AMELIORATION OF FLUORIDE TOXICITY IN SOME ACCESSORY REPRODUCTIVE GLANDS AND SPERMATOZOA OF RAT

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SUMMARY: Sodium fluoride (NaF) at a dose of 10 mg/kg body weight was administered orally to male rats (*Rattus norvegicus*) daily for 30 and 50 days to evaluate the effect on the physiology of some sex accessory glands and sperm functions. The effects of withdrawal upon cessation of NaF ingestion, and of administering ascorbic acid (AA) and/or calcium (Ca^{++}) along with NaF, were also investigated. The results revealed that the NaF treatment caused a significant elevation in serum fluoride levels with a simultaneous rise in Ca^{++} levels. This could be attributed to the formation of a calcium fluoride complex leading to calcium accumulation. The treatment resulted in structural and metabolic alterations in sperm, leading to low sperm motility, a low sperm mitochondrial activity index (SMAI), reduced viability (live:dead ratio), and changes in sperm membrane phospholipids (particularly phosphatidylinositol, phosphatidylserine and phosphatidylethanolamine, which would affect hormone receptor interaction and their functions). A significant reduction in electrolyte levels of sperm also occurred which would also affect their viability. The protein levels in cauda epididymal sperm suspension, vas deferens, seminal vesicle and prostate were significantly decreased after NaF administration, which may be due to altered protein metabolism by interference of fluoride ions. The changes in epididymal protein profile, with absence of some proteins and induction of some new ones, were probably a result of the "stress proteins" in NaF-treated rats affecting the structural and functional integrity of sperm. Glycogen accumulation in vas deferens and a decrease in fructose in seminal vesicles and vas deferens indicated disturbances in carbohydrate metabolism in these organs. However, withdrawal of treatment resulted in partial recovery. A significant recovery from NaF-induced toxic effects occurred following administration of ascorbic acid and/or calcium, while combined treatment (AA + Ca^{++}) for 70 days manifested a synergistic effect. The transient fluoride-induced effects were reversible.

The results, corroborated by earlier data from our laboratory, show that fluoride has a definite effect on male reproduction and fertility. Ascorbic acid and calcium are proposed as therapeutic agents in endemic populations for amelioration of effects of fluoride.

Key words: Ascorbic acid; Calcium; Fluoride; Rat spermatozoa; Sex accessory glands; Synergistic action; Reversibility.

Introduction

Consumption of fluoride through drinking water is known to cause several ailments, including bone and teeth disorders, among human populations in endemic areas. Investigations on the role of fluoride on reproductive status in relation to adverse ill-effects and their amelioration are very limited. We earlier reported that NaF caused alterations in testicular seminiferous tubules, such as vacuolation and denudation of spermatogenic epithelium, which disrupted spermatogenesis, reducing sperm counts and serum testosterone levels.[1-6] Changes also in the epididymal secretory epithelium impaired its metabolic and functional status, inhibited sperm maturation and motility, and reduced fertility of NaF-treated animals (rats, mice,

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rabbits and guinea pigs). Similar alterations in vas deferens and other sex accessory organs of fluoride intoxicated animals were also reported.[1-3,7-12] *In vitro* fluoride toxicity in human spermatozoa has also been found.[13]

Further work indicated that administering ascorbic acid (AA) and calcium (Ca^{++}), alone and in combination, contributed significantly towards complete recovery of reproductive and some other functions from fluoride toxicity.[6,10,11,14] The present study further explores the effects of fluoride on sperm viability and some sex accessory organs of rat, as well as the therapeutic effects of AA and Ca^{++} in the amelioration of fluoride toxicity.

Materials and Methods

Charles Foster strain male rats (*Rattus norvegicus*) weighing 225-250 g, from the National Institute of Occupational Health, Ahmedabad, were allowed to acclimatise to the new environment of our laboratory for one week, and then were divided into six groups and subgroups (see protocol below) and caged separately. Group I (control) were maintained on standard diet and water *ad libitum*. Group II were fed sodium fluoride orally (source: Loba Chemie, Bombay, 99% purity) using a hypodermic syringe at 10 mg/kg body weight dose daily for 30 and 50 days before giving food. The dose of NaF used was based on earlier data.[7,8] Since one complete spermatogenic cycle requires 48 ± 2 days in rat, the treatment period was 50 days. In Group III, the treatment was withdrawn after 50 days of NaF feeding, and the animals were maintained on standard diet and water *ad libitum* for further periods of 50 and 70 days, in order to evaluate the reversible effects, if any. Groups IV, V and VI were orally administered ascorbic acid (AR Grade, 98% purity) and calcium (Glaxo India, 99% purity), together with NaF, for 30, 50 and 70 days to study their ameliorative role in the light of earlier work.

Experimental Protocol

Group	Treatment and dose	Duration (days)	Day of autopsy	Number of animals used
I	Control, untreated	-	Sacrificed along with treated	10
II A, B	Sodium fluoride (NaF) (10 mg/kg body wt./rat/day)	30, 50	31, 51	20
III A, B	NaF withdrawal from day 51	50, 70	51, 71	20
IV A,B,C	NaF (as in Group II) + Ascorbic Acid (AA) (50 mg/animal/day)	30, 50, 70	31, 51, 71	30
V A, B, C	NaF(as in Group II) + Calcium (Ca^{++}) (62.5 mg/animal/day)	30, 50, 70	31, 51, 71	30
VI A, B, C	NaF + AA + Ca^{++} (dose same as in Gps IV & V)	30, 50 70	31, 51, 71	30

The control and treated animals were then sacrificed by cervical dislocation. Blood was collected by cardiac puncture for fluoride estimation. The vas deferens, seminal vesicle, prostate and cauda epididymis were dissected out carefully and blotted free of blood. The cauda epididymis was teased gently in normal saline, filtered through a strainer and centrifuged at 1500 rpm twice to obtain the spermatozoa. The following parameters, for control and treated groups, were studied:

Serum fluoride concentrations (ppm) were determined with an Ion Selective Fluoride Electrode (Orion Model 701A as per the Orion Instruction Manual).

Sperm motility percentages were determined by the method of Prasad *et al*[15] (vas deferens and cauda epididymal sperm suspension was prepared in normal saline).

Sperm viability (ratio of live:dead spermatozoa) was determined from the cauda epididymal spermatozoa suspension, using 1% trypan blue (supravital stain), following the method of Talbot and Chacon.[16]

Sperm mitochondrial activity index (SMAI) was evaluated by the method of Gopala Krishnan *et al*,[17] using nitroblue tetrazolium (NBT) salt. This test estimates the percent of spermatozoa having a full complement of mitochondria, which contain respiratory enzymes essential for providing energy for sperm motility.

Sperm plasma membrane phospholipids were extracted from the cauda epididymis using lipid solubilizing and protein denaturing solvents (methanol followed by chloroform) then separated using two-dimensional thin layer chromatography (TLC) by the method of Horrocks and Sun,[18] followed by quantitative estimation (percent phospholipids) by the procedure of Bartlett[19] using Amino Naphthol Sulphonic Acid (ANSA) colouring reagent (the blue colour was read at 820 nm on a Systronics UV VIS Spectrophotometer 118).

Sperm Sodium (Na^+), Potassium (K^+) and Calcium (Ca^{++}) levels (ppm) in cauda epididymal sperm suspension were determined using the Systronics Flame Photometer, Digital Unit Type 125, by the method of Dean.[20]

Cauda epididymal sperm proteins were separated by sodium dodecyl sulphate polyacrylamide disc gel electrophoresis (SDS-PAGE) by the original method of Laemmli[21] (a discontinuous buffer system, Tris-HCl in the gel and tris-glycine in the buffer compartments - proteins separated on a 10% polyacrylamide gel). The gels were scanned on a Beckman DU-40 Spectrophotometer.

Total protein levels in cauda epididymis sperm suspension, vas deferens, seminal vesicle and prostate (expressed as mg/100 mg fresh tissue weight) were determined by the method of Gornall *et al.*[22]

Glutathione concentrations (μ moles/100 ml sperm suspension) in sperm suspensions were estimated by the modified procedure of Grunert and Phillips.[23] Glutathione reacts with sodium nitroprusside in alkaline medium to give a red coloured complex, the intensity of which was measured with a Spectronic 20 Bausch and Lomb colorimeter at 520 nm within 1 minute.

Fructose concentrations (μg/mg tissue weight) in the seminal vesicle and vas deferens were estimated by the method of Foreman *et al.*[24] One ml of 0.1% resorcinol and 3 ml of 30% HCl were added to 2 ml of homogenate. For the blank 2 ml of perchloric acid was added instead of homogenate. The tubes were boiled at 80°C for 1 hour, cooled, and optical density was read at 410 nm on a Bausch and Lomb Spectronic 88 colorimeter.

Glycogen levels in vas deferens (μg/100 mg fresh tissue weight) were estimated by the method of Seifter *et al.*[25]

Statistics. For all the estimations, a minimum of 8-10 replicates were used and the data were analysed statistically using Factorial analysis of variance (ANOVA), Newman-Keuls test, and Student's T test.

Results

Results are presented in Tables 1-9. After NaF treatment, compared to control:

Serum fluoride levels were significantly elevated. (Table 1).

Sperm motility, sperm viability (live:dead ratio), and **sperm mitochondrial activity index (SMAI)** were significantly reduced (Table 2).

Sperm plasma membrane phospholipids - phosphatidylinositol, phosphatidylserine and phosphatidyl ethanolamine levels decreased, but sphingomyelin and phosphatidylcholine levels were not affected. (Table 3).

Sperm Ca^{++} levels were significantly enhanced after 50 days (Table 4).

Sperm Na^{+} levels declined (Table 5).

Sperm K^{+} levels were reduced (Table 6).

Total protein levels in cauda epididymis spermatozoa, vas deferens, seminal vesicle and prostate gland significantly decreased (Table 7).

Cauda epididymal sperm proteins - some were inhibited while some additional ones were induced (Table 8).

Glutathione levels in cauda epididymis spermatozoa and **fructose** levels in vas deferens and seminal vesicle were significantly suppressed, while **glycogen** concentrations significantly increased (Table 9).

On withdrawal of NaF treatment, for the parameters studied for Group III, there was a trend to recovery, compared to the treated group, after 70 days (Tables 1, 4, 5, and 6), but withdrawal did not improve the protein motility pattern (Table 8).

For all the above parameters, administration of AA or Ca^{++} resulted in significant recovery, greater after 70 days, while the combimed treatment (AA + Ca^{++}) resulted in even better and faster recovery, to almost control levels.

All the above changes were statistically significant ($p < 0.001$).

Table 1. Serum fluoride levels (ppm) in control and treated groups of rats

Group	Treatment	Duration 50 days	Duration 70 days
I	Control, untreated	0.58 ± 0.04	0.58 ± 0.04
II	NaF treatment	3.6 ± 0.11	–
III	NaF withdrawal	1.9 ± 0.08	1.4 ± 0.04
IV	NaF + ascorbic acid (AA)	0.98 ± 0.03	0.71 ± 0.03
V	NaF + calcium (Ca^{++})	0.97 ± 0.05	0.70 ± 0.05
VI	NaF + AA + Ca^{++}	0.75 ± 0.04	0.56 ± 0.03

Values are mean ± SE n = 5 in all groups

Table 2. Sperm motility (%) in cauda epididymis and vas deferens, sperm viability, and sperm mitochondrial activity index (SMAI) in control and treated groups of rats

Tissues	Group I	Group IIA	Group IVA	Group VA	Group VIA
Sperm motility:					
Cauda epididymis	69.67 ± 0.29	11.39 ± 0.49	64.57 ± 0.28	55.51 ± 0.51	69.50 ± 0.3
Vas deferens	66.19 ± 1.27	23.88 ± 0.47	61.84 ± 0.25	37.37 ± 1.99	64.30 ± 1.8
Sperm viability (%)	73.15:26.85 ± 0.05	11.96:88.04 ± 0.44	67.00:38.76 ± 0.54	61.33:38.67 ± 0.8	69.11:30.89 ± 0.74
SMAI (%)	41.9 ± 1.13	21.2 ± 0.77	35.6 ± 1.19	32.0 ± 1.05	40.1 ± 1.03

Values are mean ± S.E.

Group I Untreated control
Group IIA NaF treated (30 days)
Group IVA NaF treated as in group II withdrawal on day 31 and AA feeding for another 31 days
Group VA NaF treated as in group II withdrawal on day 31 and Ca^{++} feeding for another 30 days
Group VIA NaF treated as in group II withdrawal on day 31 and AA and Ca^{++} feeding for another 30 days

Table 3. Concentrations of phospholipids (%) in cauda epididymis spermatozoa of control and treated groups of rats.

Groups	Phosphatidyl-inositol	Phosphatidyl-serine	Sphingomyelin	Phosphatidyl-choline	Phosphatidyl ethanolamine
I	5.36 ± 0.42	11.57 ± 0.57	22.36 ± 1.28	34.91 ± 0.54	25.88 ± 0.37
II	2.78 ± 0.17	8.09 ± 0.04	21.41 ± 0.27	34.92 ± 0.48	18.70 ± 0.25
IVA	4.96 ± 0.13	10.12 ± 0.25	21.06 ± 0.26	35.18 ± 0.34	23.84 ± 0.50
VA	3.83 ± 0.12	9.21 ± 0.17	21.32 ± 0.26	34.95 ± 0.34	20.15 ± 0.26
VIA	5.91 ± 0.06	12.32 ± 0.21	21.19 ± 0.27	35.19 ± 0.04	24.94 ± 0.29

Values are mean ± S.E.

Group I Untreated control
Group IIA NaF treated (30 days)
Group IVA NaF treated as in group II withdrawal on day 31 and AA feeding for another 31 days
Group VA NaF treated as in group II withdrawal on day 31 and Ca^{++} feeding for another 30 days
Group IVA NaF treated as in group II withdrawal on day 31 and AA and Ca^{++} feeding for another 30 days

Table 4. Sperm Ca^{++} (ppm) levels in control and treated groups of rats

Duration (days)	Control (Group I)	NaF treatment (Group II)	NaF withdrawal (Group III)	NaF + AA (Group IV)	NaF + Ca^{++} (Group V)	NaF + AA + Ca^{++} (Group VI)
50	131±2.9	168 ±1.28	160 ± 2.01	138 ± 1.91	141 ± 3.6	139 ± 2.76
70	131±2.0	–	140 ± 1.89	130 ± 1.87	133 ± 2.8	136 ± 2.54

Values are mean ± S.E.

Table 5. Sperm sodium (Na^+) (ppm) levels in control and treated groups of rats

Duration (days)	Control (Group I)	NaF treatment (Group II)	NaF withdrawal (Group III)	NaF + AA (Group IV)	NaF + Ca^{++} (Group V)	NaF + AA + Ca^{++} (Group VI)
50	112 ± 3.4	103 ± 2.01	106 ± 1.91	104 ± 1.77	103 ± 2.1	107 ± 1.89
70	112 ± 3.0	–	110 ± 1.87	110 ± 2.7	114 ± 2.02	112 ± 1.23

Values are Mean ± S.E.

Table 6. Showing potassium (K^+) (ppm) in control and treated groups of rat

Duration (days)	Control (Group I)	NaF treatment (Group II)	NaF withdrawal (Group III)	NaF + AA (Group IV)	NaF + Ca^{++} (Group V)	NaF + AA + Ca^{++} (Group VI)
50	124 ± 4.1	95 ± 1.81	106 ± 1.28	114 ± 2.9	108 ± 1.54	107 ± 3.2
70	124 ± 4.1	–	119 ± 1.67	126 ± 3.1	121 ± 2.20	126 ± 3.8

Values are mean ± S.E.

Table 7. Protein concentration (mg/100 mg tissue weight) in the cauda epididymis spermatozoa, vas deferens, seminal vesicle, and prostate of control and treated groups of rats

Tissues	Group I	Group IIA	Group IVA	Group VA	Group VIA
Cauda epididymis spermatozoa	43.15 ± 0.19	34.39 ± 0.76	38.09 ± 1.73	37.34 ± 1.01	42.98 ± 0.71
Vas deferens	13.92 ± 0.63	8.17 ± 0.77	12.41 ± 0.56	10.29 ± 0.58	12.71 ± 0.64
Seminal vesicle	23.73 ± 0.4	8.63 ± 0.34	18.67 ± 0.39	16.63 ± 0.13	22.15 ± 0.52
Prostate	28.79 ± 0.44	14.26 ± 0.97	20.27 ± 0.73	17.26 ± 0.76	23.65 ± 0.77

Values are mean ± S.E.

Group I Untreated control
Group IIA NaF treated (30 days)
Group IVA NaF treated as in group II withdrawal on day 31 and AA feeding for another 31 days
Group VA NaF treated as in group II withdrawal on day 31 and Ca++ feeding for another 30 days
Group IVA NaF treated as in group II withdrawal on day 31 and AA and Ca++ feeding for another 30 days

Table 8. Cauda epididymal protein molecular weights (K. daltons) scanned on Beckman DU-40 Spectrophotometer

Control	NaF	NaF withdrawal	NaF + AA	NaF + AA	NaF + Ca^{++}	NaF + Ca^{++}	NaF + AA + Ca^{++}	NaF + AA + Ca^{++}
	50 days	70 days	30 days	70 days	30 days	70 days	30 days	70 days
264	263	–	258	263	258	271	269	264
250	–	247	250	256	246	255	252	243
–	–	–	–	247	241	–	–	–
–	–	115	–	123	–	125	121	–
–	–	90.9	94.8	89.7	–	75.9	79	–
–	68.5	–	60.5	68	–	–	61	–
–	57	–	–	–	–	–	–	–
–	53	54.6	–	–	–	–	53	–
46.6	–	–	47	–	47.9	49.6	47	49.6
40	42	–	40	–	40	–	–	42.3
30.8	32	36	33	37.5	–	39.5	38	33.6
25	29	–	28.5	27.5	29	24.5	27	28.0
21	23	22.5	–	24	23	–	22.7	22.8
–	–	19.9	18.5	21.7	19.9	–	–	–
16.5	17	15.9	–	–	17	16.2	14.8	16.8
–	15	–	–	–	15	–	–	–
13.7	14	12	–	13.7	13	–	–	11.5
11.6	12.6	–	–	10.8	–	–	–	–
9.5	10	9.8	8.0	8.1	8.8	9.9	–	9.3
7.7	–	7.5	–	–	–	7.5	7.0	7.0
6.9	–	–	–	–	–	6.4	–	–
5.7	5.7	–	5.9	–	–	5.5	5.5	5.5
5.6	5.0	–	–	–	–	–	–	–
4.6	–	–	–	–	–	–	–	4.7
4.1	–	–	–	–	–	–	–	–

NaF = Sodium Fluoride AA = Ascorbic Acid Ca^{++} = Calcium

Table 9. Levels of glutathione (GSH) in cauda epididymis spermatozoa, fructose concentrations (µg/mg fresh tissue weight) in the vas deferens and seminal vesicle and glycogen (µg/100 mg fresh tissue weight) in vas deferens of control and treated groups of rats.

Parameters	Group I	Group II	Group IVA	Group VA	Group VIA
Glutathione	36.78 ± 0.43	8.85 ± 0.66	19.43 ± 1.08	14.79 ± 1.00	28.04 ± 0.85
Fructose					
Vas deferens	13.14 ± 0.27	7.42 ± 0.37	12.57 ± 0.27	12.12 ± 0.33	13.01 ± 0.11
Seminal vesicle	13.32 ± 0.41	7.59 ± 0.14	11.06 ± 0.17	10.90 ± 0.17	13.12 ± 0.03
Glycogen					
Vas deferens	1144 ± 30.59	1998 ± 12.43	991 ± 28.58	941 ± 17.31	1062 ± 32.59

Values are mean ± S.E.

Group I Untreated control
Group IIA NaF treated (30 days)
Group IVA NaF treated as in group II withdrawal on day 31 and AA feeding for another 31 days
Group VA NaF treated as in group II withdrawal on day 31 and Ca++ feeding for another 30 days
Group VIA NaF treated as in group II withdrawal on day 31 and AA and Ca++ feeding for another 30 days

Discussion

In this study, serum fluoride levels of rats were enhanced significantly after 50 days NaF treatment. Active sequestration of fluoride at the beginning and reduced elimination are known to occur in mice on prolonged NaF ingestion, due to structural kidney damage and altered function.[26]

Schoff and Lardy[27] have demonstrated the effects of fluoride (30 mM) and caffeine on the metabolism and motility of ejaculated bull sperm *in vitro*. Similarly, human spermatozoa lost their motility *in vitro* in the presence of 250 mM NaF within 20 minutes incubation.[13] In the present study too, the motility of cauda epididymal and vas deferens spermatozoa was decreased significantly in NaF-treated rats. Similar results were reported earlier in NaF-treated mice, rats, rabbits and guinea pigs under different experimental conditions.[1,6-8,10-12]

The present study also revealed a significant decrease in the sperm mitochondrial activity index (SMAI), indicating a decline in mitochondrial enzymes in NaF-treated animals, especially succinate dehydrogenase which is an oxidative enzyme involved in the Krebs cycle. Earlier studies support these results.[12] Hence it is likely that sperm mitochondrial structure and metabolism are affected by NaF. Further studies in this direction are under way.

The evaluation of treated spermatozoa stained with Trypan blue showed a large number dead, probably due to loss of membrane integrity. The effect of fluoride on membrane permeability might be another major factor in the loss of sperm motility.

Phospholipids, one of the major components of the lipid bilayer of plasma membrane, play an important role in maintaining membrane integrity and its semi-permeable nature. This study showed a significant decrease in phosphatidylinositol, phosphatidylserine and phosphatidylethanolamine levels due to NaF treatment, while the other two phospholipids, phosphatidylcholine and sphingomyelin, were not affected. Similar results have been obtained in NaF-treated testis and epididymis of rats and guinea pigs.[12,28] To the best of our knowledge these are the first reports of this kind. Phosphatidylinositol is involved in hormone receptor function at the plasma membrane,[29] so is likely to be a factor in the changes in sperm metabolism after NaF treatment. Detailed studies are needed on hormone receptor action in target tissues in fluoride treated animals.

The NaF-induced enhancement in sperm calcium levels could be due to the affinity of fluoride to form Ca^{++} complexes, and to the Ca^{++} influx caused by direct action of fluoride. Thus, it is possible that fluoride induces Ca^{++} accumulation and thereby suppresses sperm motility. The optimal concentration of Ca^{++} ions play an important role in various cellular functions, including sperm motility through the Ca^{++}-calmodulin complex at optimal concentrations.[30] High calcium levels affect sperm function via its interaction with c-AMP.[31]

The reduction in sperm electrolyte levels (Na^+ and K^+) after NaF treatment might be due to suppression of Na^+ and water reabsorption in rat epididymis influenced by adrenal hypofunction.[32] Susheela and Das[33] have reported adrenal hypofunction in patients afflicted with fluorosis as well as in fluorotic rabbits. It is well known that the Na^+ and K^+ levels of cauda epididymis are androgen-dependent.[34,35] Sodium is actively transported out of the epididymal lumen and the circulating androgens maintain the active transport system.[35] It is further known that Na-K-ATPase, an androgen-sensitive enzyme, maintains the Na^+ levels and Na^+-dependent water

reabsorption in epididymis.[34] Hence the changes in the present study, as well as alterations in cauda epididymal and sperm ATPase activity reported in other studies.[6,9] Fluoride has earlier been reported to cause K^+ efflux from intact cells,[13] as in the present study. These alterations suggest either a change in circulating androgens in rats[6] or insensitivity of androgen target organs to the hormone.

Fluoride inhibits biosynthesis of protein *in vitro* and *in vivo*. Such inhibition is mainly due to impairment of peptide chain initiation.[36] Fluoride treatment caused a significant decrease in total protein levels in cauda epididymal spermatozoa, vas deferens, seminal vesicle and prostate, a finding in line with our earlier studies on mice, rats and rabbits.[1,2,4,7-10]

The disappearance of some cauda epididymal proteins in NaF-treated rats might be due to inhibition of protein synthesis.[37] The present study shows that proteins of molecular weights 4.6 KD and 4.1 KD seem to be very sensitive to NaF treatment. Such alterations in sperm protein caused by NaF could contribute to reduction of sperm motility and consequent low fertilizability, while a decline in protein in seminal vesicle and prostate would affect their secretions. The induction of new (probably "stress") proteins might be related to increase in DNA and RNA contents after NaF treatment.[28,38] This might in turn be the result of an increase in the transcriptional rate thus inducing new proteins. These induced proteins (53 KD to 68 KD) might also be the result of induction of new DNA polymerases, as the molecular weights of induced proteins fall in the range of 30 KD-300 KD which are the same for DNA polymerases (Table 8). These polymerases probably ultimately increase the transcriptional rate for specific "stress proteins". Shashi[38] hypothesized that DNA polymerases were increased in ovary and uterus of NaF-treated rabbits but did not present any evidence. To the best of our knowledge, our reported data are the first of their kind. A decrease in epididymal sperm protein suggests that epididymal changes led to altered synthesis of specific proteins,[39] which are important as sperm antigens and for sperm viability.[40]

Glutathione (GSH) is involved in the mechanism of detoxification of various xenobiotics. Meister and Anderson[41] noticed a primary defence mechanism in cells by GSH against the effects of toxic chemicals. In the present study, sperm GSH significantly decreased after 30 days NaF administration, which suggests its rapid oxidation. The extremely suppressed GSH levels would further aggravate the toxic effects of NaF. The depleted sperm GSH by NaF treatment strongly suggests that, like several exogenous compounds, fluoride is largely dependent upon glutathione for detoxification.[42]

NaF treatment caused significant accumulation of glycogen in the vas deferens. In fluorotic rats, glycogen turnover was depressed and levels of glucose-6-phosphate dehydrogenase were decreased.[43] Other studies have also shown such accumulation after NaF treatment in vas deferens, liver, skeletal muscle and uterus of mice and rat as well as in fish liver and muscle.[9,14,44-46]

The decline in fructose levels of seminal vesicle and vas deferens after 30 days NaF exposure further supports our observation on alteration in carbohydrate metabolism of NaF-treated animals. As fructose has a vital role providing energy to the sperm, rendering them active, it is evident that the decreased fructose level might be another factor causing reduced sperm motility and metabolism.

The results of the present study, corroborated by earlier data from our laboratory, show that fluoride has a definite effect on male reproduction and fertility.

In the withdrawal group of animals the NaF-induced effects were not restored to normal after 50 days withdrawal, while a trend of recovery was observed after 70 days. However, in groups of animals treated with AA and/or Ca^{++} along with NaF, almost complete recovery from fluoride toxicity was obtained, which supports earlier investigations.[6,10,14,47] The ameliorative action of ascorbic acid might also be due to elevation of c-AMP levels by inhibition of phosphodiesterase (PDE).[31]

In the present study, AA ingestion resulted in a significant recovery in all the parameters studied. AA is known to bind with macromolecules like protein and nucleic acids by a charge transfer complex formation and influence metabolism of several organs and cells including reproductive organs and sperms. This also occurs via formation of ascorbate free radical, monodehydroascorbic acid, which participates in oxido-reduction reactions and acts as a supplementary source of electron energy thereby activating several metabolic processes.[42,48,49] It is, therefore, likely that AA by its active detoxification and the antioxidant properties helps in overcoming fluoride toxicity and manifests an ameliorative action.

Calcium is an ideal antidote for fluoride due to its binding to form the insoluble complex, calcium fluoride, which reduces fluoride absorption. Further, the Ca^{++}-calmodulin complex influences various cellular and biological processes, especially in epididymal and vas deferens smooth muscle contraction as well as activation of some enzymes in sperm.[30] It has also been shown that the action of calcium and c-AMP are interdependent on each other and they could control the rate of calcium transfer across the plasma membrane.[30] Thus, Ca^{++} administration brought about a significant recovery in electrolyte levels as well as protein motility pattern, which elucidates its beneficial effects against fluoride toxicity. Administration of AA and Ca^{++} together with NaF resulted in a more complete and faster recovery than the individual treatments. This synergistic action of AA and Ca^{++} is possibly due to inhibition of phosphodiesterase (PDE), as they are known inhibitors of this enzyme - and thereby augmented c-AMP levels occur, which are involved in activation of several kinases.[6,31] The increase in c-AMP might also be due to induction of its synthesis by new stress proteins (68.5 KD, 57 KD, 53 KD) formed in cauda epididymis after NaF treatment. It is known through recent promotor mapping studies that in c-AMP responsive genes, a DNA sequence called c-AMP responsive element (CRE) is present, which confers c-AMP inducibility on heterologous promoters.[50] Hence it is suggested that the stress proteins might be inducers of c-AMP synthesis and result in increased phosphorylation which could be correlated with transcriptional induction of c-AMP-dependent genes. Thus increased c-AMP levels trigger the hormonal mechanism at cellular level and recovery is better with combined administration than with AA and Ca^{++} alone.

The data reveal that both ascorbic acid and calcium are promising therapeutic agents, whose beneficial effects on reversal of fluoride toxicity among human population in endemic areas need to be further investigated.

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References

1 Chinoy NJ, Sequeira E. Effects of fluoride on the histoarchitecture of reproductive organs of male mouse. *Reproductive Toxicology 3* (4) 261-268 1989.
2 Chinoy NJ, Sequeira E. Reversible fluoride induced fertility impairment in male mice. *Fluoride* 25 (2) 71-76 1992.
3 Chinoy NJ, Roa MV, Narayana MV, Neelakanta E. Microdose vasal injection of sodium fluoride in the rat. *Reproductive Toxicology 5* (6) 505-512 1991.
4 Chinoy NJ, Pradeep PK, Sequeira E. Effect of fluoride ingestion on the physiology of reproductive organs of male rat. *Journal of Environmental Biology 13* (1) 55-61 1992.
5 Narayana MV, Chinoy NJ. Effects of fluoride on rat testicular steroidogenesis. *Fluoride 27* (1) 7-12 1994.
6 Narayana MV, Chinoy NJ. Reversible effect of sodium fluoride ingestion in spermatozoa of rat. *International Journal of Fertility 39* (6) 337-346 1994.
7 Chinoy NJ. Effects of fluoride on physiology of animals and human beings. *Indian Journal of Environmental Toxicology 1* (1) 17-32 1991.
8 Chinoy NJ. Effects of fluoride on some organs of rats and their reversal. *Proceedings of Zoological Society Calcutta 44* (1) 11-15 1991.
9 Chinoy NJ, Sequeira E. Fluoride induced bio-chemical changes in reproductive organs of male mice. *Fluoride 22* (2) 78-85 1989.
10 Chinoy NJ, Sequeira E, Narayana MV. Effects of vitamin C and calcium on the reversibility of fluoride induced alterations in spermatozoa of rabbit. *Fluoride 24* (1) 29-39 1991.
11 Chinoy NJ, VVPC Reddy, Mathews Michael. Beneficial effects of ascorbic acid and calcium on the reproductive functions of sodium fluoride treated prepubertal male rats. *Fluoride 27* (2) 71-79 1994.
12 Chinoy NJ, Bhavana Patel, Dhruva Patel. Fluoride toxicity in the testis and cauda epididymis of guinea pig and reversal by ascorbate. *Reproductive Toxicology.* Submitted 1994.
13 Chinoy NJ, Narayana MV. *In vitro* fluoride toxicity in human spermatozoa. *Reproductive Toxicology 8* (2) 155-159 1994.
14 Chinoy NJ, Sharma M, Mathews Michael. Beneficial effects of ascorbic acid and calcium on reversal of fluoride toxicity in male rats. *Fluoride 26* (1) 45-56 1993.
15 Prasad MRN, Chinoy NJ, Kadam KM. Changes in succinic dehyrogenase levels in rat epididymis under normal and physiological condition. *Fertility Sterility 2* 186-190 1972.
16 Talbot P, Chacon RS. A triple stain technique for evaluating normal acrosome reaction of human sperm. *Journal of Experimental Zoology 215* 201-208 1981.
17 Gopalakrishnan K, Hinduja IN, Mehta RH, Anandkumar RC. Laboratory manual for human semen analysis. *Molecular Andrology 3* 243-253 1990.
18 Horrocks LA, Sun GY. Ethanolamine plasmogens. In: Marks N (Ed). *Research Methods in Neurochemistry.* Rodnight 1 1972 pp 223-231.
19 Bartlett GR. Phosphorus assay in column chromatography. *Journal of Biological Chemistry 234* 466-468 1959.
20 Dean JA. *Flame photometry.* McGraw 1960 pp 113-115.
21 Laemmli UK. Clevage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature 227* 680-685 1970.
22 Gornall AG, Bardavill CJ, David MM. Determination of serum proteins by Biuret reagent. *Journal of Biological Chemistry 177* 751-766 1949.
23 Grunert RR, Phillips PH. A modification of the nitroprusside method of analysis for glutathione. *Biochemistry 30* 217-225 1951.

24 Foreman D, Gaylor L, Evans E, Trella C. A modification of Roe procedure for determination of fructose with increased specificity. *Analytical Biochemistry 56* 584-590 1973.
25 Seifter S, Dayton S, Novic B, Muntwyler E. The estimation of glycogen with anthrone reagent. *Archives of Biochemistry* 25 191-200 1950.
26 Kour K, Singh J. Histological findings in kidneys of mice following sodium fluoride administration. *Fluoride 13* (4) 163-167 1980.
27 Schoff P K, Lardy M A. Effects of fluoride and caffeine on metabolism of ejaculate bovine spermatozoa. *Biology of Reproduction* 37 1037-1046 1987.
28 Chinoy NJ, Shukla Shruti, Amita Walimbe, Bhattacharya S. Reversible induction of stress proteins and alterations in membrane phospholipids and gluthione by fluoride in testis and epididymis of rat. *Reproductive Toxicology.* Submitted 1994.
29 Fain JN. Involvement of phosphatidylinositol breakdown in elevation of cytosol Ca^+ by hormones and interrelationships to prostaglandin formation. In: Kohn LD (Ed). *Hormone Receptors.* John Wiley, New York 1982 pp 237-276.
30 Rasmussen H. Interactions between the c-AMP and calcium messenger systems. In: Kohn LD (Ed). *Hormone Receptors.* John Wiley, New York 1982 pp 175-197.
31 Pasternak CA. *Introduction to Biochemistry.* Oxford University Press, New York 1979 pp 199-219.
32 Au CL, Ngai HK, Yeung CH, Wong PYD. Effect of adrenalectomy and hormone replacement of sodium and water transport in the perfused rat cauda epididymis. *Journal of Endocrinology 77* 265-266 1978.
33 Susheela AK, Das TK. Effect of chronic fluoride toxicity on glucocorticoid levels in plasma and urine. *Fluoride 24* (1) 23-28 1991.
34 Wong PYD, Yeung CH. Hormonal regulation of fluid reabsorption in isolated rat cauda epididymis. *Endocrinology 101* 1391-1397 1977.
35 Jenkins AD, Lechene CP, Howards SS. The effects of cyproterone acetate on the elements in the intraluminal fluids of the testis and epididymis of the rat. *Archives of Andrology 11* 151-155 1983.
36 Hoerz W, McCary KS. Inhibition of protein synthesis in rabbit reticulocyte lysate system. *Biochimica Biophysica Acta 228* 526-535 1971.
37 Holland RI. Fluoride inhibition of protein and DNA synthesis in cells *in vitro. Acta Pharmacologica et Toxicologica 45* 96-101 1979.
38 Shashi A. Preliminary observations on alterations on rabbit ovary DNA and RNA content in experimental fluorosis. *Fluoride 27* (2) 76-80 1994.
39 Prasad MRN, Rajalakshmi M. Recent advances in the control of male reproductive functions. In: Greep RO (Ed). *International Review of Physiology. Reproductive Physiology II.* University Park Press, Baltimore 1977 pp 153-199.
40 Chinoy NJ. Structure and function of epididymis in relation to vulnerable points of intervention for male fertility regulation. *Indian Review of Life Sciences 4* 37-68 1984.
41 Meister A, Anderson ME. Glutathione. *Annual Review of Biochemistry 52* 711-760 1983.
42 Chinoy NJ. Metabolic significance of ascorbic acid in animal and human tissues. *Journal of Animal Morphology and Physiology, Silver Jubilee Volume* 68-75 1978.
43 Carlson JR, Suttie JW. Pentose phosphate pathway enzymes and glucose oxidation in fluoride-fed rats. *American Journal of Physiology 210* 79-83 1966.
44 Sheikh YA, Hiradhar PK. Fluoride induced changes in blood glucose, tissue glycogen and SDH activity in the mud skipper, *Boleophthalmus dussumeria.* Proceedings, Symposium on Assessing Environmental Pollution, 1985 pp 93-99.

45 Chinoy NJ. Fluroide toxicity in female mice and its reversal. In: Saxena AK, Ramamurthy R, Srirama Reddy G, Saxena VL (Eds). *Recent Advances in Life Sciences* (Indian Society of Life Sciences). Manu Publications, Kanpur UP 1992 pp 39-50.
46 Chinoy NJ, Suresh Patel, Nikunj Bhatt, Mathews Michael. Effects of fluoride on some soft tissue function of fresh water fish, *Channa punctatus. Proceedings, Academy of Environmental Biology 3* (2) 191-196 1994.
47 Wadhwani TK. Prevention and mitigation of fluorosis (Endemic) II. *Journal of Indian Institute of Sciences 36* 64-69 1954.
48 Chinoy NJ, Buch RP. Metabolic significance of ascorbic acid in human semen. *Indian Journal of Experimental Biology 15* 921-923 1977.
49 Robaire B, Hermo L. Efferent ducts, epididymis and vas deferens: structure, functions and their regulations. In: Knobil E, Neill J (Eds). *The Physiology of Reproduction.* Raven Press, New York 1988 pp 999-1065.
50 Montiminy MR, Bilezikjian LM. Binding of nuclear protein to the cAMP responsive element of the somatostatin gene. *Nature 328* 175-178 1987.

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