

PROTECTIVE EFFECT OF MELATONIN ON FLUORIDE-INDUCED OXIDATIVE STRESS AND TESTICULAR DYSFUNCTION IN RATS

Mandava V Rao,^{a,b} Rajendra N. Bhatt^a

Ahmedabad, Gujarat, India

SUMMARY: Daily dosages of 5 and 10 mg NaF/kg bw were administered orally to male rats (15 per group) for 60 days to evaluate the effect on the testis in relation to oxidative stress. A significant decrease in body and organ weights occurred compared to controls. Alterations in the antioxidant indices in the testis were confirmed by increased lipid peroxidation (LPO) along with decrements in antioxidant parameters such as glutathione peroxidase (GPx), glutathione (GSH), total ascorbic acid (TAA), glutathione-S-transferase (GST), glutathione reductase (GR), superoxide dismutase (SOD), and catalase (CAT) levels affecting testis function as indicated by histopathological study. Supplementation of the NaF-treated rats by the antioxidant melatonin (10 mg/kg bw) revealed a significant protection to the gonadal function, thus indicating a mitigating effect by melatonin against NaF-induced testis toxicity and oxidative stress in a rat model.

Keywords: Fluoride testis toxicity; Melatonin as antioxidant; Oxidative stress; Rat testis; Testicular dysfunction.

INTRODUCTION

Fluoride (F) is a well-known soil, water, and air contaminant, and its toxicity to humans has been widely studied. Intake of excess F through drinking water, food, or inhalation causes a wide range of toxic effects known as fluorosis.¹ Various forms of F are commonly used in toothpastes, mouth rinses, and even in certain processed beverages and public supplies to help prevent dental caries.² However, excessive intake of F leads to fluorosis, weakened antioxidant defense systems, and increased oxidative stress in rat liver.³ Thus, it is toxic to living cells because it can generate reactive free radicals and causes alteration in biochemical indices including oxidative stress in a variety of animal species.⁴ The F ion is able to exert powerful effects on various enzymes and endocrine gland functions that affect or control the status of oxidant/antioxidant systems in living organisms.⁵ In this regard, the discovery of the pineal hormone melatonin heralded a new field of research in reproductive physiology.⁶ The pineal gland hormone melatonin (N-acetyl-5-methoxytryptamine) has therefore become a substance of interest after its powerful antioxidant potential was proven by several *in vivo* and *in vitro* studies.^{7,8} Melatonin also appears to be beneficial in its free-radical scavenging actions beyond its stimulatory effects on antioxidant enzyme systems.^{9,10}

The present work was undertaken to examine, in the light of earlier work from our laboratory,¹¹ the effects of F toxicity on some antioxidant indicators of the rat testis and the influence of melatonin on various aspects of testicular function after F ingestion.

MATERIALS AND METHODS

Animals: Mature male Wistar rats (*Rattus norvegicus*) weighing between 250 and 350 g were procured from Zydus-Cadila Health Care, Ahmedabad, under the

^aZoology Department, School of Sciences, Gujarat University, Ahmedabad 380 009, India.

^bFor correspondence: Dr MV Rao, Professor and Head, Zoology Department, School of Sciences, Gujarat University, Ahmedabad 380 009, India. E-mail: zooldeptgu@satyam.net.in.

Animal Maintenance and Registration No. 167/1999/CPCSEA from the Ministry of Social Justice and Empowerment, Government of India. The animals were housed at standard temperature ($24\pm 1^{\circ}\text{C}$) with a 12-hr dark/light cycle. They were fed standard rodent food (Pranav Agro Industries, Vadodara, India) and water *ad libitum*.

Experimental design: After a 15-day adaptation period, the animals were divided into five different groups (Table 1) of 15 each and caged separately.

Table 1. Experimental Protocol

Group	Treatment and daily dose (15 rats in each group)	Duration (days)	Day of autopsy
I	Untreated control	-	Sacrificed with treated
II	Melatonin alone (10 mg/kg bw, ip)	60	61 st
III	Sodium fluoride (Low dose: 5 mg/kg bw, orally)	60	61 st
IV	Sodium fluoride (High dose: 10 mg/kg bw, orally)	60	61 st
V	NaF treated (High dose: 10 mg/kg bw) + Melatonin (10 mg/kg bw, ip) 30-min prior	60	61 st

Based on our earlier studies,¹² the following doses were given for 60 days. Group I (control) rats were maintained on standard diet. Group II was treated with melatonin alone (10 mg/kg bw) intraperitoneally. Group III was administered a low dose of sodium fluoride (5 mg/kg bw) orally. High dose of sodium fluoride (10 mg/kg bw) was given to Group IV. Because of the rapid metabolism of melatonin,^{12,13} Group V was given melatonin ip 30 min before the administration of the 10 mg/kg bw high dose of NaF given to Group IV.

After 60 days, the rats were fasted overnight and sacrificed under mild ether anesthesia. Besides their body weight, the weight of the testis was recorded and utilized for estimation of different biochemical parameters.

Biochemical Analysis: Testis tissue lipid peroxidation (by increased malondialdehyde [MDA] concentration), superoxide dismutase (SOD, E.C.1.1.1.15.11), catalase (CAT, E.C.1.11.1.6), glutathione peroxidase (GPx, E.C.1.11.1.9), glutathione reductase (GR, E.C.1.8.1.7), glutathione-S-transferase activities (GST, E.C.2.5.1.18), glutathione (GSH), and total ascorbic acid (TAA) were determined according to standard methods.¹⁴⁻²¹

Statistical Analysis: Data are presented as mean \pm SEM. One-way analysis of variance (ANOVA) with Tukey's significant difference post hoc test was used to compare differences among groups. Data were analyzed statistically by Graph Pad Prism 5.0 statistical software. p values <0.05 were considered significant.

RESULTS

Body and organ weights: As shown in Table 2, body and testis weights of the rats treated with NaF (Groups III and IV) were significantly ($p < 0.001$) decreased as compared to the control animals (Group I) and the animals administered melatonin alone (Group II). The combined NaF-melatonin Group V did not show any significant changes compared to the Group I controls.

Table 2. Body weight (g) and organ weight (mg) of control and treated groups of rats

Group	Body weight (g)	Organ weight (mg)
I	375 ± 14.32	1695.31 ± 20.46
II	388 ± 14.88 ^{NS}	1626.00 ± 32.46 ^{NS}
III	312 ± 12.4 [*]	1560.68 ± 29.92 [*]
IV	297 ± 9.03 [†]	1527.08 ± 35.51 [†]
V	363 ± 7.68 ^{NS}	1684.82 ± 19.46 ^{NS}

Values are Mean ± S.E. * $p < 0.01$, [†] $p < 0.05$, NS = Non Significant.

Groups I = Untreated Control; II = Melatonin alone; III = Low dose NaF treated;

IV = High dose NaF treated; V = High dose NaF treated + Melatonin.

Antioxidant indices: Antioxidant indices in the testis fell markedly after NaF treatment. All antioxidant enzyme activities, SOD, CAT, GR, GSH-Px, and GST activity declined in the NaF-treated groups (III, IV). Decrements were also seen in the non-enzymatic antioxidant GSH and TAA levels from NaF treatment. Moreover, NaF treatment also produced markedly elevated levels of lipid peroxidation and glutathione S-transferase (GST) as compared to the control group (I). Administration of melatonin along with NaF (Group V) showed no significant differences in anti-oxidant indices as compared to control and melatonin alone treated groups (Table 3).

Table 3. Effects of sodium fluoride on testis lipid peroxidation and antioxidant profiles

Group/profile	I	II	III	IV	V
LPO (nM MDA/mg)	25.32 ± 1.16	22.80 ± 1.56 ^{NS}	37.27 ± 2.65 [‡]	47.36 ± 2.10 [‡]	28.79 ± 1.07 ^{NS}
SOD (U/mg protein)	1.22 ± 0.08	1.27 ± 0.02 ^{NS}	0.95 ± 0.04 [*]	0.61 ± 0.10 [‡]	1.11 ± 0.04 ^{NS}
CAT (µM H ₂ O ₂ consumed/min/mg protein)	34.53 ± 1.35	36.05 ± 0.89 ^{NS}	26.03 ± 1.74 [†]	24.58 ± 1.58 [‡]	32.92 ± 1.13 ^{NS}
GSH (µM/mg)	57.05 ± 0.74	57.26 ± 0.66 ^{NS}	48.73 ± 0.43 [‡]	42.47 ± 0.51 [‡]	54.96 ± 0.44 ^{NS}
GPx (µM GSH consumed/min/mg protein)	20.22 ± 1.02	20.90 ± 1.23 ^{NS}	15.03 ± 0.93 [†]	11.67 ± 0.83 [‡]	18.97 ± 1.22 ^{NS}
GR (nM NADPH oxidized/min/mg protein)	31.50 ± 1.17	31.88 ± 1.08 ^{NS}	24.44 ± 1.27 [†]	18.74 ± 1.14 [‡]	29.44 ± 1.56 ^{NS}
GST (U/mg protein)	0.00616 ± 0.00063	0.00680 ± 0.00046 ^{NS}	0.00932 ± 0.00049 [†]	0.01264 ± 0.00077 [‡]	0.00586 ± 0.00062 ^{NS}
TAA (mg/g)	2.04 ± 0.06	2.10 ± 0.06 ^{NS}	1.70 ± 0.01 [†]	1.32 ± 0.04 [‡]	1.92 ± 0.04 ^{NS}

Values are Mean ± S.E. * $p < 0.01$, [†] $p < 0.05$, [‡] $p < 0.001$, NS = Non Significant. Groups: I = Untreated Control;

II = Melatonin alone; III = Low dose NaF treated; IV = High dose NaF treated; V = High dose NaF treated + Melatonin.

Testis histology: Various stages of spermatogenesis in the seminiferous tubules of control animals were noted (Figure 1). Leydig cells were seen between the seminiferous tubules (Figure 2).

Figure 1. Transverse section of testis of control (Group I) rat showing seminiferous tubules and Leydig cells. HE staining (x210).

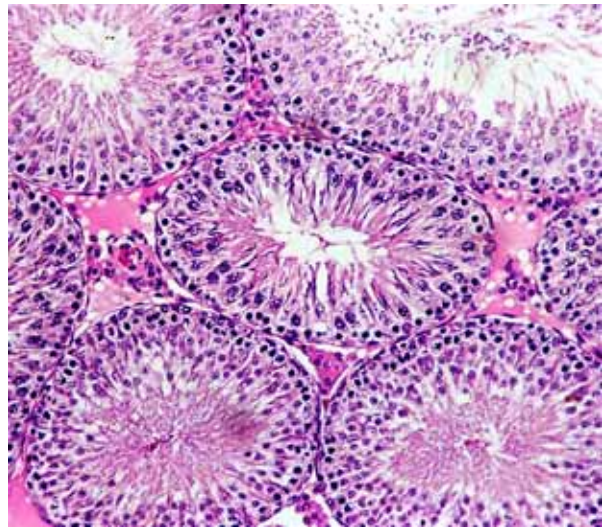
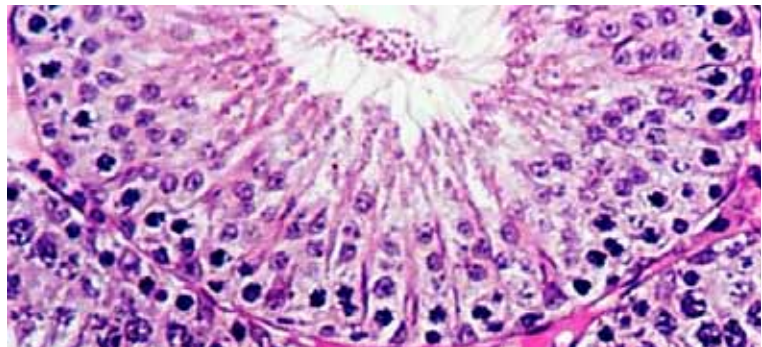
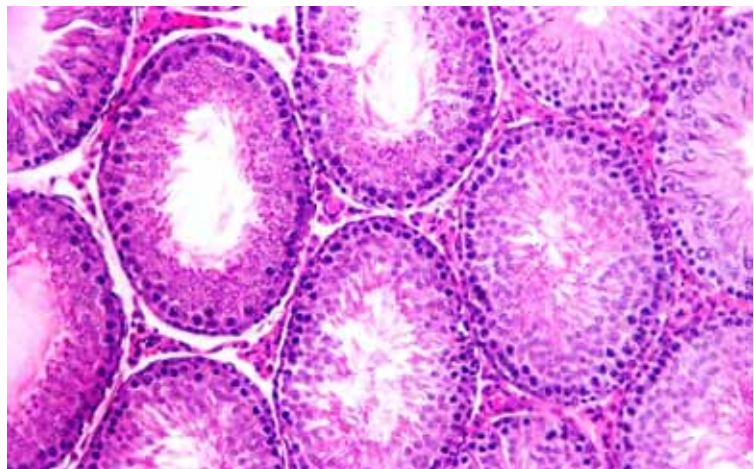


Figure 2. Transverse section of testis of control (Group I) rat. HE staining (x840).



The melatonin alone group had the same histological structure as the control group (Figures 3 and 4).

Figure 3. Transverse section of testis of melatonin alone (Group II) rat showing seminiferous tubules and Leydig cells. HE staining (x210).



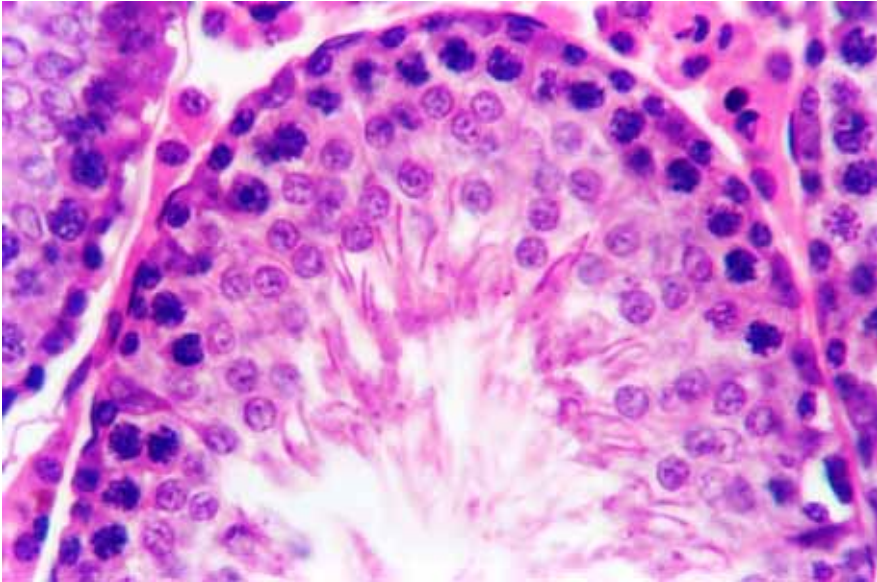


Figure 4. Transverse section of testis of melatonin alone (Group II) rat. HE staining (x840).

As a result of F feeding, stages of spermatogenic elements were disrupted. Exfoliation of germinal epithelium was noted in some tubules (Figure 5).

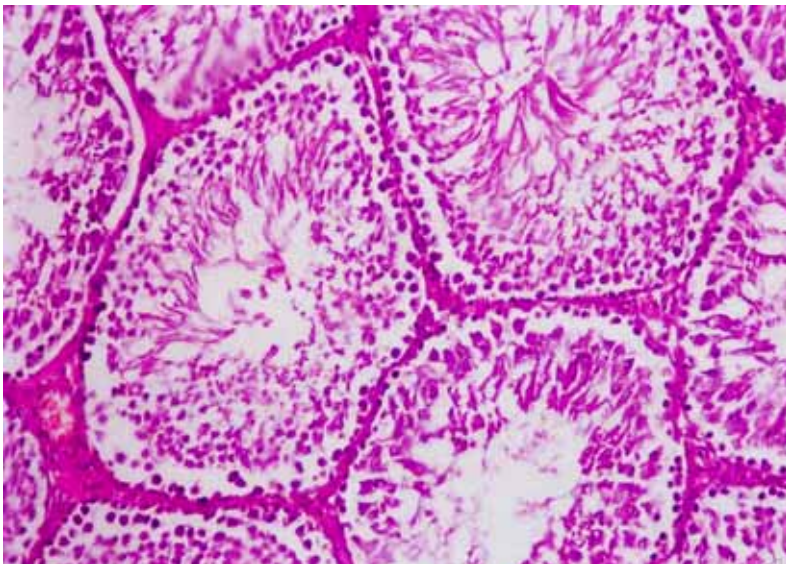


Figure 5. Transverse section of testis of NaF (Group IV) treated rat showing disorganized germinal epithelium and vacuolation in the cell. HE staining (x210).

Basement membrane of the tubules was also affected, losing its tubular shape, and pyknotic changes were also noted. The appearance of the Leydig cells indicated atrophy (Figure 6).

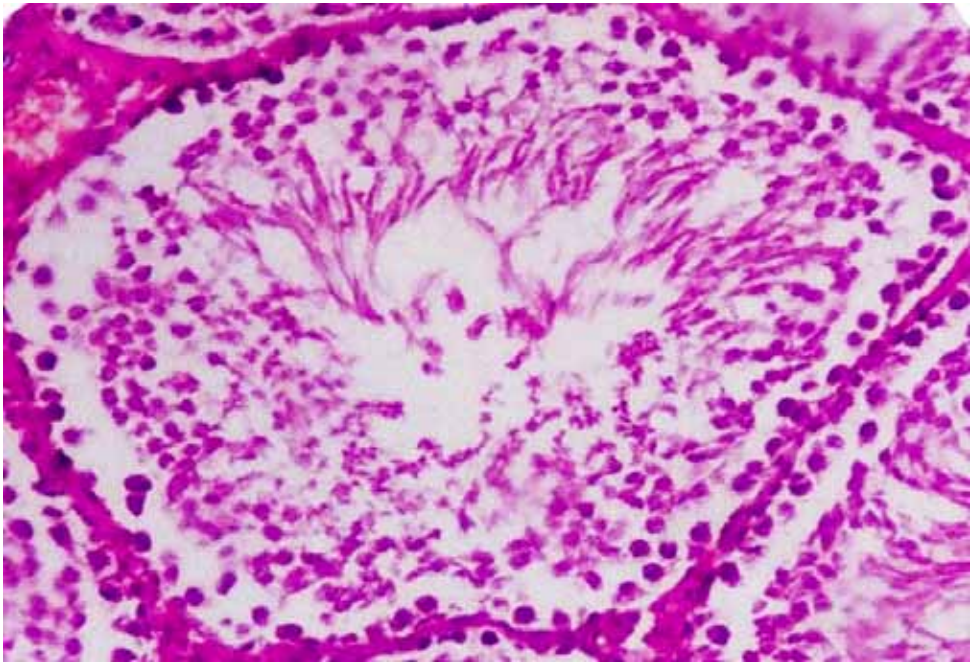


Figure 6. Transverse section of testis of NaF (Group IV) treated rat showing Leydig cell atrophy. HE staining (x840).

With melatonin supplementation, these changes were less prominent and nearly normal spermatogenic stages were observed in some tubules (Figures 7 and 8).

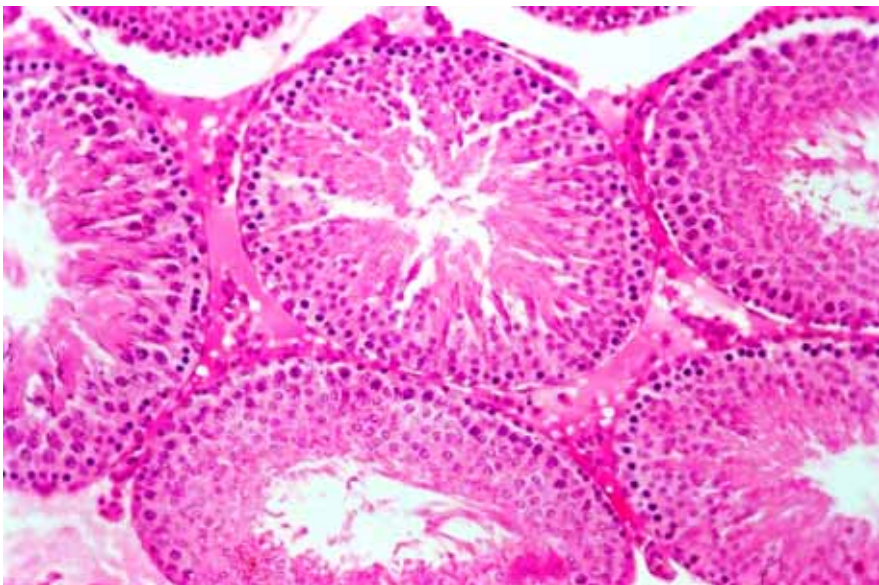


Figure 7. Transverse section of testis of NaF + Melatonin (Group V) rat showing normal cell structure. HE staining (x210).

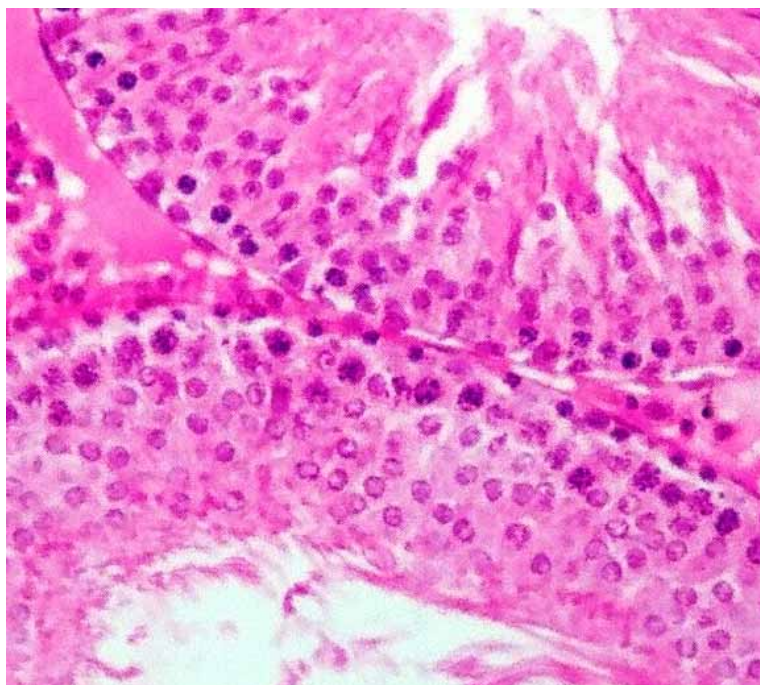


Figure 8. Transverse section of testis of NaF + Melatonin (Group V) rat. HE staining (x840).

DISCUSSION

In this investigation, adverse effects of NaF and their amelioration by melatonin of testicular dysfunction in male rats have been observed in relation to F-induced oxidative stress. These effects involve Reactive Oxygen Species (ROS) as a natural by-product of the normal metabolism of oxygen and have an important role in cell signaling and homeostasis with a dramatic increase during conditions of environmental stress.²² Our results indicated that F treatment caused severe oxidative stress in the testis as evidenced by reduced antioxidant enzyme activities and levels of glutathione and total ascorbic acid. These alterations were accompanied by a significant increase mainly in LPO and GST levels. Various studies in different organs of mammals have also shown that F induces oxidative stress.²³⁻²⁶ The significantly lower levels of glutathione and TAA after 60 days treatment of NaF appear likely to be due to the stress imposed by NaF. Glutathione is known to have a role in the formation of reduced AA from dehydroascorbic acid. The reduced AA together with its free radical, monodehydroascorbic acid (MDHA), is a powerful reducing agent that assists in overcoming stress in several tissues.^{27,28} Hence, alterations in the glutathione and TAA levels after NaF treatment would be expected to affect testicular metabolism and function. Moreover, oxidative stress produced by free radicals and hydrogen peroxide is greater if F impairs the production of the free radical indices of the defense system.²⁹ Reduction in these indices has been found in people living in areas of endemic fluorosis^{30,31} as well as in the tissues of experimental animals subjected

to F intoxication, thus supporting our observation of higher rather than lower levels of LPO and GST.³²

The F ion acts as enzymatic poison, inhibiting enzyme activity, ultimately, interrupting metabolic processes such as glycolysis, synthesis of proteins, and antioxidative pathways. These changes, along with reduced food intake, caused reduction in the body weight and loss of testis organ weights of our rats.^{33,34} Any alteration in androgen level may also result in changes in the weight of these organs.³⁵ Besides inhibition of protein synthesis by NaF treatment, the reduction in the testis weight by treatment is probably further related to the loss of spermatozoa and spermatids, which make up a substantial proportion of testicular volume.^{36,37} Furthermore, testis histopathology following F treatment revealed loss of spermatogenesis, pyknosis, vacuolization, disorganization of germ cells, and atrophic Leydig cells,³⁸ supporting the view that oxidative stress induced by F affected gonadal functions.

With melatonin supplementation to the F-intoxicated rats, enzymatic and non-enzymatic parameters and testis anatomy were maintained as compared to control rats. It is well known that melatonin and its subsequent metabolites are powerful antioxidants to prevent free radical production and quenching of these radicals by enhancing the defense system during stress to combat tissue destruction and safeguard cellular structure and function.³⁹ Thus melatonin has been found to ameliorate F-induced reproductive organ toxicity in male rats.

ACKNOWLEDGEMENTS

This work was supported by University Grants Commission (UGC), New Delhi, in the form of a Major Research Project grant. We also thank Dr. Sunita Chawla for her assistance in this study.

REFERENCES

- 1 Rao MV, Vyas DD, Meda RB, Chawla SL. *In vitro* protective role of melatonin against hemolysis induced by sodium fluoride in human red blood cells. *Fluoride* 2011;44(2):77-82.
- 2 Buzalaf M, de Almeida BS, Cardoso VE, Olympio KP, Furlani Tde A. Total and acid-soluble fluoride content of infant cereals, beverages and biscuits from Brazil. *Food Addit Contam* 2004;21:210-5.
- 3 Rupal VA, Narasimhacharya AVR. Alleviation of fluoride-induced hepatic and renal oxidative stress in rats by the fruit of *Limonia Acidissima*. *Fluoride* 2011;44(1):14-20.
- 4 Chawla SL, Yadav R, Shah D, Rao MV. Protective action of melatonin against fluoride-induced hepatotoxicity in adult female mice. *Fluoride* 2008;41(1):44-51.
- 5 Burgstahler AW. Recent research on fluoride and oxidative stress [editorial]. *Fluoride* 2009;42(2):73-4.
- 6 Lerner AB, Case JD, Takahashi Y, Lee TH, Mori W. Isolation of melatonin, the pineal gland factor that lightens melanocytes. *J Am Chem Soc* 1958;80:2587.
- 7 Tan DX, Reiter RJ, Manchester LC, Yan M, El-Sawi M, Sainz RM, et al. Chemical and physical properties and potential mechanisms: melatonin as a broad spectrum antioxidant and free radical scavenger [review]. *Curr Top Med Chem* 2002;2:181–97.
- 8 Allegra M, Reiter RJ, Tan DX, Gentile C, Tesoriere L, Livrea MA. The chemistry of melatonin's interaction with reactive species [review]. *J Pineal Res* 2003;34:1–10.
- 9 France EL, Feliciano ND, Silva KA, Ferrari CKB, Honorio-Franca AC. Modulatory role of melatonin on superoxide release by spleen macrophages isolated from alloxan-induced diabetic rats. *Bratisl Lek Listy* 2009;110(9):517-22.
- 10 Rao MV, Chawla SL, Patel N. Melatonin reduction of fluoride-induced nephrotoxicity in mice. *Fluoride* 2009;42(2):110-6.
- 11 Chinoy NJ. Effects of fluoride on physiology of some animals and human beings. *Indian J Environ Toxicol* 1991;(1):17-32.

- 12 Chinoy NJ. Fluoride toxicity in female mice and its reversal. In: Saxena AK, Ramamurthy R, Reddy SG, Saxena VL, editors. Recent Advances in Life Sciences. Kanpur: Indian Society of Life Sciences, Manu Publishers;1992. p. 39-50.
- 13 Vakkuri O, Leppäluoto J, Kauppila A. Oral administration and distribution of melatonin in human serum, saliva and urine. *Life Sci* 1985;37:489-95.
- 14 Roe JH, Kuether CA. The determination of ascorbic acid in whole blood and urine through the 2, 4-dinitrophenyl hydrazine derivative of dehydroascorbic acid. *J Biol Chem* 1943;147:399-407.
- 15 Kakkur P, Das B, Viswanathan PN. A modified spectrophotometric assay of superoxide dismutase. *Indian J Biochem Biophys* 1984;4:130-2.
- 16 Sinha AK. Colorimetric assay of catalase. *Anal Biochem* 1972;47(2):389-94.
- 17 Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG. Selenium: biochemical role as a component of glutathione peroxidase. *Science* 1973;179:588-90.
- 18 Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 1959;82:70-7.
- 19 Habig WH, Pabst MJ, Jakoby WB. Glutathione S-Transferases: the first enzymatic step in mercapturic acid formation. *J Biol Chem* 1974;249:7130-9.
- 20 Carlberg I, Mannervik B. Glutathione reductase. *Methods Enzymol* 1985;113:484-90.
- 21 Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissue by thiobarbituric acid reaction. *Anal Biochem* 1979;95:351-8.
- 22 Devasagayam TPA, Tilak JC, Bloor KK, Sane KS, Ghaskadbi SS, Lele RD. Free radicals and antioxidants in human health: current status and future prospects [review]. *Journal of Association of Physicians of India (JAPI)* 2004;52:796-804. Available from: <http://www.panelamonitor.org/media/docrepo/document/files/free-radicals-and-antioxidants-in-human-health.pdf>
- 23 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.
- 24 Shivarajashankara YM, Shivashankara AR, Bhat PG, Rao SH. Brain lipid peroxidation and antioxidant systems of young rats in chronic fluoride intoxication. *Fluoride* 2002;35(3):197-203.
- 25 Chinoy NJ, Shah SD. Biochemical effects of sodium fluoride and arsenic trioxide toxicity and their reversal in the brain of mice. *Fluoride* 2004;37(2):80-7.
- 26 Chinoy NJ, Mehta D, Jhala DD. Beneficial effects of a protein rich diet on fluoride induced free radical toxicity in the liver of male mice. *Fluoride* 2005;38(4):276-83.
- 27 Chinoy NJ. Ascorbic acid turnover in animal and human tissues. *J Anim Morphol Physiol. Silver Jubilee Volume.* 1978;68-85.
- 28 Chinoy NJ, Sharma M, Michael M. Beneficial effects of ascorbic acid and calcium on reversal of fluoride toxicity in male rats. *Fluoride* 1993;26(1):45-56.
- 29 Rzeuski R, Chlubek D, Machoy Z. Interactions between fluoride and biological free radical reactions [review]. *Fluoride* 1998;31(1):43-5.
- 30 Shanthakumari D, Srinivasalu S, Subramanian S. Antioxidant defense systems in red blood cell lysates of men with dental fluorosis living in Tamil Nadu, India. *Fluoride* 2006;39(3):231-9.
- 31 Li JX, Cao S. Recent studies on endemic fluorosis in China. *Fluoride* 1994;27:125-8.
- 32 Shanthakumari D, Srinivasalu S, Subramanian S. Effect of fluoride intoxication on lipid peroxidation and antioxidant status in experimental rats. *Toxicology* 2004;204:219-28.
- 33 Chlubek D. Fluoride and oxidative stress [editorial review]. *Fluoride* 2003;36:217-28.
- 34 Kumar A, Tripathi N, Tripathi M. Fluoride-induced biochemical changes in fresh water catfish (*Clarias batrachus*, Linn). *Fluoride* 2007;40(1):37-41.
- 35 Jhala DD, Chinoy NJ, Rao MV. Mitigating effects of some antidotes on fluoride and arsenic induced free radical toxicity in mice ovary. *Food Chem Toxicol* 2008;46:1138-42.
- 36 Chatterjee A, Adhikari P, Banerji I, Choudhury D, Jana S, Sengupta A. Antifertility effect of *piper betle* Linn (Stalk) in adult male rats. *J Indian Chem Soc* 1994;71:81-4.
- 37 Mathur P, Vyas DK, Sharma N, Jacob D. Antifertility efficacy of leaves of the common 'Gonads' *Chordia sicutima* in the male swiss albino mouse. In: International symposium on male contraception: present and future. New Delhi: AIIMS; 1995. p. 27-9.
- 38 Chinoy NJ, Sorathia HP, Jhala DD. Fluoride+aluminium induced toxicity in mice testis with giant cells and its reversal by vitamin C. *Fluoride* 2005;38(2):109-14.
- 39 Bharti VK, Srivastava RS. Effect of pineal proteins and melatonin on certain biochemical parameters of rats exposed to high-fluoride drinking water. *Fluoride* 2011;44(1):30-6.