# ANTIOXIDANT DEFENSE SYSTEMS IN RED BLOOD CELL LYSATES OF MEN WITH DENTAL FLUOROSIS LIVING IN TAMIL NADU, INDIA

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**SUMMARY: The status of lipid peroxidation (LPO) and antioxidants was studied in red cell blood lysates of male subjects, aged 41–50, living in an endemic fluorosis area, Vellore district, Tamil Nadu, India. The men were divided into four groups: 1) normal healthy individuals (n=10); 2) individuals with mild dental fluorosis (n=13); 3) individual with moderate dental fluorosis (n=8); 4) individuals with severe dental fluorosis (n=7). In the groups with dental fluorosis, the concentration of thiobarbituric acid reactive substances (TBARS) was higher in the red blood cell lysates along with a concomitant decrease in the levels of both enzymatic and nonenzymatic antioxidants. Statistical analysis of all the group data revealed that increased lipid peroxidation and altered antioxidant status induced by fluoride were strongly associated with the prevalence of dental fluorosis.**

Keywords: Antioxidant status; Dental fluorosis; Endemic fluorosis area; Free radicals; Male subjects; Oxidative stress; Red blood cell lysates; Tamil Nadu, India.

## INTRODUCTION

Fluorosis is a serious health problem in many parts of the world caused by excessive intake of fluoride (F) present in water, food, and air. In general, drinking water is the main carrier of F. The most obvious early toxic effects of F in humans are dental and later skeletal fluorosis, both of which are endemic in areas with elevated F exposure.[1] F is known to cross cell membranes and enter soft tissues. Impairment of soft tissue function has been demonstrated in F-intoxicated animals.[2,3] In addition, F has been shown to inhibit many enzymes such as those involved in the pentose phosphate pathway, antioxidant defense systems, and the myosin ATPase path.[4,5]

Reactive oxygen species (ROS) are implicated as important pathologic mediators in many disorders. Increased generation of ROS and enhanced lipid peroxidation are considered responsible for the toxicity of wide range of compounds.[6] Increased lipid peroxidation and altered levels of antioxidants in the blood of children with endemic skeletal fluorosis[7] and in the liver of young rats exposed to high levels of F in drinking water during the early stages of life[8] have been observed. In the present study the status of lipid peroxidation and antioxidant defense parameters in red blood cell lysates of human male subjects with different degrees of dental fluorosis residing in Vellore district of Tamil Nadu, India were evaluated.

## MATERIALS AND METHODS

*Collection of water samples:* Ground water sampling was carried out systematically from dug wells, shallow hand pump wells, and overhead tanks

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throughout the entire area of Poongulam and Sowedakuppam panchayats of Tirupattur Subdivision and Marimanikuppam and Narasingapuram panchayats of Alangayam Subdivision of Vellore district in the northern part of Tamil Nadu, India. The water samples were collected in pre-cleaned 500-mL polyethylene bottles with air-tight lids. The F concentration in the water and also in blood samples was determined with a F ion selective electrode on an Orion ion analyser (Orion Model 720 pH-ISE Fluorimeter, USA).

*Subjects:* After some preliminary selection of subjects for examination, a total of 391 children and adults living in the water sample area were examined as per norms of the World Health Organization[9] for dental fluorosis. From these individuals, 28 of 30 males between the ages of 41 and 50 exhibiting mild, moderate, or severe dental fluorosis, all of whom who gave formal consent for collection of blood samples, were selected for further biochemical studies. Ten age-matched healthy males residing in other parts of Alangayam Subdivision of Vellore district, with permissible levels of F in the drinking water (< 1.0 ppm) served as controls.

The four groups of men selected for study were: Group I: normal healthy males (Control; n=10); Group II: males with mild dental fluorosis (n=13); Group III: males with moderate dental fluorosis (n=8); Group IV: males with severe dental fluorosis (n=7).

Blood samples of the patients were collected by venipuncture, and the plasma was separated by centrifugation at 1500 g for 15 min using EDTA as an anticoagulant. Blood samples were collected without anticoagulant for the estimation of serum F level. Red blood cell lysate was prepared[10] for biochemical estimations. The total erythrocyte count[11] and blood corpuscles[12] were determined. Haemoglobin (Hb) was also measured.[13]

Red blood cell lysate was used to estimate malondialdehyde (MDA),[14] superoxide dismutase (SOD),[15] glutathione peroxidase (GPx),[16] catalase (CAT),[17] and glutathione-S-transferase (GST).[18] Reduced gluathione was also estimated.[19]

Plasma was used to estimate the levels of ascorbic acid,[20] α-tocopherol,[21] retinol,[22] ceruloplasmin,[23] and uric acid.[24]

*Statistical Analysis*: All the grouped data were statistically evaluated with SPSS/10 software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference (LSD) test. P values of less than 0.05 were considered to indicate statistical significance. All the results are expressed as Mean ± SD for the number of individuals in each group.

## RESULTS

As seen in Table 1, the mean concentration of F in the water samples exceeded the national recommended maximum of 1.0 ppm. The location of the study area in northern Tamil Nadu (Figure 1) shows that the prevalence of fluorosis is higher in Tirupathur and Alangayam Subdivision of Vellore district. The incidence and severity of dental fluorosis among different age and sex groups of Narasingapuram panchayat were greater in males than females (Table 2).

**Table 1.** Fluoride concentration in the groundwater of Vellore district, Tamil Nadu, India

| Panchayat | No. of samples | Fluoride concentration range (mg/L) | Mean fluoride concentration (mg/L) |
|---|---|---|---|
| Poongulam | 29 | 0.90 –3.86 | 1.93 |
| Sowdekuppam | 16 | 0.97 – 3.05 | 1.82 |
| Marimanikuppam | 15 | 1.04 – 3.24 | 1.58 |
| Narasingapuram | 9 | 2.32 – 4.59 | 3.35 |

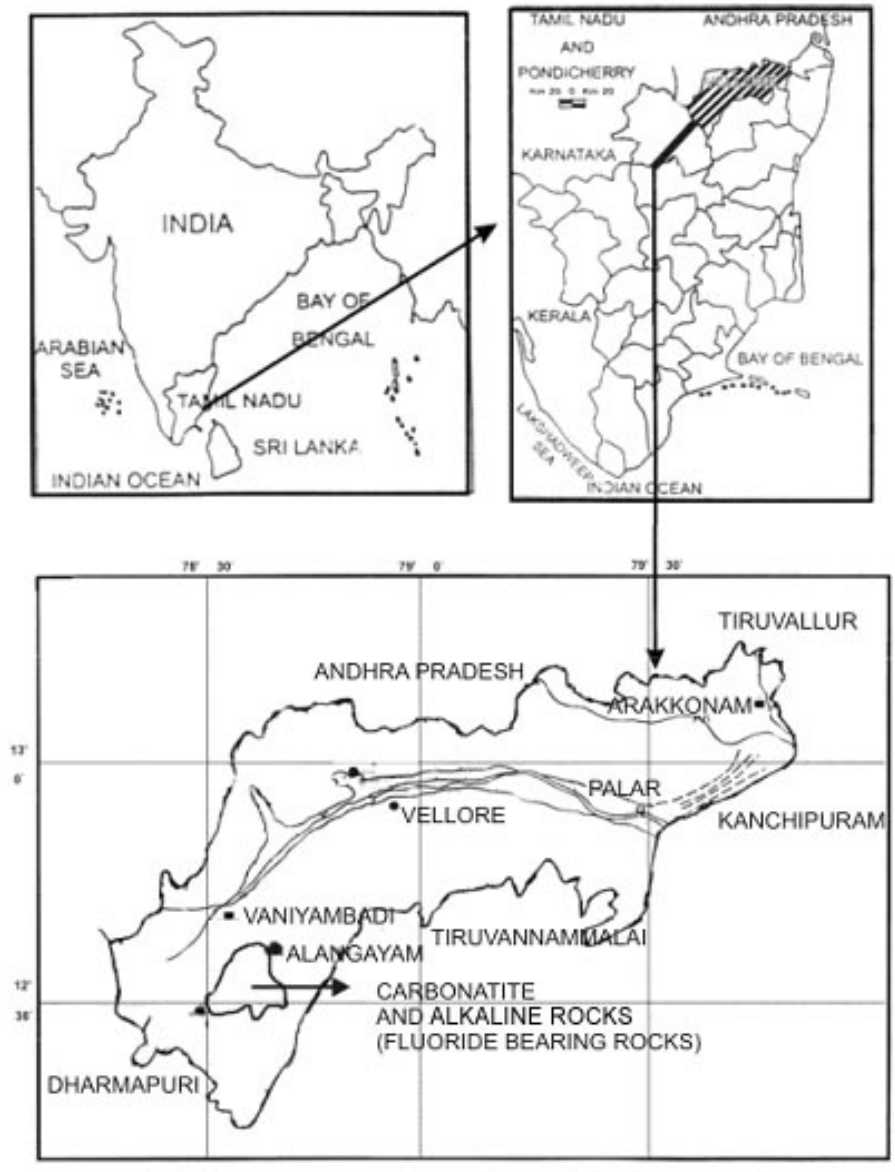


**Figure 1.** Location map of study area

**Table 2.** Incidence and severity of dental fluorosis in Narasingapuram panchayat of Alangayam Subdivision, Vellore district, Tamil Nadu, India

| Age group, years | No. of individuals examined | Total number Affected | No. with dental fluorosis | | |
|---|---|---|---|---|---|
| | | | Mild | Moderate | Severe |
| Males | | (72.0)[a] | | | |
| 7 – 9 | 21 | 13 (61.9) | 8 (61.5) | 2 (15.3) | 3 (23.0) |
| 10 – 12 | 19 | 16 (84.2) | 8 (50.0) | 5 (31.3) | 3 (18.8) |
| 13 – 15 | 23 | 11 (47.8) | 7 (63.6) | 3 (27.3) | 1 (9.0) |
| 16 – 20 | 25 | 13(52.0) | 6 (46.2) | 4 (30.8) | 3 (23.0) |
| 21 – 30 | 15 | 10 (66.7) | 6 (60.0) | 3 (30.0) | 1 (10.0) |
| 31 – 40 | 27 | 20(74.0) | 10 (50.0) | 6 (30.0) | 4 (20.0) |
| 41 – 50 | 30 | 28(93.3) | 13 (46.4) | 8(28.6) | 7(25.0) |
| > 50 | 44 | 36 (81.8) | 20 (55.6) | 13 (36.1) | 3 (18.3) |
| Total | 204 | 147 | 78 | 44 | 25 |
| Females | | (50.8) [a] | | | |
| 7 – 9 | 18 | 7 (38.8) | 4 (57.1) | 2 (28.6) | 1 (14.2) |
| 10 – 12 | 16 | 8 (50.0) | 4 (50.0) | 3 (37.5) | 1 (12.5) |
| 13 – 15 | 20 | 9 (45.0) | 5 (55.6) | 3 (33.3) | 1 (11.1) |
| 16 – 20 | 21 | 11 (52.4) | 6 (54.5) | 4 (36.4) | 1 (9.0) |
| 21 – 30 | 19 | 8 (42.1) | 5 (62.5) | 2 (25.0) | 1 (12.5) |
| 31 – 40 | 24 | 10 (41.6) | 5 (50.0) | 4 (40.0) | 1 (10.0) |
| 41 – 50 | 30 | 16 (53.3) | 7 (43.7) | 6 (37.5) | 3 (18.7) |
| > 50 | 39 | 26 (66.6) | 15 (57.6) | 8 (30.7) | 3 (11.5) |
| Total | 187 | 95 | 51 | 32 | 12 |

[a]Figures in parenthesis indicate percentages. Mild fluorosis: the white opaque areas in the enamel of the teeth are more extensive; Moderate fluorosis: all enamel surfaces affected and brown stain is frequently a disfiguring feature; Severe fluorosis: discrete confluent pitting with brown stains widespread and teeth often present a corroded appearance.

The total RBC count, WBC count, and Hb content were significantly lower ($p<0.05$) in males with dental fluorosis (Groups II–IV) than in the controls (Group I). Serum F levels in Groups II–IV were significantly higher ($p<0.05$) than in Group I (Table 3).

**Table 3.** Serum fluoride and haematological parameters in control and fluorotic male subjects 41–50 years old in Narasingapuram panchayat of Alangayam Subdivision, Vellore district, Tamil Nadu, India

| Subjects | RBC ($10^6/\mu m^3$) | WBC (No./$mm^3$) | Hb (g/dL) | Serum fluoride (mg/L) |
|---|---|---|---|---|
| Group I (n=10) | 4.35 ± 0.45 | 7500 ± 352 | 14.42 ± 1.52 | 0.031 ± 0.002 |
| Group II (n=13) | 3.06 ± 0.26* | 6420 ± 280* | 11.04 ± 1.16* | 0.043 ±0.003* |
| Group III (n=8) | 2.73 ± 0.18* | 6110 ± 298* | 10.89 ± 0.09* | 0.058 ±0.004* |
| Group IV (n=7) | 2.16 ± 0.11* | 5630 ± 273* | 9.04 ± 0.8* | 0.067 ±0.005* |
| Reference values | 4.5-6.5[35] | 4,500-11,000[36] | 13.5-17.5[37] | 0.020-0.080[38] |

Values are expressed as mean ± SD.
*Values are statistically significant ($p<0.05$) compared with Group I.

Lipid peroxidation was greater ($p<0.05$) in the red blood cells of subjects with dental fluorosis (Groups II–IV) as evidenced by their elevated MDA levels (Figure 2 and Table 4). Significantly lower levels ($p<0.05$) were observed in the activities of SOD, catalase, and GST in the red blood cell lysates of Groups II–IV (Table 4). On the other hand, the activity of GPx was significantly higher ($p<0.05$) in Groups II–IV than in the control Group I.

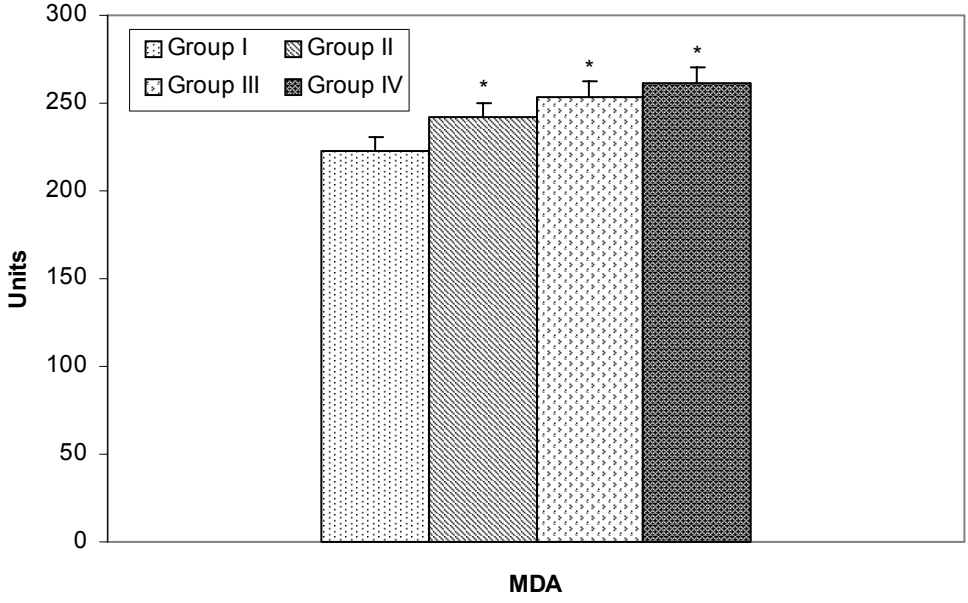


**Figure 2.** Malondialdehyde (MDA) levels in red blood cell lysates of control and fluorotic male subjects 41-50 years old in Narasingapuram panchayat of Alangayam subdivision, Vellore district, Tamil Nadu, India. *Values are expressed as mean ± SD and are statistically significant at $p<0.05$ compared to Group I. Units are in nmoL/mL RBC. Reference value: 240 - 270 nmoL/mL RBC.[37]

**Table 4.** Activities of superoxidedismutase (SOD), catalase, glutathione peroxidase GPx), and glutathione-S-transferase (GST) in red blood cell lysates of control and fluorotic male subjects 41–50 years old in Narasingapuram panchayat of Alangayam Subdivision, Vellore district, Tamil Nadu, India

| Subjects | SOD (U/mL RBC) | Catalase (U x $10^{-4}$/mL RBC) | GPx (U/g Hb) | GST (U/mL RBC) |
|---|---|---|---|---|
| Group I (n=10) | 1551 ± 62.56 | 1.20 ± 0.12 | 31.39 ± 1.87 | 1.86 ± 0.32 |
| Group II (n=13) | 1473 ± 56.12* | 0.98 ± 0.10* | 33.86 ± 1.89* | 1.45 ± 0.28* |
| Group III (n=8) | 1446 ± 46.21* | 0.87 ± 0.10* | 36.53 ± 1.84* | 1.36± 0.24* |
| Group IV (n=7) | 1435 ± 43.11* | 0.72 ± 0.08* | 38.01 ± 1.72* | 1.16 ± 0.21* |
| Reference values | ~1500[39] | ~ 3.57[39] | ~30[7] | ~1.5[39] |

Values are expressed as mean ± SD.
*Values are statistically significant ($p<0.05$) compared with Group I.

The GSH level and ceruloplasmin contents were significantly ($p<0.05$) lower in the fluorotic subjects (Group II–IV) than in the Group I control subjects (Figures 3 and 4). The level of plasma ascorbic acid was significantly ($p<0.05$) elevated in

the fluorotic subjects compared to the normal control subjects (Table 5), whereas, the content of uric acid, α-tocopherol, and retinol in plasma of fluorotic subjects was lower ($p<0.05$).

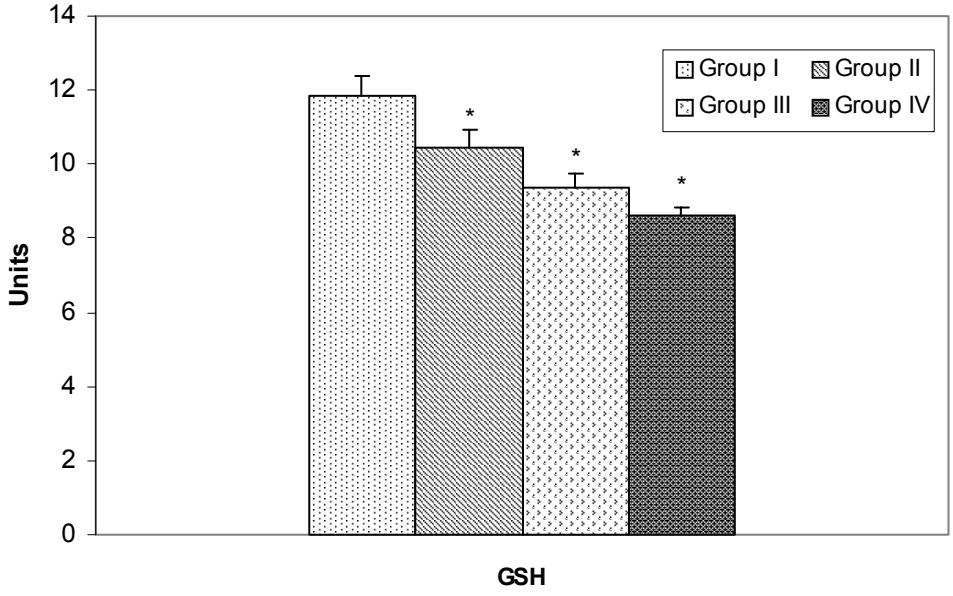


**Figure 3.** Glutathione (GSH) levels in red blood cell lysates of control and fluorotic male subjects 41-50 years old in Narasingapuram panchayat of Alangayam subdivision, Vellore district, Tamil Nadu, India. *Values are expressed as mean ± SD and are statistically significant $p<0.05$ compared to Group I. Units μmoL/mL RBC. Reference value: ~ 12.96 μmoL/mL RBC[7]

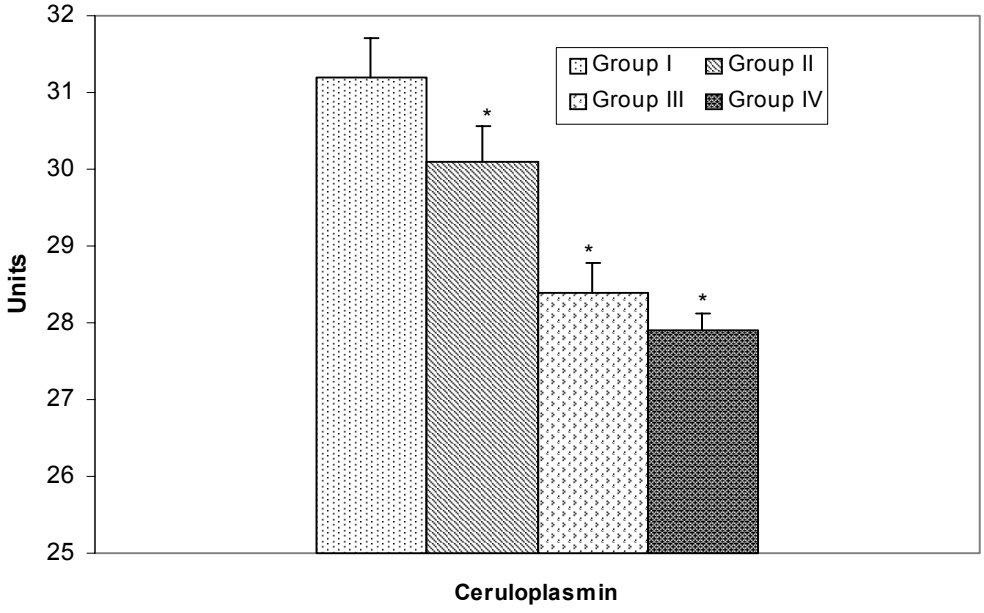


**Figure 4.** Ceruloplasmin levels in the plasma of control and fluorotic male subjects 41-50 years old in Narasingapuram panchayat of Alangayam subdivision, Vellore district, Tamil Nadu, India. *Values are expressed as mean ± SD and are statistically significant at $p<0.05$ compared to Group I. Units, mg/dL plasma. Reference value: 25 - 45 mg/dL plasma.[36]

**Table 5.** Levels of ascorbic acid, uric acid, α-tocopherol, and retinol in the plasma of control and fluorotic male subjects 41–50 years old in Narasingapuram panchayat of Alangayam Subdivision, Vellore district, Tamil Nadu, India

| Subjects | Ascorbic acid (mg/dL) | Uric acid (mg/dL) | α Tocopherol (μmol/dL) | Retinol (μg/dL) |
|---|---|---|---|---|
| Group I (n=10) | 0.71 ± 0.12 | 2.82 ± 0.21 | 16.3 ± 0.12 | 28.3 ± 2.6 |
| Group II (n=13) | 1.71 ± 0.36* | 2.28 ± 0.16* | 13.4 ± 0.08* | 23.6 ± 1.9* |
| Group III (n=8) | 1.84 ± 0.42* | 2.09 ± 0.12* | 11.6 ± 0.06* | 21.5 ± 1.6* |
| Group IV (n=7) | 1.98 ± 0.48* | 1.93 ± 0.07* | 10.2 ± 0.04* | 17.2 ± 1.2* |
| Reference values | ~0.62[7] | ~ 2.95[7] | 12-48[37] | 40-140[37] |

Values are expressed as mean ± SD.
*Values are statistically significant ($p<0.05$) compared with Group I.

## DISCUSSION

The mean F content of the drinking water sources of Narasingapuram panchayat of Vellore district, Tamil Nadu, was higher than the recommended permissible level of 1 ppm according to the World Health Organisation.[9] The relationship between the levels of F in drinking water and the incidence of dental fluorosis varies from place to place. Enamel mottling at 0.5 ppm and 0.9–1.0 ppm F levels has been reported.[25] Grade 2 dental fluorosis prevalences of 25.6% and 84.4% in children at F levels of 1.4 ppm and 6.04 ppm, respectively, have been observed.[26] Among 391 children and adults examined in our water sample area, 72.0% of males and 50.8% of females were affected with dental fluorosis. Clearly, males showed a higher prevalence of dental fluorosis than females.

In an area of endemic fluorosis, decreased RBC and Hb have been observed.[27] F toxicity also affects the immune system which, in turn, reduces the total WBC count during fluorosis. Over the last several years, numerous reports from China, India, and elsewhere indicate that F in varying concentrations induces free radical toxicity in both animals and in people living where there is endemic fluorosis.[28] There is much evidence that superoxide free radicals and lipid peroxidation play an important role in fluorosis.[28] Elevated MDA in fluorotic patients could be due to F-induced generation of reactive oxygen species (ROS). F has been demonstrated *in vivo* and *in vitro* to cause increased lipid peroxidation in erythrocytes of humans[29] and in blood and tissues of experimental animals.[30] Increase in the accumulation of MDA and conjugated dienes in the cells can result in cellular degradation, biochemical and functional changes, and even cell death in mice.[5]

Studies have shown a decrease in the activities of SOD, CAT, and GPx in people living in areas of endemic fluorosis[31] and in the tissues of experimental animals subjected to F toxicity.[30] These effects may be due to oxidative stress exerted by F intoxication. The decreased levels of GSH observed in red blood cells may be due to increased utilization of GSH by GPx in detoxification of hydrogen peroxide generated by F-induced oxidative stress. In children with endemic skeletal

fluorosis, decreased GSH levels and increased GPx activity in red blood cells have been noted.[7]

Increased plasma ascorbic acid levels from increased utilization and/or mobilization of ascorbic acid from storage in response to F-induced stress has been observed in experimental animals[32] and in humans.[7] The decrease in the level of uric acid suggests that F toxicity may involve a reduction of certain intrinsic scavengers resulting in a increased vulnerability to oxygen ($O_2$) free radical toxicity. The antioxidant nature of retinol can be attributed to its activity of trapping free radicals.[33] The decrease in the retinol in the fluorotic subjects may be due to the oxidative stress produced during F toxicity. α-Tocopherol is more lipophilic and a potent antioxidant. It protects cellular components against peroxidative damage by a free-radical scavenging mechanism or as a constituent of the membrane.[34] The decrease in α-tocopherol in the fluorotic subjects may therefore be due to increased free radical production.

In conclusion, this work has shown that long-term elevated F intake by individuals through drinking water not only increases serum F levels but also lipid peroxidation activity associated with free-radical-mediated oxidative stress as demonstrated by increased levels of MDA and decreased levels of both enzymatic and nonenzymatic antioxidants.

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