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Fluoride ingestion in excess and its effect on organic and certain inorganic constituents of soft tissues

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Introduction: Although fluorosis was regarded in the past as a disease affecting only bone [1] and teeth [2], recent studies have provided ample evidence to suggest that chronic fluoride intoxication causes pathological manifestations in various soft tissues and organs [3–6]. Gross and radiological examination of patients suffering from fluorosis have revealed calcification of ligaments, tendons and blood vessles [7–9].

Our recent study provided ultrastructural evidence for the presence of calcified loci in the aorta of rabbits treated with fluoride [10]. However, to date, the effect of fluoride on chemical constituents of soft tissues has not been reported. In view of the fact that pathological calcification is accompanied by changes in certain inorganic constituents [11, 12] we established the present study to elucidate the changes in certain relevant biochemical parameters of soft tissues of rabbits after fluoride ingestion in excess.

Materials and methods: Normal healthy rabbits weighing 500–700 g were housed individually in the Central Animal Facility of the All India Institute of Medical Sciences. Animals were given a standard animal diet (Lipton India Ltd) and municipal water containing fluoride less than 0.5 ppm.

The animals were assigned to two groups and kept under identical laboratory conditions. Animals in one group were fed 10 mg NaF/kg body weight daily (Glaxo Laboratory (India) Ltd, Bombay, India) through the intragastric route. Control animals were pair-fed but did not receive daily fluoride.

After 24 months of NaF treatment the animals were anaesthetised using ether. Ligament, tendon and the aorta were dissected out and wet weight was estimated. Tissues were then defatted in an ether:acetone mixture (1:1 v/v), dried in acetone and the dry weight estimated. The tissues were ashed in a muffle furnace at 550°C for 24 h. The ash was used for the estimation of various inorganic constituents.

For the estimation of organic, inorganic and water content of tissues, all chemicals were procured from Glaxo Laboratories and from E. Merck (India), Ltd. Delhi, India.

Total water, organic and inorganic contents of the tissues were determined as follows:

Water content = Wet weight − dry weight

Organic content = Dry weight − ash weight

Inorganic content = Ash weight

Fluoride content was estimated using an ION 85 ion analyser (Radiometer, Copenhagen, Denmark) according to the method of Singer and Armstrong [13]. A known amount of ash was dissolved in 1.5 mL of 0.25 N NaOH. Out of this, 0.5 mL of the solution was pipetted into a test tube and the total volume was made up to 5 mL by addition of sodium acetate buffer *p*H 5.2.

Ash obtained from a known amount of dry defatted tissue was dissolved in 1 mL of 1N nitric acid at 80°C for 2 h. After appropriate dilution calcium and magnesium contents were estimated using Atomic Absorption Spectrophotometer (GBC 902) [14].

Phosphorous content was estimated by the method of Fiske and SubbaRow [15] using potassium dihydrogen phosphate as standard. Ash obtained from a known amount of dry defatted tissue was dissolved in 0.5 mL of 72% perchloric acid, followed by addition of 0.5 mL H_2SO_4 and 0.5 mL of 2.5% ammonium molybdate solution. The above solution was then treated with 0.1 mL of reducing reagent (mixture of 0.2 g of 1-amino-2naphthol-4-sulfonic acid + 1.2 g of sodium bisulfite + 1.2 g of sodium sulfite). The absorbance was measured after 20 min at 660 nm using Spectronic 2000 Spectrophotometer.

Pyrophosphate in the soft tissues was estimated according to the method of Putnins and Yamada [16]. Dry defatted tissues were ashed at 550°C, ash was dissolved in 0.1 N HCl and 0.42 N sodium acetate buffer was added. To samples containing 0–20 nmoles of PPi, 50 µL of molybdate reagent (2.5% ammonium molybdate in 5 N H_2SO_4) and 50 µL of thiol reagent (0.5 M 2-mercaptoethanol were added, followed immediately by 20 µL of Eikonogen (1 g pack of Ekinogen contains 0.25 g of sodium sulfite and 14.65 g of sodium sulfite) in a total volume of 0.5 mL. Absorbance was read after 10 min at 580 nm using Spectronic 2000 Spectrophotometer. The reading provides information on pyrophosphate content as well as phosphate content.

To determine phosphate content, additional samples were treated as above but without the thiol reagent. Absorbance was read after 10 min at 580 nm. Pyrophosphate content was calculated by subtracting the concentration of phosphate from pyrophosphate + phosphate content.

Results are expressed as means ± SD. For statistical analysis Student's *t*-test was used and p values < 0.05 were considered significant.

Results and discussion: From the results reported in Tables 1a, b and c, it is evident that prolonged ingestion of fluoride resulted in its accumulation in the soft tissues *viz.* ligament, tendon and the aorta. However, the amount of fluoride deposited in the different tissues varied, suggesting that different tissues have different affinity for fluoride. The maximum increase in fluoride content was observed in the aorta followed by ligament and tendon in that order. The accumulation of extraordinary amounts of fluoride in the aorta of patients suffering from flurosis has been reported by Geever *et al.* [17] and Call *et al.* [18].

Total organic content was significantly lower in ligament and the aorta and this could be due to the decrease in protein content of the tissue. According to Kathpalia and Susheela [19], the protein content of the soft tissues decreases on fluoride ingestion. Another organic constitutent of the soft tissues, glycosminoglycan (GAG), has also been reported to be decreased in fluoride-treated animals [20].

The increase in the total inorganic content in ligament and the aorta of treated animals could be due to deposition of the mineral in these tissues. The enhanced calcium content and Ca/P ratio observed in the present investigation are suggestive of the deposition of calcium salts following fluoride

Table 1a: Effect of fluoride toxicity on chemical constituents of rabbit ligament (means ± SD: n = 6).

	Organic content (%)	Water (%)	Inorganic content (%)	Fluoride (ppm)	Calcium (μm/100 mg)	Phosphorus (μM/100 mg)	Ca/P	Magnesium (μm/100 mg)	Pyrophosphate (mg/gddt†)
Control	35.31 ± 3.64	65.08 ± 4.31	1.03 ± 0.48	10.42 ± 2.86	1.05 ± 0.30	1.14 ± 0.36	0.88 ± 0.30	3.04 ± 0.91	0.64 ± 0.26
24 Months NaF^- treated	29.58 ± 2.16*	64.12 ± 2.61	5.46 ± 0.74***	43.21 ± 9.60**	1.89 ± 0.76*	1.47 ± 0.22	1.24 ± 0.15*	5.86 ± 0.78*	0.11 ± 0.16**

*As compared with control, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. † Indicate gram dry defatted tissue*

Table 1b: Effect of fluoride toxicity on chemical constituents of rabbit tendon (means ± SD: n = 6).

	Organic content (%)	Water (%)	Inorganic content (%)	Fluoride (ppm)	Calcium (μm/100 mg)	Phosphorus (μM/100 mg)	Ca/P	Magnesium (μM/100mg)	Pyrophosphate (mg/gddt†)
Control	31.25 ± 6.12	66.32 ± 5.44	1.99 ± 0.97	14.86 ± 5.48	0.64 ± 0.25	1.00 ± 0.24	0.68 ± 0.24	4.16 ± 1.67	0.49 ± 0.13
24 months NaF^- treated	32.25 ± 5.20	65.85 ± 4.70	2.08 ± 0.92	28.11 ± 6.53*	0.73 ± 0.15	1.28 ± 0.45	0.65 ± 0.36	3.69 ± 1.51	0.17 ± 0.09*

** As compared with control, $p < 0.05$. † Indicate gram dry defatted tissue.*

Table 1c: Effect of fluoride toxicity on chemical constituents of rabbit aorta (means ± SD: n = 6).

	Organic content (%)	Water (%)	Inorganic content (%)	Fluoride (ppm)	Calcium (μm/100 mg)	Phosphorus (μm/100 mg)	Ca/P	Magnesium (μM/100mg)	Pyrophosphate (mg/gddt†)
Control	27.04 ± 4.31	69.91 ± 8.24	3.41 ± 0.74	397.14 ± 29.06	2.17 ± 0.22	2.36 ± 0.23	0.94 ± 0.18	6.54 ± 1.21	1.04 ± 0.18
24 months NaF^- treated	21.63 ± 2.63*	71.24 ± 5.48	6.81 ± 1.08**	862.58 ± 98.0***	3.55 ± 0.35*	2.40 ± 0.22	1.50 ± 0.08*	9.52 ± 1.60*	0.21 ± 0.14***

** As compared with control, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. † indicate gram dry defatted tissue.*

administration. However, it appears that all the calcium salts deposited may not be in the form of hydroxyapatite. Certain other calcium salts such as octacalcium phosphate, dicalcium phosphate dehydrate or amorphous calcium phosphate may be present. This can be explained by the fact that the above-mentioned slats have lower Ca/P ratios, ranging from 1 to 1.5 than that of crystalline apatite *i.e.* 1.607 [21]. In the present study the Ca/P ratio was 1.24 ± 0.15 in ligament and 1.5 ± 0.08 in the aorta of 24 months fluoride treated animals respectively.

Calcium salts other than hydroxyapatite possibly represent the initial mineral phase of intermediate precursors that precede the formation of apatite. The reduced level of PPi shown in the present investigation is likely to provide a favourable meileu for mineralisation; PPi is an inhibitor of calcification [22, 23].

The increased magnesium content in ligament and the aorta in fluoride-treated animals could be one of the factors responsible for the deposition of calcium salts other than apatite, as a high concentration of Mg^{2+} is known to favour the formation of amorphous calcium phosphate [24] and prevent the transformation of amorphous calcium phosphate to apatite [25]. Second, the higher fluoride content in the aorta and ligament as compared to tendon might also be due to the presence of higher Mg^{2+} concentration in these tissues following fluoride ingestion.

Increased magnesium in soft tissues facilitates the retention of fluoride, possibly due to the formation of MgF_2 [25]. The increased Mg^{2+} content is probably related to the secondary hyperparathyroidism, reported to occur in fluorosis [26, 27].

Among the tissues investigated, the changes in the inorganic constituents of ligament and the aorta are highly significant as compared to tendon. This suggests that although fluoride is deposited in all the three tissues, ligament and the aorta are more severely affected. Radiologically, calcification of ligament is detected earlier than in any other tissue [1, 7] and is used as a parameter for the diagnosis of the disease.

In conclusion, we have shown that fluoride accumulates in the ligament, tendon and the aorta following fluoride administration and alters the chemical composition of the tissues, resulting in the deposition of calcium salts in ligament and the aorta.

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