affects the serum lipid profile and causes degenerative histopathological changes in rat liver. Simultaneous treatment with L-ascorbic acid partially improved nickel-induced hepatocellular damage and serum lipid profiles, but a mild counterproductive effect on the histopathology of rat liver occurred with L-ascorbic acid supplementation alone.

ACKNOWLEDGEMENT

The authors greatly acknowledge the Director, Defence Institute of Physiology and Allied Sciences, New Delhi (DRDO, Ministry of Defence, Government of India) for providing financial assistance *(Ref.No. TC/260/TASK-91 (KKD)/DIPAS/2004 dt.07/06/2004)*. The authors also acknowledge Dr. B.S. Patil, Dean of Al-Ameen Medical College, Bijapur for his kind cooperation and Mr. Saleem Pathan, Animal House Incharge and Mr. Abid Jahagirdar, Computer operator, Al-Ameen Medical College, Bijapur, Karnataka, India for their technical assistance in this study.

REFERENCES

1. Luckey CD, Venugopal B. Metal Toxicity in Mammals, Volume 1. New York, NY, USA, Plenum Press, 1974; 2.
2. Nielsen FH, Shuler TR, Mcleod TG, Zimmerman TJ. Nickel influences iron metabolism through physiologic, pharmacologic and toxicologic mechanisms in rats. J Nutr 1984; 114: 1280–1288.
3. Agency for Toxic Substances and Disease Registry (ATSDR). Toxicological Profile for Nickel. U.S. Department of Health and Human Services, Public Health Service, ATSDR. Atlanta, Georgia, USA: U.S. Government Printing Office 2003; 5–16.

4. Das KK, Dasgupta S. Studies on the role of nickel in the metabolism of ascorbic acid and cholesterol in experimental animals. Ind J Physiol Allied Sci 1998; 52: 58–62.
5. Onkelinx C, Becker J, Sunderman FW Jr. Compartmental analysis of the metabolism of Ni (II) in rats and rabbits. Res Commun Chem Pathol Pharmacol 1973; 6: 663.
6. Scansetti G, Maina G, Botta GC, at al. Exposure to cobalt and nickel in the hard metal production industry. Int Arch Occup Environ Health 1998; 71: 60–63.
7. Das KK, Dasgupta S. Alteration of testicular biochemistry during protein restrict ion in nickel treated rats. Biol Trace Elem Res 1997; 60: 243–248.
8. Das KK, Dasgupta S. Effect of nickel sulfate on testicular steroidogenesis in rats during protein restriction. Environ Health Perspect 2002; 110: 923–926.
9. Whanger PD. Effect of dietary nickel on enzyme activities and mineral content in rats. Toxicol Appl Pharmacol 1973; 25: 323–331.
10. Dieter MP, Jameson CW, Tucker AN, Luster MI, French JE, Hong HL, et al. Evaluation of tissue deposition myelopoietic and immuno-logic responses in mice after long term exposure to nickel sulfate in drinking water. J Toxicol Environ Health 1988; 24: 357–372.
11. Bucher JR, Hailey JR, Roycroft JR, Haseman JK, Sills RC, Grumbein SL, et al. Inhalation toxicity and carcinogenicity studies of cobalt sulfate. Toxicol Sci 1999; 49: 56–67.
12. Das KK, Das SN, Dasgupta S. The influence of ascorbic acid on nickel in duced hepatic lipid peroxidation in rats. J Basic Clin Physiol Pharmacol 2001; 12: 187–194.
13. Doreswamy K, Shrilatha B, Rajeshkumar T, Muralidhara. Nickel induced oxidative stress in testis of Mice: Evidence of DNA damage and Genotoxic effects. J Androl 2004; 25: 996–1003.
14. Dasgupta S, Ghosh S, Das KK. Transaminase activities in some metabolically active tissues of nickel treated rats under protein restriction. Ind J Physiol Allied Sci 1996; 50: 27–33.
15. Salinikow K, Blagosklonny MV,Ryan H, Johnson J and Costa M. Carcinogenic nickel induces genes involved with hypoxic stress. Cancer Research 2000; 60: 38 - 41
16. Salnikow K, Donald SP, Bruick RK, Zhitkovich A, Phang JM, Kasprzak KS. Depletion of intracellular ascorbate by the carcinogenic metal nickel and cobalt results in the induction of hypoxic stress. J Biol Chem 2004; 279: 40337–40344.

17. Knowles HJ, Raval RR, Harris AL, Ratcliffe PJ. Effect of ascorbate on the activity of hypoxia inducible factor in cancer cells. Cancer Res 2003; 63: 1764–1768.
18. Das S, Yadav D, Narang R, Das N. Interrelationship between lipid peroxidation and ascorbic acid and superoxide dismutase in coronary artery disease. Current Sei 2002; 83: 488–491.
19. Terasawa Y, Ladha Z, Leonard SW, Morrow JD, Newland D, Sanan D, et al. Increased atherosclerosis in hyperlipidemic mice deficient in alpha-tocopherol transfer protein and vitamin E. Proc Natl Acad Sei U S A 2000; 28: 830–834.
20. Santillo M, Mondola P, Milone A, Gioielli A, Bifulco M. Ascorbate administration to normal and cholesterol-fed rats inhibits in vitro TBARS formation in serum and liver homogenates. Life Sei 1996; 58: 1101–1108.
21. Ercal N, Gurer-Orhan H, Aykin-Burns N. Toxic metals and oxidative stress part I: mechanisms involved in metal-induced oxidative damage. Curr Top Med Chem 2001; 1: 529–539.
22. Das KK, Das SN. Studies on the role of ascorbic acid on nickel induced hepatic nucleic acid concentrations in rats. J Basic Clin Physiol Pharmacol 2004; 14: 185–195.
23. Bordes E, Papillion VV. Myocardial change induced by nickel and in association with cadmium. Rev Ig Bacteriol Virusal Parazitol Epidemol Pneumotizol 1983; 32: 51–56.
24. Husain K, Sugendran K, Pant SC, Sharma VP, Vijayaraghavan R. Biochemical and pathological changes in response to hyperoxia and protection by antioxidants in rats.Indian J Physiol Pharmacol 1992; 36: 97-100.
25. Declaration of Helsinki (1964), amended by World Medical Assembly, Venice, Italy, 1983. Br Med J 1996; 313(7070): 1448–1449.
26. Bartis.CA, Ashwood E (edited). Text Book of Clinical Chemistry, 3rd edition. Philadelphia, Pennsylvania, USA: WB Saunders Co, 1999; 840–841.
27. [No authors listed] Summary of the second report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel II) JAMA 1993; 269: 3015–3023.
28. Steel RGD, Torrie JH. Principles and Procedures of Statistics, New York, NY, USA: McGraw-Hill 1960; 99–131.
29. Lichtenstein AH, Deckelbaum RJ. AHA Science Advisory. Stanol/sterol ester-containing foods and blood cholesterol levels. A statement for healthcare professionals from the Nutrition

Committee of the Council on Nutrition, Physical Activity, and Metabolism of the American Heart Association. Circulation 2001; 103: 1177–1179.

30. Kantola T, Kivisto KT, Neuvonen PJ. Grapefruit juice greatly increases serum concentration of lovastatin and lovastatin acid. Clin Pharmacol Ther 1998; 63: 397–402.
31. Kojima M, Masui T, Nemoto K, Degawa M. Lead nitrate induced development of hypercholesterolemia in rats; Sterol independent gene regulation of hepatic enzymes responsible for cholesterol homeostasis. Toxicol Lett 2004; 154: 35–44.
32. Tofler GH, Stec JJ, Stubbe I, Beadle J, Feng D, Lipinska I, et al. The effect of vitamin C supplementation on coagulability and lipid levels in healthy male subjects. Thromb Res 2000; 100: 35–41.
33. Aulinskas TH, Van der Westhnuyzen DR, Coetzee GA. Ascorbate increases the number of low density lipoprotein receptors in cultured arterial smooth muscle cells. Atherosclerosis 1983; 47: 159–171.
34. Harwood Jr., Greene YJ, Stacpoole PW. Inhibition of human leukocyte 3-hydroxy 3-methyl glutaryl Coenzyme A reductase activity by ascorbic acid. An effect mediated by the free radical monohydroascorbate. J Biol Chem 1986; 261: 7127–7135.
35. Brody T. Nutritional Biochemistry. San Diego, California, USA: Academic Press, 1998.