EFFECTS OF DIFFERENT PROTEIN DIETS ON FLUORIDE INDUCED OXIDATIVE STRESS IN MICE TESTIS

NJ Chinoy,[a] D Mehta, DD Jhala

Ahmedabad, India

SUMMARY: Adult male Swiss strain mice were fed a control protein diet, a protein deficient diet, and a protein enriched diet with and without sodium fluoride (5, 10, and 20 mg NaF/kg body wt for 30 days) to study the effects on fluoride induced oxidative stress in the testis. Ingestion of the protein deficient diet together with NaF resulted in a significant decrease in the activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) as well as in levels of reduced ascorbic acid (RAA) and glutathione (GSH). By contrast, the serum and testis levels of lipid peroxides (LPO), total ascorbic acid (TAA), dehydroascorbic acid (DHA), and fluoride increased significantly compared to the controls. Similar results were obtained in mice with the control protein diet plus the same doses of NaF. However, in mice fed the protein enriched diet along with the same doses of NaF, essentially no change in these parameters occurred, which were almost the same as in the controls. These results clearly indicate that protein supplementation in the diet is conducive to overcoming fluoride induced oxidative stress in mice testis and that analogous dietary improvement could play a major role in overcoming human fluoride toxicity in endemic areas.

Keywords: Mice testis and fluoride; Oxidative stress; Protein deficient diet; Protein enriched diet.

INTRODUCTION

Toxic levels of fluoride occur naturally in many foods and drinking water supplies, especially in fluoride endemic areas.[1,2] Experimentally, Chinoy and Mehta[3] reported that a protein deficient diet ingested by male mice treated for 30 days with sodium fluoride (5, 10, and 20 mg/kg body wt) caused a significant decline in protein levels in testis, cauda epididymis, and vas deferens. The activities of succinate dehydrogenase (SDH), 3β,17β-hydroxysteroid dehydrogenases (3β,17β-HSDs) and adenosine triphosphatase (ATPase) in cauda epididymis and phosphorylase in vas deferens decreased as compared to control mice fed a normal protein diet. On the other hand, glycogen in the vas deferens and cholesterol in the testis were increased as compared to controls. A control protein diet + NaF (same doses) also caused similar effects, but with a protein supplemented diet these parameters were almost the same as in the controls.

The occurrence of oxidative stress in various organs of rats, mice, and humans has been reported earlier.[4-8] However, studies on the effects of simultaneous feeding of NaF to male mice in different doses together with protein deficient and protein supplemented diets on oxidative stress in mice testis have not been reported previous to this new work.

MATERIALS AND METHODS

Animals: Healthy adult male Swiss strain mice *(Mus musculus)* weighing between 30 and 40 g were obtained from the National Institute of Occupational Health (NIOH), Ahmedabad, Gujarat, India, under the Animal Maintenance and

[a]For correspondence: Reproductive Endocrinology and Toxicology Unit, Department of Zoology, School of Sciences, Gujarat University, Ahmedabad-380 009, India.
E-mail: zooldeptgu@satyam.net.in

Registration No. 167/1999/CPCSEA from the Ministry of Social Justice and Empowerment, Government of India, and the Committee for the Purpose of Control and Supervision of Experiments on Animals, Chennai, India. The mice were housed in an air-conditioned animal unit at 26ºC ± 2ºC with 10–12 hr of daylight/day. They were fed standard chow and given drinking water containing 0.6–1.0 ppm F available *ad libitum*.

Exposure: The mice were divided into twelve groups as shown in the Experimental Protocol.

Experimental Protocol

Group	Treatment and dose (10-15 animals used in each group)	Duration (days)	Day of autopsy
I	Control protein diet (20% protein)	-	*
II	Protein deficient diet (5% protein)	30	31
III	Protein enriched diet (40% protein)	30	31
IV	Control protein diet + NaF (5 mg/kg bw†)	30	31
V	Protein deficient diet + NaF (5 mg/kg bw)	30	31
VI	Protein enriched diet + NaF (5 mg/kg bw)	30	31
VII	Control protein diet + NaF (10 mg/kg bw)	30	31
VIII	Protein deficient diet + NaF (10 mg/kg bw)	30	31
IX	Protein enriched diet + NaF (10 mg/kg bw)	30	31
X	Control protein diet + NaF (20 mg/kg bw)	30	31
XI	Protein deficient diet + NaF (20 mg/kg bw)	30	31
XII	Protein enriched diet + NaF (20 mg/kg bw)	30	31

*Sacrificed with treated groups. †bw = body weight

Sodium fluoride (NaF) (Loba Chemie, Bombay, 99% purity) was administered orally using a feeding tube attached to a hypodermic syringe. The NaF was given in three doses of 5, 10, or 20 mg/kg body weight (bw) to the different groups. The dose was based on the oral LD_{50} value of fluoride, which is 54.4 mg F/kg bw in male mice.[9] Oral administration was preferred since water is the main source of fluoride among human populations in endemic areas.

Diets: The control protein diet, the protein enriched, and the protein deficient diets (corresponding to the amount of casein present) were prepared according to the protocol of the National Institute of Occupational Health (NIOH), Ahmedabad, India. The diets had the following composition:

Diet	Protein (%)	Casein (%)	Starch powder (%)	Salt mixture (%)	Vitamin mixture (%)	Ground nut oil (%)
Control	20	23.53	63.47	4	2	7
Protein deficient	5	5.88	81.12	4	2	7
Protein enriched	40	47.06	39.94	4	2	7

Data collection: The control and treated groups of animals were weighed on an animal weighing balance (Ohaus, USA) and sacrificed by cervical dislocation after the respective treatments. The testes were carefully dissected out and utilized for conducting the biochemical tests. The enzyme activities of catalase (CAT) (E.C.1.11.1.6),[10] superoxide dismutase (SOD) (E.C.1.15.1.1),[11] and glutathione peroxidase (GSH-Px) (E.C.1.11.1.9)[12] were assayed by the methods cited. Similarly, the levels of lipid peroxide,[13] total, dehydro-, and reduced ascorbic acid (TAA, DHA, and RAA),[14] and glutathione (GSH)[15] were estimated by the standard methods cited. Fluoride levels in testis and serum were determined with an Orion Ion Analyser, model 920A, and a fluoride ion selective electrode.

Statistical analysis: For all biochemical parameters, a minimum of five or six replicates were made, and the data were analyzed by Student's t test and ANOVA.

RESULTS

As seen in Tables 1–3, the activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) as well as the levels of glutathione (GSH) and reduced ascorbic acid (RAA) were significantly decreased in mice of Groups IV, VII, and X (Control protein diet + 5, 10, and 20 mg NaF/kg body wt) as well as in Groups II, VIII, and XI (Protein deficient diet alone and with 10 and 20 mg NaF) as compared to controls.

Table 1. Catalase (CAT) (units/min/mg protein), superoxide dismutase (SOD) (units/mg protein), and lipid peroxide (LPO) levels (nanomoles of MDA/mg tissue wt/60 min) in testis of Groups I–XII mice[a]

Group	Treatment	CAT	SOD	LPO
I	Control protein diet (20% protein)	21.33 ± 0.47	0.35 ± 0.030	67.41 ± 1.59
II	Protein deficient diet (5% protein)	17.92 ± 0.57‡	0.27 ± 0.006‡	181.44 ± 2.36‡
III	Protein enriched diet (40% protein)	21.32 ± 0.62	0.36 ± 0.009	67.34 ± 1.18
IV	Control protein diet + NaF (5 mg/kg bw)	17.59 ± 0.39‡	0.25 ± 0.007‡	166.24 ± 2.04‡
V	Protein deficient diet + NaF (5 mg/kg bw)	15.57 ± 0.82	0.20 ± 0.005‡	186.23 ± 0.94
VI	Protein enriched diet + NaF (5 mg/kg bw)	21.10 ± 0.23	0.36 ± 0.009	66.80 ± 1.31
VII	Control protein diet + NaF (10 mg/kg bw)	15.06 ± 0.93§	0.20 ± 0.008§	186.67 ± 2.56§
VIII	Protein deficient diet + NaF (10 mg/kg bw)	13.23 ± 0.65‡	0.16 ± 0.007§	194.35 ± 1.02§
IX	Protein enriched diet + NaF (10 mg/kg bw)	20.94 ± 0.49	0.35 ± 0.006	67.09 ± 1.47
X	Control protein diet + NaF (20 mg/kg bw)	13.48 ± 0.27§	0.16 ± 0.009§	195.41 ± 1.87§
XI	Protein deficient diet + NaF (20 mg/kg bw)	10.49 ± 0.72§	0.13 ± 0.009§	198.46 ± 0.48§
XII	Protein enriched diet + NaF (20 mg/kg bw)	21.70 ± 0.51	0.35 ± 0.004	67.27 ± 1.07

[a]Data are expressed as mean ± SE; *P<0.05; †P<0.02; ‡P<0.01; § P<0.001; no sign = not significant. Comparisons between: Group I with Groups II or III or IV or VII or X individually; Group II with Groups V or VIII or XI individually; Group III with Groups VI or IX or XII individually.

Table 2. Total, dehydro, and reduced ascorbic acid (TAA, DHA, and RAA) (mg/g fresh tissue weight) levels in testis of Groups I-XII mice[a]

Group	Treatment	TAA	DHA	RAA
I	Control protein diet(20% protein)	3.59 ± 0.18	1.33 ± 0.27	2.26 ± 0.52
II	Protein deficient diet (5% protein)	4.03 ± 0.19*	1.98 ± 0.28*	2.05 ± 0.26*
III	Protein enriched diet (40% protein)	3.58 ± 0.12	1.32 ± 0.34	2.26 ± 0.22
IV	Control protein diet + NaF (5 mg/kg bw)	3.38 ± 0.15	2.09 ± 0.35‡	1.29 ± 0.15‡
V	Protein deficient diet + NaF (5 mg/kg bw)	3.91 ± 0.05	2.09 ± 0.34	1.82 ± 0.09
VI	Protein enriched diet + NaF (5 mg/kg bw)	3.56 ± 0.15	1.32 ± 0.54	2.24 ± 0.19
VII	Control protein diet + NaF (10 mg/kg bw)	3.97 ± 0.08*	2.92 ± 0.21§	1.05 ± 0.12‡
VIII	Protein deficient diet + NaF (10 mg/kg bw)	4.26 ± 0.10*	3.03 ± 0.62‡	1.23 ± 0.09‡
IX	Protein enriched diet + NaF (10 mg/kg bw)	3.54 ± 0.15	1.31 ± 0.45	2.14 ± 0.20
X	Control protein diet + NaF (20 mg/kg bw)	4.30 ± 0.10§	3.32 ± 0.24§	0.98 ± 0.15§
XI	Protein deficient diet + NaF (20 mg/kg bw)	4.51 ± 0.03*	3.46 ± 0.13§	1.05 ± 0.08‡
XII	Protein enriched diet + NaF (20 mg/kg bw)	3.47 ± 0.07	1.31 ± 0.54	2.16 ± 0.24

[a]Data are expressed as mean ± SE; *P<0.05; †P<0.02; ‡P<0.01; §P<0.001; no sign = not significant. Comparisons between: Group I with Groups II or III or IV or VII or X individually; Group II with Groups V or VIII or XI individually; Group III with Groups VI or IX or XII individually.

Table 3. Glutathione peroxidase (GSH-Px) activity (nanomoles of NADPH oxidized/min/mg protein) and glutathione (GSH) (µg/100 mg fresh tissue weight) levels in testis of Groups I–XII mice[a]

Group	Treatment	GSH-PX	GSH
I	Control protein diet (20% protein)	4.82 ± 0.06	51.15 ± 0.81
II	Protein deficient diet (5% protein)	2.94 ± 0.16§	40.01 ± 1.14§
III	Protein enriched diet (40% protein)	4.81 ± 0.09	51.46 ± 0.79
IV	Control protein diet + NaF (5 mg/kg bw)	2.76 ± 0.38§	43.05 ± 1.57§
V	Protein deficient diet + NaF (5 mg/kg bw)	2.54 ± 0.19	37.12 ± 0.90
VI	Protein enriched diet + NaF (5 mg/kg bw)	4.76 ± 0.13	51.18 ± 0.99
VII	Control protein diet + NaF (10 mg/kg bw)	2.59 ± 0.33§	38.17 ± 0.55§
VIII	Protein deficient diet + NaF (10 mg/kg bw)	2.07 ± 0.14‡	27.39 ± 1.76‡
IX	Protein enriched diet + NaF (10 mg/kg bw)	4.25 ± 0.17	50.83 ± 0.97
X	Control protein diet + NaF (20 mg/kg bw)	1.95 ± 0.17§	22.79 ± 1.04§
XI	Protein deficient diet + NaF (20 mg/kg bw)	1.82 ± 0.21§	18.09 ± 1.96§
XII	Protein enriched diet + NaF (20 mg/kg bw)	4.71 ± 0.22	50.40 ± 0.61

[a]Data are expressed as mean ± SE; *P<0.05; †P<0.02; ‡P<0.01; §P<0.001; no sign = not significant. Comparisons between: Group I with Groups II or III or IV or VII or X individually; Group II with Groups V or VIII or XI individually; Group III with Groups VI or IX or XII individually.

With the protein deficient diet + 5 mg NaF/kg body wt, the Group V mice exhibited a significant decline only in the activity of SOD, indicating that this was the most sensitive parameter.

On the other hand, the levels of total ascorbic acid (TAA), dehydroascorbic acid (DHA), and lipid peroxides (LPO) were significantly increased in Groups IV, VII, X and II, VIII, and XI but not in Group V (Tables 1 and 2). Amongst these three parameters, TAA was affected less than DHA and LPO. In Groups III, VI, IX, and XII (Protein enriched diet alone and with three doses of NaF), all the parameters studied were unaffected, and their activities/levels were almost same as in controls (Tables 1–3)

Fluoride levels in serum and testis showed almost the same trend as obtained for DHA and LPO (Table 4).

Table 4. Fluoride content (ppm) in testis and serum of Groups I–XII mice[a]

Group	Treatment	Testis	Serum
I	Control protein diet (20% protein)	0.0012 ± 0.0002	0.052 ± 0.003
II	Protein deficient diet (5% protein)	0.0008 ± 0.0003	0.051 ± 0.006
III	Protein enriched diet (40% protein)	0.0009 ± 0.0001	0.051 ± 0.002
IV	Control protein diet + NaF (5 mg/kg bw)	0.0021 ± 0.0001§	0.286 ± 0.033§
V	Protein deficient diet + NaF (5 mg/kg bw)	0.0022 ± 0.0004§	0.266 ± 0.079§
VI	Protein enriched diet + NaF (5 mg/kg bw)	0.0008 ± 0.0002	0.051 ± 0.005
VII	Control protein diet + NaF (10 mg/kg bw)	0.0032 ± 0.0004	0.352 ± 0.043§
VIII	Protein deficient diet + NaF (10 mg/kg bw)	0.0030 ± 0.0007§	0.360 ± 0.027§
IX	Protein enriched diet + NaF(10 mg/kg bw)	0.0008 ± 0.0003	0.052 ± 0.002
X	Control protein diet + NaF (20 mg/kg bw)	0.0042 ± 0.0004§	0.378 ± 0.084§
XI	Protein deficient diet + NaF (20 mg/kg bw)	0.0044 ± 0.0005§	0.402 ± 0.074§
XII	Protein enriched diet + NaF (20 mg/kg bw)	0.0008 ± 0.0003	0.051 ± 0.003

[a] Data are expressed as mean ± SE; *P<0.05; †P<0.02; ‡P<0.01; §P<0.001; no sign = not significant.
Comparisons between: Group I with Groups II or III or IV or VII or X individually; Group II with Groups V or VIII or XI individually; Group III with Groups VI or IX or XII individually.

DISCUSSION

In monkeys with experimentally produced fluorosis, administration of a low protein diet appeared to accelerate the development of bone fragility and cause a higher incidence of bone rarefaction.[16] The present findings also indicate that protein deficiency coupled with fluoride treatment caused severe oxidative stress in the testis as evident by the decrease in antioxidant enzyme activities and levels of glutathione and reduced ascorbic acid. These changes were accompanied by a significant increase mainly in LPO and DHA levels. The enhancement in TAA concentrations, however, could be the result of the presence of extra vitamins in the special diets, while changes in testicular and serum fluoride are likely to be due

to fluoride ingestion. These results suggest that structural alterations affect testicular functions under protein deficiency and fluoride treatment.[1-3] Various studies in different organs of animals and humans have also indicated fluoride induced oxidative stress[4-8, 17] accompanied by changes in structure and metabolism of the affected organs.

Table 5. ANOVA of various parameters of mice testis and serum fluoride levels

Parameter	Source of variation	SS	df	MS	F-cal	F-tab
Catalase	Between groups	766.1296	11	69.64814	205.3341	1.994579
	Within groups	16.28132	48	0.339194		
SOD	Between groups	0.454989	11	0.041363	297.787	1.994579
	Within groups	0.006667	48	0.000139		
LPO	Between groups	212509	11	19319	5729.927	1.994579
	Within groups	161.8366	48	3.371597		
TAA	Between groups	99.38113	11	9.034648	549.525	1.994579
	Within groups	0.78916	48	0.016441		
DHA	Between groups	39.47538	11	3.588671	86.82093	1.994579
	Within groups	1.98404	48	0.041334		
RAA	Between groups	47.184	11	4.316727	3.184927	1.994579
	Within groups	24.83286	48	1.975685		
GSH-Px	Between groups	77.2605	11	7.023682	155.4628	1.994579
	Within groups	2.1686	48	0.045179		
GSH	Between groups	7565.996	11	687.8179	361.2289	1.994579
	Within groups	84.3886	48	1.758096		
Testis fluoride level	Between groups	0.000111	11	1.05	30.0172	1.994579
	Within groups	1.6105	48	3.3507		
Serum fluoride level	Between groups	1.219407	11	0.110855	12.32164	1.994579
	Within groups	0.431846	48	0.008997		

SS=Sum of squares; df=degrees of freedom; MS=Mean sum of squares; F-cal=Fisher calculated; F-tab=Fisher tabulated.

The present data support many epidemiological and experimental studies which have shown that dietary factors such as proteins, vitamins, and amino acids can mitigate the toxic effects of fluoride.[1,2] The results also suggest that a protein enriched diet has beneficial effects, whereas protein deficiency aggravates fluoride

toxicity. As such, these findings have important implications where protein malnutrition and fluorosis co-exist. More detailed studies in this direction are solicited in the future using different fluoride doses, durations, and animal models.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the help given by the Director, National Institute of Occupational Health (NIOH), Ahmedabad, India, for supplying the protocols for preparing the special diets used in this study.

REFERENCES

1 Chinoy NJ. Studies on fluoride, aluminium and arsenic toxicity in mammals and amelioration by some antidotes. In: Tripathi G, editor. Modern trends in experimental biology. New Delhi: CBS Publisher; 2002. p. 164-93.
2 Chinoy NJ. Fluoride in the environment. In: Chlubek D. editor. Fluoride in medicine, biology and toxicology. Warsaw, Poland: Katedra i Zaklad Biochemii i Chemii Pomorskiej Akademii Medycznej; 2003. p. 5-33.
3 Chinoy NJ, Mehta D. Effects of protein supplementation and deficiency on fluoride induced toxicity in reproductive organs of male mice. Fluoride 1999;32(4):204-14.
4 Guo X-Y, Sun G-F, Sun Y-C. Oxidative stress from fluoride induced hepatotoxicity in rats. Fluoride 2003;36(1):25-9.
5 Wang A, Xia T, Ran P, Bai Y, Yang K, Chen X. Effects of selenium and fluoride on apoptosis and lipid peroxidation in human hepatocytes. Zhonzhua Yu Fang Yi Xue Za Zhi 2002;36(4):235-8. [in Chinese]. (Abstract in Fluoride 2003;36(1):45-6).
6 Chinoy NJ, Patel DK. Influence of fluoride on biological free radicals in ovary of mice and its reversal. Environ Sci 1998;6(3):171-84.
7 Shivarajashankara YM, Shivashankara AR, Bhat GP, Rao HS. Brain lipid peroxidation and antioxidant systems of young rats in chronic fluoride intoxication. Fluoride 2002;35(3):197-203.
8 Chinoy NJ, Shah SD. Biochemical effects of sodium fluoride and arsenic trioxide and their reversal in the brain of mice. Fluoride 2004;37(2):80-7.
9 Pillai KS, Mathai AT, Deshmukh PB. Effect of subacute dosage of fluoride on male mice. Toxicol Lett 1988;44:21-9.
10 Luck H. A spectrophotometric method for the estimation of catalase. In: Bergmeyer HU, editor. Methods of enzymatic analysis. London: Academic Press; 1963. p. 886-7.
11 Kakkar P, Das B, Viswanathan PN. A modified spectrophotometric assay of superoxide dismutase. Ind J Biochem Biophys 1984;21(4):130-2.
12 Pagila DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 1967;70:158-69.
13 Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:351-8.
14 Roe JH, Küether CA. The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenyl- hydrazine derivatives of dehydroascorbic acid. J Biol Chem 1943;147:399-407.
15 Grünert RR, Philips PH. A modification of the nitroprusside method of analysis for glutathione. Arch Biochem 1951;30:217-25.
16 Reddy GS, Srikantia SG. Effect of dietary calcium, vitamin C and protein in development of experimental skeletal fluorosis. 1. Growth, serum chemistry and changes in composition and radiological appearance of bones. Metabolism 1971;20(7):642-9.
17 Chinoy NJ, Mehta D, Jhala DD. Beneficial effects of a protein rich diet on fluoride induced free radical toxicity in mice liver. Fluoride 2005;38(4):276-283.