CHAPTER - III

Methodology
The present study was undertaken in order to compute the prevalence of dental and skeletal fluorosis and to assess fluoride status, bone mineral status, biochemical markers of bone metabolism status, hormonal status, oxidative stress and antioxidant enzyme status in adults living in endemic fluorosis areas in comparison with adults living in non-fluorotic area, who served as controls.

3.1 Inclusion criteria: Males and females of age group 21-50 years living in endemic fluorosis areas (>5 ppm since birth) were considered as study group (fluorotic group). Males and females of same age group living in non-endemic fluorotic area (<1 ppm) were considered as control group (non-fluorotic group).

3.2 Exclusion criteria: The subjects who have diabetes mellitus, liver diseases, hypertension, cardiovascular disease, chronic renal failure and carcinoma were excluded from the study and control groups (data not presented). Patients who received treatment with calcium, vitamin D, calcitonin, and hormone replacement therapy that can affect bone metabolism were also excluded from the study and control groups.

3.3 Clinical assessment: Dental examination was carried out in broad day light, for both males and females, for dental fluorosis. Likely symptoms for skeletal fluorosis were also noted down. Examination of pulse, blood pressure, anemia, jaundice, lymph nodes, skin lesions, secondary sex characters, tetany, etc., was recorded in each patient (data not presented). Body weight and height were also recorded, and the body mass index (BMI) was calculated for each patient as weight (Kg)/ height (m)².

3.4 Selection of the area: In Nalgonda district of Andhra Pradesh, the Nagarjunasagar water project supplies safe drinking water to certain highly fluorotic villages. Yet there are certain other fluorotic villages in the district not covered by the project. Sarampet village is one such with drinking water fluoride levels of 5.1 to 6.4 ppm. The Vaillapally village drinking water fluoride level is 7.2 to 8.2 ppm. Sarampet and Vaillapally villages of Munugode and Narayanpur mandals of Nalgonda district, Andhra Pradesh, India, with different water F-levels were selected for the study.
The total population of the two villages was 2250 (all age groups) comprising of 1077 males and 1173 females.

Table 6: Population distribution of two villages

<table>
<thead>
<tr>
<th>Village Name</th>
<th>Total population</th>
<th>Males</th>
<th>Females</th>
<th>Households</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarampet</td>
<td>615</td>
<td>291</td>
<td>324</td>
<td>146</td>
</tr>
<tr>
<td>Vaillapally</td>
<td>1635</td>
<td>786</td>
<td>849</td>
<td>373</td>
</tr>
<tr>
<td>Total</td>
<td>2250</td>
<td>1077</td>
<td>1173</td>
<td>519</td>
</tr>
</tbody>
</table>

3.5 Prevalence study: From the total households, sixty-five families from Sarampet village and one hundred and twenty families from Vaillapally village were selected randomly. A survey was carried out for eliciting information from all the members of all the selected families for the presence or absence of symptoms of dental and skeletal fluorosis as per the norms of WHO (1970), based on which the prevalence of dental and skeletal fluorosis was calculated. Age-wise and sex-wise distribution of the subjects was included in this study.

3.6 Selection of the subjects: A survey was conducted in two fluorotic endemic villages. Out of 328 subjects, 132 subjects affected with clinically proven fluorosis were selected randomly from each of the two villages with different water fluoride levels i.e., Sarampet (5.1-6.4 ppm) and Vaillapally (7.2-8.2 ppm) of Munugode and Narayanpur mandals of Nalgonda district, Andhra Pradesh, India, matched for age and sex with 88 controls living in a non-endemic area of the city of Hyderabad (> 1 ppm). The remaining subjects were removed from the study based on the criteria of exclusion.

The data was discussed with comparison between fluorotics and non-fluorotics, between males and females and between two levels of fluorosis i.e., Fluorotic group I (5.1 - 6.4 ppm) and Fluorotic group II (7.2 - 8.2 ppm) based on drinking water fluoride levels.
A survey was conducted to study prevalence status and Anthropometric data i.e., height, weight and BMI was recorded.

Biochemical Analysis

Fluoride status
1. Serum F
2. Urine F

Bone status
1. Serum Ca
2. Serum P
3. Serum ALP

Mineral status

Biochemical markers of bone metabolism
1. Serum TRAP-5b
2. Urinary CTX

Calcitropic hormonal status
1. Serum BAP
2. Serum Vit D
3. Serum PTH
4. Serum CTX

Thyroid status
1. Serum TSH
2. Serum T4
3. Serum T3
4. Serum CAT
5. Serum GST

Oxidative and Antioxidant status
1. Serum MDA

Statistical Analysis
3.7 Study Design
Map 3: Map showing selected mandals in Nalgonda District
3.8 Specimen collection and storage: Fasting samples of blood were drawn from the study and control groups after taking informed consent. 2 ml of EDTA blood and 18 ml of plain blood samples were drawn from each subject at their respective villages and transported to Vijaya diagnostic center, Hyderabad using cool packs. Plasma, cells and serum were separated the same day into different aliquots and stored at -80°C. The subjects were asked to collect 24 hours urine samples in sterilized, calcium-free containers for the determination of urinary creatinine levels. Simultaneously, all the subjects were asked to collect second morning void urine samples separately in sterilized 10.0 ml vials which were preserved at -20°C for the estimation of C-terminal telopeptides of type-1 collagen. Water from different sources of drinking water in both villages was also collected for water fluoride estimations.

3.9 Determination of fluoride content in drinking water samples: The concentration of fluoride in drinking water was measured using ion selective electrode method using Orion’s pH/ISE meter, model 940A. 18 ml of sample and 2 ml of TISAB III were mixed in a beaker. Electrode was rinsed with distilled water and placed into beaker. Concentration was noted when “Ready” was displayed on the instrument. The values of fluoride in water were calculated.

3.10 Statistical analysis: Data were analyzed using the statistical software program SPSS for windows version 11.5. Descriptive results were represented as mean ± standard error (SE). Comparison between two groups was based on student’s t-test. Among three or more groups, comparison was done using one way analysis of variance followed by Duncan’s multiple range test (DMRT). The p-values <0.05 were considered as significant and very small p-values are reported as p<0.01. Association between fluoride status and other parameters were identified by Pearson correlation.

3.11 Methods:

3.11.1 Serum Analysis:

3.12.1.1 Estimation of Bone-specific alkaline phosphatase (BAP) activity: Bone-specific alkaline phosphatase (BAP) activity was measured by using Enzyme linked immunosorbent assay (supplied by ImmunoDiagnostic Systems limited, U.K.). The
inter- and intra-assay coefficients of variations (CV) were 5.5 and 4.2 respectively. The control A (range: 6.9-10.4) and control B (range: 27.0-40.5) values obtained in BAP assay were 8.8 and 31.2 μg/L respectively. This assay procedure is explained in detail in appendix I.

3.11.1.2 Estimation of Tartrate-resistant acid phosphatase -5b (TRAP-5b) activity: Tartrate-resistant acid phosphatase-5b (TRAP-5b) activity was measured by using Enzyme linked immunosorbent assay kit (supplied by Suomen Bioanalytiikka Oy, Oulu, Finland). The inter- and intra-assay coefficients of variations (CV) were 6.3 and 9.5 respectively. The reference range was 1-10 U/L. The control value (range: 2.6-4.0) obtained in TRAP-5b assay was 3.2 respectively. This assay procedure is explained in detail in appendix II.

3.11.1.3 Biochemical tests performed by auto-analyzer: Serum calcium, phosphorus, total alkaline phosphatase, creatinine, bilirubin, serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT) and 24- hours creatinine was measured using automated analyzer.

3.11.1.4 Estimation of serum intact-parathyroid hormone (intact-PTH) levels: Immuno Chemiluminescence Microparticle Assay (ICMA) was used for the quantitative determination of parathyroid hormone levels in human serum samples. The reference range was 15-65 pg/ml respectively (Bayer Centaur Auto analyzer).

3.11.1.5 Estimation of 25-hydroxy vitamin D (25-OH vitamin D) levels: 25-hydroxy vitamin D (25-OH vitamin D) levels were measured using Radioimmunoassay kits (supplied by Bioline S.A.-Rue Andre Fauchille 17-B-1150 Bruxelles-Belgium). The control A value (range: 10.1- 31.6 ng/ml), control B value (range: 44.7-82 ng/ml), inter- and intra- assay coefficients of variations (CVs) obtained in 25-OH vitamin D assay were 8.3 and 7.1 respectively. The reference range was 7.6-75 ng/ml. This assay procedure is explained in detail in appendix III.

3.11.1.6 Estimation of Thyroid stimulating hormone (TSH) levels: Immuno Chemiluminescence Microparticle Assay (ICMA) was used for the quantitative determination of thyroid stimulating hormone levels in human serum samples. The reference range was 0.35 – 5.50 μIU/ml (Bayer Centaur Auto analyzer).
3.1.1.7 **Estimation of Thyroxine (T4) levels:** Immuno Chemiluminescence Microparticle Assay (ICMA) was used for the quantitative determination of thyroxine levels in human serum samples. The reference range was 4.50-11 μg/dl (Bayer Centaur Auto analyzer).

3.1.1.8 **Estimation of Triiodothyronine (T3) levels:** Immuno Chemiluminescence Microparticle Assay (ICMA) was used for the quantitative determination of triiodothyronine levels in human serum samples. The reference range was 0.60-1.80 ng/ml (Bayer Centaur Auto analyzer).

3.1.1.9 **Estimation of serum fluoride levels:** Serum fluoride levels were estimated by using Ion selective electrode method. 4.5 ml of sample and 0.5 ml of TISAB III were mixed in a beaker. Electrode was rinsed with distilled water and placed into beaker. Concentration was noted when “Ready” was displayed on the instrument. The values of fluoride in serum were calculated.

3.1.1.10 **Estimation of Lipid peroxidation products- Malondialdehyde levels:** Malondialdehyde levels in serum were estimated by the method of Okhawa et al (1974). This assay procedure is explained in detail in appendix IV.

3.1.1.11 **Estimation of Erythrocyte catalase activity (CAT):** Catalase activity was estimated by the method of Aebi et al (1984). Hemoglobin content in the blood was determined by the method of Drabkin (1932). This assay procedure is explained in detail in appendix V.

3.1.1.12 **Estimation of Glutathione S- transferase activity (GST):** Glutathione-S-transferase activity in serum was estimated by the method of Habig et al (1974). This assay procedure is explained in detail in appendix VI.

3.1.2 **Urine Analysis:**

3.1.2.1 **Estimation of C-terminal telopeptides of type-1 collagen (CTX) levels:** Urinary C-terminal telopeptides of type-1 collagen levels were measured by using Enzyme linked immunosorbent assay (supplied by Nordic Biosciences Diagnostics, Denmark). The inter- and intra-assay coefficients of variations (CV) were 4.4 and 6.7 respectively. The control A (range: 892-1339 μg/L) and control B (range: 1843-
2764 μg/L) values obtained in CTX assay were 1056 and 2289 μg/L respectively. This assay procedure is explained in detail in appendix VII.

3.11.2.2 Estimation of urinary fluoride levels: Urinary fluoride levels were estimated by using Ion selective electrode method. 9 ml of sample and 1ml of TISAB III were mixed in a beaker. Electrode was rinsed with distilled water and placed into beaker. Concentration was noted when “Ready” was displayed on the instrument. The values of fluoride in urine were calculated.