TRANSIENT AND REVERSIBLE FLUORIDE TOXICITY IN SOME SOFT TISSUES OF FEMALE MICE

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SUMMARY: The effects of NaF ingestion (10 mg/kg body weight) and possible therapeutic effects of vitamin C (25 mg/animal/day) and/or calcium (25 mg/animal/day) were investigated on some soft tissues of female mice (Mus musculus). The decrease in protein levels in liver, muscle (pectoralis and gastrocnemius), and small intestine suggested an alteration in metabolism by fluoride and a possible change in the osmotic balance. The decline in the succinate dehydrogenase activity in muscle elucidates disturbances in oxidative metabolism and contractility. The significant accumulation of glycogen levels in muscle and liver led to decreased glycogen turnover, probably due to the reduction in activity of phosphorylase. The Ca²⁺ levels of muscle were elevated, which would create an ionic gradient across the sarcolemma affecting muscle contraction. The Na⁺ and K⁺ levels in the kidney were decreased significantly, indicating an electrolyte imbalance. However, the cholesterol levels in the ventricle were not affected. The administration of ascorbic acid and calcium to NaF-treated mice revealed marked recovery from fluoride toxicity in all above parameters, showing that fluoride toxicity is reversible and transient with ameliorative effects of ascorbic acid and calcium alone and/or in combination. The recovery was more pronounced in the animal group treated with both ascorbic acid and calcium, thus indicating their synergistic action.

Key words: Ascorbic acid; Calcium; Fluoride; Mice; Reversibility; Soft tissues; Synergistic effect; Toxicity.

Introduction

Fluoride toxicity is increasingly becoming a matter of grave concern as many countries have been declared endemic for fluorosis. This makes it imperative for scientists to focus on the precise toxic effects of fluoride on various soft tissues, so that therapeutic agents can be effectively used. The toxicity of fluoride compounds administered orally differs from species to species. Every phase of metabolism can be affected critically by fluoride under certain conditions. Though the mechanism of fluoride toxicity is not known clearly, generally it is shown that fluoride kills in acute poisoning by blocking the metabolism of cells, either by inhibiting the enzyme or by intervening with the nerve impulse. Impairment of organ function is observed, due to cell damage and necrosis.

The non-essential role of fluoride in reproduction was observed by Tao and Suttie (1). NaF treatment to mice, rats, rabbits and guinea pigs caused significant alterations in the structure and function of testis, internal milieu of epididymis, vas deferens, and also affected the morphology and metabolism of their contained spermatozoa (2-12). Human spermatozoa also lost their motility after 20 minutes incubation in vitro with 250 mM of NaF (13). However, there is a paucity of data on effects of fluoride in the females.
In this study the effects of fluoride on some soft tissues of female mice, and the possible reversibility of the induced toxicity, by ingestion of ascorbic acid (AA) and/or calcium (Ca++) as possible therapeutic agents, were investigated.

**Materials and Methods**

Healthy, adult female albino mice (*Mus musculus*) of Swiss strain, weighing about 35-45 gm, were housed in animal cages under standard conditions, maintained on standard diet, and given water *ad libitum*.

The animals were divided into five groups. All animals except the controls were subjected to NaF at a dose of 10 mg/kg body weight/day, selected on the basis of the previous work on rodents (2,3) as well as the LD₅₀ value of fluoride which is 51.6 mg F⁻/kg body weight in female mice (14).

The experimental protocol was as follows:

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment and dosage</th>
<th>No of animals</th>
<th>Duration (days)</th>
<th>Day of autopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>15</td>
<td>-</td>
<td>Sacrificed along with NaF treated</td>
</tr>
<tr>
<td>II</td>
<td>NaF treated (10 mg/kg body weight)</td>
<td>15</td>
<td>30</td>
<td>31st</td>
</tr>
<tr>
<td>III</td>
<td>NaF treatment as in group II, withdrawal after 30 days and from day 31st administration of AA (25 mg/animal/day) for another 30 days</td>
<td>15</td>
<td>30</td>
<td>61st</td>
</tr>
<tr>
<td>IV</td>
<td>NaF treatment as in group II, withdrawal after 30 days and from day 31st administration of Ca^{++} (25 mg/animal/day) for another 30 days</td>
<td>15</td>
<td>30</td>
<td>61st</td>
</tr>
<tr>
<td>V</td>
<td>NaF treatment as in group II, withdrawal after 30 days and from day 31st administration of AA + Ca^{++} (dosages same as in Groups III and IV)</td>
<td>15</td>
<td>30</td>
<td>61st</td>
</tr>
</tbody>
</table>

At the end of each treatment, the animals were sacrificed by cervical dislocation and the liver, muscle (gastrocnemius and pectoralis), small intestine and kidney were dissected out, blotted free of blood and utilized for the following biochemical estimations:
Protein: The protein levels in liver, muscle and small intestine of control and treated mice were determined by the Biuret method (15) and expressed as mg protein/100 mg fresh tissue weight. Protein present in the tissue homogenate reacts with Biuret reagent to give a bluish-violet colour which can be measured spectro-photometrically at 540 nm.

Phosphorylase: Phosphorylase activity in liver and gastrocnemius muscle of control and treated mice was assayed by the method of Fiske and Subbarow (16) as modified by Cori, Cori and Green (17).

The phosphorylase enzyme catalyses the substrate (glucose-1-phosphate) used under standard assay conditions which brings about the liberation of inorganic phosphorus. This inorganic phosphorus reduces the molybdic acid to phosphomolybdate in the presence of reducing agent, 1:2:4 aminonaphthol sulphonic acid (ANSA) which gives a blue coloured solution. The intensity of the blue colour is directly proportional to the amount of inorganic phosphorus released. The enzyme activity was expressed as mg phosphorus released/100 mg fresh tissue weight/15 minutes.

Glycogen: Glycogen content in tissue was estimated by the method of Seifter et al (18). Glycogen is precipitated and converted into glucose which gives a green colour on boiling with anthrone reagent. The intensity of the green colour could be measured on % transmittance scale. The levels were expressed as µg glycogen/100 mg fresh tissue weight.

Succinate dehydrogenase: The succinate dehydrogenase activity in the pectoralis muscle of control and treated animals was assayed by the modified tetrazolium reduction reaction method (19) using INT [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium] as an electron acceptor. The electrons released by the action of the enzyme from the substrate, sodium succinate, are accepted by the electron acceptor (INT) which is reduced to a red coloured formazan. The resultant formazan was extracted in ethyl acetate and the colour intensity was measured on a 103 colorimeter at 420 nm. The activity was expressed as µg formazan formed/15 minutes/100 mg fresh tissue weight.

Na⁺, K⁺, and Ca⁺⁺ levels by flame photometry: The Na⁺ and K⁺ levels in the kidney and Ca⁺⁺ levels in the muscle were estimated by using the Systronics flame photometer, digital type 125, according to the method of Dean (20). The solutions under analysis were sprayed as a fine mist into a nonluminous flame which becomes coloured according to the characteristic emission of the metal. A very narrow band of wavelength corresponding to the element being analysed was selected by a light filter and allowed to fall on a photometer which is a measure of the element obtained on the digital display. The levels were expressed as ppm.

Cholesterol: Cholesterol in the ventricle of control and treated animals was estimated by the method of Pearson et al (21). Cholesterol present in the homogenate reacts with acetic anhydride in the presence of sulphuric acid to give a brownish green coloured complex which can be measured colorimetrically. The concentration of cholesterol was expressed as mg/100 mg fresh tissue weight.
Results

Protein: The protein levels in liver, muscle, and small intestine of mice decreased by NaF treatment (group II). The decrease was significant in liver (p<0.001) and gastrocnemius muscle and pectoralis muscle (p<0.001), respectively. Significant recovery was obtained (p<0.01) in each tissue when animals were administered ascorbic acid as compared to calcium (group III and IV), respectively. On the other hand, administration of ascorbic acid and calcium in combination as in group V showed a synergistic effect, and recovery was almost complete as levels were almost same as in control (Table 1).

Glycogen: A significant (p<0.001) accumulation in glycogen concentration occurred in both liver and gastrocnemius muscle after NaF treatment. A significant (p<0.001) recovery was obtained in glycogen levels after ascorbic acid treatment as compared to calcium administration (groups III and IV), respectively. The recovery was most pronounced in group V in which animals were fed ascorbic acid + calcium (Table 2).

Phosphorylase: A decrease in phosphorylase activity in liver and muscle in group II animals (NaF treated) was observed. The decrease in muscle phosphorylase in NaF treated animals was more significant (p<0.001) than in liver phosphorylase (p<0.01). The activity of phosphorylase was recovered by AA and calcium treatments (groups III and IV respectively) (p<0.01). The combined treatment of AA and calcium resulted in more significant recovery rather than AA and calcium alone in the activity of phosphorylase (liver p<0.01; muscle p<0.001) (Table 3).

Succinate dehydrogenase (SDH): SDH activity in pectoralis muscle of NaF treated mice decreased significantly (p<0.001) as compared to control. Administration of AA alone caused greater recovery than calcium administration. However, combined treatment with AA + calcium resulted in a synergistic effect for the recovery of SDH activity (Table 4).

Na\(^+\), K\(^+\) and Ca\(^{++}\) levels: Sodium and potassium levels in kidney decreased after NaF treatment whereas calcium levels in the present study were enhanced in muscle as compared to control (p<0.001). However, sodium and potassium levels of animals fed with AA were almost as similar to control as those animals fed with calcium. But combined treatment of AA along with calcium caused much better recovery than individual treatments (p<0.001) (Table 5).

Cholesterol: Cholesterol in ventricle was not significantly affected, but a significant decrease (p<0.001) occurred in its level in group IV which were treated with calcium during the recovery period (Table 6).

Discussion

The aim of the study was to investigate the effects of fluoride toxicity and its reversal, if any, through ascorbic acid and/or calcium administration on some soft tissues of female mice, Mus musculus of Swiss strain, in the light of earlier data.

The results revealed decreases in protein levels which were attributed to impairment of polypeptide chain inhibition (22), weak incorporation of amino acids into proteins, and abnormal accumulation (23) or possibly inhibition (24) of DNA synthesis. Irrespective of high (50 mg NaF/kg body weight) or low (10 mg NaF/kg
### TABLE 1. Levels of protein (mg protein/100 mg fresh tissue weight) in liver, muscle (gastrocnemius and pectoralis) and small intestine of control and treated groups

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>GROUPS</th>
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<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
<td>IV</td>
<td>V</td>
</tr>
<tr>
<td>Liver</td>
<td>26.48±0.18</td>
<td>22.20±0.45</td>
<td>25.05±0.79</td>
<td>23.15±0.76</td>
<td>26.10±0.61</td>
</tr>
<tr>
<td>Gastrocnemius muscle</td>
<td>28.76±0.83</td>
<td>17.71±0.62</td>
<td>22.47±1.29</td>
<td>20.85±0.58</td>
<td>28.21±0.73</td>
</tr>
<tr>
<td>Pectoralis muscle</td>
<td>32.30±3.14</td>
<td>19.36±0.88</td>
<td>28.26±0.39</td>
<td>25.43±0.43</td>
<td>30.80±0.37</td>
</tr>
<tr>
<td>Small intestine</td>
<td>16.90±0.49</td>
<td>15.08±0.17</td>
<td>16.60±0.41</td>
<td>14.70±0.92</td>
<td>16.52±0.61</td>
</tr>
</tbody>
</table>

### TABLE 2. Showing concentration of glycogen (µg/100mg fresh tissue weight) in liver and gastrocnemius muscle of control and treated groups (I - V)

<table>
<thead>
<tr>
<th>TISSUE</th>
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<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
<td>IV</td>
<td>V</td>
</tr>
<tr>
<td>Liver</td>
<td>1332.46±12.32</td>
<td>1735.91±20.51</td>
<td>1540.81±30.25</td>
<td>1613.33±46.47</td>
<td>1382.49±29.74</td>
</tr>
<tr>
<td>Muscle</td>
<td>901.67±33.17</td>
<td>1255.64±22.11</td>
<td>1052.87±22.29</td>
<td>1170.13±32.25</td>
<td>919.01±28.07</td>
</tr>
</tbody>
</table>

### TABLE 3. Showing activity of phosphorylase (mg phosphorus released/100 mg fresh tissue weight/15 min) in liver and gastrocnemius muscle of control and treated groups (I-V)

<table>
<thead>
<tr>
<th>TISSUE</th>
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<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
<td>IV</td>
<td>V</td>
</tr>
<tr>
<td>Liver</td>
<td>26.49±0.92</td>
<td>20.52±0.81</td>
<td>25.77±0.82</td>
<td>22.45±1.51</td>
<td>26.68±0.96</td>
</tr>
<tr>
<td>Muscle</td>
<td>44.18±0.96</td>
<td>17.42±0.52</td>
<td>30.05±1.11</td>
<td>26.96±1.77</td>
<td>41.05±2.53</td>
</tr>
</tbody>
</table>

### TABLE 4. Showing activity of SDH (µg formazan/100 mg fresh tissue weight/30 min) in pectoralis muscle in control and treated groups (I - V)

<table>
<thead>
<tr>
<th>TISSUE</th>
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<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
<td>IV</td>
<td>V</td>
</tr>
<tr>
<td>Pectoralis muscle</td>
<td>223.62±4.69</td>
<td>129.00±2.15</td>
<td>184.96±4.20</td>
<td>169.13±2.90</td>
<td>189.76±2.12</td>
</tr>
</tbody>
</table>

### TABLE 5. Showing levels of Na⁺, K⁺ from kidney and Ca++ in muscle (ppm) of control and treated groups (I-V)

<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>ORGANS</th>
<th>GROUPS</th>
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<tr>
<td></td>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
<td>IV</td>
<td>V</td>
<td></td>
</tr>
<tr>
<td>Na⁺</td>
<td>Kidney</td>
<td>4.95±0.75</td>
<td>2.33±0.19</td>
<td>3.06±0.26</td>
<td>3.15±0.45</td>
<td>3.66±0.18</td>
<td></td>
</tr>
<tr>
<td>K⁺</td>
<td>Kidney</td>
<td>3.45±0.25</td>
<td>2.50±0.10</td>
<td>3.00±0.20</td>
<td>2.80±0.55</td>
<td>3.20±0.15</td>
<td></td>
</tr>
<tr>
<td>Ca++</td>
<td>Muscle</td>
<td>3.20±0.10</td>
<td>4.70±0.17</td>
<td>3.80±0.30</td>
<td>4.05±0.35</td>
<td>3.50±0.10</td>
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</tr>
</tbody>
</table>
TABLE 6. Showing concentration of cholesterol (mg cholesterol/100 mg fresh tissue weight) in ventricle in control and treated groups (I - V)

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>GROUPS</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ventricle</td>
<td></td>
<td>0.71±0.09</td>
<td>0.87±0.08</td>
<td>0.64±0.05</td>
<td>0.42±0.03</td>
<td>0.63±0.05</td>
</tr>
</tbody>
</table>

TABLES 1-6.
For all the above Tables: Values are mean ± S.E.

Group I Untreated control.
Group II NaF treated (30 days).
Group III NaF treatment as in Group II, withdrawal after 30 days and administration of ascorbic acid from day 31st for another 30 days.
Group IV NaF treatment as in Group II, withdrawal after 30 days and administration of calcium from day 31st for another 30 days.
Group V Combined treatment as in Group III and IV.

body weight) doses of NaF administration, collagen biosynthesis in NaF treated animals was reduced as compared to control (25), affecting the contractile properties of muscle. The results obtained in this study also revealed that the concentrations of protein in muscle (gastrocnemius and pectoralis), liver, and intestine were reduced after NaF treatment. The muscle protein was more significantly affected than liver. A similar decrease in the protein levels in various reproductive tissues as well as in liver and muscle of fluoride intoxicated experimental animal models (male) has also been reported (2,3,5,7-9,26). This was attributed to the decline in protein synthesis and its metabolism. Induction of some “stress proteins” have also been found in testis and epididymis of rat (27).

Dramatic changes occurred in carbohydrate metabolism after fluoride ingestion. Significant accumulation of glycogen in liver might be due to inhibition of glycolysis either by enolase mediated inhibition (28) or by decrease in isocitrate dehydrogenase. Hence accumulation of citrate occurs which is a negative modulator of phosphofructokinase (29). Alterations in catecholamine levels could also be responsible for the accumulation of glycogen and disturbed carbohydrate metabolism (2,3). Another factor causing accumulation of glycogen might be the inhibition of phosphorylase activity. In the present study, phosphorylase activity showed a significant decrease in muscle and a moderate decline in liver. These results concur with data earlier reported from our laboratory (2).

Ingestion of fluoride produces a specific metabolic alteration in rapidly growing cells by decreasing the cellular ATP and to a lower level than other metabolic inhibitors. Secondly, the extracellular calcium may be lost due to the formation of insoluble salts of calcium fluoride, calcium thus becoming unavailable for phosphorylase activation. The fluoride induced effects could also be due to internal injury to the cell membrane, resulting in receptors failing to receive hormone-mediated signals. In another study of fluoride toxicity from our laboratory a significant decrease in inner-membrane phospholipids (phosphatidyl serine, phos-
phatidyl ethanolamine, and phosphatidyl inositol) levels were obtained in testis and cauda epididymis after NaF treatment. This would affect the activity of membrane-bound enzymes like Na+K+-ATPase by disturbing membrane fluidity and its integrity. Since the phosphatidyl inositol is involved in hormone receptor interaction, the androgen target organs would be affected in fluoride treated animals (27).

An oral dose of NaF (10 mg/kg body weight) caused a significant decrease in the activity of muscle SDH. Chinoy et al (26) have also reported inhibitory action of fluoride on muscle SDH. As SDH is known to be a mitochondrial enzyme involved in oxidative metabolism in the muscle, it is likely that the structure of mitochondria of muscle fibres might be altered by NaF, resulting in a change in the enzyme and thereby the contractile properties of muscle. Further ultrastructural studies in this direction are needed.

In the present study, the calcium levels were enhanced in pectoralis muscle, which might be due to transport of extracellular Ca++ to the intracellular region due to elevated catecholamine levels in the muscle. This may create an ionic gradient across the sarcolemma, thus hampering the overall muscle contraction. Calcium also inhibits the enzyme, phosphodiesterase, thus elevating C-AMP levels and disturbing Ca++/C-AMP interaction in regulating mechanisms of muscle contraction (26).

According to McIvor (30), fluorosis is known to produce a marked K+ efflux from intact cells. Clearance of F− decreases as damage to the kidney increases (31). The fall in Na+ and K+ levels in the kidney and their subsequent rise in the urine was reported earlier (2, 3) and could be attributed to change in electrolyte balance in the intercellular and intracellular fluids. Suketa and Terui (32) also reported disturbances in Na+ and K+ levels in urine and serum of fluoride-intoxicated rats. Recently, Das and Susheela (33) reported that the fluorotic human population and NaF treated animals suffer from adrenal hypofunction. The loss of electrolytes brings about a decrease in body weight due to loss of water along with salts. Similar data have been obtained in fluoride-treated male rats, mice and fluorotic human populations (2,9).

Saralakumari (34) observed unaltered serum cholesterol levels in rats supplemented with sodium fluoride in drinking water for two months. Ectopic calcification of aorta in rabbits chronically treated with sodium fluoride is also known (35). On the other hand, in testis and ovary of sodium fluoride treated rats and rabbits, as well as in serum of fluorotic individuals, it has been reported (2-5) that, at least in the initial stages of fluoride toxicity, the animals or individuals do not suffer from marked alterations in cholesterol metabolism nor steroidogenesis in the gonads. Chronic treatment, however, causes alteration in testicular steroidogenesis and Leydig cell functions (11). In the present study, cholesterol levels in the ventricle were not significantly affected after 30 days of treatment.

All the above alterations in the various parameters were transient, and 30 days of oral treatment with AA and calcium along with NaF feeding reversed the toxic effects almost to control levels. AA administration was more potent than calcium administration, but the combination of AA and calcium caused almost complete recovery, suggesting a synergistic effect. Cholesterol levels in the ventricle
decreased within the recovery period after administration of calcium, suggesting restoration of cardiac lipid metabolism. The combined use of ascorbic acid and calcium as therapeutic agents, to help ameliorate fluoride toxicity, concurs with our finding that the combination reversed induced fluoride toxicity. The almost complete recovery appears to be due to their active participation in several metabolic processes, growth, and in overcoming stress conditions (2,3,36).

It is known that vitamin C and calcium deficiency, especially under fluoride toxicity, poor nutrition, and hard labour, aggravate endemic fluorosis (37,38). Data obtained from dietary surveys suggested that inadequate ascorbic acid and calcium were related to the severity of fluorosis (39). That calcium and vitamin C are necessary for amelioration of fluoride toxicity has been reported (7,10,26).

The present study makes clear that calcium and ascorbic acid have a significant role in overcoming fluoride toxicity, and have a synergistic effect on the recovery from NaF induced alterations in female mice, in line with earlier studies on males from our laboratory. Vitamin C, due to its active antioxidant as well as detoxification properties, is a promising and potent agent in suppressing fluoride toxicity. Similarly, calcium is known to form insoluble complexes with fluoride, and reduce its absorption and thereby its action. In addition, both these agents are involved in various metabolic processes. The synergistic effect of ascorbic acid and calcium might be due to inhibition of phosphodiesterase (which is a known inhibitor of C-AMP) and resulting augmentation of C-AMP levels, which is involved in activation of several kinases. We believe high priority should be given to the combined use of these two agents for fluoride endemic populations, at least for the children. However, monitoring of doses is necessary because high concentrations of these agents, particularly calcium, can be harmful.

References

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