Research Article

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Correlation of Water Fluoride with Body Fluids, Dental Fluorosis and FT$_4$, FT$_3$ –TSH Disruption among Children in an Endemic Fluorosis area in Pakistan

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Abstract: In the present study 134 children were studied for comparison and correlation between an endemic fluorotic village Rukh Mudke (RM), $n = 74$, and a non-fluorotic village Ottawa (OTW), $n = 60$. The children were aged between 7 -18 years and selected for the estimation of fluoride in their household water, body fluids (urine-serum), dental fluorosis and thyroid hormones (Free tri-iodothyronine (FT$_3$) free tetra iodothyronine (FT$_4$) and thyroid stimulating hormone (TSH) respectively. Mean concentration of water fluoride in subjects of RM was $4.6 \times 10^6$ ng/L, urine fluoride $2.59 \times 10^6$ ng/L, serum fluoride $6.0 \times 10^4$ and dental fluorosis 90.5% respectively. Significant elevation ($P = 0.000$) in the concentration of three out of these four variables ($P < 0.01$) was observed (except in serum fluoride) in subjects of RM compared to those in the control group (OTW). Mean FT$_4$, FT$_3$ and TSH concentrations in RM subjects was 18.3 pmol/L, 5.06 pmol /L and 3.2 mIU/L respectively. No marked difference in FT$_3$ and FT$_4$ ($P = 0.17$ and $P = 0.7$) was found compared to the control (OTW) group, while significant elevation in TSH ($P < 0.05$) was found in 22% of the children in the RM group, portrayed well defined thyroid hormonal aberrations. A negative correlation between water fluoride - FT$_4$ ($r = - 0.24$); a strong positive between water, urine, serum, dental fluorosis and TSH ($r = 0.94$, 0.87, 0.88, 0.74 and 0.8) and moderate correlation between water fluoride - FT$_3$ ($r = 0.52$) was observed. Results of this study indicate that the fluoride intoxication through drinking water is not only increasing fluoride level in body fluids and deteriorating teeth but also destroying thyroid function in a large number of children.

Keywords: Water fluoride; Urine and Serum fluoride; Dental Fluorosis; FT$_3$, FT$_4$, TSH.

1 Introduction

The problem of elevated levels of fluoride in groundwater is prevalent in many regions of Pakistan. It was very first recognized in Punjab by Wilson in 1941 in the town of Raiwind [1]. Currently, fluorosis is endemic in many areas of Sindh, Balochistan and Punjab, affecting millions of people with a remarkable ratio of children [2]. However, the problem of excessive fluoride in ground water is widespread throughout the world and approximately 200 million individuals among 25 countries are under the terrible providence of fluorosis including India, China, Sri Lanka, Spain, Italy, West Indies and America [3-7]. For example in India alone 21 states out of 29 and 65 million people including 6 million children are affected by fluorosis [8-9]. In Pakistan, out of 29 major cities 34% had water fluoride greater than $1.5 \times 10^6$ ng/L including Lahore, Quetta and Tehsil Mailsi with maximum values of 23.6 $\times 10^6$ and 24.48 $\times 10^6$ ng/L, respectively [7]. A large number of human activities increase fluoride in the environment, including chemical production plants, waste pools, production of aluminum, steel, glass, enamel, brick, tile, pottery, and cement, manufacture of fluoride containing chemicals, phosphate fertilizers and
metal casting, welding, and brazing [10]. In 2002, a study reported in India showed fluorosis in 10% of children, even when the concentration of fluoride in water was 1.0 ×10^-6 ng/L [11].

Excretion of urinary fluoride is considered as the primary indicator of fluoride toxicity, not only in the fluoride susceptible workers, but also in natives of fluoride contaminated territories [12]. Disintegration of renal activity causes the decrease in the excretion of fluoride: increasing the probability of its deposition within the kidney [13]. As kidneys are the main excretory path of fluoride from the body, 50% fluoride is excreted within 24 hours of its consumption, while the remaining 93-97% get accumulated in hard tissues like teeth and bones, causing dental and skeletal fluorosis [14,15]. Excessive fluoride in teeth changes the rate of disintegration of amelogenin (enamel matrix protein): alters the function of protease by decreasing the availability of calcium ion in mineralization environment, giving rise to hypomineralized enamel which presents itself as a sheenless and opaque look [16] with brown streaks due to fluorohydroxy apatite formation. A large number of factors influence the accumulation of fluoride in the hard tissues, like endemic pollution, nutritional status, supplementation of water supply with fluoride, usage of bottled water, carbonated drinks, age and gender etc. [17,18]. Dental fluorosis is also a developmental abnormality, which indicates disturbed thyroid hormonal secretion [1]. In fact the most sensitive organ of the body affected by fluoride is the thyroid gland [19]. Desun in 1994, demonstrated potential effects of fluoride on thyroid hormonal production [20]. Yaming in 2005 described that long term ingestion of fluoride is a risk factor for the progression of thyroid abnormalities [21]. Interference of fluoride in thyroid hormone metabolism is a potential cause of degeneracy in central nervous system, deterioration in brain function and anomalous growth in children [22].

Fluoride exists in human serum in the form of ionic (F^-) and non-ionic fluoride (NF) where non-ionic makes up 80-90% of total fluoride. Numerous studies are available showing a positive dose relationship between water and serum fluoride [23,24].

Few studies from Punjab, especially from the villages of district Kasur are available depicting concentration of fluoride in water, urine and serum [25-27]. Consumption of fluoride, primarily through drinking water as (75% of daily intake) causes different types of fluorosis at different stages [28]. To prevent fluoride toxicity, it is very important to investigate the ground water of fluorosis affected territories and examine its consequences on the natives, especially children, which are most adversely affected.

One such area, (Manga Mandi and its surroundings) is examined in the present study, where fluorosis has been documented previously [2,28]. Rukh Mudke is a village; 3.69 km east of Manga Mandi where water contamination, excessive browning of teeth and common problem of joint pain (all signs of excess fluoride consumption) in children have been found. In this territory, the effect of high fluoride intake on the human body in a particular age group has tried to explore in every possible detail. Similar studies have also been performed in India and China in endemic fluorosis rural areas to understand the long term influence of fluoride intake on the human body, particularly in children [29,47].

2 Material and Method

2.1 Reagents and Chemicals

The following reagents and instruments were used during the course of fluoride quantification,

Fluoride Ion Selective (combination) Electrode; HI 4110: Hanna Instruments, Rhode Island, USA. pH/ISE/mv meter ; HI 4222: Hanna, Electrode Holder ; HI 76404: Hanna, Magnetic Stirrer ; HI 180: Hanna, Plastic Beakers ; HI 740036P : Hanna , Gamma Counter ; Cap-RIA 16 : Capintec Inc. New Jersey, USA. Vortex type Mixer; M 63210-33: Barnstead Internationals, Texas USA. Orbital Shaker OSM-747; Gallenkamp: Leicestershire UK, 500 UL semi programmed pipette; Eppendorf International: Hamburg Germany, Small test tubes (Specially designed 5 cm long, φ 14 mm). Fluoride standard solution 1000 ppm; HI 4010-03: Hanna, Total ionic strength adjustment buffer; (TISAB II) HI 4010-00: Hanna, Reference fill solution; HI 7075: Hanna, FT-r, FT-y, TSH kits by Immunotech Inc. Beckman; Prague Czech Republic. Deionized water (<18MΩ/cm) for dilutions.

2.2 Selection of subjects

For the purpose of the study, a village around Manga Mandi namely Rukh Mudke (RM) was selected as a sampling group because of evidence suggesting excessive mottled enamel and high water fluoride content in the nearby sites [26]. The area of manga Mandi (MM) is located towards the North West boundary of the district of Kasur; geographically situated at 31° 30´ N latitude and 74°06´E longitude: 43.4 km from Kasur. Natives of this village use groundwater as a primary source of drinking water.
and hand pumps, motor pumps and tube wells are the main sources of drinking water. Initial survey of selected villages showed a mean water fluoride concentration of 5 mg/L. For comparison, 60 healthy individuals of the same age group, living in a non-fluorosis village Ottawa (65 km from Lahore) with identical socioeconomic conditions were selected as a control group. A total of 134 male and female participants (74 from RM and 60 from OTW) with or without dental fluorosis: with groundwater consumption as drinking water and only by birth residents were randomly selected for comparison between fluorotic (RM) and non fluorotic (OTW) territories.

2.3 Sample collection

For the collection of water samples, clean, transparent plastic bottles (200 ml capacity) with caps were used. Urine samples were collected in plastic containers of 50-100 ml capacity. Collection of blood samples was done with syringes containing metal free needles and plastic capped vials of 2 ml volume. The clinical protocol was approved by the ethical committee of Institute of Chemistry, University of the Punjab. “Written and informed Consent for participation” was taken from each child or his/her guardian. Those who did not agree or were not a resident by birth were excluded from the study.

All glassware and polyethylene bottles were initially washed with deionized water, soaked in 2 molar HNO₃ overnight, rinsed again with ultra-pure water and dried before use [30].

2.4 Water Sampling

Collection of water samples was done between 9 am and 3 pm in four sequential visits from sampling sites during May-August 2017. Children aging 12-18 provided samples themselves while guardians, usually their mothers, helped to collect samples from those less than 11 years old. Collection of water samples was done from a subject’s household tap, either hand pump or electric motor, getting supplies from the water and sewer authority (WASA) tube well in that area. Each water bottle was rinsed three times with sampling water and emptied downstream. Before collecting the sample, the tap or hand pump was kept open for 3 minutes to flush the pipe sufficiently to get a representative sample. In order to avoid turbulence and air bubbles, the bottles were filled slowly and gloves were provided to avoid any hand contamination (a volunteer helped in collection of each sample). At the sampling location the color, odor, taste and PH of the water samples was noted. All the water samples were transported to the laboratory on the same day and stored frozen. Each samples was analyzed for fluoride concentration within a week by Fluoride ion selective electrode (F ISE [25, 27]).

2.5 Urine collection

Participants were requested to provide “on the spot” causal urine samples in pre-washed, pre-coded polyethylene containers, containing 0.2 g EDTA as a preservative and during 9:00 am to 1:00 pm in three successive visits. Collection from younger children was done with the help of their mothers. Each urine sample was packed in a separate polyethylene bag and placed in an ice box. All samples were transported to the laboratory on the same day and analyzed by fluoride ISE [27].

2.6 Blood sampling

5 ml of blood was withdrawn intravenously with the help of a sterilized syringe by a qualified nurse in the presence of a General physician along with urine collection. Blood was transferred into a pre-washed, clean, open glass vial at room temperature for 30 minutes, then it was centrifuged at 4000 rpm for 10 minutes and the serum was separated into another vial and capped [31]. Serum vials were kept frozen at -20°C and analyzed within one week using fluoride ISE [25].

2.7 Survey of dental fluorosis

Due to the very common existence of the browning of teeth in the studied area, a dentist was arranged who analyzed the teeth of sampling participants with mouth mirror, forceps, and probe under sunlight. Dean’s fluorosis index was used to examine the level of dental fluorosis according to the scale. Revised six point scale (1942) according to the levels (0-5) was used for this purpose. The individual score depends on the two most severely affected teeth. Due to its simplicity, it is most commonly used in prevalence studies.

2.8 Fluoride analysis by Ion Selective Electrode

Determination of fluoride in water, urine and serum was done using fluoride ion selective (combination) electrode.
It is a solid state electrode with a lanthanum fluoride crystal membrane and a reference electrode incorporated in it to complete its electrolytic circuit. The lanthanum fluoride crystalline pellet is practically insoluble in the test solutions being measured and produces a potential change due to changes in the sample’s ion activity. When the ionic strength of the sample is fixed, the voltage is proportional to the concentration of fluoride ions in solution and the electrode follows the Nernst equation: \[ E = \frac{E_a + 2.3RT/nF \log A_{\text{ion}}}{A_{\text{ion}} - \text{Reference and fixed internal voltages}, R = \text{gas constant} (8.314 \text{ J/K Mol}), n = \text{Charge on ion} (1), A_{\text{ion}} = \text{ion activity in sample}, T = \text{Absolute Temperature in K} \text{ and } F = \text{Faraday constant} \text{ (9.648 x 104 C/equivalent).}

Deionized water with resistivity < 18MΩ/cm was used for dilutions. Fluoride ISE was calibrated first with various fluoride standards and then samples were analyzed one by one. After each reading the electrode was washed and dried with absorbent paper before testing the next sample [32].

For determination of water fluoride, 7:3 ratios of water sample and TISAB were mixed. The concentration of fluoride standard for analysis was kept within the 0.1-10 mg/L range. Water fluoride results were expressed as less than 10% coefficient of variation of impression profile. For accuracy and validity of the applied method, water samples were spiked with standard fluoride solutions and recoveries were measured in the range 0.2-3.2 ug which appeared to be 99% (range 96.7-99.8%). For urine fluoride analysis, 10:3 ratios of urine sample and TISAB were used with the fluoride standard kept between 0.5-5 mg/L. Urine fluoride results were expressed as less than 10% coefficient of variation of impression profile. For precision, urine samples was spiked with standard fluoride solutions and found to be in the range of 98-101.3% (mean percentage recovery 99.1%).

For the determination of fluoride in serum, 1:1 ratios of serum and TISAB were combined and the Fluoride standard solution used for calibration was prepared in the range 0.01-1 mg/L. Mean percentage Co-efficient of variance appeared to be 0.22% and 0.23% in case of intra and inter assay precision. For precise measurement and validation of the applied methodology, percentage recoveries exhibited the range of 96 - 101.4% (mean 98%).

2.9 \( FT_4 \), \( FT_3 \) and TSH analysis

Determination of free \( T_4 \) and free \( T_3 \) was carried out by competitive Radioimmunoassay (RIA) and thyroid stimulating hormone (TSH) by a sandwich type Immunoradiometric assay (IRMA) using commercial kits of Immunotech Inc. (Beckman, Czech Republic). Measurement of radioactivity, fitting of a standard curve and analysis of samples was carried out using a computerized gamma counter (Cap-RIA 16, CAPINTEC; Inc. USA). Assay reliability was determined by the use of commercially derived control sera of low, medium and high concentrations, which were included in every run. All assays were carried out in duplicate. At least 10% of coefficient of variation (CV) of impression profile was used to present the outcomes of RIA and IRMA.

2.10 Statistical analysis

The comparison of the mean water, urine, serum fluoride, dental fluorosis, \( FT_4 \), \( FT_3 \), and TSH between children of RM and OTW was done using student t-test (unequal variances) by Microsoft Excel at confidence interval (CI) of 95%: \( P \)-value ‘less than 0.05’ was considered statistically significant. Normality distribution of the data was checked by the Kolmogorov- Smirnov test before application of this parametric test. In order to find correlation between water fluoride with urine/serum fluoride, \( FT_4 \), \( FT_3 \), TSH and dental fluorosis Pearson correlation at 95% CI and \( P < 0.05 \) was applied. Group wise comparison of dental fluorosis with \( FT_4 \), \( FT_3 \) and TSH was done using mean, standard deviation and student t-test under same CI and \( P \)-value.

3 Results

A total of 134 children were examined during the course of this study; 74 from RM (31 male and 43 females) and 60 from the control group i.e. OTW (30 male and 30 females). The average age of the participants in RM and OTW was 11.23 ±2.51 and 12.13 ± 3.2 respectively.

In the subjects of RM, 75.67% samples had fluoride in water greater than the WHO limit [1.5 x10⁶ ng/L (33)] while from OTW all water samples appeared within WHO standard range. Results (Table 1) indicate that the level of fluoride in drinking water of RM was significantly \( (P = 0.000) \) higher than in OTW.

The average concentration of fluoride in the urine samples of the two villages is presented in the same Table. Concentration of urinary fluoride in the children of RM appeared to be clearly elevated compared to the control \( (P = 0.000 \text{ (student t test)}) \). The value of ‘harmless fluoride concentration in human urine quoted in literature differs depending upon the age of population, level of fluoride in drinking water and additional sources of fluoride intake [34].
Correlation of Water Fluoride with Body Fluids, Dental Fluorosis and FT₄, FT₃ – TSH Disruption...

Figure 1 presents the scatter plot between water and urine fluoride of RM Participants. Pearson correlation coefficient (r = 0.94) showed that a very strong relationship exists between water and urine fluoride thereby an increase in the amount of fluoride in water is the main source of fluoride elevation in urine.

No significant difference in serum fluoride level of RM and OTW groups (P = 0.05) is noticed (Table 1). Mean urine fluoride of the subjects of OTW also show higher than safe limits: indicating other potential sources of fluoride intake than water. Normal limit of fluoride in urine and serum are 1.0 x 10⁶ ng/L and 0.15 x 10⁶ ng/L respectively [35].

Figure 2 shows the scatter plot of water fluoride with serum fluoride of subjects from RM. Water fluoride exhibited strong positive dose relation with serum fluoride (r = 0.86). Urine fluoride also portrayed identical relation with serum fluoride (r = 0.86: Figure 3), clearly showing that the presence of excessive fluoride in water as main source of over fluoride consumption by human body.

The normal laboratory range of FT₄, FT₃, and TSH is 11.5 - 23 pmol/L, 2.5 - 5.8 pmol/L and 0.3 - 5 mIU/L correspondingly. The concentration of free T₄ in the subjects of RM and OTW is summarized in Table 2. Student t-test of two groups showed no significant difference (P = 0.17) in the mean value of the two groups. Table 2 is also shows the concentration of free T₃ and free T₄ in subjects of RM versus OTW. There appeared no significant difference (P = 0.77) in the mean FT₃ and FT₄ of the two groups. When comparing the concentration of TSH in both groups its level in RM children was clearly elevated (P < 0.05) than in subjects of OTW (Table 2).

The correlation of FT₄, FT₃, and TSH in participant’s serum with drinking water fluoride between the subjects of sample (RM) and control (OTW) groups by Pearson Correlation is shown in Table 3. There is no significant difference observed between water fluorides - FT₄ whereas the significant positive dose relation was noted between

Table 1: Level of Fluoride in Water, Urine and Serum of Children from RM and OTW.

<table>
<thead>
<tr>
<th>Rural Area</th>
<th>Number of samples</th>
<th>Water Fluoride (ng/L)</th>
<th>Urine Fluoride (ng/L)</th>
<th>Serum Fluoride (ng/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RM</td>
<td>74</td>
<td>4.66 x 10⁶ ± 2.83 x 10⁶ (a)</td>
<td>2.59 x 10⁴ ± 1.33 x 10⁴ (b)</td>
<td>6.0 x 10³ ± 5.0 x 10³</td>
</tr>
<tr>
<td>OTW</td>
<td>60</td>
<td>0.54 x 10⁶ ± 0.15 x 10⁶</td>
<td>1.52 x 10⁴ ± 0.43 x 10⁴</td>
<td>5.0 x 10³ ± 1.0 x 10³</td>
</tr>
</tbody>
</table>

(a) = Mean ± Standard Deviation
(b) = Range
Note: Water Fluoride: P < 0.001 (student t test using unequal variances between RM and OTW), Urine Fluoride: (P < 0.001), Serum Fluoride: (P > 0.05).

Figure 1: Relationship between Water and Urine Fluoride of RM Participants. Pearson correlation coefficient r = 0.94.

Figure 2: Relationship between Water and Serum Fluoride of RM Participants r =0.86.

Figure 3: Relationship between Urine and Serum fluoride of RM Participants r = 0.86.
To examine the level of dental fluorosis (D.F), all 134 subjects were analyzed. 90.5% from RM and 7% from OTW exhibited various degrees of D.F. Its various stages among the study group were white flecks, small to medium opaque paper white areas covering 25-50% of tooth surfaces, marked wears on biting surfaces, brown staining up to confluent pitting and “all tooth surfaces affected” were observed.

In RM moderate (score 4) dental fluorosis is most commonly observed (Figure 4); suggesting that the endemic fluorosis area is greatly affecting tooth development even the minimum influence is so high that it produces level 3 dental fluorosis, as a result of fluoride toxicity. Water fluoride shows a positive relation with (r =0.74) fluorosis index (Figure 5) in subjects of RM suggesting strong impact of fluoride consumption on teeth derangements.

The number of children from RM: falling in various D.F categories as per Dean’s Index and their corresponding mean FT\textsubscript{4}, FT\textsubscript{3} and TSH concentrations respectively is summarized in Table 4. Comparison of mean FT\textsubscript{4} in different D.F scores (Student t- test using unequal variances) indicate that it differs significantly (P < 0.00) between mild - severe whereas FT\textsubscript{3} and TSH differs in very mild - moderate (P < 0.05 for FT\textsubscript{3} and TSH) and very mild - severe (P < 0.00 for FT\textsubscript{3} and TSH) grades correspondingly.
**Table 4:** Relationship between D.F grades and mean FT$_4$, FT$_3$ and TSH in RM Participants.

<table>
<thead>
<tr>
<th>D.F group (According to Dean’s index)</th>
<th>No. of samples</th>
<th>FT$_4$ pmol/L (M ± SD)</th>
<th>FT$_3$ pmol/L (M ± SD)</th>
<th>TSH mIU/L (M ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>18.75 ± 1.86</td>
<td>5.0 ± 0.6</td>
<td>2.42 ± 0.96</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>18.81 ± 2.06</td>
<td>4.8 ± 0.38</td>
<td>2.0 ± 0.37</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>17.75 ± 1.6</td>
<td>5.23 ± 0.62</td>
<td>2.36 ± 0.88</td>
</tr>
<tr>
<td>4</td>
<td>24</td>
<td>17.93 ± 2.31</td>
<td>5.25 ± 0.51</td>
<td>3.19 ± 0.71</td>
</tr>
<tr>
<td>5</td>
<td>14</td>
<td>17.67 ± 3.33</td>
<td>5.3 ± 0.33</td>
<td>5.93 ± 3.69</td>
</tr>
</tbody>
</table>

4 Discussion

Punjab is one of the provinces of Pakistan where fluoride is endemic among many regions especially in Kasur district and its surroundings. Presence of fluoride containing salt range, excessive use of phosphate fertilizers and large number of brick kiln units, causing tremendous increase in fluoride toxicity [36,37]. In sample (RM) and control (OTW) territories, due to poor socio-economic conditions; infrequent travelling to other residential sites and rare usage of mineral water defines the fluoride ingestion history.

Consumption of drinking water is directly proportional to atmospheric temperature. In Punjab the average summer temperature is 41°C [37] and, according to WHO [38], the maximum concentration of fluoride in drinking water under such extreme climatic conditions should not be more than $0.7 \times 10^6$ ng/L. An average water consumption per child from April to August is 2.5 L and 86-97% fluoride from water gets absorbed directly into the human body [39,40]. In the current study concentration of drinking water fluoride at RM is $4.66 \times 10^6$ ng/L (mean, Table 1) which is three times as much as than the recommended safe limit: setting an elevated baseline for other investigating parameters. Farooqi found water fluoride range of $2.47 \times 10^6$ - $21.1 \times 10^6$ ng/L in a nearby village Kalalanwala while Ahmed observed 86% water samples with fluoride concentration > WHO limit in the same village [36,41].

Level of fluoride in bio fluids (urine and serum) is considered to be the most authentic measure of fluoride toxicity [42]. Kidneys are the main excretory organs for removal of absorbed fluoride via urine [21]. Chronic kidney problems often seen in early aging society shows the gradual decline of kidney function that may lead to permanent kidney failure, or end-stage renal disease: excessive serum fluoride on the other hand directly influence the production and function of thyroid hormones. Our findings i.e. higher concentration of fluoride in urine ($2.59 \times 10^6$ ng/L) and serum from RM subjects (Table 1) is indicating the same potential hazards. Identical urine fluoride level ($1.78 \times 10^6$ ± 1.20 $\times 10^6$ ng/L) was observed by Isaac in 100 primary school children aged 6-13 years in Kaiwara village while similar serum fluoride concentration range was also observed by Kumar in India [43,44].

In the present analysis water fluoride shows a positive dose relationship with urine fluoride ($r = 0.94$, Figure 1) and serum fluoride ($r = 0.86$, Figure 2). Urine fluoride also presented a positive correlation with serum fluoride ($r = 0.86$, Figure 3) therefore showing the link of fluoride between these parameters. This fact also advocated the potential role of water fluoride in increasing fluoride concentration within body fluids in this particular region. Similar results were also observed by Rathee and M. Qayyum [25,27,45].

Approximately half of the fluoride ingestion becomes a part of hard tissues within 24 hours [46]. Absorption of fluoride by skeletal tissues is more active in adolescents and decreases with age [47]. The present analysis indicates the same high absorption of fluoride by calcified tissue (i.e. teeth in the form of Fluorapatite) with increase in ground water fluoride ($r = 0.74$, Figure 5): responsible for their unpleasant brown look which further causes teeth deterioration in early stages of life. Identical elevation in teeth browning with increase in ground water fluoride was also noticed in South India by Viswanathan [47]. In this area (RM) this phenomena is so pronounced that it is causing 90.5% D.F with 66.4% children suffering from mild to severe degree of fluorosis (Figure 4). 94.63% and 85.37% teeth fluorosis was also found in Dadanpur and Wamiao respectively, the two endemic fluorosis villages in India and China [44,48] thereby confirming this relationship. D.F is also an indication of abnormal thyroid metabolism [49]. Also the risk factors known to influence teeth fluorosis are similar to those observed in thyroid dysfunction.

When mean serum FT$_4$, FT$_3$, and TSH were compared between RM and OTW no significant difference in FT$_4$ and FT$_3$ of the two groups was noticed, but serum TSH (which
is stimulator of thyroid gland for $T_4$ and $T_3$ production) showed a clear elevation in the children of RM than OTW ($P < 0.05$). Therefore TSH is the first hormone getting disturbed by excess fluoride intake. Similar outcomes were reported by Xiang in children from an endemic fluorosis village compared to a non-fluorosis village in China [48].

The mean FT$_4$, FT$_3$, and TSH of subjects of two groups is compared with their corresponding mean W.F (Table 3). A positive relationship is observed between W.F - FT$_4$ and W.F- TSH in RM thereby indicating that drinking W.F is playing direct or indirect role in elevating the level of FT$_3$ ($r = 0.52$) and TSH ($r = 0.8$) at this endemic fluorosis site. Such elevation in FT$_3$ and TSH level in 8-12 years old children was observed by Xiaoli in chronically fluorosis areas [50]. An increment in TSH with excess water fluoride was also observed by Susheela in Delhi India [49].

22% children at RM showed well defined thyroid hormonal aberration with 11% depicting high TSH and normal FT$_3$ - FT$_4$: the first sign of thyroid malfunctioning known as “subclinical hypothyroidism”. 3% showed elevated FT$_3$ with normal FT$_4$ and TSH which is an indication of cellular resistance to FT$_4$ that can lead to “hypothyroidism” because active $T_3$ cannot get to the cell to do its job. 3% with high FT$_4$ - normal FT$_3$ TSH which are the first signs of “Hyperthyroidism” leads to an elevated metabolic rate: characterized by rapid heart rate, high blood pressure, weight loss, nausea and vomiting etc. 1.35% with low FT$_3$, high TSH and normal FT$_4$: the main indicators of “primary hypothyroidism and iodine deficiency disorder” resulting in lethargy, depression and weight gain in later stages of development. Identical hormonal abnormalities are also noticed in children of Dehli India exposed to excess fluoride consumption [49]. So all these facts are a part of the reason that why millions of children have fragile physical and mental health status in Punjab Pakistan.

In the present work a significant correlation was observed between serum fluoride and FT$_3$ ($r = 0.3$) FT$_4$ ($r = 0.35$) and TSH ($r = 0.8$); demonstrating how these hormonal production increase or decrease with increment or decline in serum fluoride. Therefore by controlling fluoride level in body fluids, disturbance in thyroid hormones can be significantly reduced. Singh et al. studied the same correlation in 8-15 years old children living in endemically fluorosis and non-fluorosis territories [29]. Also Wu found such serum thyroid correlation in rats [51].

When comparing various degrees of D.F (score 1-5) with their corresponding mean FT$_3$, FT$_4$, and TSH (Table 4), it was observed that FT$_3$ differs markedly between mild-severe while FT$_4$ and TSH between very mild-moderate and very mild – severe fluorosis Index respectively; supporting a significant relationship between these degrees of D.F and mean thyroid hormone concentrations. So this study could be helpful in predicting the magnitude of thyroid abnormalities in children merely from their dental status. Singh also reported similar differences in various scores of teeth fluorosis and their corresponding mean FT$_3$, FT$_4$ and TSH levels [29].

Although this was a comprehensive study there were some limitations that could not be overcome e.g.: 1. While the two fluorotic and non-fluorotic villages were selected for the comparison, inhabitants from both villages had higher than normal fluoride levels in urine and serum, indicating other potential sources of fluoride than water.

2. Fluoride measurement in liquid samples - water, urine and serum was performed using the same fluoride ion selective electrode; however the samples were kept till the final analysis and processed using quite different conditions.

3. Over fluoride consumption also leads to skeletal fluorosis along with effects on stomach lining, nausea and other complication which were not included in this study.

Due to limited resources and the availability of children for sampling, these constraints could not be avoided.

5 Conclusion

It is obvious from the results that the concentration of fluoride in drinking water samples is evidently higher than control samples. Consequently the level of fluoride in body fluids (urine and serum) is elevated; resulting in disturbance of thyroid hormone production and function, as it is the most fluoride sensitive organ in human body and has effect on every cell of human anatomy. Positive correlation between these parameters advocates the massive role of fluoride toxicity towards disintegration of hard tissues. 90% teeth deterioration and 22% thyroid hormones abnormalities in apparently health teenagers raise many questions about the health of the future generation in this region. So in light of the current investigations, it is strongly recommended that a detailed study should be carried out to mark the area with excessive fluoride in ground water along with mapping of this region according to fluoride concentration in water. Also a comprehensive health survey should be accomplished.
to investigate the victims of endemic fluorosis so that the suitable preventive measures can be taken rapidly.

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