Effect of Nickel Sulfate on Testicular Steroidogenesis in Rats during Protein Restriction

Kusal K. Das[1] and Shakuntala Dasgupta[2]

[1]Department of Physiology, Al-Ameen Medical College, Bijapur, Karnataka, India; [2]Department of Physiology, University of Calcutta, Calcutta, India

Nickel, a widely used heavy metal, exerts potent toxic effects on peripheral tissues as well as on the reproductive system. Low dietary protein coupled with exposure to this metal induces more severe changes, including biochemical defects, structural disorders, and altered physiologic functions. This study was designed to assess the effects of nickel sulfate on testicular steroidogenesis and to ascertain whether such alterations are reversible with normal protein and protein-restricted dietary regime. Nickel sulfate [2 mg/100 g body weight (bw)] dissolved in double-distilled water was administered on alternate days for 10 doses in a normal protein diet (18% casein) and a protein-restricted diet (5% casein) to Wistar male albino rats (bw 160 ± 5 g). Two groups, one with a normal protein diet and the other with a protein-restricted diet, served as controls. Twenty-four hours after the last treatment, all the animals except those in withdrawal groups were sacrificed by decapitation. We observed a significant reduction in the activities of the testicular steroidogenic enzymes and plasma testosterone concentration accompanied by a significant elevation in cholesterol and ascorbic acid level in both dietary groups. After 15 days of withdrawal from the nickel sulfate treatment, the testicular steroidogenic enzymes, along with plasma testosterone level, improved significantly in both normal protein-fed and protein-restricted dietary groups. The effects of nickel on testicular cholesterol and ascorbic acid concentration were also reduced after withdrawal. Our results indicate that nickel sulfate affects the steroidogenic enzymes, causing alteration in the formation of testosterone in both dietary groups, which was manifested in the elevated cholesterol and ascorbic acid level with decreased activities of steroidogenic enzymes in adult rats testes. However, these alterations were reversible in both groups of animals fed normal protein diets and protein-restricted diets. ***Key words:*** **hydroxysteroid dehydrogenase, nickel sulfate, protein restriction, testes, testosterone.** ***Environ Health Perspect*** **110:923–926 (2002). [Online 13 August 2002]**

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Modern industrialization has introduced harmful metals into the environment by redistributing them from immobilized ores and minerals, thereby exposing humans and animals to more metal salts. The toxicity of a metal depends on its inherent capacity to adversely affect any biologic activity. Among the myriad environmental pollutants, nickel, a heavy metal, merits special consideration as a potential toxic element (*1,2*), even though it helps in hemopoiesis because it functions as a cofactor that facilitates the intestinal absorption of ferric ion (*3*). Humans are exposed to nickel via food, water, and air produced from such sources as mining, extraction and refining, electroplating, grinding and polishing, nickel powder metallurgy, nickel alloys, nickel cadmium batteries, the chemical industry, food processing, and nickel waste disposal (*4*). The primary toxic effects of nickel sulfate are expressed in the myeloid system. Nickel causes dose-related decreases in bone marrow cellularity and in granulocyte-macrophage and pluripotent stem cell proliferative responses (*5*).

A number of studies on the carcinogenicity of nickel compounds in experimental animals have been reported (*6,7*). Generally, tumors are induced at the site of administration of the nickel compound. Several nickel compounds induce injection-site sarcomas (*2*). Bone, kidney, and liver are the main organs in which nickel accumulates. In addition, the skin, heart, spleen, testes, and intestine also store large amounts of nickel (*8*). Nickel is excreted mainly through the bile and urine. The nickel concentration in urine of normal human subjects is between 0.1 and 13.3 µg/L, whereas in urine of welders, nickel was found at concentrations > 18.5 µg/L (*9,10*).

At present there is much concern about the nature of toxicity of nickel. Strict controls have been instituted, and top priority is being given to setting the safe limits to which the population may be exposed. The level of dietary protein intake can markedly influence the severity of toxicity manifestations from chronic exposure to nickel (*11*). Metal ions interact with amino acids and protein in the biologic system in the form of coordinate complexes and chelates, participating in biosynthesis and degradation as well as maintaining the conformation of macromolecules. Not only do they enhance the catalytic activity of enzymes, but they also increase the stability of the protein moiety to metabolic turnover (*12*). In our earlier investigations, we reported infertility and varying degrees of testicular dysfunction with exposure to nickel according to dietary protein intake (*13,14*). As protein deficiency is widespread in most industrial and rural workers in India and in other developing countries, the present study was designed to elucidate the effect of nickel sulfate ($NiSO_4 \times 6H_2O$) on testicular steroidogenesis during protein restriction and also to ascertain whether these changes are permanent or reversible.

Materials and Methods

Animals and treatment. Adult (age 60–70 days) laboratory-bred male Wistar rats, weighing 160 ± 5 g, were initially maintained on standard laboratory stock diet and water *ad libitum*. They were acclimatized for 7 days to the laboratory conditions at 22–24°C and a 12-hr light:dark cycle. Rats were then divided into six equal groups of 10 each, and five animals were kept in each metabolic wire cage (24 in × 12 in × 8 in). Three groups were fed a normal protein diet (18% casein), and the other three groups were fed a protein-restricted isocaloric diet (5% casein; Table 1). The low-protein diet (5% casein) used in the present study was prepared by replacing a portion of the protein source (casein) in the normal stock diet with starch.

After 3 weeks of acclimatization, in the above mentioned dietary regime group 1 (18% casein) and group 4 (5% casein) served as controls and received the appropriate volume of the vehicle injected intraperitoneally. Groups 2 and 5 animals were treated with nickel sulfate (Sigma, Chemicals, St. Louis, MO) intraperitoneally in double-distilled water at a dose of 2 mg/100 g body weight (bw) on alternate days for 10 doses (*15*). This was an effective dose, and it does not cause any deleterious effect on the kidney because it is far below from the LD_{50} value of nickel sulfate. Groups 3 and 6 animals received the

Address correspondence to K. K. Das, Department of Physiology, Al-Ameen Medical College, Bijapur 586108, Karnataka, India. Telephone: 91-8352-70055 (extension nos. 224, 225). Fax: 91-8352-70184. E-mail: kusaldas@yahoo.com

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same dose of nickel sulfate as groups 2 and 5, but they were given an additional recovery period of 15 days after dose 10. The dietary status was maintained by pair feeding. The chances of nickel toxicity occurring under these circumstances by the oral route are remote because a large amount of nickel is required to produce a toxic effect by ingestion. In contrast to nickel salts administrated orally, nickel salts administered intraperitoneally or subcutaneously are highly toxic (*3*). Hence, nickel sulfate was administered intraperitoneally in this study.

We recorded the body weights of all rats on day 1 of the dietary treatment, on day 1 of the nickel sulfate injection, and just before sacrifice. At the end of the nickel sulfate treatment, animals of all groups except those of groups 3 and 6 were fasted overnight and sacrificed by decapitation from 0700 to 1100 hr to avoid any possible diurnal variation. The entire experimental protocol was approved by the Calcutta University ethical committee on animal research, and utmost care was taken during the experimental procedure as well as at the time of sacrifice as per the Helsinki Declaration, 1984 (*16*). At the end of a 15-day recovery period, animals of groups 3 and 6 were also sacrificed. Blood was collected in heparinized tubes, and plasma was separated by centrifugation immediately for estimation of testosterone. The testes and seminal vesicles of each group were separately dissected out, trimmed of fat, wiped clean, and weighed immediately.

Study of testicular steroidogenic enzymes, cholesterol, and ascorbic acid content. We used the right testes of each animal to estimate the activity of the steroidogenic enzymes 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) and to estimate cholesterol and ascorbic acid content. For enzymatic study, testicular tissue was homogenized (20% glycerol, 5 mM potassium phosphate, 1 mM EDTA) and then centrifuged at 10,000 rpm at 4°C for 30 min. The supernatant was decanted. Enzyme activities were measured by optical measurement (absorbance at 340 nm) of the rate of reduction of pyridine nucleotides (NAD or NADP) according to the method of Talalay (*17*) and Jarabak (*18*). The assay system contained 1 mL sodium pyrophosphate buffer, 0.5 mL of 5% bovine serum albumin, 0.04 mL steroid (dehydroepiandrosterone for 3β-HSD and testosterone for 17β-HSD), and 1 mL of the supernatant fluid. All chemicals were purchased from Sigma Chemicals, except the steroid (Organon India Ltd., Kolkata, India). Duplicate samples and blanks (without steroid) were run each time. One unit of enzyme activity is the amount that causes a change in absorbance of 0.001 U/min using the steroids as substrate. We measured testicular protein by the method of Lowry et al. (*19*). The enzyme activity was expressed in units per milligram of protein. We estimated the cholesterol (*20*) and ascorbic acid (*21*) content spectrophotometrically.

Study of plasma testosterone. We carried out radioimmunoassay of plasma testosterone according to the method of Coombs (*22*) using ^{125}I-testosterone (Diagnostic Products Corp., Los Angeles, CA, USA) and used 13% polyethylene glycol to separate antibody-bound and free hormone according to the method of Anderson et al. (*23*). All samples were assayed in duplicate. Because chromatographic purification of the samples was not performed, the testosterone values are the sum of testosterone and dehydrotestosterone (plasma levels of dehydrotestosterone are only about 1/10 of testosterone levels, and the cross-reactivity is typically less than 5%). The coefficient of variance within the assay was 6.2%. Between-assay variance was not determined because all samples were measured in a single assay. The average sensitivity of the assay was 7.5 pg testosterone/tube at the 95% confidence level. The blank plasma value was 40 pg testosterone/mL of plasma (n = 20), as determined by the assay of a pool of adult male rat plasma after treatment twice with 0.2 mL dextran-coated charcoal (2% charcoal, 0.5% dextran)/mL plasma. We determined the accuracy of the assay by adding 1 mg testosterone/ mL (nonradioactive) to 20 plasma samples.

Table 1. Composition of normal and protein-restricted diets.

Dietary components	Normal diet (%)	Protein-restricted diet (%)
Carbohydrate (amylum IP)	70	83
Fat (peanut oil)	7	7
Protein (casein)	18	5
Salt mixture[a]	4	4
Vitamin mixture[b]	1	1

IP, industrial production.
[a]Data from U.S. Pharmacopeia (*25*). [b]Data from the American Institute of Nutrition (*26*).

Table 2. Changes in body weight after nickel sulfate treatment (2 mg/100 g bw, for 10 days).

	Body weight (g)			
Group	Day of 1st injection	Day of sacrifice	p-Value	Percent change
1	190 ± 34[a]	203 ± 4.2[a]	< 0.01	+ 6.84
2	195 ± 4.8[a]	162 ± 3.4[b]	< 0.001	– 16.92
3	194 ± 5.4[a]	174 ± 4.8[c]	< 0.001	– 10.30
4	160 ± 3.4[b]	150 ± 4.2[d]	< 0.01	– 6.25
5	165 ± 4.2[b]	128 ± 4.2[e]	< 0.001	– 22.42
6	170 ± 3.8[b]	144 ± 3.8[f]	< 0.001	– 15.29

Each value is mean ± SEM of 10 observations in each group. In each column, values with different superscripts were significantly different from each other ($p < 0.05$). Group 1: normal protein diet (18% casein); group 2: normal protein diet + $NiSO_4$; group 3: normal protein diet + $NiSO_4$ + withdrawal; group 4: protein-restricted diet (5% casein); group 5: protein-restricted diet + $NiSO_4$; group 6: protein-restricted diet + $NiSO_4$ + withdrawal.

Statistical analysis. We calculated the mean ± SEM values for each group. To determine the significance of the intergroup differences, each parameter was analyzed separately and one-way analysis of variance (ANOVA) was carried out at 5% of Fisher's distribution. To find out which of the groups differed among themselves, we applied Duncan's multiple range test with the level of significance fixed at $p < 0.05$ (*24*).

Results

The nickel sulfate-treated rats showed a significant decrease in the growth rate, as indicated by the body weights (Table 2) of the treated rats in both the normal protein-fed and protein-restricted groups. In case of withdrawal groups (groups 3 and 6), marked improvement in body weights were observed. Table 3 shows that the relative weights of testes significantly decreased in all the experimental groups (groups 2, 3, 5, 6) compared to their respective controls (groups 1 and 4). Withdrawal groups in both dietary regimes showed a significant improvement in testicular weight. An insignificant decrease of seminal vesicle weights was observed after nickel

Table 3. Changes in wet weight to testicular and seminal vesicular tissues after nickel sulfate treatment (2 mg/100 g bw for 10 days).

Group	Testes weight (g)	Seminal vesicle weight (mg)
1	1.741 ± 0.082[a]	274.61 ± 24.24[a]
2	1.502 ± 0.075[b]	270.22 ± 25.32[a]
3	1.632 ± 0.081[c]	273.43 ± 22.48[a]
4	1.320 ± 0.082[d]	180.32 ± 24.32[b]
5	1.024 ± 0.081[e]	178.41 ± 25.4[b]
6	1.220 ± 0.074[f]	174.30 ± 20.47[b]

Each value is mean ± SEM of 10 observations in each group. In each column, values with different superscripts are significantly different from each other ($p < 0.05$). Group 1: normal protein diet (18% casein); group 2: normal protein diet + $NiSO_4$; group 3: normal protein diet + $NiSO_4$ + withdrawal; group 4: protein-restricted diet (5% casein); group 5: protein-restricted diet + $NiSO_4$; group 6: protein-restricted diet + $NiSO_4$ + withdrawal.

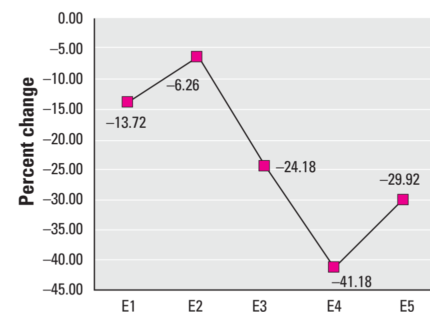

Figure 1. Percentage change in testicular cholesterol weight after nickel treatment. E1, group 1 vs. group 2; E2, group 1 vs. group 3; E3, group 1 vs. group 4; E4, group 1 vs. group 5; E5, group 1 vs. group 6.

sulfate treatment in all the experimental groups. The percent changes in testicular weight in both normal protein-fed and protein-restricted groups after nickel sulfate treatment are depicted in Figure 1.

Table 4 depicts the changes in the testicular cholesterol and ascorbic acid concentration after nickel sulfate treatment in the same experimental condition. Significant increases in ascorbic acid and cholesterol level occurred after nickel sulfate administration in all the experimental groups compared to their respective controls, whereas the withdrawal groups in both dietary regimes showed significant reductions compared to control groups.

Nickel sulfate treatment significantly reduced the activities of the two testicular steroidegenic enzymes 3β-HSD and 17β-HSD and plasma testosterone level (Table 4) in both dietary experimental groups. In contrast, 15 days after withdrawing nickel sulfate from rats in both dietary regimes (groups 3 and 6), the two steroidogenic enzymes and plasma testosterone level showed a significant recovery compared to nickel sulfate-treated groups. Percent changes in testicular cholesterol, ascorbic acid, 3β-HSD, 17β-HSD, and plasma testosterone level in both normal protein-fed and protein-restricted groups after nickel treatment are depicted in Figures 2 and 3.

We also observed that protein restriction itself produced a deleterious effect on body weight, organ weight, concentration of cholesterol, ascorbic acid, 3β-HSD, and plasma testosterone, but not on 17β-HSD activities (Tables 2–4).

Discussion

Our results indicate that nickel sulfate has an adverse effect on total body weight and on weights of testes under both normal and protein-restricted conditions, but a partial recovery took place in withdrawal groups under both dietary conditions. Loss of body weight after nickel sulfate administration indicates the interference of protein metabolism, presumably by inhibiting enzymatic pathways (*1*). The reduction in weights of testes and accessory glands is likely caused by lowered production of testicular androgen, possibly as a result of loss of mass of Leydig cells (*27,28*). Nickel sulfate induced a decrease in testicular weight, with decreased sperm concentration and motility (*14*). Nickel sulfate degenerated the germinal epithelium of testes and reduced testicular weight (*29*).

Steroidogenesis in the testes is under the physiologic control of two dehydrogenases. A constant supply of cholesterol and ascorbic acid is required for the synthesis of steroid hormones (*30*). Both dehydrogenases are directly involved in biosynthesis of testosterone from pregnenolone as well as androstenedione. Any alteration in the activity of these two enzymes affects androgen production. Reduced activities of these steroidogenic enzymes in mature testes of adults rats indicate reduced steroidogenesis (*30*).

The role of cholesterol as a precursor molecule in the synthesis of steroids hormones is well established (*31*). In this study, the cholesterol content in the testes of experimental groups of rats showed significant increases compared to their respective dietary control groups. This high accumulation of cholesterol may suggest that cholesterol is not used in testosterone biosynthesis and thereby corroborate nickel sulfate-induced reduction in steroidogenesis.

Ascorbic acid, an easily diffusible water-soluble biologic reductant, is found in abundance in testes (*32*), where it plays an important role in testicular hormonogenesis (*33*). Nickel sulfate induced an increase in ascorbic acid concentration in both normal and protein-restricted groups, reflecting that ascorbic acid is not used in the process of testicular steroidogenesis (*33*). Nickel might have some influence on the distribution and concentration of ascorbic acid, which in turn influences the physiologic fate of the metallic ions by a separate type of mechanism (*34*). The alteration of testicular steroidogenic activities and concentration of cholesterol and ascorbic acid in the protein-restricted condition is higher than that in normal protein condition, which indicates that the toxic effects of nickel were aggravated by protein restriction.

Our investigation further revealed that nickel sulfate induced a decreased plasma testosterone level in both dietary experimental groups. Nickel also affects the hypothalamic–pituitary–testicular axis (*29*). Metal exposure decreased the neurotransmitter content in anterior and mediobasal hypothalamus and decreased circulating levels of lutenizing hormone (LH) and testosterone (*35*). Our investigations show that nickel sulfate affects the hormonal milieu of the testes in both dietary groups. The hormonal milieu originates in the hypothalamus, which releases gonadotropin-releasing hormone in a pulsatile manner. This results in secretion of LH, which in turn stimulates the Leydig cell production of testosterone.

The production of testicular testosterone requires cholesterol and ascorbic acid, along with 3β-HSD and 17β-HSD (*36*). Low level of plasma testosterone may be due to a direct effect of nickel on the testicular hormonogenesis, which is supported by the decrease in steroidogenic enzyme activities with a concomitant increase in cholesterol and ascorbic acid levels (Table 4, Figures 2 and 3). The decrease in plasma testosterone level in protein-restricted experimental groups after nickel sulfate treatment is higher than in the normal dietary protein experimental groups (Table 4). Possibly, the low level of dietary

Table 4. Effect of nickel (2 mg/100 g bw) on testicular cholesterol, ascorbic acid, 3β- and 17β-hydroxysteroid dehydrogenase, and plasma testosterone concentration in rats.

Group	Cholesterol (mg/g tissue)	Ascorbic acid (mg/g tissue protein)	3β-HSD (U/mg)	17β-HSD (U/mg protein)	Testosterone (ng/mL)
1	86.35 ± 4.80^a	147.42 ± 8.32^a	8.97 ± 0.18^a	6.50 ± 0.29^a	3.27 ± 0.06^a
2	142.48 ± 8.34^b	194.32 ± 9.48^b	6.57 ± 0.23^b	5.10 ± 0.21^b	2.43 ± 0.10^b
3	100.28 ± 7.32^c	175.32 ± 9.21^c	7.83 ± 0.21^c	6.04 ± 0.22^a	2.84 ± 0.07^c
4	105.34 ± 8.28^c	170.46 ± 6.78^c	7.51 ± 0.22^c	6.10 ± 0.21^a	2.97 ± 0.11^c
5	168.32 ± 9.40^d	205.48 ± 10.41^d	4.21 ± 0.18^d	4.04 ± 0.11^c	2.17 ± 0.28^d
6	138.48 ± 10.30^b	188.32 ± 7.81^b	6.20 ± 0.21^b	5.34 ± 0.28^b	2.52 ± 0.12^b

Each value is mean ± SEM of 10 observations in each group. In each column, values with different superscripts were significantly different from each other ($p < 0.05$). Group 1: normal protein diet (18% casein); group 2: normal protein diet + $NiSO_4$; group 3: normal protein diet + $NiSO_4$ + withdrawal; group 4: protein-restricted diet (5% casein); group 5: protein-restricted diet + $NiSO_4$; group 6: protein-restricted diet + $NiSO_4$ + withdrawal.

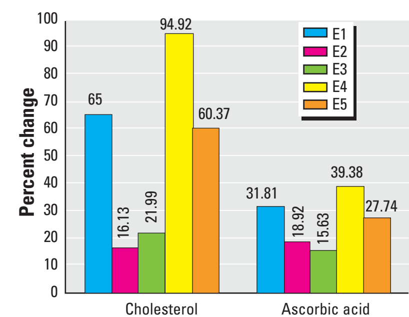

Figure 2. Percent change in testicular cholesterol and ascorbic acid concentration after nickel treatment. E1, group 1 vs. group 2; E2, group 1 vs. group 3; E3, group 1 vs. group 4; E4, group 1 vs. group 5; E5, group 1 vs. group 6.

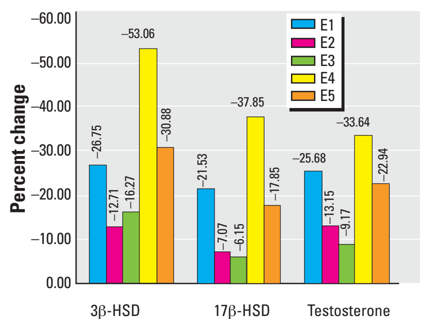

Figure 3. Percent change in testicular 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase and plasma testosterone concentration after nickel treatment. E1, group 1 vs. group 2; E2, group 1 vs. group 3; E3, group 1 vs. group 4; E4, group 1 vs. group 5; E5, group 1 vs. group 6.

protein intake decreases synthesis of testicular enzymes, lowering the testosterone level, which is further aggravated by exposure of nickel (*37*). After 15 days of withdrawal from the nickel sulfate treatment, the effects of nickel on testicular cholesterol, ascorbic acid, 3β-HSD, 17β-HSD, and plasma testosterone content were reduced in normal protein-fed as well as protein-restricted dietary groups. This may be explained by the fact that withdrawal from nickel sulfate causes an improvement of testicular steroidogenesis.

Simple dietary protein restriction induced reduction of body weight, organ weight, testicular 3β-HSD, and plasma testosterone level with a concomitant increase of testicular cholesterol and ascorbic acid concentration reflecting biochemical defects, structural disorders, and altered physiologic function. The organism depends essentially on dietary protein that directly or indirectly regulates biochemical processes (*38*). It has been reported that nickel sulfate adversely affects the expression of genetic information by reducing DNA, RNA, and protein concentration in the testes of albino rats fed normal protein and protein-restricted diets (*14*). A relationship between the threshold level of nickel toxicity and dietary protein levels may be considered (*14*). Nickel sulfate appears to adversely affect the biochemical microenvironment of the testes of albino rats fed a normal protein diet, which is further aggravated in protein-restricted diets. However, all these alterations may be reversible.

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