TOXICOLOGICAL SCIENCES **72,** 363–368 (2003)
DOI: 10.1093/toxsci/kfg030

Antioxidant Defense System and Lipid Peroxidation in Patients with Skeletal Fluorosis and in Fluoride-Intoxicated Rabbits

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Received September 19, 2002; accepted January 3, 2003

Fluorosis is a serious public health problem in many parts of the world where drinking water contains more than 1 ppm of fluoride. The main manifestations of skeletal fluorosis are crippling bone deformities, spinal compressions, and restricted movements of joints. Although fluorosis is irreversible, it could be prevented by appropriate and timely intervention through understanding the process at biochemical and molecular levels. As in the case of many chronic degenerative diseases, increased production of reactive oxygen species (ROS) and lipid peroxidation has been considered to play an important role, even in the pathogenesis of chronic fluoride toxicity. However, there is inconclusive proof for an altered oxidative stress and antioxidant balance in fluorosis, and the existing data are not only conflicting but also contradictory. In the present communication we have evaluated the antioxidant defense system (both enzymatic and nonenzymatic) and lipid peroxidation in both humans from an endemic fluorosis area (5 ppm fluoride in the drinking water) and in rabbits receiving water with 150 ppm of fluoride for six months. There was no significant difference in lipid peroxidation, glutathione, and vitamin C in the blood of human fluorotic patients and fluoride-intoxicated rabbits as compared to respective controls. Neither were there any changes in the activities of catalase, superoxide dismutase, glutathione peroxidase, or glutathione *S*-transferase in the blood due to fluoride intoxication (of rabbits) or fluorosis in humans. The results together do not subscribe to oxidative stress theory in fluorosis. Thus, in the absence of clear proof of oxidative damage and to counter toxic effects of fluoride through supplementation of antioxidants, extensive investigations are needed to conclusively prove the role of oxidative stress in skeletal fluorosis.

Key Words: **fluorosis; fluoride intoxication; RBC; rabbits; humans; oxidative stress; antioxidant system.**

Although fluoride is considered an essential trace element in view of its ascribed role in imparting stability to teeth and bone, chronic exposure to (>1 ppm) fluoride is known to cause toxic effects (Das, 1996; Krishnamachari, 1986). Fluorosis is one of the manifestations of chronic poisoning from long-term exposure to high levels of fluoride, and is a serious health problem in many parts of the world where drinking water contains more than 1–1.5-ppm of fluoride (Susheela, 1999; WHO, 1984). An estimated 62 million people in 17 states in India are affected with dental and skeletal fluorosis (Susheela, 1999). Fluorosis due to occupational exposure to fluoride, such as in aluminum smelting and in glass industries and the manufacture of fertilizers, is reported (Susheela, 1999; WHO, 1984). A variant of the severe form of skeletal fluorosis, termed genu valgum (knock–knee syndrome), has been reported from certain endemic areas including India (Krishnamachari and Krishnaswamy, 1973). Young and adolescent people were found to be the chief victims of this syndrome.

Fluorosis is irreversible, but preventable by appropriate and timely intervention. Therefore, a greater understanding, at biochemical and molecular levels, of the disease progression is very important. Interaction between fluoride, nutritional status, dietary habits, environmental factors, and the body's response to ingested or inhaled fluoride are important in understanding the nature of the disease. For example, earlier studies carried out on humans at this institute showed that sorghum-based diet retained more fluoride than rice-based diet (Krishnamachari, 1976; Lakshmaiah and Srikantia, 1977). Further studies on experimental rats also confirmed these findings (Lakshmi and Lakshmaiah, 1999).

Biochemical changes in the composition of bone, urine, and plasma, and some hormonal changes in fluorosis have been reported by many researchers (reviewed in Das, 1996; Krishnamachari, 1986). Higher concentrations of fluoride are known to affect collagen synthesis and bone mineralization (Krishnamachari, 1986; Goldhaber, 1967; Harrison *et al.*, 1990). In addition, fluoride has been shown to inhibit many enzymes such as those involved in the pentose pathway, antioxidant defense system, and the myosin-ATPase path (Carlson and Suttie, 1966; Park *et al*., 1999; Vani and Reddy, 2000). Furthermore, fluoride is also known to cross the cell membrane and enter soft tissues. Impairment of soft-tissue function has been demonstrated in fluoride-intoxicated animals (Mullenix *et al*., 1995; Singh, 1984; Vani and Reddy, 2000). Fluoride affects the brain and muscle by inhibiting some enzymes associated with energy production and transfer, membrane transport, and synaptic transmission (Krishnamachari and Krishnaswamy, 1973; Shashi *et al*., 1994; Vani and Reddy, 2000).

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Since reactive oxygen species (ROS) are implicated as important pathologic mediators in many disorders, various studies have investigated whether oxidative stress and lipid peroxidation are involved in the pathogenesis of chronic fluorosis. The results of those studies are conflicting and contradictory to one another. A decrease in the activity of free-radical scavenging enzymes, SOD and GPx, was found in people living in areas of endemic fluorosis (Li and Ca, 1994). A similar inhibitory effect of fluoride on SOD in germinating mung-bean seedlings support the above findings and indicate the possibility of greater toxicity if fluoride can impair the free-radical scavengers (Rzenski *et al*., 1998). Recently, it was reported in children aged 3 to 10 years with endemic fluorosis that there was an increased oxidative stress based on increased MDA, ascorbic acid, GPx activity, and decreased GSH levels (Shivarajashankara *et al*., 2001b). Based on similar observations in fluoride-intoxicated rats, which showed increased MDA, ascorbic acid, GPx, and GST, although GSH levels were increased (Shivarajashankara *et al*., 2001a), it is proposed that there is an increased oxidative stress in skeletal fluorosis (Shivarajashankara *et al*., 2001a,b). Contrary to decreased GSH and unaltered SOD in children with endemic fluorosis, there was an increase in GSH and a decrease in SOD in the red blood cells (RBC) of fluoride-intoxicated rats (Shivarajashankara *et al*., 2001a,b). In another study, decreased GST, SOD, and catalase activities in rat brain upon ingestion of sodium fluoride (20 mg/kg body weight/day, ip) for 14 days were observed (Vani and Reddy, 2000).

In view of these inconsistent data, we have studied the antioxidant defense system and lipid peroxidation status both in human subjects with skeletal fluorosis and in rabbits intoxicated with 150 ppm of fluoride through drinking water.

MATERIALS AND METHODS

Human Study

Subjects. Thirteen human subjects (8 females and 5 males) from the Edavalli village of Nalgonda District (India), with clinically defined skeletal fluorosis in the age group of 28–70 (mean age 54 years) were recruited for the study, with a written consent from the subjects. These subjects have been exposed to fluoride (>5 ppm) through drinking water for more than 15 years. The study protocol was approved by the Institutional Human Ethical Committee. Fourteen healthy human volunteers (5 females and 9 males) in the age range of 25–63 years (mean 40 years) from the staff members of the institute, whose drinking water contained permissible levels (<1 ppm) of fluoride, served as controls. Clinical history and bone X-rays of the subjects were recorded.

Blood collection. After overnight fasting, blood samples of the subjects were collected by venipuncture into heparinized tubes. Plasma and buffy coat were removed by centrifugation at 3000 rpm for 20 min. RBC were washed 3 times with buffered saline, and the packed cells were then aliquoted for further analysis.

Animal Experiment

Animals. Twelve 3-month-old male New Zealand White rabbits (average body weight 1430 g) were obtained from, and maintained at, the National Center for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad. The animals were randomly distributed, with six animals in each group, into control and fluoride groups. Animal care and experimental protocols were in accordance with, and approved by the Institutional Animal Ethical Committee. Rabbits were housed individually in stainless steel cages and fed purified agar gel diet and water *ad libitum*. Composition of the purified agar gel diet was according to Sivakumar *et al*. (1985). Control-group rabbits received distilled water for drinking while the experimental group received distilled water with 150 ppm of sodium fluoride from the polypropylene bottles. Water intake in each group was measured for 3 consecutive days every month and averaged for quantifying the intake of fluoride. After 6 months, the animals were sacrificed by CO_2 asphyxiation.

TABLE 1
Details of Age, Sex, and Urinary Fluoride Levels of Control and Fluorotic Subjects

Subjects	Male	Female	Mean age (years)	Fluoride levels in urine (μg/ml)
Control	9	5	40 (25–63)	0.41 ± 0.20
Fluorosis	5	8	54 (28–70)	5.94 ± 1.70*

*Statistically different from control group.

Blood collection. Before sacrificing the animals, blood was drawn from the ear veins of overnight-fasted animals into heparinized tubes. Plasma and RBC were separated as described above.

Ascorbic acid and malondialdehyde (MDA) in plasma and catalase levels in RBC were estimated on the same day. The rest of the investigations were done within a few weeks on RBCs stored at –70°C.

Biochemical Estimations

Ascorbic acid was estimated spectrophotometrically by ferric chloride and the bipyridyl method (Bhat, 1991). Lipid peroxidation (MDA) was quantified as thiobarbutyric acid reactive substances by spectrophotometric method (Bhat, 1991). Reduced glutathione (GSH) was measured fluorimetrically by the O-phthalaldehyde method (Bhat, 1991). Activities of catalase (Aebi, 1974), superoxide dismutase (SOD) (Bhat, 1991), glutathione peroxidase (GPx) (Bhat, 1991) and glutathione *S*-transferase (GST) (Habig *et al*., 1974) were measured spectrophotometrically according to the reported methods. Serum fluoride was estimated using ion selective electrode (Singer and Armstrong, 1973). Urine fluoride levels were determined according to the method of Tusl (1970).

In vitro enzyme inhibition studies. To study the direct effect of fluoride on the activity of antioxidant enzymes of RBCs, erythrocyte lysate in PBS was incubated with various concentrations of sodium fluoride for 20 min at 25°C, and activities of catalase and SOD were assayed in lysate according to the methods described above.

Statistical Analysis

Data were analyzed using SPSS Windows version 10.0. Student's *t*-test was used to compare the mean values of different biochemical parameters between control and fluorosis groups (Snedecor, and Cochran, 1967). A nonparametric test of Mann-Whitney 'U' was also utilized whenever the assumption of homogeneity of variances was violated (Snedecor, and Cochran, 1967); $p < 0.05$ is considered as statistically significant.

RESULTS

Details of control and fluorotic subjects such as age, sex, and fluoride levels in urine are given in Table 1. Signifi-

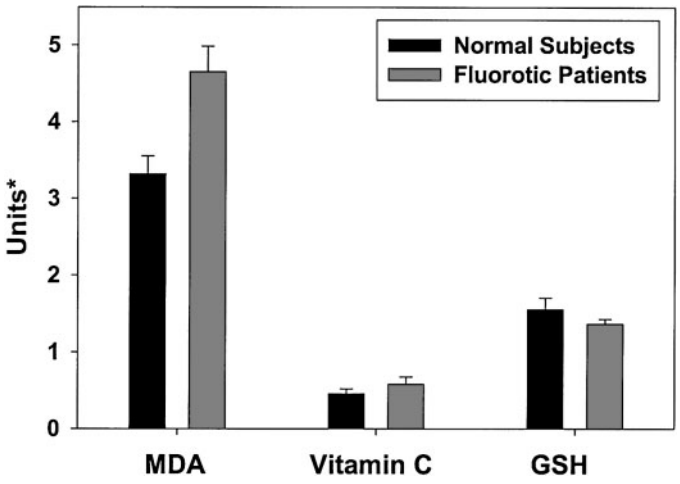

FIG. 1. Levels of malondialdehyde, ascorbic acid, and reduced glutathione in blood of control and fluorotic subjects. Data are mean ± SE ($n = 14$ for control subjects and 13 for fluorotic patients). *Units, nmol/ml plasma (MDA), mg/dl plasma (ascorbic acid), and μmol/ml RBC (reduced glutathione).

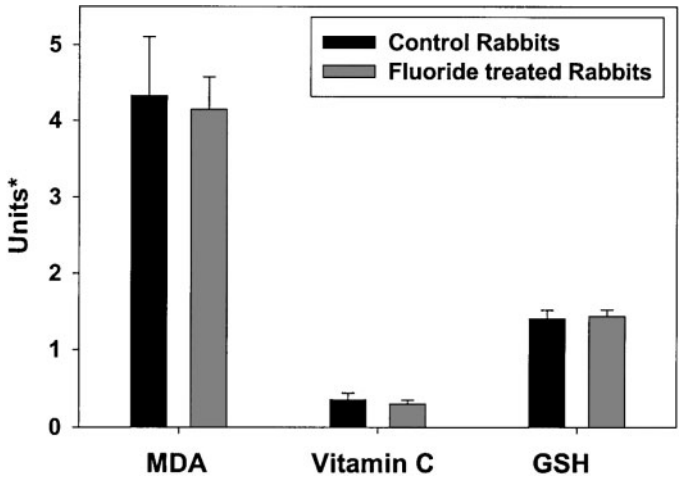

FIG. 2. Levels of malondialdehyde, ascorbic acid, and reduced glutathione in blood of control and fluoride-intoxicated rabbits. Data are mean ± SE ($n = 6$). *Units: nmol/ml plasma (MDA), mg/dl plasma (ascorbic acid), and μmol/ml RBC (reduced glutathione).

cantly higher urinary fluoride levels were found in fluorotic subjects when compared to controls. Their bone X-ray images show typical clinical features of skeletal fluorosis (not shown).

There was no significant difference in GSH and ascorbic acid content between control and fluorosis subjects (Fig. 1). Though the levels of MDA (indicator of lipid peroxidation) are marginally higher in fluorotic subjects, they are not statistically significant (Fig. 1). Activities of enzymes involved in free-radical detoxification are also not altered significantly in fluorosis subjects (Table 2). These results do not corroborate the earlier findings, which reported a decrease in GSH and altered enzyme activities in subjects with endemic skeletal fluorosis (Li and Ca, 1994; Shivarajashankara *et al*., 2001b).

There could be many variables that influence the pathogenesis of skeletal fluorosis in humans, such as fluoride absorption, bioavailability, kidney status, age, sex, and calcium intake. Dietary factors can also affect the severity of the disease. Factors such as calcium, protein, and vitamin C are concerned with bone formation, and the effects of these dietary factors on skeletal fluorosis have been studied (Das, 1996; Krishnamachari, 1986; Krishnamachari and Lakshmaiah, 1975; Lakshmi and Lakshmaiah, 1999; Reddy and Rao, 1971, 1972). Therefore, we have conducted an animal experiment wherein rabbits were given water with 150-ppm fluoride daily for six months under controlled conditions.

Intake of fluoride through water was assessed by quantifying water consumption for 3 consecutive days a month, and the fluoride intake of rabbits in the fluoride group (18.77 ± 4.76 mg/24 h) was significantly higher than control group animals (0.044 ± 0.15 mg/24 h). The bone X-ray of rabbits receiving fluoride water shows typical osteofluorosis characteristics (not shown). In addition, serum fluoride (mean ± SD) levels were significantly higher in fluoride-treated rabbits ((0.857 ± 0.204 ppm) as compared to control animals (0.188 ± 0.88 ppm), suggesting the existence of fluorosis. However, there was no significant change in either lipid peroxidation or nonenzymatic antioxidants, ascorbic acid content and GSH levels in fluoride intoxicated rabbits (Fig. 2). The activities of antioxidant enzymes, catalase, SOD, GPx, and GST were also not affected by fluoride intoxication (Table 3).

Further, to investigate whether fluoride inhibits the activity of antioxidant enzymes if given directly *in vitro*, we have estimated the activities of catalase and SOD in RBC derived from normal subjects in the presence of various concentrations of fluoride. The activities of catalase and SOD are not altered when assayed in the presence of various concentrations of sodium fluoride, up to a 500-μM concentration (Fig. 3).

TABLE 2
Activities of Antioxidant Enzymes in RBC of Control and Fluorotic Subjects

Subjects	Catalase (U/ml RBC)	SOD (U/ml RBC)	GPx (U/ml RBC)	GST U/ml RBC
Control	$3.57 \times 10^4 \pm 0.148 \times 10^4$	1495 ± 91.45	8.29 ± 0.419	1.57 ± 0.214
Fluorosis	$3.32 \times 10^4 \pm 0.103 \times 10^4$	1490 ± 95.16	8.70 ± 0.464	1.79 ± 0.109

Note. Data are mean ± SE ($n = 14$ for control and 13 for fluorotic subjects).

TABLE 3
Activities of Antioxidant Enzymes in RBC of Control and Fluoride Intoxicated Rabbits

Rabbits	Catalase (U/ml RBC)	SOD (U/ml RBC)	GPx (U/ml RBC)	GST (U/ml RBC)
Control group	$3.2 \times 10^4 \pm 0.31 \times 10^4$	1946 ± 166.19	20.47 ± 0.885	0.29 ± 0.076
Fluoride group	$2.3 \times 10^4 \pm 0.22 \times 10^4$	1775 ± 316.19	21.43 ± 2.1	0.21 ± 0.06

Note. Data are mean ± SE ($n = 6$).

DISCUSSION

Chronic fluorosis can severely damage many systems of the human body, but its pathogenesis is poorly understood (Das, 1996; Krishnamachari, 1986). As in the case of many chronic degenerative diseases, increased productions of reactive oxygen species (ROS) and lipid peroxidation have even been considered to play an important role in the pathogenesis of chronic fluoride toxicity. Interactions between fluoride and free-radical reactions have been studied in various biological systems including fluorosis (Rzenski *et al*., 1998). However, the relationship between fluorosis/fluoride toxicity and oxidative stress is still not clear. In the absence of conclusive evidence for an increased oxidative stress in fluorosis, some studies suggest the use of antioxidants and antioxidant-rich foods for the management of fluorosis (Susheela, 1999) and also for the beneficial effects of antioxidants as antidotes for fluoride toxicity (Chinoy and Memon, 2001; Chinoy and Patel, 2001). In view of numerous conflicting and contradictory reports with regard to fluorosis and the oxidative/antioxidant system, we assessed the antioxidant defense system: enzymatic and nonenzymatic and lipid peroxidation in humans from the endemic fluorosis area. Studies were also conducted in rabbits intoxicated with 150-ppm fluoride (in drinking water) for six months, to confirm the findings of the human study.

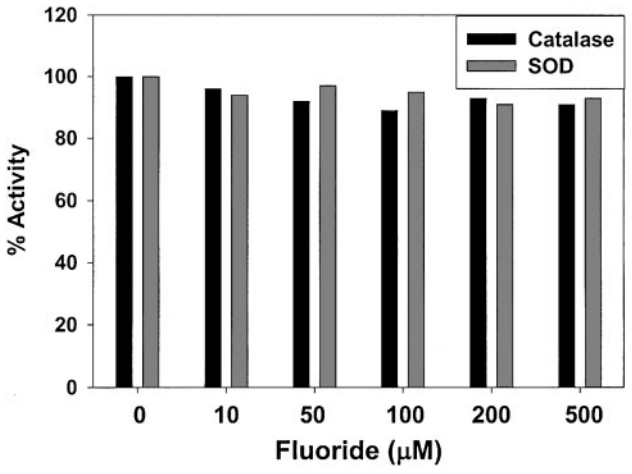

FIG. 3. Activities of catalase and superoxide dismutase in RBC of normal subjects in the absence and presence of varying concentrations of sodium fluoride. Data are the average of three independent assays each time in duplicates. Activity in the absence of sodium fluoride is considered as 100%.

Initially, we assessed a set of known antioxidant molecules in the blood of human fluorotic subjects. We did not find a significant change in any of the antioxidant parameters tested, which included vitamin C, GSH, catalase, SOD, GPx, and GST. In agreement with this result, we did not observe a change in lipid peroxidation compared to control subjects. A severe decline in an antioxidant system with greater oxidative stress is to be expected in these fluorotic subjects, if oxidative stress is the major factor in the pathogenesis of skeletal fluorosis. The adult fluorotic subjects selected in the study have been suffering from fluoride toxicity for many years (~15 years) while the fluorotic children in whom an increased oxidative stress was reported (Shivarajashankara *et al*., 2001) were exposed to fluoride for much shorter periods. Age, sex, calcium intake in the diet, dose and duration of fluoride intake, and renal efficiency in fluoride handling are some of the major factors that influence the fluoride toxicity in humans (Das, 1996; Krishnamachari, 1986) and bring about variations in the clinical presentation (Krishnamachari, 1986). Interaction between fluoride and nutrients is considered very important in understanding the nature of the disease. Earlier studies showed that osteoporosis developed in rats fed a low-calcium diet with fluoride, while calcium supplementation mitigated the low-calcium effect (Reddy and Rao, 1972). A low-calcium and low-vitamin C diet was also shown to increase the cumulative retention of labeled calcium (Reddy and Rao, 1972), but supplementation of vitamin C had no effect on urinary excretion of fluoride in fluorosis patients (Krishnamachari and Lakshmaiah, 1975).

Therefore we assessed the antioxidant defense system in experimental animals wherein we could control most of the above-mentioned variables to the extent possible. The data obtained in rabbits on antioxidant enzymes catalase, SOD, GPx, and GST and ascorbic acid and GSH also suggest that fluoride ingestion (150 ppm through water) for six months had no effects on the antioxidant defense system. There was no significant change in lipid peroxidation status as well, due to fluoride intoxication in rabbits. In the previous animal experiments (Shivarajashankara *et al*., 2001a; Vani and Reddy, 2000) the fluoride was given for shorter periods (14–120 days) and the amounts of fluoride given also were less (20–100 ppm) as compared to the six-month duration and 150 ppm fluoride-containing water given in the present study.

Since fluoride was reported to inhibit many enzymes (Carlson and Suttie, 1966; Krishnamachari and Krishnaswamy, 1973; Mullenix *et al*., 1995; Park *et al*., 1999; Sashi *et al*., 1994; Singh, 1984; Vani and Reddy, 2000), we have investigated whether it also inhibits the antioxidant enzymes if added directly to the assay system. The results clearly suggest that fluoride does not inhibit the activity of catalase and SOD in RBC collected from normal human subjects at concentrations as high as 500 μM (Fig. 3), and this substantiates the *in vivo* observations.

In communities where genu valgum is prevalent, it is observed that the staple diet is generally sorghum and the calcium intake is low (Krishnamachari, 1976; Krishnamachari and Krishnaswamy, 1973; Lakshmaiah and Srikantia, 1977). Sorghum contains high levels of molybdenum as compared to other cereals (Deosthale *et al*., 1977). High molybdenum intake is known to cause secondary deficiency of copper, an essential element for bone development (Arthur, 1965). It is also observed that copper deficiency along with high fluoride intake is associated with high prevalence of genu valgum (Krishnamachari, 1976, 1986; Krishnamachari and Krishnaswamy, 1973). Therefore, we have also conducted a study on rabbits fed either supplemented molybdenum (0.1% in the diet) or a copper-deficient diet and given drinking water with 150 ppm fluoride for six months However, we were unable to find a significant difference with regard to lipid peroxidation and antioxidant parameters in the RBC of these animals compared to control animals (G. B. Reddy and A. L. Khandare, unpublished data.) Results obtained with human subjects and rabbits together indicate that fluorosis/fluoride intoxication is unlikely to shift the oxidant/antioxidant balance towards oxidative stress at least in RBC. However, studies are underway to investigate the oxidative stress/antioxidant system in other tissues such as brain, liver, kidney, etc., in rabbits intoxicated with 150 ppm fluoride in water in validating the above findings in other organs/tissues.

Earlier studies of Krishnamachari and Lakshmaiah (1975) showed that supplementation with massive doses of vitamin C did not bring any beneficial effects on the prevention of skeletal fluorosis, lending indirect support to our findings. Studies elsewhere also reported that vitamin C is not effective in treating skeletal fluorosis (Shangguan *et al*., 1995). Although there is inconclusive proof for an altered oxidative stress and antioxidant balance in fluorosis/fluoride toxicity, some studies reported beneficial effects of antioxidants in combating the toxic effects of fluoride (Chinoy and Memon, 2001; Chinoy and Patel, 2001; Susheela, 1999). Therefore, in the absence of increased oxidative stress and with a normal antioxidative system in RBCs, extensive studies are necessary to investigate the mechanism(s) of fluoride toxicity in various tissues and organs, and strategies may be decided accordingly to counter the toxic effect of fluoride.

ACKNOWLEDGMENT

We thank N. Lakshmaiah for his invaluable suggestions and critical comments during the course of the study.

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