



## Sodium Fluoride Induced Toxicity in Testes of Swiss Albino Mice and its Reversal by Ascorbic Acid and Calcium

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Accepted on: 14-11-2013; Finalized on: 31-01-2014.

### ABSTRACT

Fluorosis, which was considered to be a problem related to teeth and bone only, has now turned up to be a serious health hazard. The aim of this study is to see the effect of fluoride on the reproductive system and to see the role if any of Ascorbic acid (A.A) and Calcium supplementation. The healthy, adult Swiss albino mice (*Mus musculus*) were divided into eight equal groups. Group I (fed on standard diet) Group II (NaF 5ppm and 50ppm) Group III (withdrawal of treatment) Group IV (NaF 5ppm and 50 ppm+25ppm A.A) Group V (NaF 5ppm and 50 ppm+25ppm Ca) Group VI (NaF 5ppm and 50 ppm+25ppm A.A+25ppm Ca) Group VII(25ppm A.A) and Group VIII(25ppm Ca). Recovery was also observed in Group IV, V and VI with simultaneously administration of A.A, Ca and A.A +Ca respectively. Animals from each group were autopsied by cervical dislocation at each post treatment intervals of 10, 20, and 30 days. Quantitative histological study of the spermatogenic events after various periods of drug administration was made from randomly selected sections of the testis. The changes were dose and duration dependent. The value of spermatogonia 'A', resting spermatocytes, pachytene spermatocytes, stage VII spermatids and stage XVI spermatids decreased in all experimental groups. It has been reported that sodium fluoride (NaF) affects the histoarchitecture of testis also causing disintegration of the germinal epithelium, vacuolization and exfoliation of cells in the lumen, some of which were giant cells. Thus, in conclusion, sodium fluoride has a definite effect on reproduction. However, the fluoride induced effects are reversible and transient and could be effectively reversed by withdrawal of treatment and subsequent supplementation of A.A and Calcium.

**Keywords:** Ascorbic acid, Calcium, Fluoride, Seminiferous epithelial cells, Spermatogenesis, Testis.

### INTRODUCTION

Fluoride is one of the important life elements to human health. It is essential for normal mineralization of bones and formation of dental enamel with presence in small quantity<sup>1</sup> when fluoride is taken up more than permissible limit (WHO 1.5ppm) it becomes toxic and causes clinical and metabolic disturbance in animals and human being such as dental and skeletal Fluorosis.<sup>2-7</sup> The main sources of fluoride (F) intake include drinking water, foodstuffs, Industrial dust, smoke, pesticides and F-containing dental products.<sup>8-12</sup>

The effect of fluoride on the structure and metabolism of several soft tissues has been reported recently. Earlier studies reported that ingestion of 10 or 20mg sodium fluoride in mice caused alterations in the histology of reproductive organs, morphology of sperm and induced biochemical changes.<sup>13-14</sup> Recent investigations showed that fluoride interferes with the structural and functional integrity of testis, internal milieu of epididymis, vas deferens and also affected the metabolism and morphology of spermatozoa thus reduces fertility.<sup>15-16</sup> Role of diet in fluorosis has a double sword action. Intake of high fluoride in diet increases the toxic manifestations of fluorosis, whereas intake of diet rich in calcium and vitamin C helps in overcoming the toxicity of fluorosis.<sup>17</sup> The present study was undertaken to elucidate<sup>18-20</sup> the effect of fluoride toxicity on histological parameters of mice testis and its possible reversibility by feeding ascorbic acid (A.A) and or calcium (Ca<sup>+2</sup>). The quantitative

studies<sup>21</sup> (sperm kinetics) were also undertaken to observe any changes in number of spermatogonial cells.

### MATERIALS AND METHODS

#### Subjects

Adult healthy, six to seven weeks old male Swiss albino mice (*Mus musculus*) weighing between 30 to 40gm were procured for the experiments under the Animal Maintenance act and Registration No--/1066/ac/07/ CPCSEA Chennai, India. The animals were kept in polypropylene cages, saw dust was put on the bottom of cages. The cages were cleaned daily. Water bottles and nipples were autoclaved periodically. Mice were fed with standard pellet feed. Water was given *ad-libitum*.

The animals were divided into following groups:-

#### Group I (Normal)

This group comprised the control group. These animals were provided with standard pellet feed and they received distilled water *ad-libitum*.

#### Group II (Sodium Fluoride treated animals)

The animals of this group received sodium fluoride at the dose rate of different levels in distilled water (*ad-libitum* until autopsy). This group was further divided into different sub groups on the basis of sodium fluoride doses:

Sub Group I - 5ppm

Sub Group II - 50ppm



The animal of these two sub-groups were given sodium fluoride and were sacrificed after 10, 20 and 30 days of treatment.

### **Group III (After Withdrawal of Sodium fluoride treatment)**

The animals were divided into following sub groups

Sub Group I - 5ppm

Sub Group II - 50ppm

In these sub groups animals were treated with sodium fluoride for 30 days as in group II and were sacrificed after 10, 20 and 30 days of cessation of treatment.

### **Group IV (This group comprised of 2 different sub groups)**

#### **Group IV (A) (Sodium Fluoride + Ascorbic Acid)**

Sub Group I - 5ppm NaF + 25ppm Ascorbic Acid

Sub Group II - 50ppm NaF + 25ppm Ascorbic Acid

In these sub groups, animals were treated with sodium fluoride + Ascorbic acid and were sacrificed after 10, 20 and 30 days of treatment.

#### **Group IV (B) (Sodium fluoride + Ascorbic acid treatment followed by continuation of ascorbic acid in recovery groups)**

In this subgroup, animals were treated and sacrificed as in group IV(A) but during recovery period the ascorbic acid alone was given continuously until autopsy.

### **Group V (This group comprised of 2 different sub groups)**

#### **Group V (A) (Sodium Fluoride + Calcium phosphate (Ca))**

Sub Group I - 5ppm NaF + 25ppm Ca

Sub Group II - 50ppm NaF + 25ppm Ca

In these sub groups, animals were treated with sodium fluoride + Calcium and were sacrificed after 10, 20 and 30 days of treatment.

#### **Group V (B) (Sodium fluoride + Calcium treatment followed by continuation of Calcium in recovery groups)**

In this subgroup animals were treated and sacrificed as in group V (A) but during recovery period Calcium alone was given continuously until autopsy.

### **Group VI (This group comprised of 2 different sub groups)**

#### **Group VI (A) (Sodium Fluoride + Ascorbic Acid + Calcium)**

Sub Group I - 5ppm NaF + 25ppm Ca + 25ppm Ascorbic Acid

Sub Group II - 50ppm NaF + 25ppm Ca + 25ppm Ascorbic Acid

In these sub groups, animals were treated with sodium fluoride+ Ascorbic Acid + Calcium and were sacrificed after 10, 20 and 30 days of treatment.

### **Group VI (B) (Sodium fluoride+ Ascorbic Acid + Calcium treatment followed by continuation of Ascorbic Acid + Calcium in recovery groups)**

In this subgroup, animals were treated and sacrificed as in group (VIA) but during recovery period the ascorbic acid + Ca alone was given continuously until autopsy.

### **Group VII [Ascorbic acid alone (25ppm)]**

In this subgroup, animals were given 25ppm Ascorbic acid alone and were sacrificed after 10, 20 and 30 days of treatment

### **Group VIII [Calcium alone (25ppm)]**

In this subgroup, animals were given 25ppm Calcium alone and were sacrificed after 10, 20 and 30 days of treatment.

### **Histological Studies**

After sacrificing the mice, the pieces of tissues (testis) from control as well as experimental groups were put in the Bounis fixative for overnight at room temperature. The material was washed thoroughly with 50% alcohol and dehydrated by passing through 70%, 90% and absolute alcohol. Finally the tissues were cleared in xylol and embedded in paraffin wax at 58-60°C. The blocks were made and sections were cut at 7µ (micron) with the help of rotary microtome and stained with Haematoxyline-eosin and PAS - Haematoxyline stains for histological and quantitative (sperm kinetics) studies.

### **Chemicals**

Analytical laboratory-grade sources of sodium fluoride (NaF), ethanol, xylene, picric acid, glacial acetic acid, formaldehyde, embedding wax; DPX, haematoxylin, eosin etc were used for various experimental preparations in this study.

### **Preparation of NaF solutions**

Analytical grade sodium fluoride was used to prepare the required aqueous NaF solutions. A 1000ppm of F stock solution was prepared by dissolving 2.21g of NaF in 1L of water. Feeding dilutions of 50ppm and 5ppm were prepared by adding 95ml of water to 5ml stock solution and 99.5ml of water to 0.5ml stock solution as per requirement respectively.

### **Statistical Analysis**

The Statistical results were expressed as mean ± S.E. The characteristics were compared by the student t-test

## **RESULTS AND DISCUSSION**

The histological sections in the control group showed seminiferous tubules surrounded by dense interstitial tissue. All along the basement membranes, in each seminiferous tubule, spermatogonia were arranged in concentric layers (typically representing the zone of mitosis), followed by similar concentric layers of spermatocytes immediately inward to the spermatogonial layers (representing the zone of meiosis), while the core

area contained differentiating spermatozoa (identified as the zone of spermiogenesis). The most central luminal part of the seminiferous tubules was occupied by mature spermatozoa (Figure 1).

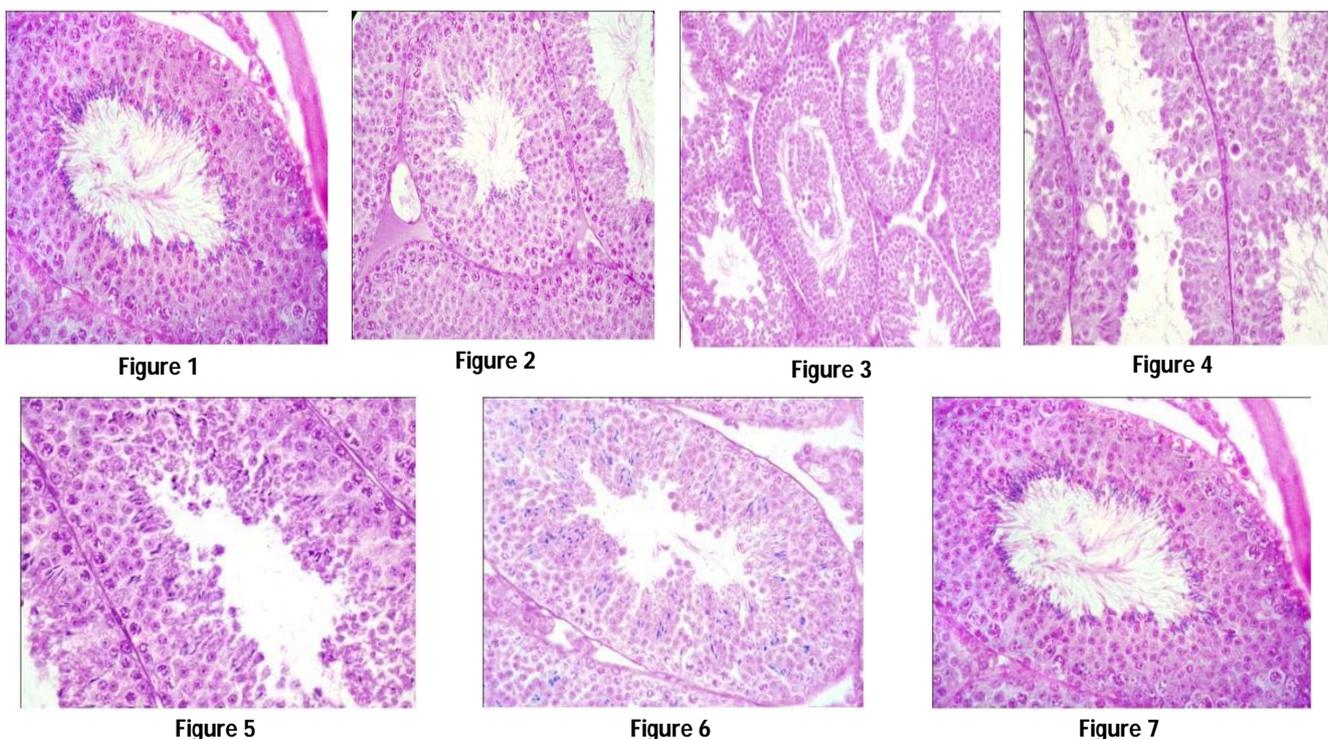
5ppm treated group showed very few histological changes with an impression of increase in the thickness of the zone of mitosis and denser luminal space filled with spermatozoa. The mice treated with high dose of fluoride, testis tubules showed irregularly arranged spermatocytes were seen. Wide luminal spaces with a few scattered clusters of spermatozoa were observed in the centre of each seminiferous tubule.

In 50ppm sodium fluoride treatment after 30 days, the seminiferous tubules of mice showed damaged tubules displaying of injury in cells and a thick, irregular tubular basement membrane. The multinucleate giant cells appeared to be one of ultimate atrophy, and increased vacuolization, sloughing off of the spermatogenic cells in the luminal region of the seminiferous tubules of the testis, leading to disorganization of their epithelium.

Recovery with A.A+ Ca (Group VI B) was much better compared to when animals were treated with ascorbic acid or calcium alone. Lower dose treated groups were almost recovered. In 50ppm dose group giant cell, vacuolization, thickening of basement membrane were decreased and tubules showed few scattered clusters of spermatozoa. (Fig 2-7)

In the present quantitative study, quantitative morphometric information on the testis of sodium fluoride treated mice revealed that the number of resting spermatocytes, pachytene spermatocytes, stage VII and stage XVI spermatids decreased gradually from 10 to day 30 in 50ppm NaF treated group while in recovery groups the number of these cells recovered quantitatively.

But it was more pronounced in those animals in which A.A and Ca were given during recovery period. The tubular diameter and germinal height also showed a decreasing trend. in recovery groups especially with A.A and Ca these parameters registered significant recovery but the values did not reach to the normal. (Table 1)



**Figure 1:** T.S Control testis showing normal arrangement of seminiferous epithelium in a tubules. X 200; **Figure 2:** T.S of Swiss albino mice after 30 days of NaF (5ppm) no significant changes. X200; **Figure 3:** T.S of Swiss albino mice after 10 days of NaF (50ppm) Showing vacuolization, intertubular oedema, dead cells and sloughing of the spermatogenic cells in the luminal region. X200; **Figure 4:** T.S of Swiss albino mice after 30 days of NaF (50ppm) showing vacuolization, multinucleate giant cells confluences of tubules at places, and exfoliation of germinal epithelium. X200; **Figure 5:** T.S of Swiss albino mice after 30 days of NaF (50ppm) withdrawal of treatment (Group IV B) showing increase in number of germ cells. X200; **Figure 6:** T.S- of Swiss albino mice after 30 days of NaF (50ppm) withdrawal of treatment (Group V B), showing mild recovery as compared to those treated group (Group IV B) where A.A was given continuously during recovery. X200; **Figure 7:** T.S of Swiss albino mice after 30 days of NaF (50ppm) withdrawal of treatment (Group VI B), showing decreased intertubular oedema, vacuolization and histoarchitecture was more or less near to normal. X200.

**Table 1:** Changes in different spermatogonial cells in various experimental groups

Dose	A type		Resting spermatocyte		Pachytene		Cap stage (VII)		Stage XVI	
	10 Days	30 Days	10 Days	30 Days	10 Days	30 Days	10 Days	30 Days	10 Days	30 Days
Control	1.56±0.30		15.47±1.12		17.74±1.94		48.32±0.34		42.84±0.35	
5ppm NaF	1.56 ±0.28	1.54 ±0.21	14.25 ±1.22	13.07 ±1.16	17.35 ±3.92	15.94 ±4.05	47.92±0.29	46.54±0.35**	42.60±0.32	42.02±0.27
Recovery with water	1.54 ±0.22	1.54 ±0.39	13.18 ±1.36	13.75 ±1.20	16.30 ±4.08	17.28 ±3.93	46.96±0.28*	47.54±0.32	42.20±0.22	42.37±0.25
NaF+A.A	1.56 ±0.28	1.55 ±0.25	14.65 ±1.14	13.28 ±0.96	17.56 ±3.99	16.67 ±3.77	47.96±0.29	46.72±0.27*	42.69±0.30	42.07±0.27
Recovery with A.A	1.55 ±0.36	1.55 ±0.32	13.48 ±1.10	14.76 ±1.13	16.83 ±3.75	17.54 ±3.14	47.12±0.28*	47.74±0.26	42.16±0.35	42.42±0.38
NaF+Ca	1.55 ±0.33	1.54 ±0.29	14.38 ±1.08	13.18 ±.86	17.47 ±3.28	16.52 ±3.58	47.88±0.25	46.64±0.24*	42.65±0.30	42.05±0.28
Recovery with Ca	1.53 ±0.29	1.55 ±0.32	13.37 ±1.02	14.21 ±1.37	16.68 ±4.09	17.42 ±3.12	46.94±0.27	47.54±0.29	42.14±0.39	42.39±0.33
NaF+A.A+Ca	1.56 ±0.38	1.55 ±0.29	14.82 ±1.08	13.65 ±1.28	17.60 ±4.04	17.19 ±4.08	47.97±0.28	47.23±0.22	42.74±0.32	42.74±0.26
Recovery with A.A+Ca	1.55 ±0.35	1.56 ±0.32	14.45 ±1.07	15.24 ±1.15	17.38 ±3.92	17.62 ±3.57	47.49±0.18	47.98±0.34	42.68±0.32	42.80±0.33
50ppm NaF	1.55 ±0.23	1.49 ±0.25	13.64 ±1.05	11.67 ±0.82*	14.54 ±3.59	11.47 ±0.88*	44.90 ±0.31***	43.06 ±0.17****	40.12 ±0.34***	38.92 ±0.20****
Recovery with water	1.51 ±0.23	1.52 ±0.29	12.20 ±1.17	13.51 ±1.20	11.62 ±2.42	12.93 ±4.06	43.46 ±0.19	43.65±0.30	39.21±0.21	40.20±0.32*
NaF+A.A	1.53 ±0.31	1.52 ±0.31	13.92 ±.87	11.73 ±0.62*	14.84 ±3.15	12.42 ±3.14	44.98 ±0.26***	43.97 ±0.15****	40.72 ±0.27***	39.18 ±0.18****
Recovery with A.A	1.51 ±0.29	1.52 ±0.30	12.72 ±0.87	14.32 ±0.46	13.02 ±3.98	1392 ±2.92	44.82 ±0.19*	46.16 ±0.19***	39.54 ±0.15	40.78 ±0.17**
NaF+Ca	1.51 ±0.28	1.51 ±0.35	13.22 ±0.92	11.70 ±0.051*	13.67 ±3.42	12.27 ±3.53	44.03 ±0.21****	43.88 ±0.29****	40.55 ±0.31***	39.06 ±0.24****
Recovery with Ca	1.54 ±0.23	1.54 ±0.28	12.69 ±1.03	14.28 ±0.80	12.80 ±3.52	13.15 ±2.53	44.32 ±0.27	45.68 ±0.31**	39.48 ±0.23	40.58 ±0.27**
NaF+A.A+Ca	1.54 ±0.28	1.55 ±0.28	13.15 ±0.72	11.97 ±0.58*	12.97 ±3.44	12.86 ±3.85	45.32 ±0.26****	44.90 ±0.18****	40.82 ±0.24**	39.33 ±0.31***
Recovery with A.A+Ca	1.54 ±0.31	1.55 ±0.26	12.92 ±0.89	14.62 ±0.62*	17.74 ±3.39	14.39 ±3.51	46.36 ±0.09***	47.34 ±0.21****	39.72 ±0.31	40.85 ±0.18**
Alone A.A	1.56±0.31	-----	15.45±1.20	-----	17.72±3.34	-----	48.12±0.22	-----	42.83±.034	-----
Alone Ca	1.56±0.32	-----	15.46±1.18	-----	17.73±3.91	-----	48.18±0.23	-----	42.80±0.28	-----
A.A+Ca	1.56±0.34	-----	15.50±1.26	-----	13.54±2.16	-----	48.20±0.32	-----	42.84±0.26	-----

Values are Means ±S.E; \*= $p < 0.5$ ; \*\*= $p < 0.02$ ; \*\*\*= $P < 0.01$ ; \*\*\*\*= $p < 0.001$ .

The present investigation was carried out to explore the effects of fluoride (NaF) and the possible ameliorative role of A.A and Calcium ingestion on testis of mice. Elevated intake of F has been shown to interfere with the anatomical structure and physiological activity of testis, epididymis, and associated duct system.<sup>22</sup> Experimental data have showed NaF exposure-related degenerative changes mainly affecting shape, motility, viability, and capacitation of spermatozoa in laboratory animals.<sup>23-24</sup>

Sodium fluoride (NaF) affects the histoarchitecture of testis causing disintegration of the germinal epithelium, exfoliation of cells in the lumen, some of which were giant cells. Sperm bundles were absent and vacuolization seen in the seminiferous epithelial cells. The testes of male albino mice intoxicated with Fluoride alone showed damaged tubules displaying of injury in cells and a thick, irregular tubular basement membrane. The multinucleate giant cells appeared to be one of ultimate atrophy, and increased vacuolization, sloughing off of the spermatogenic cells in the luminal region of the seminiferous tubules of the testis, leading to disorganization of their epithelium.

The present data showed that AA administration during the recovery period (group IVB) was beneficial and revealed a much better recovery, in almost all the parameters, than by  $Ca^{+2}$  treatment alone (group VB). The combined treatment of  $Ca^{+2}$  and AA (group VIB) resulted in prominent recovery than by individual treatments (group IV and V).

Earlier studies carried out have revealed that the treatment of A.A, D and calcium showed a significant improvement in the skeletal, clinical fluorosis and biochemical parameters in children consuming water containing 4.5 ppm of fluoride.<sup>25</sup> The above reports and earlier work carried out by Chinoy and associates have elucidated that therapeutic agents like amino acids, A.A and  $Ca^{+2}$  could mitigate fluoride induced effects.<sup>26-27</sup>

The occurrence of giant cells in the lumen of mice testis after 30 days of treatment with fluoride and arsenic has also been reported.<sup>28</sup> These giant cells could be the result of faulty or failed chromosomal replication or cell division.<sup>29</sup>

Our results demonstrated significant decrease in the frequency of pachytene spermatocytes, stage VII and

stage XVI spermatids. These cells appear to be susceptible to sodium fluoride treatment. The decrease in the number of these cells is found to be dose and duration dependent.<sup>30-32</sup> Decrease in the stage XVI spermatids indicates increased permeability to stage VII tubules in addition to a greater vulnerability of sodium fluoride to germ cells themselves. Similar studies have been made by many researcher employing rat as an animal model.<sup>33-34</sup>

Our finding confirm the studies made by earlier worker<sup>35,36</sup> suggesting that NaF exposure via the drinking water route of exposure does not at the dose used in present study adversely affect spermatogenesis in mice. In our experiment, 30 days treatment was inadequate to deplete the testis of all spermatocytes and spermatids.

Earlier reports<sup>37-39</sup> have indicated that AA and Ca<sup>+2</sup> can mitigate the fluoride toxicity effects and bring about reversal. Ascorbic acid is a biological antioxidant which is known to activate numerous hydroxylating enzymes, participates in metabolic processes as a supplementary source of energy in several tissues including sperms<sup>4</sup>. It also inhibits phosphodiesterase and hence enhances C-AMP levels which would bring about activation of several kinases and have an indirect metabolic effect.

The Ca<sup>+2</sup> probably made the fluoride less soluble through the formation of CaF<sub>2</sub>. This has been shown frequently in another mechanism by which Ca<sup>+2</sup> could have aided in recovery might be through inhibition of phosphodiesterase (PDE) activity like ascorbate, leading to enhanced C-AMP levels, since it is a known inhibitor of this enzyme.<sup>39</sup>

## CONCLUSION

In conclusion, sodium fluoride has a definite effect on reproduction. Thus A.A and Calcium can be used as therapeutic agents for the mitigation of fluoride induced toxicity in endemic areas all over the world. Hence, these results have important implications for amelioration of fluorosis in endemic regions.

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Source of Support: Nil, Conflict of Interest: None.

