

TOE NAILS AS A BIOMARKER OF CHRONIC FLUORIDE EXPOSURE SECONDARY TO HIGH WATER FLUORIDE CONTENT IN AREAS WITH ENDEMIC FLUOROSIS

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SUMMARY: There is a need to monitor the body burden of fluoride (F) in endemic areas without resorting to bone biopsy. For a biomarker of F exposure to be applicable in a large population, it should be easily collectible without objections from the donors and be an effective, a reliable, and a valid method of F estimation. The present study was designed as a population based observational cross-sectional study. The F content in toe nail clippings was determined by the dry ashing method in a total of 40 local residents residing in three F endemic areas of the Jaipur district of Rajasthan, India, with mean drinking water F levels of 4.1, 4.8, and 5.6 ppm. The F levels of serum and 24 hr urine were also estimated in the same population. The mean nail ash F levels were extremely high with a range of 82.38±6.89 – 103.92±16.89 ppm. Highly significant relationships were present between the water F levels and the F levels in toe nail ($p<0.001$), serum ($p<0.001$), and urine ($p<0.001$). Similarly, significant relationships were present between the toe nail F levels and the F levels in serum ($p<0.001$ and $p<0.01$) and urine ($p<0.001$ and $p<0.01$). The presence of F in toe nail indicates that F is obtained from the systemic circulation to the nail beds and deposited in the nails by either continuous incorporation or secondary concentration. Toe nail F is a useful biomarker for both subchronic and chronic F exposure.

Keywords: Biomarkers; Dry ashing; Fluoride; Fluorosis; Toe nail clippings.

INTRODUCTION

Chronic endemic fluorosis in humans secondary to high levels of drinking water fluoride (F) is widely prevalent in many states of India.¹⁻³ At present, the impact of exposure to environmental F can be estimated through analysis of several biological tissues (nails, hairs, bone, dentin, etc.) or fluids (blood serum and urine) with varying degrees of accuracy depending on the chronicity of exposure and other physiological variables.⁴⁻⁸ In a time perspective classification,⁹ biomarkers are divided into contemporary (plasma and urine), recent (nails and hair), and historical (bone and dentin) with the last two determining exposure to F in the medium and long term, respectively. In a community setting, an ideal biomarker should be easily retrievable in a non-invasive manner, with measured F values showing a temporal and dose response relationship with F intake. The method used for analyzing F concentration in any biological sample should be reliable, valid, easy to perform, and incur minimum expenditure.

F levels in serum and urine provide reliable estimations of changes due to acute exposure^{5,6,9,10} and indicate its utilization in the short-term but their value as a marker of chronic F exposure is less precise. Although F levels in fasting serum

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samples may be taken as a marker of total body load,^{6,7} the determination of a reliable fasting history and obtaining the required blood sample are difficult in an epidemiological study covering a large population. Levels of F in urine are affected by its intake in the previous few hours along with differences in age, glomerular filtration, urinary pH, and flow rate.^{7, 9, 11}

As more than 99% of the total body F is concentrated in calcified tissues,⁹ the level of chronic F exposure is best assessed from its concentration in bone and dentin. These tissues, however, are rarely collected as, intravital, their biopsy entails an operative procedure. Finger nails and toe nails have been used as biomarkers of acute, subchronic, and chronic exposure to F since they offer a simple and noninvasive bioassay method which is easily consented to by virtually all donors.^{5,8,10,12–17} The storage of the samples does not require any sophisticated methods and there is a minimal risk of decay.

Although bioindicators for fluoridated water and endemic hydrofluorosis have recently been reported,¹⁸ epidemiological studies using toe nail clippings as a biomarker of chronic F exposure in areas with endemic fluorosis are limited. Therefore, the present investigation was undertaken to ascertain the effectiveness and feasibility of using F concentration in toe nail clippings as a biomarker of chronic F toxicosis, the relationship between the F concentration in toe nail clippings and those in serum and urine, and the degree of individual differences in F concentration in different biological samples secondary to varying levels of drinking water F level using regression models.

MATERIALS AND METHODS

Study areas: The study was conducted in the villages (a) Mohanpura and Nanglia of Sanganer tehsil, (b) Jairajpura and Prahaladpura of Chaksu tehsil, and (c) Madhorajpura of Phagi tehsil of Jaipur district of Rajasthan, India with drinking water F levels of 4.1, 4.8, and 5.6 ppm, respectively (unpublished data, the Public Health and Engineering Department, Government of Rajasthan, Jaipur). Except for the drinking water, there were no other sources of F exposure in the villages.

Inclusion and exclusion criterion: Eligibility criterion included individuals older than 20 years of age, those constantly residing for more than fifteen years in the study areas where drinking water F level was greater than 4 ppm, and those willing to give their consent for the study. Exclusion criterion included exposure to any F source other than drinking water in last two weeks prior to collection of the sample, those on medication for any acute or chronic illness, and a history suggestive of acute or chronic involvement of the kidneys.

Study population and pre-enrollment evaluation: One hundred and fifty individuals out of the combined population of 1222 in the selected villages met the eligibility criterion. Prior to the final definition of the sample, the purpose of the study and the nature of the samples required were explained to the prospective participants and written consent was taken which reduced the sample size to 40. A detailed questionnaire regarding their demographic details, duration of F exposure, and other sources of F intoxication, if any, was completed.

Sample collection: A 25 mL venous blood sample was collected from each selected subject after overnight fasting in a plain plastic vial without any anticoagulant. A 24 hr urine sample, collected in a small clean polythene bag provided to the participant, was also retrieved at the same time. Toe nail clippings from both the feet were collected in plastic pouches and all samples were stored at 4°C.

Fluoride analysis: Whole blood was centrifuged at 3000 rpm for 10 min to separate the serum. 1mL of TISAB II was added to 10 mL of each serum and urine sample with pH held at 5–5.5.¹⁹ During the measurement, solutions were constantly mixed with a magnetic stirrup, and the temperature was kept constant at 25°C.

To quantitatively transfer the F within the nail clippings into a solution which can be placed in contact with the F electrode, the direct acid extraction method was used.¹⁶ For this, each nail clipping was cleaned with deionized water using an interdental brush to remove surface contamination and kept in a muffle furnace at 400°C for 12 hr. The resultant ash was weighted up to 5 mg and dissolved in 1 mL of 0.25 M HCl for 2 hr. The solution was then neutralized and buffered with addition of 1 mL TISAB II.¹⁹ The final volume of the solution was built to 10 mL with deionized water held at a pH 5–5.5.

F concentration in each of the prepared solutions was estimated with the help of a F ion specific electrode (THERMO-ORION 960900). Deionized water was used for all measurements. For calibration, three standard solutions of 100, 10, and 1 ppm F concentration were prepared by serial dilution. 1 mL of TISAB II was added to each 10 mL of standard solution and the instrument was calibrated. When calibrating, it was assumed that the added TISAB II had no effect on the standard concentration.

Ethical approval: The protocol for this study was approved from the Ethical Committee of SMS Medical College, Jaipur. All work was performed according to the ICMR guidelines, New Delhi, for human experimentation in biomedical research. Before the sample collection a written consent was obtained from each participant.

RESULTS AND DISCUSSION

The study group of 40 individuals, 23 men and 17 women, was further divided into three groups based on their mean drinking water F concentration of 4.1, 4.8, and 5.6 ppm (Table 1). As a preliminary analysis of the data did not reveal any significant effect of sex distribution with regard to any of the study parameters, the results are presented with both the sexes combined. The majority of the individuals were aged 41–60 yr.

The F concentration (ppm) found in serum, urine, and nail ash tended to increase with increased F exposure from using drinking water with higher F levels (Table 1). The Kruskal Wallis test of significance for multiple independent samples showed a statistically significant difference in serum, urine, and nail ash F concentrations between the three groups (Table 1). The variability within-group as

indicated by standard deviation is substantial for all three samples, but not excessive in the context of biological variations.

Table 1. Mean fluoride concentrations (ppm) in serum, urine and nail ash samples with different water fluoride (WF) levels. Values are mean±SD with the range in parentheses

Sample	WF 4.1 ppm (n=13)	WF 4.8 ppm (n=14)	WF 5.6 ppm (n=13)	Level of significance ^a
Serum	0.29±0.03 (0.22–0.34)	0.40±0.08 (0.29–0.61)	0.52±0.03 (0.34–0.91)	p<0.001
Urine	4.07±0.71 (2.8–5.1)	4.72±1.38 (3.4–7.8)	6.69±2.86 (3.90–14.0)	p=0.003
Nail ash	82.38±6.89 (67–94)	86.14±9.77 (71–106)	103.92±16.89 (79–131)	p=0.001

^aKruskal Wallis test of significance.

A significant positive linear correlation was found between the water F levels and the serum, urine, and nail ash F levels. A similar correlation was also found between the nail ash and the serum and urine F levels in both bivariate and partial correlation analysis. Further regression analysis estimated the effect of drinking water F in determining the level of F in each of the three samples studied (Table 2).

Table 2. Correlation between fluoride concentrations (ppm) in nails, serum, urine and water fluoride (WF) levels

Samples		Spearman's correlation coefficient (r)	Partial correlation coefficient ^a	Equation ^b
y	x			
Nails	Serum	0.689 (p<0.001)	0.417 (p<0.01)	y = 69.5x + 62.0
Nails	Urine	0.692 (p<0.001)	0.493 (p<0.01)	y = 4.52x + 67.0
Nails	WF	0.535 (p<0.001)		y = 14.59x + 19.9
Serum	WF	0.788 (p<0.001)		y = 0.15x – 0.3
Urine	WF	0.527 (p<0.001)		y = 1.77x – 3.4

^aAdjusted for water F level; ^bLinear regression model

In this study, the mean F concentration in the three samples differed significantly depending on water F levels and showed a strong positive linear correlation with it. The difference was significant although the variation in water F levels in between the three groups was not very high. Individual F levels are seen to overlap

between the three groups in all three biological samples. This indicates the presence of confounding variables other than water F level which might have accounted for these individual differences.

The F concentrations in the fasting serum samples were significantly higher than the normal serum F levels of 0.01–0.03 ppm (at water F levels of 1 ppm).⁷ It is worth noting that the F levels in the serum samples are lower than the associated drinking water F levels in all the three groups reflecting a steady state concentration achieved in the serum by exchange with the body F stores. Although the F levels in urine also reveal a similar trend, the observed day to day and individual variations in the urinary F levels²⁰ and the variables^{7,9,11} affecting them, make urinary F levels an imprecise tool to estimate the effect of environmental fluoride.

In the present investigation, the mean F content of toe nail clippings always exceeded that of water by a significant margin, perhaps due to a very long duration of F exposure in the study areas. This increases the validity of nail F level as a biomarker of chronic F exposure and even as a potential indicator for clinical fluorosis. Although a significant correlation between nail F levels and environmental F exposure has also been observed in other studies,^{8,13,15,16,21,22} such high nail F concentrations have not been found previously. The reasons for such a difference could be variations in the duration and frequency of the F exposure as well as differences in the susceptibility to F toxicosis.²³⁻²⁵ Another possible contributing factor could be using different methods for the determination of the nail F levels.

In summary, as well as the nail F levels being positively correlated with the serum and urine F levels, the high nail F levels found in the present study indicate that F obtained from the systemic circulation to the nail beds is deposited in the nails, either by secondary concentration or by continuous incorporation, thus making toe nail F a useful biomarker for both subchronic and chronic F exposures. The toe nail F values are also responsive to even slight differences in drinking water F concentration. The user-friendly technique for the measurement of nail F and its ease of establishment at a basic rural/urban laboratory setting suggest that toe nail clippings have a strong potential for use as a biomarker in epidemiological surveys. However, due to the many limitations in this study, more research with larger sample sizes is needed to arrive at any definite conclusion.

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