Material and Methods
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Selection of problematic (affected) areas

Nellore district is the coastal area of South India, which seems to be one of the most fluorosis threaten area of Andhra Pradesh state. From the data of water quality department as well as information from news papers, analysis has been initiated in the Udayagiri Mandal, Nellore district. In the selected mandal, ten villages have been reported to be affected areas of fluorosis. Particularly villagers belongs to these areas are prone to get the renal failures in recent years (Evidence from local medical reports). Thus the analysis was started with the ten villages for the identification of water fluoride content. Finally three villages having high fluoride levels were taken for further detailed analysis.

Analysis of water quality and fluoride content

A total of 10 samples were collected from the selected locations of each village representing the water quality of the whole area. Water samples from various drinking water sources were collected in polythene bottles, which were cleaned and finally washed with acid water, followed by rinsing twice with distilled water. The water samples collected were chemically analyzed. The parameters include pH, alkalinity, hardness, Ammonia, Calcium and magnesium and most importantly fluoride. The analysis of water was done using procedures
of standard methods (APHA, 1995) and all analysis was done in triplicate. Fluoride concentration was determined spectrophotometrically using Alizarin red-S and SPADNS reagents (Bellack and Schouboe, 1958). The Alizarin red-S method was found useful in higher fluoride range while SPADNA reagent was employed in low fluoride range (Gupta et al., 1993). Sodium fluoride was used to prepare the standard solution. The main sources of drinking water in these villages are open wells and hand pumps.

Selection of samples

Five hundred individuals from 10 villages in Udayagiri mandal, Nellore district, Andhra Pradesh State were chosen randomly for survey work, which was highlighted by the newspapers. A door to door survey with face-to-face interviews was carried out. The information collected was entered on a pre-coded questionnaire. Health complaints related to dental fluorosis, skeletal fluorosis and non-skeletal manifestations, including gastrointestinal complaints, were recorded. The teeth were examined for characteristic mottling and pigmentation, viz., yellow-white patches, brown streaks or black patches on the enamel surface and pitted, perforated or chipped-off enamel. Information was recorded on complaints of severe pain and rigidity of backbone, joints and hip region.
Present study was constructed to analyze the samples that are having the renal disorders with the association of fluoride intake. Further to isolate the selected samples of interest people were interviewed for the health problems related to renal function like delayed urination, frequent urination and general urine analysis for protein was also done. Peoples suffering with regular renal failure with diabetes and hypertension were separated and omitted from the analysis.

Subjects

This study was conducted on around 90 villagers, who were suffering with renal problems and without Diabetes and hypertension. They were provided with explanations for all experimental procedures and informed consent was obtained before the beginning of the study. Blood and urine samples were collected from the subjects and preceded for further hematological and biochemical analysis.

Control subjects

To compare each and every component or biological parameters, a group of normal healthy individuals were chosen from the same areas, who are not suffering with any of these disorders. Biological parameters were compared with standard clinical parameters.
Measurement of physical parameters

From the isolated test population (People suffering with renal problems without association of hypertension and/or diabetes) physical parameters like height, weight, waist, hip ratio and other details were taken at the time of blood and urine samples collection.

Estimation of serum and urine fluoride content

From the selected individuals blood and urine samples were collected in non-reactive plastic containers and brought to the laboratory in an ice box. To analyze the level of fluoride in serum, blood was centrifuged and serum separated. Fluoride content of serum and urine was analyzed through SPADNS method (Gupta et al., 1993).

Hematological findings

Only those who volunteered were included. An early morning visit was made on a mutually agreed date by a laboratory technician. Physical details were recorded and blood samples were drawn by venipuncture from each member into a heparinized tube. The whole blood was transported at 4°C to the laboratory where all the analyses were conducted on the day of blood extraction. Hemoglobin (Hb) was determined spectrophotometrically (540 nm) using cyanomethemoglobin method and expressed as g/100ml. Red blood cell counts (RBC) were made in Neubauer chamber with Hayem dilution
solution. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated according to the method of Ranzani - Paiva (1991). The red cells were separated from the plasma by centrifugation and washed twice with cold physiological saline. Complete blood picture (CBP) was got through the Semi auto analyzer (Model CHEM 400), Electronics India, India.

**Estimation of random blood glucose**

Consecutive villagers attending a general practice of random sugar estimation were asked to provide their consent to take part in this present study exploring their estimations of their blood glucose. Following consent, drawn the blood and their blood glucose was measured routinely using a ‘One Touch Ultra’ blood glucose meter. Regular calibration and quality control were by means of samples sent to the local hospital biochemistry laboratory. Normal random blood sugar level should be less than 160mg/dl (Hayavarein et al., 1962)

**Estimation of serum creatinine**

Blood was collected in to the sterile micro tubes and stand at room temperature to clot. Centrifuge the tube at 3000 rpm for separation of blood clot with the serum. The supernatant was collected and analyzed for the presence of creatinine. Serum creatinine levels were all assayed with the rate- Jaffé reaction on a Hitachi 747 auto
analyzer (Roche Diagnostics Corp., Indianapolis, Indiana). This assay was calibrated daily with a C fas calibrator (Roche Diagnostics Corp.) by using the uncompensated method during the study period. Normal range of adult males is 0.8 - 1.4 mg/dl: values are slightly higher in males due to larger muscle mass. In case of adult females, 0.6 - 1.1 mg/dl: creatinine clearance is increased in pregnancy, resulting in lower serum levels. In children, 0.2 - 1.0 mg/dl: slight increases with age because values are proportional to body mass (Bartles et al., 1972).

Collection of blood for lipid profile

1ml of heparin solution was added to 9ml of normal saline. Approximately 0.25ml of this solution was taken in a disposable syringe and blood was collected in the morning after overnight fasting from a peripheral vein in the same syringe under aseptic precautions to make it to 5ml. The sample was kept at room temperature for 15 minutes and then centrifuged at 3000rpm for 30 minutes. Plasma was separated and kept in plastic vials at -70°C till tested. Triglycerides were estimated according to the method of Buckley et al. (1966). Total cholesterol was estimated in plasma by the method described by Annino and Giese (1976). HDL, LDL, and VLDL were measured using the method of Kostner (1976) and Lopes et al. (1977). Normal values of triglycerides is <150 mgs/dl, total cholesterol is up to 239 mgs/dl, for HDL 30-60 mgs/dl, LDL is 100-190 mgs/dl, VLDL is 20-40 mgs/dl (Watson, 1960).
Evaluation of liver function tests (LFT)

Fasting blood samples were taken from the control and problematic subjects. Part of each serum sample was used for the usual tests performed by the preventive medicine center, and the remainder was conserved at -20°C until assay related to the current study (Saralakumari et al., 1988).

Estimation of serum bilirubin

Bilirubin is an endogenous anion derived from hemoglobin degradation from the RBC. The classification of bilirubin into direct and indirect bilirubin are based on the original van der Bergh method of measuring bilirubin. Bilirubin is altered by exposure to light so serum and plasma samples must be kept in dark before measurements are made. Serum levels of total and conjugated bilirubin were measured by Jendrassik and Grof’s (1938) method and serum albumin level was measured by Rodkey’s (1965) method.

Total bilirubin: This is measured as the amount, which reacts in 30 minutes after addition of alcohol. Normal range is 0.2-0.9 mg/dl (2-15μmol/L). It is slightly higher by 3-4 μmol/L in males as compared to females. It is this factor, which helps to diagnose Gilbert syndrome in males easily. The diazo method of bilirubin estimation is not very
accurate especially in detecting low levels of bilirubin (Jendrassik and Grof, 1938).

**Direct Bilirubin:** This is the water-soluble fraction. This is measured by the reaction with diazotized sulfanilic acid in 1 minute and this gives estimation of conjugated bilirubin. Normal range 0.3mg/dl (5.1μmol/ L) (Jendrassik and Grof, 1938).

**Indirect bilirubin:** This fraction is calculated by the difference of the total and direct bilirubin and is a measure of unconjugated fraction of bilirubin (Jendrassik and Grof, 1938).

**Estimation of hepatic marker enzymes**

The aminotransferases (Transaminases) are the most frequently utilized and specific indicators of hepatocellular necrosis. These enzymes- aspartate aminotransferase (AST, formerly serum glutamate oxaloacetic transaminase-SGOT) and alanine amino transferase ( ALT, formerly serum glutamic pyruvate transaminase-SGPT) catalyze the transfer of the amino acids of aspartate and alanine respectively to the keto group of ketoglutaric acid. The basic principle involves the determination of amount of pyruvic acid in case of SGPT and oxaloacetic acid in case of SGOT are determined after incubation by the formation of hydrazone with the dinitrophenyl hydrazine reagent which
is highly coloured in alkaline medium. ALT is primarily localized to the liver but the AST is present in a wide variety of tissues. Serum alkaline phosphatase activity was measured using the method of Bessey et al. (1946), serum ALT activity was measured using the method of Henry et al. (1960). All of the methods described were adapted for automated analysis (Automatic analyzer). Serum AST activity was measured by a technique derived from the technique of the Societe’ Franc¸aise de Biologie Clinique (Mathieu et al., 1982). Enzyme activity values are expressed as IU/L at 30°C.

**Estimation of serum proteins**

Albumin is quantitatively the most important protein in plasma synthesized by the liver and is a useful indicator of hepatic function. Normal serum values range from 3.5g/dl to 4.5 g/dl. The average adult has approximately 300 to 500 g of albumin (Robinson et al., 1938). Albumin is generally measured by a dye-binding technique that utilizes the ability of albumin to form a stable complex with bromocresol green dye. The BCG-albumin complex absorbs light at a different wavelength from the unbound dye. This method may overestimate albumin by binding to other proteins. The total globulin fraction is generally determined by subtracting the albumin from the total protein.
**Estimation of electrolytes**

Electrolytes are positively and negatively charged molecules called ions that are found within the body cells and extracellular fluids, including blood plasma. A test for electrolytes includes the measurement of sodium, potassium etc. Determination of electrolytes in serum was made in the normal and selected subjects. All analyses were done in duplicate. The determination of sodium and potassium was done by an internal standard flame photometer (Berry et al., 1946). Electrolyte concentrations are similar whether measured in serum or plasma. Normal serum sodium concentration is 135–145 mmol/l, alert levels. In case of serum potassium is 3.6–5.4 mmol/l.

**Estimation of glomerular and tubular markers**

**Estimation of transferrin**

The serum was obtained from healthy donor. In previous investigations, the sample was selected from plasma, but to exclude the various coagulation factors of plasma, we selected serum as the origin sample. Furthermore, the other privilege of using serum was its lack of any anti-coagulating factors such as chelators which may interact with ferric ion and limit iron saturation. Since, the amount of transferrin estimated by SRID kit in the first sample was 7.5 g, we used 10.5 mg Fe and 11.44 mg anion bicarbonate (7 μL of 0.1 M or 54 μg NaHCO3 and 7 μL of 0.1 M or 153.16 ferric citrate in pH=8, 4oC, 1 h).
**Estimation of IgG**

Serum was separated and stored at 4°C till further use. The serum and urinary IgGs were measured by sandwich ELISA (Enzyme Linked Immunosorbent Assay) within one week of storage using reagents supplied by Bangalore Genei, India. The 96 wells ELISA plate (Tarsons) were coated with 100μl of capture antibody (goat anti human IgG, 10μg/ml) in carbonate buffer (50 mM pH 9.6). The plates were incubated at 4°C under humid conditions over night. The wells were aspirated out and washed thrice with PBST (Phosphate buffer saline tween - 20 - 0.1 M pH 7.2). The vacant sites were blocked with 100ml of blocking solution (3% BSA in PBST), incubated at room temperature for 1 hour and washed thrice with PBST. 100μl of samples and standards were added to wells in conjugate diluent (0.1% BSA in PBST). The plates were incubated at 37°C for 1 hour, and washed thrice with PBST. Goat anti human IgG HRP 1:1000 diluted in PBST was added 100μg/well and incubated at room temperature for 1 hour. The plates were washed four times with PBST, 100μl of substrate TMB/ H₂O₂ was added and the reaction was stopped with 50μl/well 1N sulphuric acids after 5 minutes and the O.D. was read at 450 nm in ELISA reader (Molecular device model spectra max 190). The urine samples were centrifuged in refrigerated centrifuge at 1500 r.p.m. to remove cell debris and stored at- 20°C with sodium azide (0.2%) as preservative. Further analysis was made as earlier.
Estimation of antitrypsin

Oxidative antitrypsin (AT) in urine and serum was analyzed using an ELISA with a monoclonal antibody against oxidized α₁-AT in which chloramine T-oxidized α₁-AT was the antigen (Ueda et al., 2002). The sensitivity of oxAT measurement was 1.0 ng/ml with an inter CV of <6.7%.

β2-microglobulin assay

Under aseptic precautions venous blood was drawn and serum separated. The samples were frozen at -70° C until assay. The serum and urine was analyzed by Enzyme linked immunosorbent assay (β2-microglobulin EIA kit, Immunotech, France). 2.4mg/ L was used as the upper limit, when 97% of normal values are below this cut off value.

Serum and urine ACE Level Measurement

Serum or urine ACE level was measured by a colorimetric method (colorimetric assay kit, Fujizoki Assay, Tokyo, Japan) using p-hydroxyhippuryl- L-histidyl-L-leucine as the substrate (Kasahara and Ashihara, 1981).

Determination of ACE Genotypes

The D and I alleles were identified on the basis of polymerase chain reaction (PCR) amplification of the respective fragments from
intron 16 of the ACE gene and size fractionation and visualization by electrophoresis. DNA was extracted from peripheral leukocytes with standard techniques. PCR was performed with 20 pmoles of each primer: sense oligo 5'CTGGAGACCACACTCCCATCCTTTCT3' and anti-sense oligo: 5'GATGGGCCATCACATTGTCAGAT3' in a final volume of 25 µl, containing 1.5 mM MgCl$_2$, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 0.2 mM of each dNTP, and 1.25 unit of Taq polymerase (Perkin Elmer-Cetus, Norwalk, CT). The DNA was amplified for 30 cycles with denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 1 min, followed by final extension at 72°C for 5 min (DNA Thermal Cycler 480, Perkin Elmer-Cetus) (Saiki et al., 1988; Rigat et al., 1992). PCR products were electrophoresed in 2% agarose-gel with 5 µg ethidium bromide per milliliter. The amplification products of the D and I alleles were identified by 300-nm ultraviolet trans-illumination as distinct bands (D allele: 191 bp; I allele: 478 bp) Because the D allele in heterozygous samples is preferentially amplified, each sample found to have the DD genotype was subjected to a second independent PCR amplification with a primer pair that recognizes an insertion-specific sequence (hace 5a, 5'TGGGACCACAGCGCCGCCTAC3';hace 5c, 5' TCGCCAGCCTCCCA TGCCCATAA3'), with identical PCR conditions except for an annealing temperature of 67°C. The reaction yields a 335-bp amplicon only in the presence of an I allele and no
product in samples homozygous for DD (Shanmugam et al., 1993; Lindpaintner et al., 1995).

**Statistical Analysis**

Statistical analysis was carried out using SPSS for windows 10.0 software (SPSS Inc., Chicago, IL, USA) and Microsoft Excel. Values were reported as mean ± standard deviation. SD was not more than 10%. The difference between groups was compared by Pair wise Multiple Comparison Procedures (Duncan's Method). p value <0.001 was considered statistically significant.