CORRELATION BETWEEN PLASMA AND NAIL FLUORIDE CONCENTRATIONS IN RATS GIVEN DIFFERENT LEVELS OF FLUORIDE IN WATER

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SUMMARY: Changes in plasma and nail fluoride concentrations in rats given different concentrations of fluoride in drinking water were investigated. Six groups of weanling male Wistar rats were provided drinking water containing NaF at 0, 7, 14, 28, 56, or 100 ppm F^- for 42 days. The nails were clipped on days 21, 28, 35, and 42; plasma was collected on day 42. The nails and plasma were analyzed with a fluoride ion electrode following HMDS-facilitated diffusion. At the end of the study, a direct relationship was evident between the nail and plasma fluoride concentrations ($r = 0.67$, $p < 0.001$), indicating that nails can be used as indicators of plasma fluoride concentrations during chronic fluoride intake in rats.

Keywords: Biomarkers, Fluoride intake, Nail fluoride, Plasma fluoride, Rat nails.

INTRODUCTION

At a 1999 dental workshop in Bethesda, Maryland, USA,[1] the participants proposed an international research agenda on fluorides. One of the topics was the development of fluoride biomarkers that are easy to collect and analyze, in order to determine the body burden and to measure acute and chronic fluoride exposure in relation to bone fluoride levels and fluoride in plasma, ductal saliva, dentin, and fingernails.

The concentration of inorganic fluoride in plasma varies according to the level of intake and several physiological factors, especially kidney function.[2] In general the numerical value of the plasma concentration in μmol/L of healthy adults whose main source of fluoride is the diet is roughly equal to that in drinking water expressed in mg/L or ppm.[3] Many animal studies have examined the association between plasma fluoride concentrations and dental fluorosis.[4-6] However, similar studies in humans are difficult to conduct because of the inconvenience and expense of blood collection.

Nail sampling is simple and non-invasive, and there are many reports suggesting the use of nails as biomarkers for fluoride exposure in humans.[7-13] However, we are unaware of any animal studies correlating plasma and nail fluoride concentrations. In this work we used the rat as a model to determine the relationship between plasma and nail fluoride concentrations in animals exposed to different levels of fluoride in their drinking water.

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MATERIALS AND METHODS

Male Wistar rats were received as weanlings from the Central Vivarium of Bauru Dental School (University of São Paulo, Bauru-SP) and housed in plastic cages with six (control group) or eight (experimental groups) rats per cage. The animals had free access to a regular diet (Purina, 25.9 ppm total fluoride soluble in 1 M HCl) and deionized water. At 33 days of age (body weight around 70 g) the animals were assigned to six groups (16 per group, except for 18 in the control group) that differed according to the concentration of fluoride (as sodium fluoride) in the drinking water (Table 1). The water was placed in plastic dropper bottles, which assured minimal water spillage, and the experiment was conducted for 42 days (6 weeks).

Table 1. Mean (± sd) fluoride concentration in nails according to treatment group and time

Group ppm F in DW	Fluoride concentration in nails (μg/g ± sd) 3rd week	4th week	5th week	6th week
1: 0 (n=6)	2.85 ± 0.76^{a,A}	3.17 ± 1.28^{a,A}	2.77 ± 2.61^{a,B}	1.78 ± 0.73^{a,A}
2: 7 (n=8)	6.68 ± 6.12^{a,A}	4.06 ± 3.32^{a,A}	3.75 ± 1.38^{a,B}	4.81 ± 2.59^{a,A}
3: 14 (n=8)	8.52 ± 4.79^{a,A}	5.66 ± 3.17^{a,A}	3.96 ± 1.46^{a,B}	5.78 ± 2.59^{a,A}
4: 28 (n=8)	9.50 ± 4.81^{a,A}	5.47 ± 1.02^{a,A}	4.88 ± 2.81^{a,B}	6.81 ± 2.43^{a,A}
5: 56 (n=8)	13.01 ± 7.39^{b,A}	13.83 ± 2.15^{b,A}	8.53 ± 4.77^{b,B}	9.86 ± 3.82^{b,A}
6: 100 (n=8)	19.62 ± 10.21^{c,A}	25.92 ± 11.30^{c,A}	16.83 ± 6.96^{c,B}	15.69 ± 5.21^{c,A}

Means followed by different letters are statistically significant ($p < 0.05$). Lower case and upper case letters show, respectively, differences among groups and time.

Each week body weights were determined. Three weeks after initiation of fluoride treatment, the edges of the fore-feet and hind-hand nails were clipped. This procedure was repeated weekly for 3 additional weeks, totaling 4 clippings for each rat. Every 2 days water intake was measured. The total volume of water initially added was recorded, and after 2 days the volume that had not been consumed was measured.

At the end of the study, the rats were fatally anaesthetized with diethyl ether. A heart blood sample was collected into a lightly heparinized syringe for the determination of plasma fluoride.

Analytical Procedure: In groups 2-6, the nail clippings of two animals were pooled to get enough material to be analyzed (n = 8); in group 1 three animals were pooled (n = 6). Each nail clipping was cleaned briefly with deionized water using a piece of cloth and then sonicated in deionized water for 10 minutes, dried at 60 ± 5°C for 2 hr and weighed. The weights of the pooled samples ranged from 3.52 to 8.05 mg (± 0.01 mg).

The fluoride concentrations in the nail clippings were determined after overnight hexamethyldisiloxane (HMDS)-facilitated diffusion[14] as modified by Whitford[2] using a fluoride ion-specific electrode (Orion Research, Cambridge, Mass., USA, model 9409) and a miniature calomel reference electrode (Accumet, #13-620-79), both coupled to a potentiometer (Orion Research, model EA 940). During the diffusion process, which was conducted at room temperature, the solutions in the non-wettable Petri dishes (Falcon, No. 1007) were gently swirled on a rotatory shaker. Fluoride standards were prepared in triplicate and diffused in the same manner as the nail clippings. In addition, nondiffused fluoride standards were prepared with the same solutions (0.05 M NaOH, 0.20 M acetic acid, plus NaF) that were used to prepare the diffused standards and samples. The nondiffused standards were made up to have exactly the same fluoride concentrations as the diffused standards. Comparison of the millivolt readings demonstrated that the fluoride in the diffused standards had been completely trapped and analyzed. The fluoride concentration in the plasma was analyzed in the same way after sample preparation with previously heated HMDS-H_2SO_4 in order to remove CO_2. Plasma samples were pooled the same as the nails samples.

The same person (MARB), who was unaware of the animals from which the nail clippings were taken, made all the analysis.

Statistical Analysis: The data were tested for statistically significant differences by two-way ANOVA (criterion time repeated) and by Tukey's post hoc test. A significance level of 0.05 was selected a priori.

RESULTS

The initial and final mean body weights (70 and 237 g, respectively) did not differ significantly among the groups. There was a trend toward a small decline in water intake for the groups that received 56 and 100 ppm fluoride in the drinking water (data not shown).

Table 1 shows the mean fluoride concentration in nails (μg/g) during the experimental period. Three weeks after the rats began drinking fluoridated water, an increase in fluoride concentration in nails was observed, and this pattern persisted to the end of the experimental period. Statistical analysis showed a significant difference among groups ($F = 47.41$; $p < 0.0001$) and among experimental periods ($F = 4.47$; $p = 0.0053$). Considering the different groups, despite the dose-response effect observed, only groups 5 and 6 were statistically different from the others and from each other. Regarding the experimental periods, only the 5th week fluoride concentrations in nails were significantly lower with respect to the other periods.

Mean plasma fluoride concentrations (mg/L and μmol/L) at sacrifice (42 days after initiation of fluoride treatment) are given in Table 2. Only groups 5 and 6 were statistically different from the control.

At sacrifice, a positive correlation was found between plasma and nail fluoride concentrations ($r = 0.67$; $p < 0.001$).

Table 2. Mean (± sd) plasma fluoride after 42 days in relation to treatment groups

Group ppm F in DW	mg/L	μmol/L
1: 0 (n=6)	0.013 ± 0.001	0.68 ± 0.05
2: 7 (n=8)	0.037 ± 0.014	1.95 ± 0.07
3: 14 (n=8)	0.062 ± 0.007	3.26 ± 0.37
4: 28 (n=8)	0.068 ± 0.015	3.58 ± 0.79
5: 56 (n=8)	0.170 ± 0.023[a]	8.95 ± 1.21[a]
6: 100 (n=8)	0.220 ± 0.043[a]	11.58 ± 2.26[a]

Values without superscripts or with the same superscripts are not statistically different ($p < 0.05$).

DISCUSSION

A close approximation of total fluoride intake can be calculated from the fluoride content of food, water, therapeutic materials, dust and air, and from the associated individual ingestion and parenteral intake values. However, the generally accepted indicators of fluoride absorption–urine, plasma and ductal saliva–fall short of our requirements for two reasons: both indicate short-term fluoride absorption, and plasma fluoride determination involves the inconvenient collection of blood. The concentrations of fluoride in nails and hair appear to be proportional to intake over long periods of time. To the extent they are, their concentrations can reflect the average plasma fluoride concentration over time.[15]

Many animal studies have contributed to our knowledge of the relationship between plasma fluoride levels and dental fluorosis. A study conducted in rats by Angmar-Mannson *et al*[4] concluded that dental fluorosis was the result of occasional spikes in plasma fluoride concentrations that reached values of about 10 μmol/L. That such spikes could produce fluorosis was confirmed by a study in which rats received daily fluoride injections for one week.[5] Once- or twice-daily injections that caused peak concentrations of 5 μmol/L did not produce fluorosis. These findings suggested that the total daily dose was not the important variable, but instead it was the amount of fluoride given at one time and the resulting peak plasma fluoride concentrations. The latter study, however, also included two groups that received fluoride during the week by continuous infusion from mini-osmotic pumps implanted subcutaneously. This method of administration produced slightly

elevated but relatively constant plasma concentrations that averaged 3.3 and 4.7 μmol/L during the week. Dental fluorosis was present in some of the 3.3 μmol/L rats and all of the 4.7 μmol/L rats. Therefore high peak plasma fluoride concentrations were not necessary to produce disturbances in enamel mineralization. This conclusion was confirmed by a subsequent study in which rats were infused with fluoride for eight weeks.[6] Fluorosis was evident in all the rats of both groups even though their average plasma fluoride concentrations were only 1.5 and 3.1 μmol/L. Concentrations within this range occur in humans whose drinking water contains fluoride between about 2 and 4 ppm and whose enamel also exhibits some degree of fluorosis.

The risk of dental fluorosis is therefore directly related to the interaction of circulating fluoride and time, i.e., the area under the time-concentration curve. Thus dental fluorosis can result from a range of plasma fluoride concentrations provided that they are maintained for sufficiently long periods.[16] Despite the fact that the correlation between plasma fluoride concentrations and dental fluorosis is well established, blood collection is not an ideal procedure to be conducted in humans. An indicator of plasma fluoride levels over time that could permit non-invasive sampling would be very useful.

There are many reports regarding the use of nails as biomarkers for fluoride exposure in humans. Most of them have used complicated assay methods.[7-12,17] Different studies showed different ranges of fluoride concentrations in nails, but in all of them fluoride exposure correlated with fluoride in nails.

Whitford *et al* used a simpler and less expensive method: ion-specific electrode following HMDS-facilitated diffusion.[13] These authors also tested some variables in the analytical procedure and observed that results with ashing and not ashing the nails were not statistically different. Similarly, the surface areas of fingernails clippings exposed to the H_2SO_4-HMDS solution during the process of fluoride diffusion had only minor effects on the analytical results, indicating that the analysis of the intact nail clippings by electrode following overnight HMDS-facilitated diffusion quantitatively detects all fluoride in nails. Moreover, prolonged exposure to deionized water did not reduce the amount of fluoride in the nails. This is why the authors cleaned the human nails by brushing with deionized water. In our study we could not use a brush, because the clippings were too small. We therefore cleaned with deionized water using a piece of cloth and then sonicated the samples in deionized water for 10 min. Water analyzed after sonication contained negligible amounts of fluoride (data not shown).

All the studies cited above used human nails. Our intention using rat nails was to establish a good animal model to study the relationship between plasma and nail fluoride concentrations in animals given different doses of fluoride in the drinking water. The rat seems to be a good model, because a

positive correlation was observed, despite the fact that the differences were only significant when water containing 56 and 100 ppm fluoride was consumed. The lack of statistical significance with low fluoride concentrations in water may be attributed to the high fluoride concentration in the diet we used (25.9 ppm total fluoride soluble in 1 M HCl) and also to the great variability in fluoride concentration in the rat nails. This implies that when the intention is to use the rat model to analyze the effect of chronic ingestion of fluoride on nail fluoride concentration, it is better to use a semi-purified diet with a low-fluoride content (< 1 ppm).

We used high fluoride concentrations in the rats' water compared to the levels usually present in humans' drinking water because the rat clears fluoride more rapidly from its body fluids than does the human.[2] The fasting plasma concentration of a person whose water contains 2.0 mg/L fluoride would be about 2.0 μmol/L (0.038 ppm)[17] and these levels are known to produce disturbances in enamel mineralization. However, the rat requires water fluoride levels of 10.0 ppm to produce plasma fluoride levels of about 2.0 μmol/L and to have enamel lesions similar to those found in humans drinking water containing 2.0 ppm fluoride.[6]

We started clipping the nails only three weeks after starting exposing the rats to fluoridated water because we did not expect the dose-response effect to occur before this time lag. However, after three weeks the dose-response effect was already detectable and we do not know exactly when it could be initially detected. Whitford *et al* administered 3.0 mg of fluoride daily during 30 days to an individual and observed a time lag of 3.5 months before the nail fluoride concentrations increased.[13] These authors proposed that fluoride enters fingernails via the matrix (growth end) and not through the nail bed. This may be not true for rats if the time lag is shorter than three weeks, because the velocity of nail growth in rats is around 0.1 mm/day.[18] It would be interesting to make a study in which the nails were clipped periodically shortly after starting fluoridated water use to see exactly when nail fluoride concentrations are increased in rats.

The most important finding of this study was the presence of a positive, strong correlation between fluoride concentration in plasma and nails collected at sacrifice (fluoride concentration in plasma = 0.1565 + 0.1085 X fluoride concentration in nails at sacrifice). This means that nails can be used as indicators of plasma fluoride concentration in rats subjected to chronic intake of fluoride. The extent to which this information can be transferred to human nails needs further investigation, but many authors have found human nails to be good indicators of fluoride intake[7-11,13]. Thus, if fluoride intake is chronic, human nails may be good predictors of plasma fluoride concentration over time.

These findings are important because nails from babies and young children in the age of risk for dental fluorosis can be clipped and assayed for fluoride, in order to estimate plasma fluoride concentrations and fluoride intake. This simple, non-invasive sampling, if done early, would permit prediction of the risk of dental fluorosis. The maxillary central incisors, the most important teeth in terms of aesthetics, appear most at risk to fluorosis between age 15 and 24 months for males and between 21 and 30 months for females.[20] Thus, the periodic nail analysis would need to start by age 1-2 years to have any practical prophylactic value to prevent dental fluorosis of the early erupting permanent incisors.

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