

## FLUORIDE AND/OR ARSENIC TOXICITY IN MICE TESTIS WITH FORMATION OF GIANT CELLS AND SUBSEQUENT RECOVERY BY SOME ANTIDOTES

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**SUMMARY:** Administration of sodium fluoride (NaF, 5 mg/kg body weight) and/or arsenic trioxide (As<sub>2</sub>O<sub>3</sub>, 0.5 mg/kg body weight) for 30 days caused structural alterations (including the formation of giant cells) in mice testis, affected spermatogenesis, reduced protein levels, and lowered activities of 3β- and 17β-hydroxysteroid dehydrogenases (HSDs). In turn, the latter effect resulted in accumulation of cholesterol and a decline in testosterone levels, indicating a decrease in testicular steroidogenesis. Withdrawal of NaF + As<sub>2</sub>O<sub>3</sub> for 30 days led to partial recovery, but supplementation with ascorbic acid, calcium, and vitamin E, individually or in combination, during the withdrawal period, caused significant recovery in testis.

Keywords: Arsenic and testis; Fluoride and testis; Spermatogenesis; Testicular steroidogenesis; Testis structure alterations; Toxicity reversal.

### INTRODUCTION

The occasional widespread distribution of fluoride and arsenic in the environment through drinking water and food often results in adverse health effects. Excess fluoride in drinking water leads to fluorosis, and elevated arsenic leads to arsenism.<sup>1,2</sup> Although research has been carried out on the effects of fluoride on the reproductive organs as well as fertility impairment in animal models, the results are controversial.<sup>3-9</sup> Moreover, the effects of arsenic and/or fluoride ingestion on the reproductive organs are not fully understood. Hence, an investigation was made of the toxic effects on the testis of mice ingesting fluoride and/or arsenic by mice for 30 days, followed by monitoring recovery after a 30-day withdrawal period with and without antidotes.

### MATERIALS AND METHODS

Adult Swiss strain male mice (*Mus musculus*) weighing between 20 and 30 g were procured from National Institute of Occupational Health (NIOH), Ahmedabad, under Registration Number 167/1999/CPCSEA, from the Ministry of Social Justice and Empowerment, Government of India and Committee for the Purpose of Control and Supervision of Experiments on Animals, Chennai, India. The mice were divided into thirteen groups and treated with sodium fluoride (NaF), arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) and their combination as well as with vitamins C and E and calcium phosphate as shown in the treatment protocol table.

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**Treatment protocol**

Group	Treatment and dose (10 to 12 animals in each group)	Duration (days)	Day of autopsy
I	Control + distilled water	-	*
II	Control + olive oil (0.2 mL/animal/day)	30	31 <sup>st</sup>
III	Control + ascorbic acid (AA) (15 mg/animal/day)	30	31 <sup>st</sup>
IV	Control + calcium phosphate (Ca) (25 mg/animal/day)	30	31 <sup>st</sup>
V	Control + vitamin E (vit. E) (2 mg/0.2mL olive oil/animal/day)	30	31 <sup>st</sup>
VI	NaF - treated (5 mg/kg body weight)	30	31 <sup>st</sup>
VII	As <sub>2</sub> O <sub>3</sub> - treated (0.5 mg/kg body weight)	30	31 <sup>st</sup>
VIII	NaF + As <sub>2</sub> O <sub>3</sub> treated (5 mg + 0.5mg/kg body weight)	30	31 <sup>st</sup>
IX	Same as in Group VIII then withdrawal for further 30 days	30+30	61 <sup>st</sup>
X	Same as in Group VIII+AA for 30 days	30+30	61 <sup>st</sup>
XI	Same as in Group VIII+Ca for 30 days	30+30	61 <sup>st</sup>
XII	Same as in Group VIII+vit. E for 30 days	30+30	61 <sup>st</sup>
XIII	Same as in Group VIII+AA+Ca+vit. E (Same dose as in Groups III, IV, and V, respectively) for 30 days.	30+30	61 <sup>st</sup>

\* Sacrificed along with treated mice.

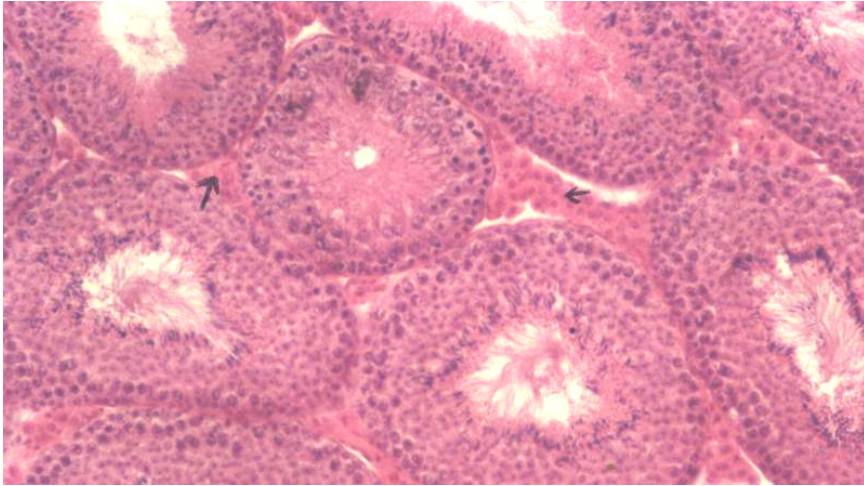
After the respective treatments the animals were sacrificed by cervical dislocation. The testes were excised, blotted free of blood, and utilized for histological studies by haematoxyline and eosin (HE) staining and some biochemical parameters.

The levels of protein<sup>10</sup> and cholesterol,<sup>11</sup> and the activities of 3 $\beta$ - and 17 $\beta$ -hydroxysteroid dehydrogenases (HSDs) (E.C. 1.1.1.51)<sup>12</sup> in testis, together with serum testosterone levels<sup>13</sup> of control and treated mice were determined using methods cited.

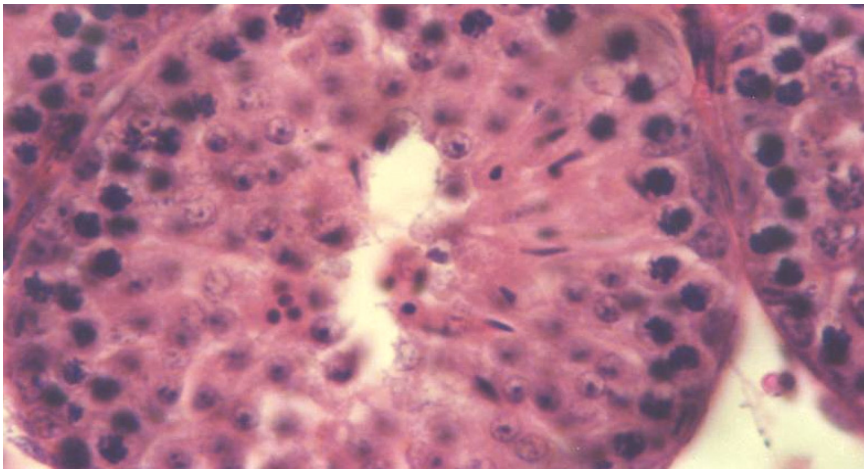
*Statistical analysis:* For all biochemical parameters, a minimum of 5–6 replicates were assayed, and the data were statistically analysed by Student's t test and ANOVA.

## RESULTS

*Testis histology:* The seminiferous tubules of normal mice showed the different germinal epithelial cells and the interstitial Leydig cells (Figures 1 and 2).

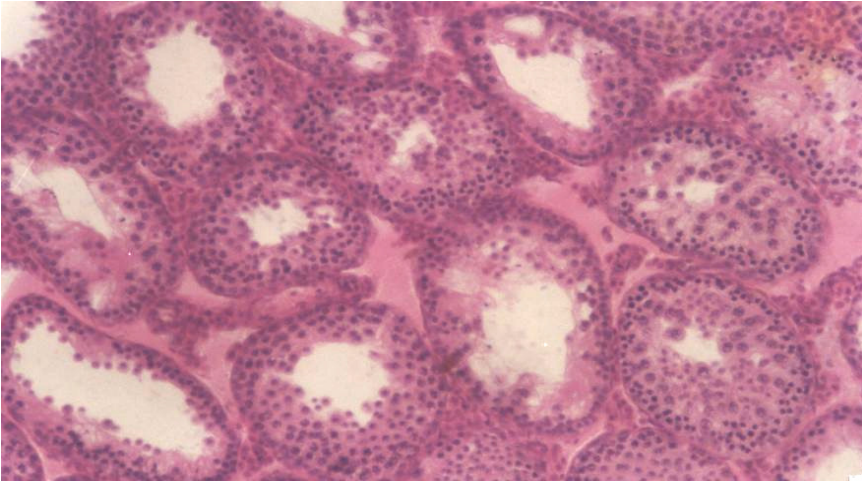


**Figure 1.** Transverse section of testis of control (Group I) mice showing seminiferous tubules and Leydig cells in the interstitium (arrow). HE staining (X 200).

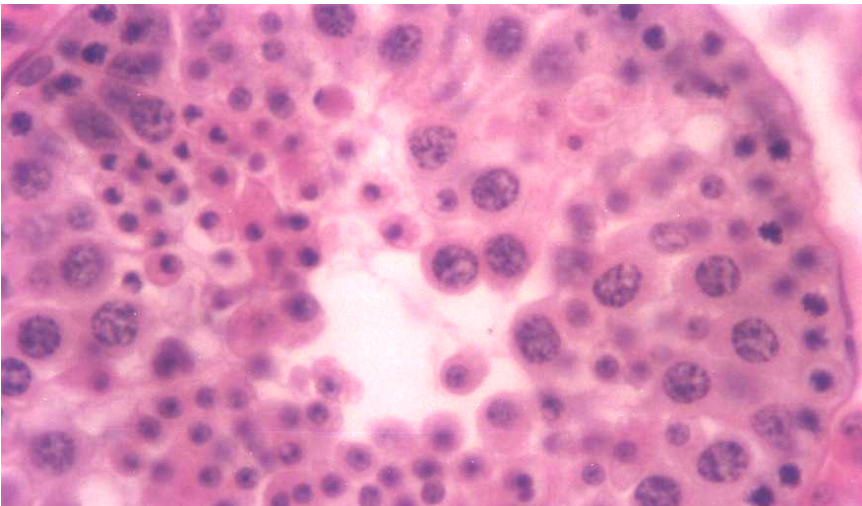


**Figure 2.** Transverse section of testis of control (Group I) mice. HE staining (X 920).

As<sub>2</sub>O<sub>3</sub> treatment (Group VII) resulted in apical degeneration and confluence of tubules, denudation of germinal epithelial cells, and absence of sperm in the lumen. In some tubules the lumen was completely obliterated. The Leydig cells were not clearly seen (Figures 3 and 4).

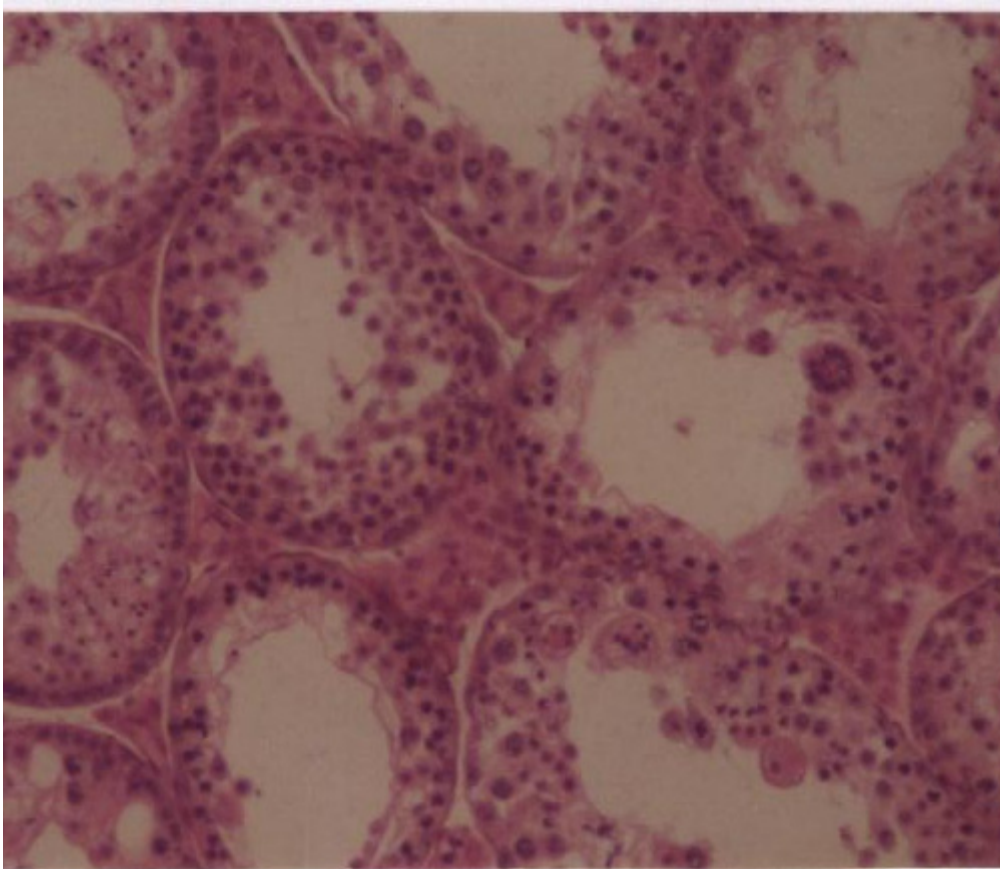


**Figure 3.** Transverse section of testis of As<sub>2</sub>O<sub>3</sub> (Group VII) treated mouse showing apical degeneration and confluence of tubules, denudation of germinal epithelial cells and some tubules with obliterated lumen and others devoid of sperms. HE staining (X 188).



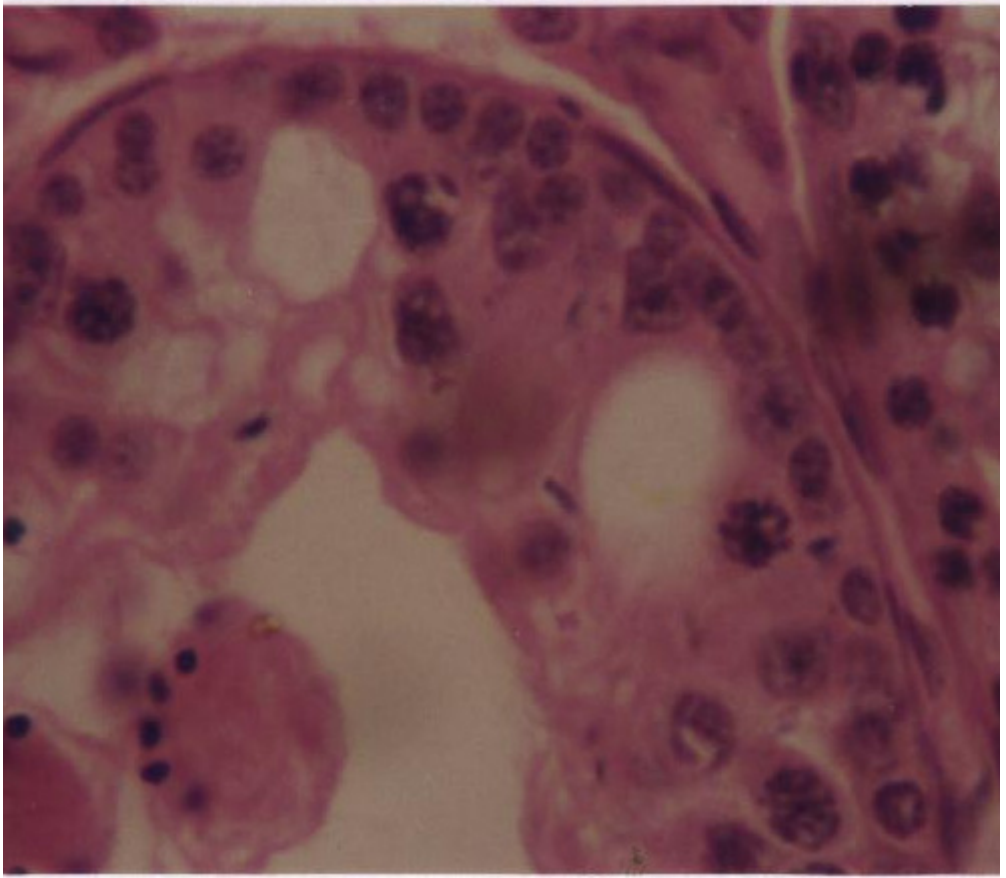
**Figure 4.** Transverse section of testis of As<sub>2</sub>O<sub>3</sub> (Group VII) treated mouse. HE staining (X 830).

The combined treatment of NaF + As<sub>2</sub>O<sub>3</sub> (Group VIII) showed many tubules with giant cells of different sizes lining the lumen (Figures 5 and 6).



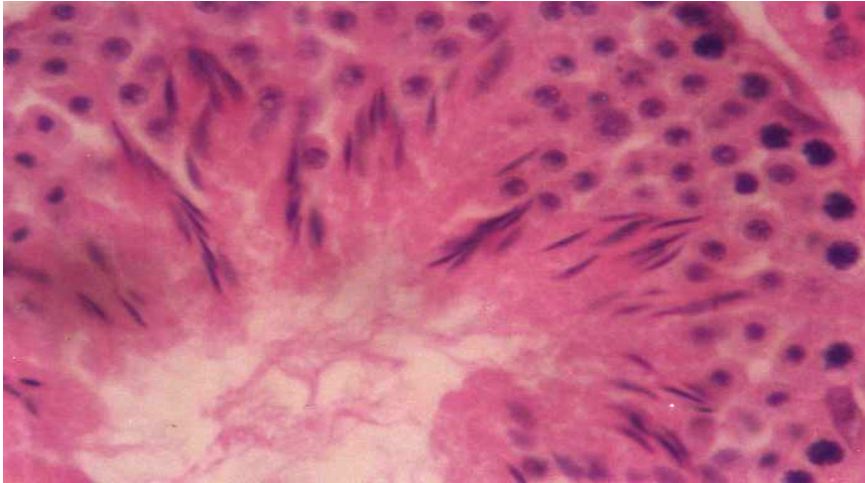
**Figure 5.** Transverse section of testis of NaF + As<sub>2</sub>O<sub>3</sub> (Group VIII) treated mouse showing denudation of germinal epithelial cells. Giant cells of different sizes lining the lumen were also seen. HE staining (X 155).



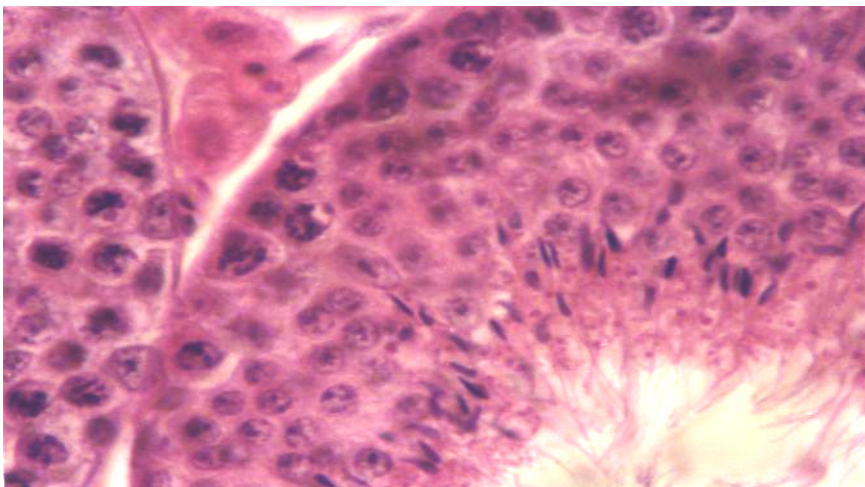


**Figure 6.** Magnified view of figure 5. HE staining (X 785).

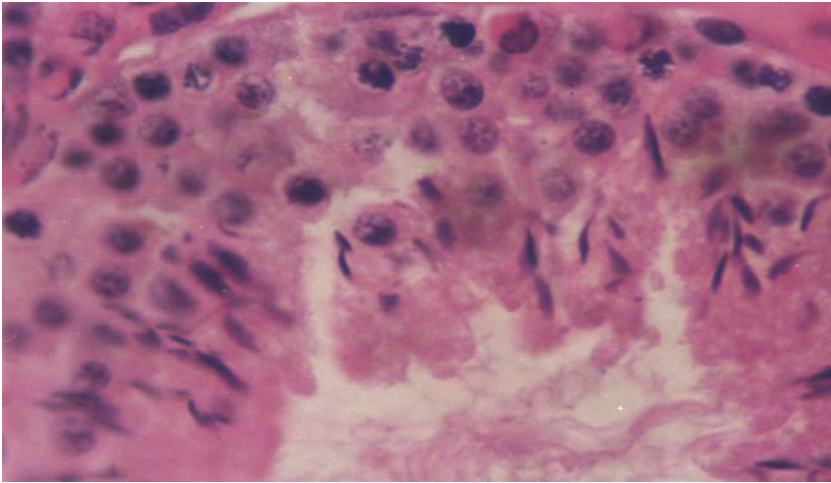
Withdrawal of treatment was not conducive for recovery. Ascorbic acid, vitamin E, and calcium treatments alone and in combination, however, brought about almost complete recovery (Figures 7–10).



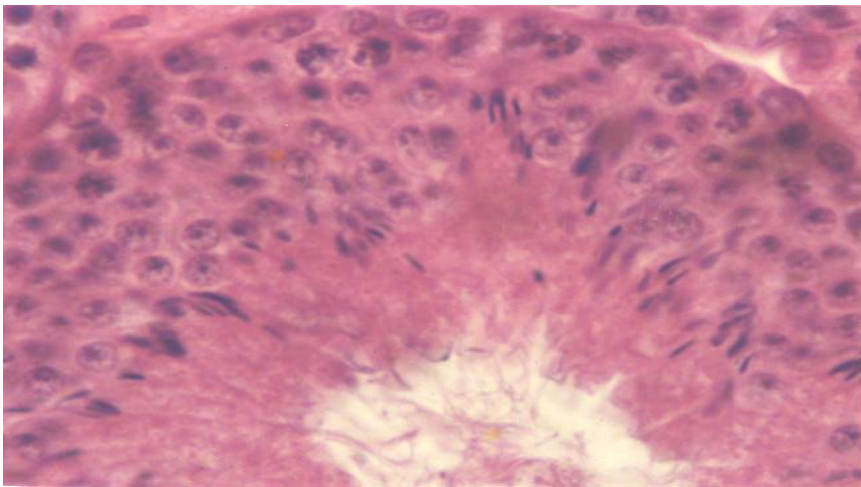
**Figure 7.** Transverse section of testis of Group X mouse showing spermatozoa in the lumen. HE staining (X 630).



**Figure 8.** Transverse section of testis of Group XI mouse showing complete recovery. HE staining (X 930).



**Figure 9.** Transverse section of testis of Group XII mouse showing recovery of seminiferous tubules. HE staining (X 630).



**Figure 10.** Transverse section of testis of Group XIII mouse treated with combined antidotes showing complete recovery. HE staining (X 685).

Testicular protein and serum testosterone levels decreased significantly ( $p < 0.001$ ) after administration of NaF,  $As_2O_3$ , and NaF +  $As_2O_3$  (Groups VI–VIII) for 30 days as compared to Groups I–V. A significant ( $p < 0.001$ ) recovery occurred after withdrawal of NaF +  $As_2O_3$  treatment (Group IX) and by administration of therapeutic agents (Groups X–XIII) singly or in combination as compared to Group VIII treated animals (Table 1).

Cholesterol levels in testis of NaF (Group VI),  $As_2O_3$  (Group VII) and NaF +  $As_2O_3$  (Group VIII) treated animals revealed a significant enhancement ( $P < 0.05$ ,  $P < 0.02$ ,  $P < 0.01$ ) as compared to control Groups I–V. An insignificant recovery



was obtained upon withdrawal of treatment in Group IX as compared to Group VIII, whereas, in mice administered therapeutic agents, alone or in combination, (Groups X–XIII) resulted in very significant ( $P < 0.001$ ) recovery (Table 1).

**Table 1.** Protein, cholesterol (mg/100 mg fresh tissue wt), activities of 3 $\beta$ - and 17 $\beta$ -hydroxysteroid dehydrogenases (HSDs) (nanomoles of androstenedione formed/mg protein/minute) and testosterone (ng/mL) in testis of Groups I–XIII mice<sup>a</sup>

Group	Treatment	Protein	Cholesterol	3 $\beta$ -HSD	17 $\beta$ -HSD	Serum testosterone
I	Control + distilled water	14.50 $\pm$ 0.48	0.47 $\pm$ 0.02	0.33 $\pm$ 0.02	0.21 $\pm$ 0.01	2.57 $\pm$ 0.08
II	Control + olive oil	14.69 $\pm$ 0.40	0.49 $\pm$ 0.01	0.34 $\pm$ 0.01	0.21 $\pm$ 0.02	2.38 $\pm$ 0.02
III	Control + ascorbic acid (AA)	14.60 $\pm$ 0.38	0.48 $\pm$ 0.01	0.34 $\pm$ 0.01	0.21 $\pm$ 0.02	2.45 $\pm$ 0.04
IV	Control + calcium phosphate (Ca)	14.40 $\pm$ 0.36	0.49 $\pm$ 0.02	0.35 $\pm$ 0.01	0.21 $\pm$ 0.01	2.50 $\pm$ 0.02
V	Control + vitamin E (Vit. E)	14.37 $\pm$ 0.36	0.49 $\pm$ 0.01	0.35 $\pm$ 0.01	0.21 $\pm$ 0.01	2.49 $\pm$ 0.06
VI	NaF	10.23 $\pm$ 0.43 <sup>§</sup>	0.53 $\pm$ 0.02 <sup>*</sup>	0.27 $\pm$ 0.01 <sup>‡</sup>	0.18 $\pm$ 0.01 <sup>*</sup>	2.20 $\pm$ 0.05 <sup>§</sup>
VII	As <sub>2</sub> O <sub>3</sub>	8.77 $\pm$ 0.23 <sup>§</sup>	0.54 $\pm$ 0.01 <sup>†</sup>	0.24 $\pm$ 0.02 <sup>§</sup>	0.16 $\pm$ 0.01 <sup>§</sup>	2.07 $\pm$ 0.04 <sup>§</sup>
VIII	NaF + As <sub>2</sub> O <sub>3</sub>	7.77 $\pm$ 0.16 <sup>§</sup>	0.56 $\pm$ 0.01 <sup>‡</sup>	0.21 $\pm$ 0.01 <sup>§</sup>	0.14 $\pm$ 0.003 <sup>§</sup>	1.8 $\pm$ 0.10 <sup>§</sup>
IX	Withdrawal of Group VIII treatment	11.01 $\pm$ 0.25 <sup>§</sup>	0.52 $\pm$ 0.02	0.23 $\pm$ 0.01	0.15 $\pm$ 0.01	2.12 $\pm$ 0.04 <sup>§</sup>
X	Withdrawal of Group VIII treatment + AA	12.64 $\pm$ 0.19 <sup>§</sup>	0.48 $\pm$ 0.004 <sup>§</sup>	0.28 $\pm$ 0.01 <sup>§</sup>	0.19 $\pm$ 0.004 <sup>§</sup>	2.40 $\pm$ 0.04 <sup>§</sup>
XI	Withdrawal of Group VIII treatment + Ca	11.68 $\pm$ 0.20 <sup>§</sup>	0.49 $\pm$ 0.004 <sup>§</sup>	0.26 $\pm$ 0.01 <sup>§</sup>	0.18 $\pm$ 0.004 <sup>§</sup>	2.30 $\pm$ 0.04 <sup>§</sup>
XII	Withdrawal of Group VIII treatment + Vit. E	12.08 $\pm$ 0.14 <sup>§</sup>	0.49 $\pm$ 0.005 <sup>§</sup>	0.26 $\pm$ 0.01 <sup>§</sup>	0.18 $\pm$ 0.003 <sup>§</sup>	2.33 $\pm$ 0.02 <sup>§</sup>
XIII	Withdrawal of Group VIII treatment + AA, Ca & Vit. E	13.56 $\pm$ 0.20 <sup>§</sup>	0.48 $\pm$ 0.005 <sup>§</sup>	0.30 $\pm$ 0.01 <sup>§</sup>	0.20 $\pm$ 0.003 <sup>§</sup>	2.52 $\pm$ 0.04 <sup>§</sup>

<sup>a</sup>Data are expressed as mean  $\pm$  SE. \* $p < 0.05$ ; † $p < 0.02$ ; ‡ $p < 0.01$ ; § $p < 0.001$ ; no sign = not significant.

Comparisons between: Group I with Groups VI or VII or VIII individually; Group VIII with Groups IX or X or XI or XII or XIII individually.

**Table 1a.** ANOVA of various parameters

Parameter	Source of variation	SS	df	MSS	F-cal	F-tab
Protein	Between Groups	6.878527	9	0.764281	0.13728	1.974829
	Within Groups	556.7325	100	5.567325		
Cholesterol	Between Groups	0.064783	12	0.005399	16.80005	1.872202
	Within Groups	0.02635	82	0.000321		
3 $\beta$ -HSD	Between Groups	0.18356	12	0.015297	60.92044	1.872202
	Within Groups	0.02059	82	0.000251		
17 $\beta$ -HSD	Between Groups	0.04225	12	0.00352	17.46354	1.872202
	Within Groups	0.01653	82	0.000202		
Serum testosterone	Between Groups	1.898887	8	0.237361	15.6311	2.305313
	Within Groups	0.41	27	0.015185		

SS=sum of squares; df=degree of freedom; MSS=mean sum of squares; F-cal=Fisher calculated; F-tab=Fisher tabulated.

The activities of 3 $\beta$ - and 17 $\beta$ -HSDs in testis of Groups VI–VIII were inhibited significantly (Group VI  $P < 0.01$  for 3 $\beta$ -HSD and  $P < 0.05$  for 17 $\beta$ -HSD; Groups VII and VIII  $P < 0.001$ ) as compared to control Groups (I–V). An insignificant recovery was observed upon withdrawal of treatment (Group IX) as compared to Group VIII. However, both enzyme activities were recovered significantly ( $P < 0.001$ ) on administration of therapeutic agents during withdrawal period (Groups X–XIII) as compared to Group VIII (Table 1).

### DISCUSSION

At the elevated levels used here, fluoride and/or arsenic trioxide treatment of adult male mice for 30 days resulted in severe alterations in the histology of testis which disturbed the process of spermatogenesis. Numerous tubules contained hypertrophic giant cells of different sizes in the lumen which may be generated either due to failure of chromosome replication or cell division causing cell death. To the best of our knowledge, this is the first report of such giant cell findings. It is known that apoptosis is a physiological process of cell death leading to the controlled elimination of single unwanted cells from the midst of a viable tissue by formation of apoptotic bodies as observed in testis of rats treated with phosphamidone.<sup>14</sup> In the present study, too, apoptotic cells/bodies were observed.

Further studies using specific tests are underway to confirm the observation. Earlier studies on mice, rats, and rabbits treated with different doses of fluoride for varied durations<sup>15-18</sup> have also reported altered histology of testis with structural defects in spermatids and sperms. Human spermatozoa showed abnormalities in head, midpiece, and tail in subjects drinking fluoride contaminated water.<sup>19</sup> The androgen-dependent enzyme activities of ram semen were significantly decreased *in vitro* by NaF.<sup>20</sup>

With respect to arsenic, degenerative changes in the tubules and varying degrees of necrosis and pyknosis were observed in testis of arsenic treated freshwater fish, *Colisa fasciatus*.<sup>21</sup> The present findings corroborate earlier work in this regard<sup>22,23</sup> and also show that fluoride and/or arsenic ingestion resulted in arrest of spermatogenesis, which is further confirmed by low sperm counts reported in the cauda epididymis.<sup>24</sup> One of the factors responsible for the arrest of spermatogenesis in the current study might be the lack of available proteins necessary for cell division, growth, and differentiation of germ cells. Studies carried out by Bano *et al*<sup>25</sup> reveal a decrease in the soluble protein in testis on administration of NaF at a dose of 10 mg/kg body weight to mice for 30, 60, and 90 days. Disruption of protein metabolism occurs in various reproductive organs of rodents treated with different doses of NaF for varied durations.<sup>3-5,22,23,26</sup> The testicular and epididymal protein profile of rats show reduction of some proteins and loss of others, but also induction of some new proteins which were not present in control animals after fluoride treatment.<sup>27</sup> This might be a response to the stress imposed by NaF.

As(III) and As(V) also inhibit protein synthesis.<sup>1</sup> Arsenic reacts with the sulphhydryl groups in certain tissue proteins and thus interferes with a number of enzyme systems essential to cellular metabolism.<sup>28</sup> Protein concentration decreased significantly in different reproductive tissues of arsenic-treated mice.<sup>3</sup> The results of the present study corroborate those findings. Lack of adequate protein turnover would have an adverse effect on testicular enzymes, receptors, and testicular secretions.

The reduction in activities of 3 $\beta$ - and 17 $\beta$ -hydroxysteroid dehydrogenases (3 $\beta$ -HSD and 17 $\beta$ -HSD) after fluoride and/or arsenic treatment correlates with accumulation of cholesterol in the testis and a decrease in the levels of circulating testosterone. Some earlier studies have also reported similar changes in rabbits, rats, and mice,<sup>4,5,23,29-31</sup> and in human populations in endemic areas of North Gujarat<sup>3</sup> and in males suffering from skeletal fluorosis.<sup>32</sup> Similarly, a significant decrease in testicular 3 $\beta$ -HSD, 17 $\beta$ -HSD activities, and testosterone levels in arsenic-treated rats<sup>33</sup> as well as decreased spermatogenesis and degenerative changes in testicular histology in arsenic-treated mice<sup>34,35</sup> are in agreement with the present study. These results suggest that fluoride and arsenic interfere with cholesterol metabolism, testicular androgenesis, and spermatogenesis.

The recovery in all the parameters by administration of vitamins C and E and calcium during the NaF + As<sub>2</sub>O<sub>3</sub> withdrawal period, could be attributed to the

antioxidant properties of the vitamins and their synergistic action with calcium.<sup>3,4,26,27,36</sup>

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