

Nail and Bone Surface as Biomarkers for Acute Fluoride Exposure in Rats

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Abstract

When acute exposure to fluoride is thought to be the cause of death, confirmation often depends on the analysis of some body fluid or tissue. The aim of this study was to evaluate the use of nails and the periosteal surface of bone as indicators of acute exposure to fluoride. Six groups of rats were given a single oral dose of fluoride (50 mg/kg body weight), while the control group was given deionized water. The rats were killed at 2, 4, 8, 16, 24, and 48 h after fluoride administration. Plasma and nails (the proximal halves) were collected and analyzed for fluoride with an ion-specific electrode after hexamethyldisiloxane-facilitated diffusion. A circular area of the femur (4.52 mm²) was etched with 0.5M HCl for 15 s, and, after the addition of a buffer, the solution was analyzed with an ion-specific electrode. Peak plasma concentration occurred at 2 h, followed by progressively declining concentrations. Peak nail fluoride concentrations occurred at 8 h. The mean nail concentrations at 8, 16, and 24 h were significantly higher than that of the control group. Bone surface concentrations were significantly higher than that of the control group at 4 h and thereafter. Thus, the proximal portion of nails and bone surface are suitable biomarkers for acute fluoride exposure in rats.

Introduction

Fluoride-induced fatalities have been caused by acute exposures to cleaning agents, hydrofluoric acid, dental products, or accidental over-fluoridation of water supplies in hemodialysis centers (1–13). When fluoride is suspected as the cause of death, confirmation often depends on the analysis of plasma (8) or urine (7). Death may occur within a few hours (5) or the victim may survive for several days (3). Because absorbed fluoride is taken up by bone and excreted rapidly by the kidneys (14), however, the fluoride concentrations in plasma and urine may approximate physiological levels before these fluids can be collected, which would impair its detection in these fluids. The uptake of fluoride from the extracellular fluids by bone in the short-term occurs largely on the periosteal and endosteal

surfaces where it is firmly bound. Thus, the analysis of bone surface fluoride has the potential of being a more reliable biomarker for acute fluoride exposures.

Nail clippings have been used as biomarkers for chronic exposures to fluoride (15–22) but not for acute exposures. Whitford et al. (21) reported that a supplemental fluoride intake of 3.0 mg per day (which doubled the intake with the diet) for 30 days resulted in increased fluoride concentrations in fingernail clippings but only after a lag time of 3.5 months. They proposed that fluoride enters fingernails via the matrix (proximal or growth portion) and not through the nail bed. Based on this, we hypothesized that analysis of the growth portion could be used as an additional postmortem test in cases of suspected fluoride poisoning.

The aim of the present study was to determine whether the fluoride concentrations in nails and the periosteal surface of the femur are elevated after an acute dose of fluoride. If so, then these tissues could serve as additional postmortem biomarkers in cases of suspected fluoride poisoning.

Materials and Methods

Male, 70-day-old Wistar rats ($n = 105$) weighing 230–290 g were obtained from the Central Vivarium of the Bauru Dental School (Bauru, Brazil). After having been denied food but allowed free access to deionized water for 12 h, the rats were randomly assigned to six groups (15 rats/group) that differed with respect to the time they were killed after having received a single fluoride dose (50 mg F/kg body weight) by gastrogavage. These groups were killed at 2, 4, 8, 16, 24, and 48 h after the administration of the fluoride doses. The dosing solution was prepared by adding sodium fluoride to deionized water. The control group was given deionized water and killed 2 h later.

While the rats were anesthetized with diethyl ether, a blood sample was collected from the heart into a lightly heparinized syringe for the determination of plasma fluoride. The nails from all four feet were removed with a hemostat, and the prox-

imal halves (considering the length of the nails) were immediately cleaned and prepared for fluoride analysis. The right femur was removed from each rat, cleaned of soft tissue with gauze, and dried for 24 h at 90°C.

Fluoride analysis

Nails and plasma

The nails were cleaned briefly with deionized water using a piece of cloth and then sonicated in deionized water for 10 min, dried at 60°C for 2 h, and weighed. The weight of the nail collections ranged from 5.48 to 27.48 mg. Nail fluoride concentrations were determined after overnight hexamethyldisiloxane (HMDS)-facilitated diffusion [Taves (23); modified by Whitford (24) using the ion-specific electrode (model 9409, Orion Research, Cambridge, MA) and a miniature calomel electrode (#13-620-79, Accumet, Hudson, MA) both coupled to a potentiometer (model EA 940, Orion Research)].

Deionized water (2.0 mL) was placed in the bottom of a nonwetable diffusion dish (1007, Falcon, Franklin Lakes, NJ), along with the nails or plasma. The trapping solution, 50 μ L 0.05M NaOH, was placed in three drops on the inside of the lid of the dish. The periphery of the lid was ringed with Vaseline and sealed to the bottom of the dish. Two milliliters 3.0M H₂SO₄ saturated with HMDS was added to the bottom through a small hole previously burned into the lid with a soldering iron. The hole was immediately sealed with Vaseline. During the diffusion process, which continued overnight at room temperature, the solutions were swirled at 45 rpm on a rotary shaker. The next day, the lid was removed and inverted, and the trapping solution was buffered to pH 5 with 25 μ L 0.20M acetic acid. The final volume was then diluted to 75 μ L by the addition of deionized water using a fixed-volume pipettor. The fluoride and reference electrodes were placed in contact with the solution with gentle agitation until a stable mV reading was obtained. Fluoride standards (0.095, 0.190, 0.475, 0.950, 1.900, and 3.800 μ g fluoride for nails and 0.019, 0.095, 0.190, 0.950, 1.900, and 4.750 μ g fluoride for plasma) were prepared in triplicate and diffused in the same manner as the nail and plasma samples. In addition, nondiffused standards were prepared with the same reagents and in the same proportions as those used to prepare the diffused standards and samples. The nondiffused standards were diluted to have exactly the same fluoride concentrations as the diffused standards. Comparison of the mV readings demonstrated that the fluoride in the diffused standards had been completely trapped and analyzed (recovery > 99%). The mV potentials were converted to μ g F using a standard curve with a coefficient correlation of $r \geq 0.99$.

Bone

The femur was sectioned transversely to obtain a 1.0-cm specimen taken from the

mid-diaphysis. The cut edges were sealed with dental wax. A circular hole (2.4-mm diameter) was punched in adhesive tape, which was applied firmly to the center of the bone specimen. The remaining surfaces of the bone were painted with acid-resistant nail varnish so that only a 4.52 mm² surface area was exposed. The bone was then placed in a plastic test tube containing 0.50 mL 0.50M HCl, and, after 15 s of being constantly agitated, the acid was buffered to pH 5 by the addition of 0.50 mL total ionic strength adjustment buffer (TISAB). This solution was analyzed along with standards containing 0.10–1.6 μ g F/mL. The mV readings were converted to μ g F using a standard curve with a coefficient correlation of 0.99. The weight of bone mineral removed was obtained by the colorimetric analysis of inorganic phosphorus (25) in the acid assuming that its concentration in dry bone is 13.5%. The fluoride concentration on the bone surface is expressed as μ g F/g bone (ppm).

Statistical analysis

The data are expressed as mean \pm standard deviation (SD). Plasma and nail fluoride concentrations as a function of time after the fluoride doses were tested for statistically significant differences by analysis of variance (ANOVA) and Tukey's post hoc test for individual comparisons. For the bone data, the Kruskal-Wallis and Dunn's multiple comparison tests were used. An alpha value of 0.05 was selected as the indicator for statistically significant differences.

Results

Table I shows the fluoride concentrations of the plasma (μ g/mL), nails, and femurs (μ g/g) as a function of time after fluoride administration. The peak plasma concentration occurred in the 2-h sample, followed by progressively declining concentrations. ANOVA revealed a significant difference among the plasma concentrations ($F = 54.9$, $p < 0.0001$). Tukey's test showed significant differences in plasma concentrations between the control group and groups that were killed at 2, 4, and 8 h ($p < 0.001$).

Table I. Mean (\pm SD; $n = 15$) fluoride Concentration in Rat Plasma, Nails, and Femur After Fluoride Administration (50 mg/kg body weight)

Experimental Groups	Plasma (μ g/mL)	Nail Growth Portion (μ g/g)	Femur Surface (μ g/g)
Control	0.025 (\pm 0.008)	35.5 (\pm 19.8)	573 (\pm 325)
2 h	3.349 (\pm 1.280)*	28.2 (\pm 16.6)	1241 (\pm 373)
4 h	2.551 (\pm 0.836)*	42.0 (\pm 29.8)	1774 (\pm 712)*
8 h	1.645 (\pm 0.691)*	107.5 (\pm 57.1)*	1480 (\pm 255)*
16 h	0.395 (\pm 0.262)	76.0 (\pm 32.2) [†]	1450 (\pm 731) [†]
24 h	0.152 (\pm 0.093)	74.6 (\pm 21.8) [†]	2024 (\pm 662)*
48 h	0.070 (\pm 0.027)	41.2 (\pm 37.8)	1542 (\pm 466)*

* $p < 0.001$ compared to control.
[†] $p < 0.05$ compared to control.

The peak nail fluoride concentration occurred at 8 h after the doses were administered and 6 h after the peak concentration was reached in plasma. As happened in plasma, the subsequent nail concentrations were lower than the peak concentration. The ANOVA showed a significant difference in nail fluoride concentrations among the groups ($F = 6.92$, $p < 0.0001$). Tukey's test revealed a significant difference between the nail concentrations of the control group and the groups killed at 8 ($p < 0.001$), 16, and 24 h ($p < 0.05$). The 48-h concentration, however, was not significantly different from the control concentration.

The fluoride concentrations in the surface mineral of the femurs also showed significant differences ($p < 0.0001$). Unlike the plasma and nail concentrations, which reached a peak and then declined, no distinct peak concentration was apparent in bone. Although, as determined by ANOVA, the 2-h concentration was not significantly higher than the control value; it was more than twice as high. The subsequent concentrations were all higher than the control concentration ($p < 0.05$).

Discussion

Whitford et al. (26) reported that the oral LD_{50} values for fluoride were 85.5 and 126.3 mg F/kg body weight when given to rats as an aqueous solution of sodium fluoride in two separate studies. An intermediate value, 98 mg F/kg, was reported by Gruninger et al. (27) in their study with rats. In the present study, we chose to use a large but nonlethal dose of fluoride because we wanted to determine tissue fluoride concentrations for up to 48 h. Although many of our rats were relatively immobile and lethargic, especially during the first day of observation, none died.

The results of our study indicate that increased fluoride concentrations are detectable in the proximal half of nails following the acute administration of fluoride. As noted in the Introduction section, there is evidence that fluoride enters human fingernails via the proximal (growth) end and not through the nail bed and that nail fluoride concentrations are determined by plasma concentrations (21). Based on this, we hypothesized that the fluoride concentrations in the proximal half of rat nails would be elevated shortly after the acute administration of a large dose of fluoride.

Our results corroborate this hypothesis. Increased nail concentrations were found in the nails collected 8, 16, and 24 h following the fluoride doses. It is not clear why the 48-h nail fluoride concentration was lower than those at 8, 16, and 24 h. It may have been due to the continuous growth of the nails and the low plasma fluoride concentrations that occurred during the second day of the study. In a previous study, increased nail concentrations were not detected when rats were killed 2 h after fluoride administration (28), nor were they found at that time in the present study. It appears that detectable increases in nail fluoride concentrations lag behind plasma concentrations by approximately 6 or 8 h in the rat.

Our results also indicate that increased fluoride concentrations in the surface mineral of the femur occur following the

acute administration of fluoride. The initial uptake of fluoride by bone occurs by iso- and heteroionic exchange on the surface of bone crystallites (29), and the rate is greatest at the endosteal and periosteal surfaces. It is for this reason that we chose to sample the first few microns of mineral on the periosteal surface and compare the bone concentrations of the fluoride-treated groups with those of the control group. This approach was reasonable because all of the rats were born and raised in the same environment, were the same age, and had been fed the same diet, thus the bone fluoride concentrations of all groups were certainly similar before the administration of fluoride.

This homogeneity is not necessarily true among humans in defined populations or regions. It is known that, within any given age range, human bone fluoride concentrations are determined by past fluoride intake (30) and, within any given range of fluoride intake, the concentrations increase with age (31,32). Because of these variables, the use of surface bone fluoride concentrations per se may not be the best indicators of acute exposure to lethal amounts of fluoride. Further, the concentration of fluoride throughout the thickness of rat and human femurs is not uniform, but, instead, it is highest at the periosteal and endosteal surfaces and lower in the interior regions (33). It is likely that ratio of surface-to-interior bone fluoride concentrations will be more instructive in this regard. The ratios for bone from persons not exposed to large, acute doses of fluoride are the subject of an ongoing study in our laboratory. The purpose of this work was to obtain normative data against which such ratios in cases of suspected fluoride fatalities can be compared.

Finally, in our 48-h study, we found that bone surface fluoride concentrations increased sooner than those in the nails and that they did not decline as they did in nails. This suggests that fluoride accumulation on the periosteal surface of the femur is faster and less likely to change as a function of time. For these reasons, we believe that this region of the femur is probably a better biomarker to confirm exposure to large doses of fluoride.

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References

1. A.A. Watson, J.S. Oliver, and J.W. Thorpe. Accidental death due to inhalation of hydrofluoric acid. *Med. Sci. Law* **13**: 277-279 (1973).
2. L.E. Church. Fluorides: use with caution. *MD. Dent. Assoc.* **19**: 106 (1976).

3. M.N.G. Dukes. Fluoride. In *Side Effects of Drugs. Annual 4*, M.N.G. Dukes, Ed. Excerpta Medica, Oxford, England, 1980, pp 354.
4. P.B. Tepperman. Fatality due to acute systemic fluoride poisoning following a hydrofluoric acid skin burn. *J. Occup. Med.* **22**: 691–692 (1980).
5. H.G. Eichler, K. Lenz, M. Fuhrmann, and K. Hruby. Accidental ingestion of NaF tablets by children: report of a poison control center and one case. *Int. J. Clin. Pharmacol. Ther. Toxicol.* **20**: 334–338 (1982).
6. S.M. Menchel and W.A. Dunn. Hydrofluoric acid poisoning. *Am. J. Forensic Med. Pathol.* **5**: 45–48 (1984).
7. J. Wiese and E. Klug. Poisoning with a wood preservative. *Beitr. Gerichtl. Med.* **47**: 103–106 (1989).
8. B.B. Randall and B.J. Fraser. Peanut butter and fatal fluoride poisoning. A case of mistaken identity. *Am. J. Forensic Med. Pathol.* **15**: 40–43 (1994).
9. B. Raikhlin-Eisenkraft, I. Nutenko, D. Kniznik, J. Merzel, and A. Lev. Death from fluoro-silicate in floor polish. *Harefuah* **126**: 258–259 (1994).
10. P.M. Arnow, L.A. Bland, S. Garcia-Houchins, S. Fridkin, and S.K. Fellner. An outbreak of fatal fluoride intoxication in a long-term hemodialysis unit. *Ann. Intern. Med.* **121**: 339–334 (1994).
11. R.O. Bost and A. Springfield. Fatal hydrofluoric acid ingestion: a suicide case report. *J. Anal. Toxicol.* **19**: 535–536 (1995).
12. L. Muriale, E. Lee, J. Genovese, and S. Trend. Fatality due to acute fluoride poisoning following dermal contact with hydrofluoric acid in a palynology laboratory. *Ann. Occup. Hyg.* **40**: 705–710 (1996).
13. M.E. Mullins, C.R. Warden, and D.W. Barnum. Pediatric death and fluoride-containing wheel cleaner. *Ann. Emerg. Med.* **31**: 524–525 (1998).
14. G.M. Whitford. Renal handling of fluoride. In *The Metabolism and Toxicity of Fluoride*, 2nd ed., H.M. Myers, Ed. Karger, Basel, Switzerland, 1996, pp 46–58.
15. C.W. Schmidt and W. Leuschke. Fluoride content in fingernails taken from persons with and without chronic fluoride exposure. *Fluoride* **23**: 79–82 (1982).
16. R.G. Schamschula, E. Sugar, P.S.H. Un, K. Toth, B.E. Barmes, and B.L. Adkins. Physiological indicators of fluoride exposure and utilization: an epidemiological study. *Comm. Dent. Oral Epidemiol.* **13**: 104–107 (1985).
17. Z. Machoy. Effects of environment upon fluoride content in nails in children. *Fluoride* **22**: 169–173 (1989).
18. W. Czarnowski and J. Krechniak. Fluoride in urine, hair and nails of phosphate fertilizer workers. *Br. J. Ind. Med.* **47**: 349–351 (1990).
19. V.L. Spate, J.S. Morris, C.K. Baskett, M.M. Mason, C.L. Reams, D.J. Hunter, and W.C. Willett. Determination of fluoride in human nails via cyclic neutral activation analysis. *J. Radioanalyt. Nucl. Chem.* **179**: 27–33 (1994).
20. D. Feskanich, W. Owusu, D.J. Hunter, W.C. Willett, A. Ascherio, D. Spiegelman, S. Morris, V.L. Spate, and G. Colditz. Use of toenail fluoride levels as an indicator for the risk of hip and forearm fractures in women. *Epidemiology* **9**: 412–416 (1998).
21. G.M. Whitford, F.C. Sampaio, P. Arneberg, and F.R. Von der Fehr. Fingernail fluoride: a method for monitoring fluoride exposure. *Caries Res.* **33**: 462–467 (1999).
22. M.A.R. Buzalaf, R. Fukushima, J.M. Granjeiro, and J.A. Cury. Correlation between plasma and nail fluoride concentrations in rats given different levels of fluoride in water. *Fluoride* **9**: 185–192 (2002).
23. D.R. Taves. Determination of submicromolar concentrations of fluoride in biological samples. *Talanta* **15**: 1015–1023 (1968).
24. G.M. Whitford. Some characteristics of fluoride analysis with the electrode. In *The Metabolism and Toxicity of Fluoride*, 2nd ed., H.M. Myers, Ed. Karger, Basel, Switzerland, 1996, pp 303–333.
25. C.H. Fiske and Y. Subarrow. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66**: 375–400 (1925).
26. G.M. Whitford, N.L. Birdsong-Whitford, and C. Finidori. Acute oral toxicity of sodium fluoride and monofluorophosphate separately or in combination in rats. *Caries Res.* **24**: 121–126 (1990).
27. S.E. Gruninger, R. Clayton, S.B. Chang, and C. Siew. Acute oral toxicity of dentifrice fluorides in rats and mice. *J. Dent. Res.* **67(Spec Issue)**: 334 (1988) (Abs 1769).
28. M.A.R. Buzalaf, M.M. Linardi, J.M. Granjeiro, and J.A. Cury. Nail and bone surface as indicators of acute fluoride exposure. *J. Dent. Res. Suppl.* **81(Spec)**: A376 (2002) (Abs 3023).
29. W.F. Neuman and M.W. Neuman. *The Chemical Dynamics of Bone Mineral*, University of Chicago Press, Chicago, IL, 1958, pp 75–100.
30. J. Charen, D.R. Taves, J.W. Stamm, and F.M. Parkins. Bone fluoride concentrations associated with fluoridated drinking water. *Calcif. Tissue Int.* **27**: 95–99 (1979).
31. F.M. Parkins, N. Tinanoff, M. Moutinho, M.B. Anstey, and M.H. Waziri. Relationship of human plasma fluoride and bone fluoride to age. *Calcif. Tiss. Res.* **16**: 335–338 (1974).
32. A. Richards, L. Mosekilde, and C.H. Sogaard. Normal age-related changes in fluoride content of vertebral trabecular bone—relation to bone quality. *Bone* **15**: 21–26 (1994).
33. N. Narita, K. Kato, H. Nakagaki, N. Ohno, Y. Kameyama, and J.A. Weatherell. Distribution of fluoride concentration in the rat's bone. *Calcif. Tissue Int.* **46**: 200–204 (1990).

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