BENEFICIAL EFFECTS OF ASCORBIC ACID AND CALCIUM ON REPRODUCTIVE FUNCTIONS OF SODIUM FLUORIDE-TREATED PREPUBERTAL MALE RATS

Niloufer J Chinoy, V V P C Reddy and Mathews Michael
Ahmedabad, India.

SUMMARY: The therapeutic effects of ascorbic acid and calcium (Ca^{2+}) supplementation on reproductive functions of fluoride-treated (10 mg/kg body weight) male rats were investigated. Sodium fluoride treatment resulted in a decrease in almost all parameters studied except concentration of testicular cholesterol, which implies that androgen synthesis might not be affected by NaF treatment. Succinate dehydrogenase activity decreased in testis suggesting that its oxidative metabolism was altered by NaF treatment. Adenosine triphosphatase activity, protein, and sialic acid levels in caput and cauda epididymides also showed a decrease. All these changes resulted in a significant decrease in sperm motility and thereby fertility rate. Glycogen concentrations in vas deferens were altered, probably due to impaired metabolic turnover. The fructose levels in vas deferens and seminal vesicle as well as the acid phosphatase activity in ventral prostate were also decreased significantly by NaF treatment.

On the other hand, simultaneous treatment of NaF along with ascorbic acid or calcium resulted in recovery in all the affected parameters studied. The recovery was more significant after treatment with ascorbic acid than with calcium. Therefore, ascorbic acid and calcium may be useful for amelioration of fluoride toxicity in endemic areas.

Key words: Ascorbic acid, Calcium, Rat, Reproductive functions, Reversibility, Sodium fluoride.

Introduction

The role of fluoride on normal reproductive functions of mice was reported by Messer *et al* (1). According to them, a low fluoride intake by female mice resulted in impaired fertility and reproductive capacity. But Tao and Suttie (2) found contradictory results. Recent reports from our laboratory have elucidated the toxic effects of fluoride on the reproductive systems (male and female) of different rodents. Chinoy and Sequiera (3) reported that 30 days administration of 10 and 20 mg/kg body weight of sodium fluoride (NaF) to mice resulted in severe disorganization and denudation of germinal epithelial cells of seminiferous tubules with absence of sperm in the lumen. The caput and cauda epididymides and vas deferens also showed marked structural alterations. Similar histological changes were observed in rats given a single microdose injection of NaF directly into the vas deferens (4). In fluoride-treated mice, rats and rabbits significant changes occurred in the activities of different enzymes, like succinate dehydrogenase, adenosine triphosphatase, hyaluronidase, acrosine and acid phosphatase, in reproductive organs and spermatozoa, as well as muscle, liver, and other tissues (3-10).

Further work in our laboratory has also elucidated the action of some therapeutic agents against fluoride toxicity (8,10). Thus the beneficial effects of ascorbic acid

Department of Zoology, School of Sciences, Gujarat University, Ahmedabad 388 009 India.

and/or calcium ingestion during the withdrawal period of NaF or fed along with NaF, resulted in reversal of fluoride toxicity and significant recovery. The purpose of the present study was to investigate further the therapeutic effects of ascorbic acid and/or calcium on some reproductive organs of fluoride-treated male rats. These studies could be important for amelioration of human suffering in endemic areas of Gujarat and elsewhere.

Materials and Methods

Healthy, colony-bred, prepubertal male rats (21-24 days old) of Charles Foster strain (*Rattus norvegicus*) weighing between 45-55 gm were used for the experiments. The animals were maintained on a standard chow, and water was given *ad libitum*. The animals were divided into four groups, and the treatments were given as shown in Table 1.

TABLE 1. SUMMARY OF TREATMENT

GROUP	TREATMENT	NO. OF ANIMALS	DURATION OF TREATMENT	AUTOPSY DAY
I	Control	20	-	31st day
II	NaF (10mg/kg body weight)	20	30 days	31st day
III	NaF + Ascorbic acid(AA) (10mg NaF/kg body weight + 50mg AA/animal/day)	20	30 days	31st day
IV	NaF + Ca^{2+} (10mg NaF/kg body weight + 62.5mg Ca^{2+}/animal/day)	20	30 days	31st day

The treatments were administered orally using a feeding tube attached to a hypodermic syringe. After the respective treatments, the animals were sacrificed by cervical dislocation and dissected out. The reproductive organs, *viz.*, testis, caput and cauda epididymides, vas deferentia, seminal vesicle and ventral prostate were excised, blotted free of blood and utilized for the various experiments. The parameters studied were:

Sperm motility and count: The cauda epididymal sperm suspension was prepared in normal saline. The percent motility and count of cauda epididymal spermatozoa of normal and NaF treated rats were determined by the method of Prasad *et al* (11) and expressed as percentage motility and millions/ml respectively.

Fertility test: This was carried out according to WHO protocol (12) and expressed as percent positive.

Biochemical parameters

Succinate dehydrogenase (SDH): Activity in the testis was determined by the modified tetrazolium reduction method of Beatty *et al* (13) and expressed as μg formazan formed/15 minutes/100 mg fresh tissue weight.

Cholesterol: Estimation of concentration in the testis was carried out by the procedure of Pearson *et al* (14) and expressed as mg/100mg fresh tissue weight.

Adenosine triphosphatase (ATPase): Activity was assayed by the method of Quinn and White (15) in the caput and cauda epididymides. The enzyme activity was expressed as μ moles of inorganic phosphorus released/100 mg fresh tissue weight/30 minutes.

Protein: Levels in the caput and cauda epididymides were determined by the method of Gornall *et al* (16) and expressed as mg/100mg fresh tissue weight.

Sialic acid: Concentration in the caput and cauda epididymides was estimated according to the method of Jourdian *et al* (17) and expressed as μg/mg fresh tissue weight.

Glycogen: Levels of proximal and distal vas deferens were estimated by the method of Seifter *et al* (18) and expressed as μg glycogen/100 mg fresh tissue weight.

Fructose: Concentration was measured by the modified Roe procedure as adopted by Foreman *et al* (19) in seminal vesicle and vas deferens (distal and proximal regions). The levels were expressed as μg fructose/mg fresh tissue weight.

Acid phosphatase: Activity in ventral prostate was estimated by employing the method of Bessey *et al* (20) and expressed as μ moles of p-nitrophenol liberated/mg fresh tissue weight/30 minutes.

Statistics: For all biochemical studies, a minimum of eight replicates were done for each tissue and parameter and the data subjected to Student's 't' test.

Results

The motility of the spermatozoa in the cauda epididymis of NaF treated (30 days) rats decreased significantly compared to control ($P < 0.001$). A significant recovery ($P < 0.001$) in the motility of the sperms occurred in the NaF + AA treated rats (Group III) but only a slight recovery occurred in NaF + Ca^{2+} treated rats (Table 2).

The sperm count in the cauda epididymis of NaF treated rats was decreased compared to control, whereas NaF + AA treated rats showed significant recovery ($P < 0.001$). However, those treated with NaF + Ca^{2+} did not show any recovery (Table 2).

The fertility rate of the NaF treated rats decreased significantly compared to control ($P < 0.001$), whereas the rate of NaF + AA treated ones showed significant recovery compared to the NaF alone treated group ($P < 0.001$). An insignificant recovery occurred in the fertility rate of NaF + Ca^{2+} treated rats (Table 2).

TABLE 2. SPERM MOTILITY, COUNT AND FERTILITY RATE IN RATS

PARAMETER	GROUP I CONTROL	GROUP II NaF ALONE	GROUP III NaF + ASCORBIC ACID	GROUP IV NaF + CALCIUM
SPERM MOTILITY (%)	61.32 ± 1.90	28.54 ± 1.56*	64.70 ± 2.26**	34.64 ± 3.70
SPERM COUNT (millions/ml)	35.73 ± 0.34	28.49 ± 0.37*	32.30 ± 0.59	26.48 ± 0.49
FERTILITY RATE (% positive)	90-95	16.66*	66.66**	33.66

Values are Mean ± S.E.
* Different from control, $P < 0.001$
** Different from Group II, $P < 0.001$

Testicular parameters

Succinate dehydrogenase (SDH): Activity in the testis decreased significantly ($P < 0.001$) after NaF treatment. However, it showed significant recovery ($P < 0.001$) in NaF + AA treated group of animals. The recovery was less in Group IV ($NaF + Ca^{2+}$ treated rats) (Table 3).

Cholesterol: Concentration in testis was not altered in NaF treated rats. However, it showed a decrease in NaF + AA and $NaF + Ca^{2+}$ treated rats (Table 3).

TABLE 3. ACTIVITY OF SUCCINATE DEHYDROGENASE (SDH) AND CHOLESTEROL IN TESTIS OF RATS

PARAMETER	GROUP I CONTROL	GROUP II NaF ALONE	GROUP III NaF + ASCORBIC ACID	GROUP IV NaF + CALCIUM
SDH (µg formazan/100mg fresh tissue wt./30 minutes)	451.25± 4.01	216.88 ± 4.35*	339.03 ± 1.39**	297.36 ± 5.38
CHOLESTEROL (mg/100mg fresh tissue wt.)	0.38 ± 0.007	0.38 ± 0.01	0.17 ± 0.06	0.14 ± 0.03

Values are Mean ± S.E.
* Different from control, $P < 0.001$
** Different from Group II, $P < 0.001$

Epididymis

Adenosine triphosphatase (ATPase): Activity in caput and cauda epididymides of NaF treated rats showed significant decrease ($P < 0.001$) compared to control. The administration of NaF + AA resulted in significant recovery in ATPase activity in both caput and cauda epididymides ($P < 0.001$ and $P < 0.02$), whereas $NaF + Ca^{2+}$ administration was not conducive for recovery (Table 4).

Protein: Concentration in caput and cauda epididymides of NaF treated rats revealed a significant decrease compared to the control. However, both NaF + AA and $NaF + Ca^{2+}$ treatments resulted in a significant recovery compared to treated ones (Table 4).

Sialic acid: Levels in caput and cauda epididymides of NaF treated rats showed a decrease compared to control (Table 4). However, the caput epididymis of NaF + AA and $NaF + Ca^{2+}$ treated rats revealed significant recovery. The cauda epididymis of ascorbic acid treated rats (Group III) showed recovery to some extent but calcium treatment did not cause any recovery (Table 4).

Vas deferens

Glycogen: Concentration increased significantly ($P < 0.001$) in the proximal and distal vas deferens of NaF treated rats compared to control. A significant recovery occurred in both regions of the vas deferens as a result of AA treatment. $NaF + Ca^{2+}$ treated rats showed less recovery compared to Group III (Table 5).

Fructose: Levels were reduced in the proximal and distal vas deferens of NaF treated rats compared to control. On the other hand, NaF + AA and $NaF + Ca^{2+}$ treatments resulted in significant recovery which was complete in $NaF + Ca^{2+}$ treated rats (Table 5).

TABLE 4. LEVELS OF ADENOSINE TRIPHOSPHATASE (ATPASE), PROTEIN AND SIALIC ACID IN CAPUT AND CAUDA EPIDIDYMIDES OF RATS

PARAMETER	TISSUE	GROUP I CONTROL	GROUP II NaF ALONE	GROUP III NaF + ASCORBIC ACID	GROUP IV NaF + CALCIUM
ATPase (μ moles i.p. released/ 100mg fresh tissue weight/30 minutes)	Caput	17.13 ± 0.82	12.54 ± 0.24*	20.08 ± 0.23**	13.38 ± 0.37
	Cauda	20.88 ± 0.39	16.12 ± 0.44*	22.52 ± 0.37**	15.80 ± 0.38
PROTEIN (mg/100mg fresh tissue weight)	Caput	16.57 ± 0.35	10.43 ± 0.18*	19.99 ± 0.19**	19.14 ± 0.49**
	Cauda	18.74 ± 0.13	11.94 ± 0.43*	21.24 ± 0.32**	20.20 ± 0.35**
SIALIC ACID (μg/mg fresh tissue weight)	Caput	0.72 ± 0.04	0.52 ± 0.01*	0.73 ± 0.014**	0.63 ± 0.02
	Cauda	1.36 ± 0.07	0.92 ± 0.01*	1.17 ± 0.03	0.82 ± 0.01

Values are Mean ± S.E. *Different from control, P<0.001. **Different from Group II, P<0.001

TABLE 5. GLYCOGEN AND FRUCTOSE LEVELS IN PROXIMAL AND DISTAL VAS DEFERENS OF RATS

PARAMETER	TISSUE	GROUP I CONTROL	GROUP II NaF ALONE	GROUP III NaF + ASCORBIC ACID	GROUP IV NaF + CALCIUM
GLYCOGEN (μg/100mg fresh tissue weight)	Proximal Vas deferens	1111.0 ± 16.89	3035.0 ± 61.8*	1586.0 ± 17.43**	2675.0 ± 50.23
	Distal Vas deferens	1142.0 ± 13.07	3215.0 ± 82.12*	1580.0 ± 7.59**	2970.0 ± 43.81
FRUCTOSE (μg/mg fresh tissue wt.)	Proximal Vas deferens	11.01 ± 0.23	7.61 ± 0.21*	16.47 ± 0.31**	11.86 ± 0.31**
	Distal Vas deferens	12.18 ± 0.26	7.68 ± 0.14*	18.80 ± 0.09**	13.36 ± 0.10**

Values are Mean ± S.E. *Different from control, P<0.001. **Different from Group II, P<0.001

Fructose in the seminal vesicle: Concentration in NaF treated rats markedly decreased compared to control, but significant recovery occurred in both NaF + AA and NaF + Ca^{2+} treated rats (Table 6).

Acid phosphatase: Activity in the ventral prostate of NaF treated rats significantly declined ($P < 0.02$) compared to control but the enzyme activity showed a significant recovery with AA and Ca^{2+} treatments (Groups III and IV) ($P < 0.02$) (Table 6).

TABLE 6. FRUCTOSE IN SEMINAL VESICLE AND ACID PHOSPHATASE IN VENTRAL PROSTATE OF RATS

PARAMETER	ORGAN	GROUP I CONTROL	GROUP II NaF ALONE	GROUP III NaF + ASCORBIC ACID	GROUP IV NaF + CALCIUM
FRUCTOSE (μg/mg fresh tissue wt.)	Seminal Vesicle	13.27 ± 0.7	9.90 ± 0.28	22.95 ± 0.44	13.62 ± 0.22
ACID PHOSPHATASE (μ moles p-nitro-phenol/mg fresh tissue wt./30 minutes)	Ventral prostate	0.504 ± 0.003	0.178 ± 0.003	0.390 ± 0.006	0.358 ± 0.008

Values are Mean ± S.E.

Discussion

The significant decline in succinate dehydrogenase (SDH) activity in testis of NaF treated rats is similar to that reported by Chinoy and Sequeira (7) in mice and by Chinoy *et al* (9) in rats. NaF treatment also reduced muscle SDH activity of rats (10,21), which suggests that the oxidative metabolism of testis and muscle was altered by NaF treatment. Since SDH is primarily a mitochondrial, oxidative enzyme, it is likely that mitochondrial structure and metabolic alterations may have occurred in NaF treated rats. Ultrastructural studies in this direction are called for to investigate structural changes.

It is well known that changes in the structural integrity of reproductive organs influence their internal milieu (22-24) and that the epididymis and vas deferens, in particular, have a higher threshold requirement for androgens than the accessory sex glands, to maintain their structure and function. In the present study, cauda epididymal sperm count and motility were reduced by NaF treatment. Kour and Singh (25) reported lack of maturation and differentiation of spermatocytes, along with necrotic seminiferous tubules in mouse testis treated with NaF. Similarly, Chinoy and Sequeira (3) reported alteration of testicular histoarchitecture in mice treated with 10 mg/kg body weight of NaF for 30 days. These changes in testis would affect spermatogenesis. The decrease in total epididymal sperm count suggests a clear relationship between fluoride intake and testicular damage which hampers spermatogenesis.

Several observations support the view that fluoride acts directly on the motile apparatus without substantially affecting other metabolic pathways, as it inhibits the dynein ATPase in sperm and cilia (26). In the present study, ATPase activity in the caput and cauda epididymides was significantly decreased after NaF treatment. Similar results were reported earlier by Chinoy and Sequeira (7) in NaF treated mice and spermatozoa of rabbits and rats administered NaF (8). This decrease in ATPase activity might be one of the causative factors leading to changes in the internal milieu of the epididymis and the reduction in sperm motility.

Fluoride inhibits biosynthesis of protein *in vitro* and *in vivo*. In the present study, the levels of protein in caput and cauda epididymides showed a significant decrease after 30 days of NaF treatment. This decrease might be due to impairment of protein metabolism. Earlier studies from our laboratory have also reported a dose-dependent decrease in protein levels in mice, rats, and rabbits (5,6,8). As some of these proteins are important for sperm motility and maturation (23), it is evident that these functions would be affected.

Sialic acid, a sialomucoprotein, is essential for the maintenance of the structural integrity of the sperm membrane. The levels of sialic acid in caput and cauda epididymides were decreased in the present study. Hence, the structural integrity of acrosomal membranes of the sperm might be affected. Moreover, sialic acid is important for sperm maturation (22). Thus it follows that sperm metabolism and motility were hampered, which would ultimately influence their fertilizing capacity. A decline in sialic acid concentration of NaF treated mouse spermatozoa has been reported earlier (7).

NaF treatment caused an accumulation of glycogen concentration in the vas deferens. It is known that fluoride causes disturbance in carbohydrate metabolism,

depression of glycogen turnover, and a decrease in the levels of glucose-1-phosphate dehydrogenase (27). Earlier work from our laboratory (7,10,21) also elucidated an increase in glycogen levels in the vas deferens, liver, and skeletal muscle after NaF treatment.

Acid phosphatase activity in the ventral prostate was also decreased by the treatment. According to Hodge and Smith (28) fluoride inhibits enzyme activities, particularly those in which divalent metal cations act as cofactors. In the present study, this finding might be attributed to the fact that the enzymes which showed decreased activity with NaF treatment, *viz.*, adenosine triphosphatase, succinate dehydrogenase, and acid phosphatase are either Mn^{2+}, Ca^{2+} or Zn^{2+} metalloproteins.

In the present study NaF treatment affected, to a variable extent, numerous androgen dependent parameters in the reproductive organs, demonstrating the differential sensitivity of various accessory organs, for maintenance of their structure and function, to circulating androgens (22-24). However, the cholesterol level did not change. Unaltered levels of serum cholesterol and testosterone have been reported earlier in fluorotic human populations (5, 29).

Hence, it is evident that fluoride affects either the hormone-receptor interaction in the target organs, or else the conversion of testosterone to its potent metabolite, 5α-dihydrotestosterone (5α-DHT), is hindered due to a probable decline in 5α-reductase activity. This would affect the utilization of the androgens by target organs. Further studies in this direction are needed to elucidate the precise mechanism of action of fluoride.

The results of the present study show that fluoride has a definite effect on male reproduction, and affects fertility. However, ingestion of ascorbic acid or calcium along with NaF manifested marked recovery in all induced effects. The recovery was more significant after ascorbic acid treatment than with calcium.

Treatment with NaF + AA and NaF + Ca^{2+} resulted in the recovery in the activity of androgen dependent enzymes like ATPase in epididymis, SDH in testis, and acid phosphatase in ventral prostate. The recovery in the activity of these enzymes is very important as they are involved in energy releasing reactions. In the present study significant recovery in sperm count and sperm motility also occurred, resulting ultimately in restoration of fertility in NaF + AA treated rats.

The beneficial and prophylactic role of ascorbic acid, and its participation in several biosynthetic processes, growth, stress conditions, and metabolism of reproductive and nonreproductive tissues, and sperm, has been extensively studied (23,24,30). Chinoy *et al* (10) reported higher levels of ascorbic acid in liver and adrenal of NaF treated rats, as a consequence of stress imposed by sodium fluoride. This elevation in concentrations of ascorbic acid might be another ameliorative role played by the vitamin in mitigating fluoride induced effects. The vitamin is known to activate numerous hydroxylating enzymes and those involved in oxidoreduction reactions in several tissues (23,24,30).

Therapeutic effects of calcium against fluoride are known (8,10). Calcium has an important role to play in several processes in the body. The calcium bound to its receptor calmodulin influences sperm motility, as well as epididymis and vas deferens smooth muscle contraction (23,24,31), and activates several enzymes, notably ATPase.

The present study supports the above findings and reveals the greater therapeutic role of ascorbic acid over calcium in overcoming fluoride toxicity - evident also from biochemical and histological observations. Further studies are under way to investigate the probable synergistic effect of administration of a combination of ascorbic acid and calcium which, as we suggested earlier (8), might be highly beneficial in the recovery of fluoride induced effects. This synergistic effect might be due to inhibition of phosphodiesterase (which is a known inhibitor of c-AMP) and thereby augmentation of c-AMP levels which are involved in activation of several kinases.

The present study further elucidates the therapeutic effects of ascorbic acid and calcium on reversal of fluoride toxicity, and so may have a bearing on the amelioration of human suffering in endemic fluorosis areas all over the world.

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