Fluoride Alters Serum Elemental (Calcium, Magnesium, Copper, and Zinc) Homeostasis Along with Erythrocyte Carbonic Anhydrase Activity in Fluorosis Endemic Villages and Restores on Supply of Safe Drinking Water in School-Going Children of Nalgonda District, India

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Abstract
The present study aimed to determine the serum trace elements (copper (Cu), zinc (Zn), calcium (Ca), magnesium (Mg)) along with erythrocyte carbonic anhydrase (CA) activity and effect of intervention with safe drinking water for 5 years in the school children of fluorosis endemic area. For this purpose, three categories of villages were selected based on drinking water fluoride (F): Category I (control, F = 1.68 mg/L), category II (affected F = 3.77 mg/L), and category III (intervention village) where initial drinking water F was 4.51 mg/L, and since the last 5 years, they were drinking water containing < 1.0 mg/L F. The results revealed that urinary F was significantly ($P$ < 0.05) higher in category II compared to categories I and III. A significant ($P$ < 0.05) increase in serum Cu and Mg was observed in category II compared to category I. Serum Zn and Ca was significantly ($P$ < 0.05) decreased in categories II and III compared to category I. The erythrocyte CA activity was decreased in the category II compared to category I. However, in the category III, erythrocyte CA activity was comparable to the control group. In conclusion, F exposure altered elemental homeostasis which has restored to some extent on intervention by safe drinking water for 5 years in school-going children.

Keywords Calcium · Copper · Fluoride · Fluorosis · Intervention · Zinc

Introduction
Exposure to high levels of F over a long period causes damage to osseous tissue, which results in dental and skeletal fluorosis [1]. Minerals and trace elements are required for both physiological and biochemical functions. Many disorders of the body are related to the altered imbalance of serum trace elements. However, deficiency or excess of these trace elements known to have adverse effects and some toxic elements disturbs the essential trace element homeostasis in the human body. Excess fluoride alters the absorption of some elements from the gastrointestinal tract; hence, its excess exposure leads to deficiencies of iron, copper, zinc, and manganese [2–5]. Calcium (Ca) and magnesium (Mg) are major bone minerals; F alters their levels in bone and serum [6].

Zinc acts as a cofactor for carbonic anhydrase (CA) as well as antioxidant enzymes. Carbonic anhydrase plays an important role in acid base balance, pH of the blood, bone resorption, urea genesis, gluconeogenesis, and production of body fluids [7, 8]. Earlier studies have suggested that the disruption of carbonic anhydrase activity with subsequent change in ionic homeostasis was associated with F toxicity [9, 10]. In various species, including rodents, domestic fowl, calves, lambs, and humans, it has been found that dietary zinc deficiency significantly reduces erythrocyte carbonic anhydrase activity [11–16]. However, studies pertaining to the changes in the serum Zn, Cu, Ca, and Mg levels along with erythrocyte carbonic anhydrase activity in fluoride-affected children and effect of intervention through safe drinking water on the same have not been studied. Hence, the present study aimed to assess serum Zn, Cu, Ca, and Mg levels along with the CA activity in fluoride endemic school-going children and its reversal through safe drinking water.

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Materials and Methods

Study Design

The cross-sectional study was undertaken in school-going children of fluoride endemic district (Nalgonda). All the participants were born and had resided in the area since their birth. Written informed consent was obtained from their School Head Master. Data collected in the study was managed to ensure the protection of individual rights and maintain confidentiality. This study was approved by the Institutional Human Ethics Committee, National Institute of Nutrition, (ICMR) Hyderabad, India (IEC No. 15/II/2014).

Based on mean drinking water fluoride levels, the villages of the district were categorized into category I control ($N = 645$; boys = 306; girls = 339, $F = 0.87 \pm 0.108$ mg/L), category II affected ($N = 416$; boys = 213; girls = 203, $F = 3.77 \pm 0.197$ mg/L), and category III intervention groups ($N = 327$; boys = 140; girls = 187, $F < 1.0$ mg/L). In the intervention category, initial drinking water $F$ was $4.51 \pm 0.077$ mg/L, which was intervened with low $F$ drinking water $< 1.0$ mg/L (reverse osmosis) for 5 years.

Clinical Examination

A total of 1388 school children from different categories were screened for dental fluorosis by clinical examination of each student, by a dental specialist (from local hospital) in the common room of the school, with the subject seated in a chair in bright daylight. The dental specialist used a mirror and a sterile dental probe for oral examination. The presence and severity of dental fluorosis were recorded. The diagnosis of fluorosis was determined using the modified Dean index criteria [17] with a range of grade 0 (normal), grade I (very mild), grade II (mild), grade III (moderate), and grade IV (severe). The index was administered to all permanent teeth in the oral cavity.

Collection of Water, Urine, and Blood Samples:

Water Collection A total of 227 drinking water samples from bore wells/hand pumps (used for drinking and cooking) from the selected villages were collected in dry season (February to April) in 50-mL pre-cleaned, dry high density plastic bottles and stored at room temperature until further use.

Urine Collection First morning spot urine samples were collected from selected school children in 25-mL plastic bottles under toluene and stored at $-4$ °C until further use.

Blood Collection Five milliliter blood was collected from subsample and transported to the laboratory under cold condition. Serum was separated by centrifugation and stored at $-80$ °C until further use.

Analysis of Water and Urine

Fluoride in water and urine was analyzed using fluoride ion-specific electrode, Orion 9609 [18], which was calibrated with fresh, serially diluted standard solutions.

Erythrocyte Carbonic Anhydrase Activity

Carbonic anhydrase (CA) activity in erythrocytes was carried out according to the electrometric method of Wilbur and Anderson [19].

In brief, erythrocytes were hemolyzed in cold distilled water and centrifuged at 10,000 rpm maintaining 4 °C for 15 min and the clear hemolysate was assayed for CA activity. The enzyme activity was calculated as units/g Hb in erythrocytes.

Serum Elemental Analysis

The serum Ca, Mg, Zn, and Cu were estimated by Atomic absorption spectrophotometer (Varian, SpectraAA) using standard procedure.

Statistical Analysis

The statistical analysis was carried out on SPSS 19.0, and the descriptive statistics were reported in mean ± SD. A $P < 0.05$ was considered significant at 5% level of significance.

Results

Dental Fluorosis

The clinical examination of dental fluorosis grade 1 to 3 in different categories is given in Fig. 1a. The results revealed that the severity of dental fluorosis was highest in category II and category III. It was found that grades I and II were highest in categories II and III compared to category I. However, about 20% children were affected with grade I in category I, whereas children were affected with grade III in categories II and III.

Figure 1b shows the age-wise differences in total dental fluorosis prevalence in different categories. In the age group 8–10 years, the prevalence of dental fluorosis was higher in categories II and III. However, it was also observed that the prevalence was lower in category III as compared to category II. The children aged 11–13 years were affected maximum in category III compared to category II. Likewise, the prevalence of dental fluorosis was more or less equal in categories II and III in the age group of > 13 years (Fig. 1b).
Urinary Fluoride

There was a significant increase in UF in the category II (affected group) as compared to the control group. However, in the category III (intervention group), the urinary fluoride was decreased and comparable with category I (Table 1).

Carbonic Anhydrase Activity

The erythrocyte CA activity was decreased in the category II compared to control group which was not statistically significant. However, in the category III, the erythrocyte CA activity was increased and comparable to the control group (Fig. 2).

Serum Calcium, Magnesium, Zinc, and Copper

There was a significant decrease in the serum Ca levels in the category II as compared to category I, whereas there was a significant increase in serum Ca in the category III (intervention group) compared to category II (Table 1). A significant increase in serum Mg levels in the categories II and III was observed compared to category I. It was also evident that there was significant decrease in serum Ca/Mg ratio in the categories II and III compared to category I. In category III (intervention group), there was a significant increase in serum Ca/Mg ratio compared to category II (Table 1). The serum zinc levels were significantly decreased in the category II compared to category I. However, the serum zinc levels were
increased in the category III group compared to category II, however, which was not comparable with category I. The serum copper level was significantly increased in the category II as compared to category I. However, in the intervention group, the serum copper level was decreased and was comparable to category I (Table 1). The serum Cu/Zn ratio was significantly increased in the category II as compared to category I. However, in the intervention group, the Cu/Zn ratio was comparable to control (Table 1).

**Discussion**

In the present study, we investigated the serum levels of trace elements Cu and Zn and minerals including Ca and Mg in school-going children of fluorosis endemic villages. The children in categories II and III were more affected by dental fluorosis; it might be due to higher fluoride in their drinking water as compared to category I, and the same was evident in earlier studies [20–22]. Even higher dental grading (grades II and III) was observed in category II compared to category I. However, we have also observed grade I dental fluorosis (about 20%) in category I (control group) which might be due to exposure to fluoride through tea [23, 24], and food which is being transported from fluoride endemic villages to non-endemic villages. Similar results were observed in one of our earlier studies, where about 10% dental fluorosis was seen at fluoride level 0.73 ppm (mg/L) in drinking water [25]. However, the dental fluorosis was reduced in 8–10 years of children in category III which might be due to intervention through supply of safe drinking water for 5 years. It was also observed that the reduction in dental fluorosis in other age groups (11–13 and > 13) was not noted may be because of exposure to high fluoride during their eruption of tooth before

![Fig. 2 Erythrocyte carbonic anhydrase activity in children of different categories](image-url)
the initiation of intervention through safe drinking water, and the same is reported in earlier studies [26–28].

There was a significant increase in UF in the category II as compared to category I, which was also observed in the earlier studies conducted [29–31]. However, in the category III, the urinary fluoride was significantly reduced compared to category II. This might be due to supply of safe drinking water for the last 5 years. In category III, the UF was reduced but it was not comparable to control, which may be due to exposure to fluoride through food as it is a fluoride endemic village.

The reduction in erythrocyte CA activity in the category II compared to category I may be due to the direct effect of fluoride on CA activity [9, 10]. Zinc is the cofactor for CA enzyme which is essential for its activity [11, 14] and it has been observed that the excretion of zinc is enhanced in the fluorotic area [32], which may be the other reason for the reduction in the CA activity in category II.

Hypocalcemia is a well-known cause of endemic skeletal fluorosis in children [32]. In a prevalence study of 260 school children living in an endemic fluorosis area in South Africa (water fluoride content 8–12 ppm), hypocalcemia was reported [37] and the same was observed in the present study in category II. However, the serum Ca levels were restored and were comparable to category I, which may be due to supply of safe drinking water in category III. In the present study, we found serum Mg was increased since Mg is the antagonist to Ca in category II.

We found significant reduction of Zn in serum of category II children may be due to enhanced urinary excretion of zinc in these fluoride-affected villages [33]. Zinc is essential for protein metabolism, as well as for cell growth, cell division, and cell function, and it is required in sufficient quantity for normal physiological function [34]. The overall worldwide deficiency of Zn is reported more than 20% [35] and it may be more in fluoride endemic areas. In fluoride poisoning cases, plasma proteins like albumin decrease [36] and albumin transports Zn in its bound form. The decrease in serum Zn levels may be due to reduction in albumin levels in fluorosis-affected subjects.

An increase in Cu levels in category II as compared to category I and intervention group category III was observed. Copper content was significantly higher in the degeneration and ossification. The increase of Cu and decrease in Zn content due to their antagonism to each other suggest the vigorous anabolism of collagen in bone degeneration [38], which is normally seen in fluoride-affected children. Copper is an important cofactor of lysyl oxidase, which is the key enzyme in anabolism of collagen. In the fluorotic area, there is a disturbance in collagen synthesis which may be the reason of bone deformities [39, 40]. In the earlier study [41], it has been reported that the high Cu/Zn ratio leads to lower BMD, elevated PTH, and low 25 OHD. We also observed high Cu/Zn ratio in category II (however, we have not done BMD analysis). In our earlier study, we also found elevated PTH and low 25OHD [42] in children from fluoride endemic villages which corroborates with earlier study [41]. However, increased serum Cu and Mg in fluoride affected children needs in-depth study.

In conclusion, fluoride exposure resulted in the alterations in the bone-related elements which may affect the bone formation and may lead to bone deformities in fluoride endemic villages. An inhibition of erythrocyte CA activity disturbs the acid base balance which may lead to decrease in urinary clearance of fluoride. Intervention with safe drinking water for 5 years in category III has shown positive effect in reversal of fluoride toxicity.

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Compliance with Ethical Standards

Written informed consent was obtained from their School Head Master. Data collected in the study was managed to ensure the protection of individual rights and maintain confidentiality. This study was approved by the Institutional Human Ethics Committee, National Institute of Nutrition, (ICMR) Hyderabad, India (IEC No. 15/II/2014).

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