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## Aortic Calcification in Chronic Fluoride Poisoning: Biochemical and Electronmicroscopic Evidence

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Fluoride is known to cause ectopic calcification. The biochemical mechanism(s) involved in the initiation of calcification is not understood and the accompanying ultrastructural changes remain to be elucidated. Therefore, certain relevant parameters have been investigated in the aorta of rabbits administered fluoride, 10 mg NaF/kg body wt, every 24 hr for 17 and 24 months. The significant findings are: (i) degeneration of smooth muscle fibers in the tunica media of the aorta, (ii) presence of electron-dense granules in the mitochondria and on the inner surface of the plasma membrane of smooth muscle cells, (iii) presence of matrix vesicles with electron-dense deposits, (iv) enhanced calcium content and the Ca/P ratio, and (v) increased total glycosaminoglycan (GAG) content with reduced dermatan sulfate. The presence of electron-dense granules in the mitochondria, on the plasma membrane and matrix vesicles is suggestive of the process of calcification. The enhanced calcium content as well as the Ca/P ratio supports the view that the aorta is undergoing mineralization. The total GAG is enhanced, possibly due to an increase in the content of GAGs other than isomers of chondroitin. The observation that conveys an important message is that the dermatan sulfate normally known to exist in high concentrations in soft tissues begins to decrease as the process of calcification sets in. This perhaps would hold true and may serve as an index in the process of ectopic calcification. © 1990 Academic Press, Inc.

### INTRODUCTION

Chronic fluoride poisoning is known to cause a variety of pathological manifestations in soft tissues in both animal and man. Skeletal muscle degeneration (Kaul and Susheela, 1974), red blood cell abnormalities (Jain and Susheela, 1986), adrenal gland dysfunction (Rao and Susheela, 1979), and connective tissue abnormalities (Susheela and Mukherjee, 1981; Susheela and Sharma, 1982) besides a wide range of structural and functional damage to the gastrointestinal system (Susheela and Taposh, 1988) due to chronic fluoride toxicity are just a few observations to record. This report focuses on the pathological manifestations of the aorta of rabbit due to chronic fluoride poisoning.

The aorta is known to accumulate the highest amount of fluoride compared to other soft tissues. The highest fluoride level in soft tissues and organs recorded to date is 8400 ppm in the aorta of a person who had lived in a fluoridated area (Greever *et al.*, 1971). Numerous investigators have reported the calcification of arteries in association with skeletal fluorosis in "natural high fluoride" areas (Kumar and Harper, 1963; Bacon, 1964; Waldbott, 1978; Mohamedally and Wix, 1983). According to Chawla *et al.* (1964) and Soriano (1968), vascular calcification takes the form of a ladder type of pattern (Monckeberg type). Contrary to this, Huo (1981) observed dense parallel lines like those caused by atherosclerosis. The above-mentioned reports on blood vessels are based on gross and radiographic examination. However, biochemical evidences of calcium ion deposition and accompanying ultrastructural changes are still unknown. This communication is

therefore aimed at revealing the biochemical and ultrastructural events leading to aortic calcification in rabbits after prolonged ingestion of fluoride. This report also provides observations on the molecular mechanism(s) involved in ectopic calcification.

## MATERIALS AND METHODS

*Animal model.* Eleven normal rabbits weighing 500–700 g each were fed 10 mg NaF/kg body wt daily through an intragastric route. Twelve age-matched controls were pair fed but deprived of the daily administration of fluoride. After 17 months of NaF administration, eight animals were sacrificed and the aortas were dissected out. Similarly, after 24 months of fluoride administration, the remaining three animals were sacrificed and the aortas were dissected out. Control animals were also sacrificed along with 17- and 24-month-treated animals and the aortas were dissected out for the various investigations.

*Electronmicroscopic studies.* One- to two-millimeter-thick pieces of the aorta were fixed for 4 hr in Karnovsky's fixative at 4°C and washed briefly in 0.1 M cacodylate buffer. Dehydration was carried out in graded concentrations of acetone, cleared in toluene, and the tissues were embedded in CY212 Araldite embedding medium using accelerator and hardener. Sections were cut and stained with uranyl acetate and lead citrate and examined under a transmission electron microscope (Philips EM-300) and electron micrographs were taken at 60 kV.

*Fluoride content.* The ionic fluoride contents of serum and aorta were estimated with the help of a fluoride ion-specific electrode according to the method adopted by Singer and Armstrong (1968). A fluoride ion analyzer, Radiometer (Copenhagen) Model ION85, was used.

*Calcium estimation.* Tissues were dried in ether-acetone mixture (1:1) digested in 1 N nitric acid at 110°C for overnight. After dilution, calcium content was estimated using an atomic absorption spectrophotometer Model GBC 902 (Parker, 1963).

*Phosphorus estimation.* The phosphorus content of dried samples was estimated by the method of Fiske and Subba Row (1925), using potassium dihydrogen phosphate as standard.

*Extraction of glycosaminoglycans and estimation of uronic acid content.* Fat free aorta was cut into 1-mm-thick pieces, digested using a digestion mixture containing 0.05 M cysteine hydrochloride and 0.2 M EDTA. An aqueous solution of papain was also added. The GAG released after digestion was diluted with 4 vol of 1% cetylpyridinium chloride (CPC) and left overnight at room temperature. The precipitate formed was collected by centrifugation for 30 min. The precipitate, CPC-GAG complex, was dissolved in a small volume of 60% aqueous propanol containing 4% CPC. To the clear solution, 3 vol of ethanol was added followed by addition of a saturated solution of sodium acetate in water. The precipitate obtained, sodium salt of GAG, was recovered by centrifugation at 4000 rpm for 30 min and dried in the air. The precipitate was dissolved in 250  $\mu$ l of 50 mM phosphate buffer, pH 7.2. The GAG thus extracted was used for the estimation of uronic acid and sulfated isomers of chondroitin.

The uronic acid content was estimated by adding 3 ml of tetraborate reagent (0.025 M sodium tetraborate in sulphuric acid to a known volume of precooled sample. The contents were mixed vigorously and heated for 10 min in a boiling

water bath. Then 0.2 ml of carbazole reagent (0.125% carbazole in absolute ethanol) was added to the samples and heated in a boiling water bath for a further 15 min for color development. Absorbance was measured at 530 nm using a Spectronic 2000 spectrophotometer (Bitter and Muir, 1962).

*Estimation of sulfated isomers of chondroitin.* The relative quantities of isomeric chondroitin sulfates, viz., chondroitin sulfates A and C and dermatan sulfate, were estimated from the extracted GAG using the method of Saito *et al.* (1968). The reaction mixture contained a volume of 75  $\mu$ l of which 15  $\mu$ l was enriched Tris buffer and contained samples (3 to 20  $\mu$ l of the purified GAG) and various combinations of enzymes as indicated below:

- Test tube 1: 0.01 unit of chondroitinase ABC  
+0.01 unit of chondro-6-sulfatase
- Test tube 2: 0.06 unit of chondroitinase AC  
+0.01 unit of chondro-4-sulfatase  
+0.01 unit of chondro-6-sulfatase
- Test tube 3: 0.02 unit of chondroitinase ABC  
+0.01 unit of chondro-4-sulfatase  
+0.01 unit of chondro-6-sulfatase

The reagent blank consisted of all the reagents except the enzyme solutions. After incubation at 37°C for 30 min, 75  $\mu$ l of tetraborate buffer was added to each mixture. The tubes were heated in a boiling water bath for 7 min and cooled in tap water. Six hundred microliters of glacial acetic acid and 250  $\mu$ l of the *p*-diaminobenzaldehyde solution were added and mixed well and the tubes were cooled. The absorbance was measured against the blank at 385 nm using a Spectronic 2000 spectrophotometer, and the results were calculated.

## RESULTS AND DISCUSSION

From the results reported in Table I, it is evident that the fluoride contents of the serum and the aorta are significantly raised compared to normal, suggesting that prolonged administration of fluoride to rabbits results in the accumulation of fluoride in the aorta.

The tunica media of the aorta consists of alternating layers of smooth muscle fibers and elastic laminae, and ultrastructurally, the changes due to chronic fluoride poisoning are prominent in the tunica media. The sarcoplasm of the smooth muscle fibers shows degenerative changes as it reveals vacuolization/fatty infiltration (Fig. 1). Intense electron-dense granules are observed within the mitochondria of smooth muscle fibers and on the inner surface of the plasma membrane (Fig. 2). Intense electron-dense granules possibly represent the deposition

TABLE I  
Fluoride Content (ppm) in the Serum and Aorta of 17-Month NaF-Treated and Control Rabbits

Tissue	Control (mean $\pm$ SD)	NaF-treated (mean $\pm$ SD)	<i>P</i> value
Serum	0.097 $\pm$ 0.02 (8)	0.837 $\pm$ 0.11 (8)	<0.001
Aorta	491.25 $\pm$ 28.04 (4)	692.75 $\pm$ 102.66 (4)	<0.05

*Note.* SD, standard deviation. The numbers in parentheses indicate the number of experiments. In aorta, in each of the experiments, material was pooled from two animals.



FIG. 1. Rabbit aorta showing alternating layers of smooth muscle cells (SMC) and elastic laminae (E) in the tunica media. Smooth muscle cells showing vacuolization and fatty infiltration can be seen ( $\times 5900$ ).

of calcium salts. It is reported that mitochondria even in soft tissues have the ability to actively accumulate  $\text{Ca}^{2+}$  (Greenawalt *et al.*, 1964; Harris, 1977; Carafoli, 1981). Soft tissues normally do not contain calcium phosphate due to the presence of a highly efficient barrier, i.e., plasma membrane. In the present study, it has been observed that fluoride disrupts the integrity of the plasma membrane of smooth muscle cells (Fig. 2). The structure of the mitochondria is also adversely affected as seen from the electron micrograph. Kaul *et al.* (1974) have reported that due to fluoride toxicity, mitochondrial structure and function are disrupted in skeletal muscle.

Yet another characteristic feature observed in the present study is that because of fluoride toxicity, matrix vesicles, i.e., round or ovoid membrane bodies in the tunica media, appear in large numbers. Matrix vesicles containing amorphous or granular material of variable electron density are observed (Fig. 3). The vesicles filled with electron-dense inclusions show an irregular contour (Fig. 4). Formation of matrix vesicles appears to be the way calcium gets out of the cells and they behave as the initial loci of calcification in the extracellular matrix. These vesicles, besides promoting calcification in tissues such as cartilage, bone, and dentine (Ali *et al.*, 1970; Eisenman and Glick, 1972; Benhur & Orney, 1984; Landis, 1986) also appear to serve as the initial loci for calcification in several pathological conditions, viz., osteosarcoma and chondrosarcoma (Lee *et al.*, 1974; Schajowicz *et al.*, 1974).

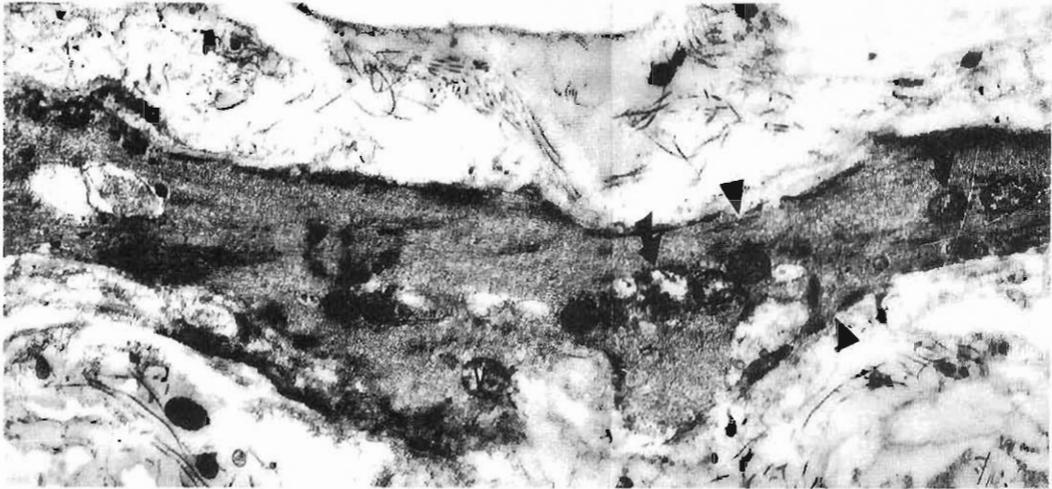


FIG. 2. A montage of a smooth muscle fiber after 17 months of exposure to fluoride, showing electron-dense granules in mitochondria (arrows) and on the inner surface of the plasma membrane. Note the presence of the matrix vesicles. (∨) and the disrupted plasma membrane (▼). ( $\times 14,490$ ).

Thus from the present study, it appears that fluoride leads to cellular toxicity, disrupting the smooth muscle cell membrane and structure of mitochondria, and thereby causing the accumulation of  $\text{Ca}^{2+}$  in the mitochondria which is later released and binds to the plasma membrane, leading to the formation of matrix



FIG. 3. Matrix vesicles of various sizes (arrows) are seen in the tunica media. Their contents vary in appearance and electron opacity ( $\times 23,000$ ).

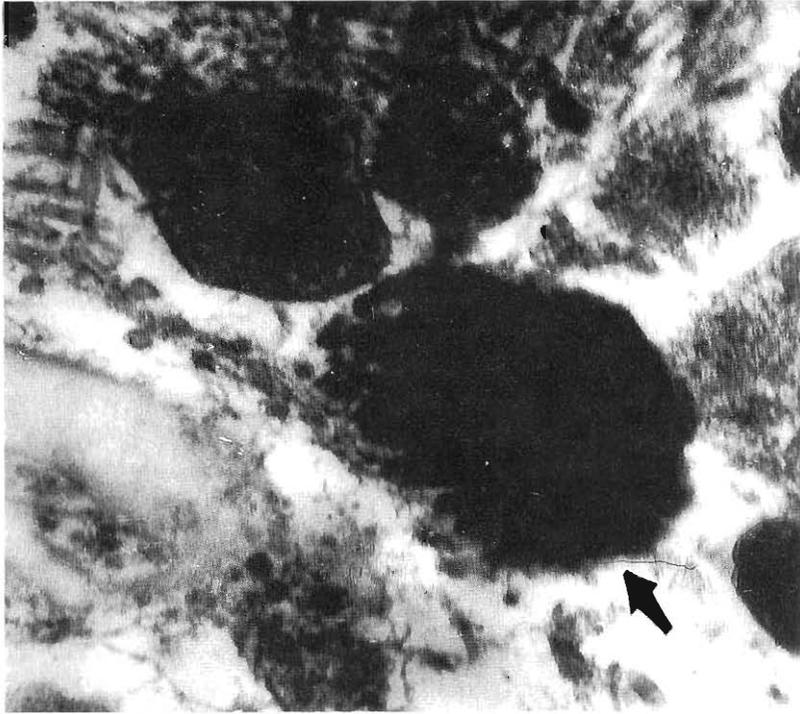


FIG. 4. A matrix vesicle with irregular contour is seen due to the presence of a greater number of crystal-like inclusions (arrow) ( $\times 54,000$ ).

vesicles. The matrix vesicles are subsequently extruded into extracellular matrix. In epiphyseal chondrocytes, there is evidence to suggest that the release of mitochondrial  $\text{Ca}^{2+}$  is coupled with the appearance of the calcium-loaded vesicles in the extracellular matrix (Martin and Matthews, 1970; Brighton and Hunt, 1978).

Calcification is brought about by a whole series of complex physiochemical and biochemical processes in which a certain concentration of calcium and phosphate is attained prior to the precipitation of calcium salts. The data obtained from this study of calcium, phosphorus, and Ca/P ratio of control and fluorosed rabbits are shown in Table II. It is evident that calcium levels are significantly increased in the aorta of animals administered fluoride, while phosphorus contents remain unaltered. Ca/P ratio is enhanced in treated animals. The results are significant at  $P < 0.01$ . These findings support the observation that the electron-dense deposits in the mitochondria and on the inner surface of the plasma membrane and matrix vesicles are loaded with calcium, although all the calcium deposited may not be in the form of hydroxyapatite and it may be present as amorphous calcium phosphate. This can be explained by the fact that amorphous calcium phosphate has a Ca/P ratio lower than that of crystalline apatite (West, 1971) and in the present study the mean value of the Ca/P ratio in treated animals is  $1.50 \pm 0.08$ . The Ca/P ratio for hydroxyapatite is 1.67 (Yu, 1974). Besides the matrix vesicles and the Ca/P ratio, the glycosaminoglycans are of great interest in physiological as well as pathological calcification.

As glycosaminoglycan content is known to increase with age, age-matched control animals have been used in the present study. The total glycosaminoglycan

TABLE II  
Calcium and Phosphorus Contents and Ca/P Ratio in Aorta of 24-Month NaF-Treated and Control Rabbits

	Control (n = 3) (mean ± SD)	NaF treated (n = 3) (mean ± SD)	P value
Calcium ( $\mu\text{M}/100\text{ mg}$ )	2.17 ± 0.22	3.35 ± 0.35	<0.01
Phosphorus ( $\mu\text{M}/100\text{ mg}$ )	2.36 ± 0.23	2.40 ± 0.22	N.S.
Ca/P ratio	0.94 ± 0.18	1.50 ± 0.08	<0.01

Note. SD, standard deviation; N.S., statistically not significant. Numbers in parentheses indicate the number of animals in that group.

content (Table III) shows a significant increase in GAG in the aorta of fluorosed animals compared to the aorta from control animals. A concomitant increase in the sulfated isomers of chondroitin has not been recorded. However, one of the isomers, dermatan sulfate, is significantly decreased in the aorta of fluoride-treated animals. This indicates that the increase in total GAG content might be due to an increase in GAGs other than the sulfated isomers.

Chondroitin sulfates including dermatan sulfate are mainly synthesized by smooth muscle cells, whereas endothelial cells synthesize predominantly hyaluronic acid and heparan sulfate (Radhakrishnamurthy *et al.*, 1985). It appears that the significant reduction in dermatan sulfate may partly be due to degenerative changes in the smooth muscle cells of the tunica media which have been observed in the present study. Decreased levels of dermatan sulfate in ligament and tendon after excess ingestion of fluoride has been reported earlier by Susheela *et al.* (1985). Dermatan sulfate normally occurs in large quantities in soft tissues (Meyer, 1956) which are not destined to be calcified, whereas calcified tissues normally contain negligible amounts of dermatan sulfate. From this study, it emerges that the reduced dermatan sulfate content in the aorta due to fluoride poisoning provides a milieu/environment for the deposition of calcium salts leading to the calcification of the aorta.

In conclusion, this report provides evidence suggesting that because of chronic fluoride poisoning the aorta is loaded with fluoride and calcium, although all the deposited calcium may not be in the form of hydroxyapatite. Ultrastructurally, the smooth muscle cells in the tunica media reveal electron-dense deposits in the

TABLE III  
Glycosaminoglycan Contents of the Aortas from Control and 17-Month NaF-Treated Rabbits

	Control (n = 4) (mean ± SD)	NaF treated (n = 4) (mean ± SD)	P value
Total glycosaminoglycan (mg/gddt)	2.55 ± 0.16	4.71 ± 0.64	<0.01
Chondroitin sulfate A (mg/gddt) <sup>a</sup>	0.59 ± 0.19	0.71 ± 0.16	N.S.
Dermatan sulfate (mg/gddt) <sup>a</sup>	0.36 ± 0.06	0.13 ± 0.07	<0.001
Chondroitin sulfate C (mg/gddt) <sup>a</sup>	0.86 ± 0.12	0.95 ± 0.17	N.S.

Note. SD, standard deviation; Numbers in parentheses indicate the number of experiments. For each experiment, material was pooled from two animals.

<sup>a</sup> Concentration is calculated by taking their average molecular weight.

mitochondria and on the inner surface of the plasma membrane. A large number of matrix vesicles filled with amorphous inclusions which are considered the initial loci of calcification in the tunica media of the aorta are also observed. This study also provides evidence on the reduction in dermatan sulfate content, indicating that soft tissue is prone to calcification.

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