

EFFECT OF FLUORIDE ON REACTIVE OXYGEN SPECIES AND BONE METABOLISM IN POSTMENOPAUSAL WOMEN

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SUMMARY: A study was made of the effects of fluoride (F) on the antioxidant defense systems of postmenopausal women residing in a fluorotic and a nonfluorotic village in Chittoor district, Andhra Pradesh, India. Twenty-five postmenopausal women (approximately 10 years postmenopause, mean age 57 years) residing in endemic fluorotic Adharam and nonfluorotic Rajanagaram (water F >2.0 ppm and <0.35 ppm, respectively) were studied for their dietary calcium (Ca), urinary F, serum levels of F, Ca, phosphorus, intact parathormone (PTH), 25-hydroxy vitamin D, and malondialdehyde (MDA), as well as the activities of serum alkaline phosphatase (SAP), glutathione S-transferase (GST), and catalase (CAT). Dietary Ca was far below the recommended daily allowance. Serum 25-hydroxy vitamin D and PTH levels were comparable in the two villages. Antioxidant and lipid peroxidation levels were significantly lower in the postmenopausal women residing in the Adharam fluorotic village. The postmenopausal women in the nonfluorotic Rajanagaram village showed a strong positive correlation between CAT and PTH ($r = 0.54$; $p < 0.01$) and a negative correlation between CAT and SAP ($r = -0.52$; $p < 0.008$). In the fluorotic village, MDA showed an inverse negative correlation with serum F ($r = -0.6$; $p < 0.001$) and a positive correlation with dietary Ca ($r = 0.43$; $p < 0.03$). GST showed a negative correlation with PTH ($r = -0.42$; $p < 0.04$). For the women of comparable age, duration of menopause, and vitamin D status, those residing in the fluorotic village showed a weaker antioxidant defense system and lower lipid peroxidation than the women residing in the nonfluorotic village. The weakened antioxidant system among women in the fluorotic village is correlated with their high serum F levels and appears to have a detrimental effect on their bone mineral metabolism and mineralization.

Keywords: Bone mineral metabolism; Chittoor district, Andhra Pradesh, India; Fluoride and reactive oxygen species; Osteoblasts; Osteoclasts; Postmenopausal women; Serum and urinary analyses.

INTRODUCTION

Endemic fluorosis (skeletal and dental) is a serious public health problem in many parts of the world, especially in India. The fluoroapatite structure in bone is poor in crystal structure and matrix strength.¹ Age, sex, dietary calcium, hormonal status, dose and duration of fluoride (F) intake, and renal efficiency in handling F all influence the outcome.^{2,3} Estrogens are reported to help prevent bone loss by increasing antioxidant defense in bone.^{4,5} Postmenopausal women residing in an endemic fluorotic area have the additional influence of F on bone in addition to that of estrogen deficiency. F supplementation stimulates new bone formation⁴ but does not decrease the risk for fracture.^{6,7}

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Oxidative stress regulates cellular function in various pathological conditions, including fluorosis. F is a powerful biological oxidation-promoting agent owing in part to its high electronegativity (4.0 on the Pauling scale) and low bond dissociation energy (126 kJ/mole). Reactive oxygen species (ROS), which are free radicals, are highly reactive atoms or molecules with unpaired electrons.⁸ They include the superoxide anion ($\text{O}_2^{\bullet-}$), the superoxide radical (HO_2^{\bullet}), organic peroxy radicals (RO_2^{\bullet}), and the hydroxyl free radical (HO^{\bullet}). In various animal studies, oxidative stress has been implicated as one of the key factors involved in fluorotoxicity.⁹⁻¹² At least one study has shown there is no clear proof of oxidative damage in skeletal fluorosis.¹³ However, antioxidants play an essential role in reversing F-induced cell injury and disease in fluorotic patients.¹⁴ Oxygen-derived free radicals stimulate osteoclastic bone resorption.¹⁵ H_2O_2 but not ROS as such has been reported to promote the differentiation of osteoclast precursor cells to osteoclasts.¹⁶ Induction of ROS generated by various stimuli inhibits osteoblastic differentiation of bone cells.^{17,18} With these reports in mind, we have studied the effects of F and the effects of ROS generated by F in postmenopausal women.

MATERIALS AND METHODS

Twenty-five postmenopausal resident women were voluntarily selected from the endemic-F village, Adharam (latitude 13.37°N, longitude 79.47°E) in Chittoor district of Andhra Pradesh, India, and the same number from the nearby non-endemic low F village, Rajanagaram (latitude 13.42°N, longitude 79.80°E). The drinking water source in each of the two villages was collected for water F determination. Women with a history of chronic diseases like diabetes mellitus, hypertension, cardiovascular disease, chronic renal failure, and liver disease, and those who smoked or were on any medications were excluded from the study.

After obtaining informed consent from each subject, venous blood samples were collected after overnight fasting between 0800 to 0900 hr from the most accessible peripheral vein in the arm without applying a tourniquet for the estimation of serum F, calcium, phosphorus, alkaline phosphatase (ALP), 25-hydroxy vitamin D (25(OH)D), and intact parathyroid hormone (PTH). The blood samples were transported in ice-cooled packs until erythrocytes and serum were separated in a refrigerated centrifuge at 4°C and stored at -20°C until analysis for the estimation of serum 25(OH)D and PTH. About 2 mL of EDTA was added to 8 mL of the blood samples drawn from each subject for the estimation of free radicals. Spot urine samples were also collected for estimation of urine Ca and F levels.

Water, serum, and urine F levels were determined using a F ion selective electrode (Orion-Ion expandable analyzer EA 940). Serum and urine calcium were measured by the O-Cresolphthalein Complexone method.¹⁹ Serum inorganic phosphorus levels were determined by the Goldenberg and Fernandez method.²⁰ Serum alkaline phosphatase (ALP) activity was measured by the 4-amino-antipyrene method.²¹ Serum 25(OH)D concentrations were measured by competitive radioimmunoassay after acetonitrile extraction (DiaSorin, Stillwater, MN; Catalog No. 68100E). The minimum detectable limit in the 25(OH)D assay is 5 ng/mL. Intact (N-tact) PTH was measured by immunoradiometric assay

(DiaSorin; Catalog no. 26100). The minimal detectable limit of the N-tact PTH assay is 13.8 pg/mL.

The lipid peroxidation product malondialdehyde (MDA) was measured as thiobarbituric acid reacting substances (TBARS).²² MDA reacts with thiobarbituric acid to form TBARS, which has a pink color and is measured by a spectrophotometer.²³ Erythrocyte catalase activity (CAT) was assayed by the method of Aebi.²⁴ Glutathione S-transferase (GST) activity was determined by the method of Habig.²⁵

Statistical analysis was performed on SPSS for Windows, version 10.0.1 (SPSS Inc, Chicago, Illinois, USA). Data are presented as mean ± standard deviation (SD). Continuous variables were analyzed with paired *t*-test. Significance was assumed at *p* = 0.05 or less. Analyses among various groups were compared using one-way ANOVA. Linear correlation was calculated Spearman’s correlation coefficient.

RESULTS

The mean ages of the resident postmenopausal women subjects in the fluorotic village and the nonfluorotic village were 57.6±11.2 years and 57.12±10.7 years, respectively (*p* = NS). The duration of menopause in both groups was 10.56±2.1 and 9.96±1.8 years (*p* = NS). Their water F levels were 2.07 ppm and 0.35 ppm, respectively. As seen in the Table below, the higher serum and urine F levels of the women in the fluorotic village compared with those in the nonfluorotic village were highly significant (*p*<0.001).

Table. Biochemical parameters of postmenopausal women in the fluorotic and nonfluorotic villages. Values are mean ± SD.

Parameter	Units	Fluorotic (n=25)	Nonfluorotic (n=25)
Serum F	ppm	0.19 ± 0.02	0.07 ± 0.01*
Urine F	ppm	2.24± 0.90	0.62 ± 0.12*
Serum Ca	mg/dL	9.15 ±1.24	9.46 ± 0.70
Serum Inorganic phosphorus	mg/dL	4.22 ± 0.47	3.27 ± 0.28*
Serum alkaline phosphatase	KA units	8.64 ± 1.81	5.61 ± 1.10*
25-OH vitamin D	ng/mL	22.54 ± 10.23	19.18 ± 7.23
Intact parathormone (PTH)	pg/mL	10 ± 7.77	8.0 ± 2.96
Urine Ca	mg/dL	11.48 ± 2.28	21.42 ± 3.44*
MDA (malondialdehyde)	nmol/mL	3.82 ± 0.35	2.79 ± 0.35*
CAT (catalase)	kU/g Hb	42.99 ± 19.22	54.37 ± 23.36
GST (glutathion S-transferase)	IU/L	40.00 ± 18.22	65.50 ± 26.88*

**p*<0.001.

Serum inorganic phosphorus and alkaline phosphatase levels of the women in the fluorotic village were also significantly higher ($p < 0.001$) than in the nonfluorotic village (Table). On the other hand, the urinary calcium level in the fluorotic group was only about half that in the nonfluorotic group. Moreover, there was no statistically significant difference between the serum Ca, PTH, and 25(OH) D levels between the two groups.

The MDA levels were higher ($p < 0.001$) (Figure 1) and GST activity was lower ($p < 0.001$) (Figure 2) in the women in the fluorotic village compared with those in the nonfluorotic village, but CAT activity was not statistically different (Table). In the women in the fluorotic village MDA showed a negative correlation with the serum F level ($r = -0.6$; $p < 0.001$), and GST showed a negative correlation with PTH ($r = -0.42$; $p < 0.04$). In the women living in the nonfluorotic village there was a strong positive correlation between CAT and PTH ($r = 0.54$; $p < 0.01$) and negative correlation with serum alkaline phosphatase (SAP) ($r = -0.52$; $p < 0.008$).

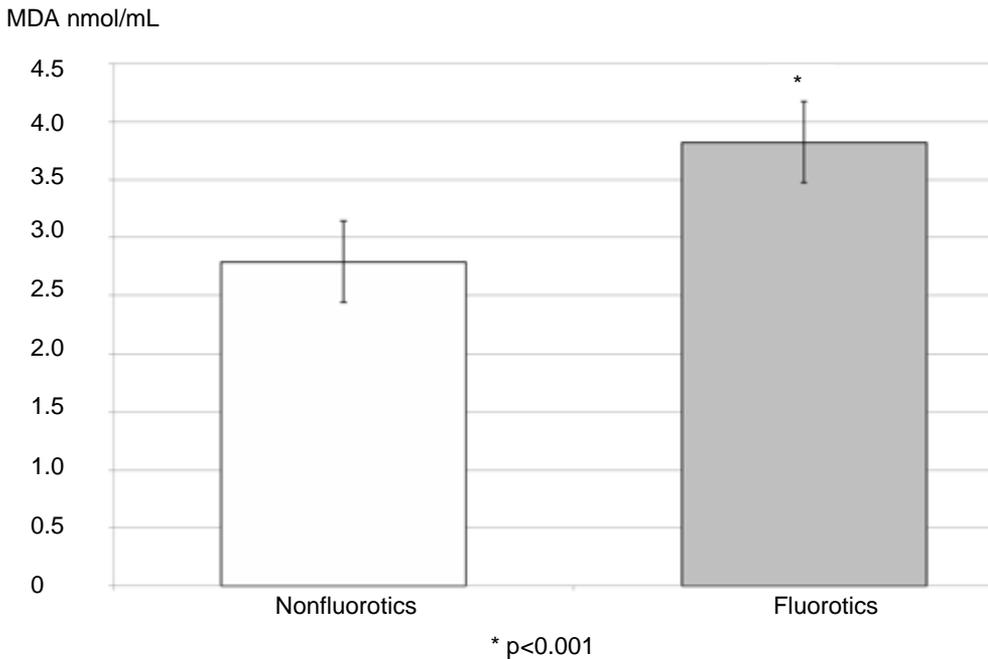


Figure 1. MDA levels in nonfluorotics and fluorotics.

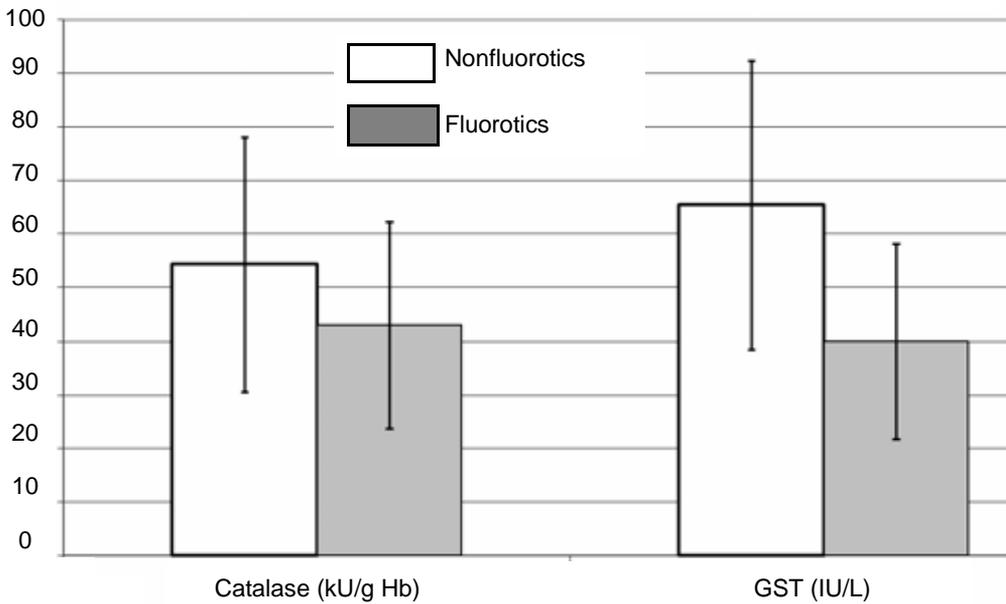


Figure 2. Catalase and GST levels in nonfluorotics and fluorotics.

DISCUSSION

In postmenopausal women estrogen deficiency is associated with bone loss. In fluorosis there is bone resorption indirectly stimulated by oxidative stress. In a normal cell, there is an appropriate pro-oxidant:antioxidant balance. F induces oxidative stress leading to the generation of reactive oxygen species (ROS), thus resulting in a shift of the pro-oxidant and antioxidant balance towards the pro-oxidants wherein the production of ROS is increased and the levels of antioxidants are diminished. Although ROS did not appear to be directly involved, H_2O_2 has been found to cause bone resorption by stimulating osteoclasts.¹⁶ H_2O_2 -induced bone resorption can be suppressed by catalase (CAT).²⁶ Various animal studies and a few human studies have reported an association between F and oxidative stress.⁹⁻¹⁴ However, so far as we are aware, there are no documented reports comparing the effects of F on oxidative stress in postmenopausal women living in endemic fluorosis and nonfluorosis areas.

In the present study, oxidative stress was studied in terms of lipid peroxidation and antioxidant enzyme activities. MDA levels were high, and the activities of the antioxidant enzymes catalase (CAT) and glutathione S-transferase (GST) were suppressed, indicating a compromised antioxidant defense system in the postmenopausal women residing in the fluorotic village. Elevated lipid peroxidation products (pro-oxidants) and diminished antioxidant defense enzyme activity suggest F plays a significant role in inducing oxidative stress in the postmenopausal women living in an area of endemic fluorosis. Thus we

hypothesize that F increases MDA levels and decreases CAT and GST activity levels, thereby increasing the extent of pro-oxidant and decreasing the antioxidant activity. This oxidative stress increases H_2O_2 levels and thus can stimulate the osteoclast precursor cells leading to increased osteoclast proliferation. Because of the decreased CAT activity level, the inhibitory effect on osteoclasts is not seen, thereby leading to increased bone resorption. Hence it can be postulated that, by inducing oxidative stress, F can indirectly stimulate bone resorption (Figure 3).

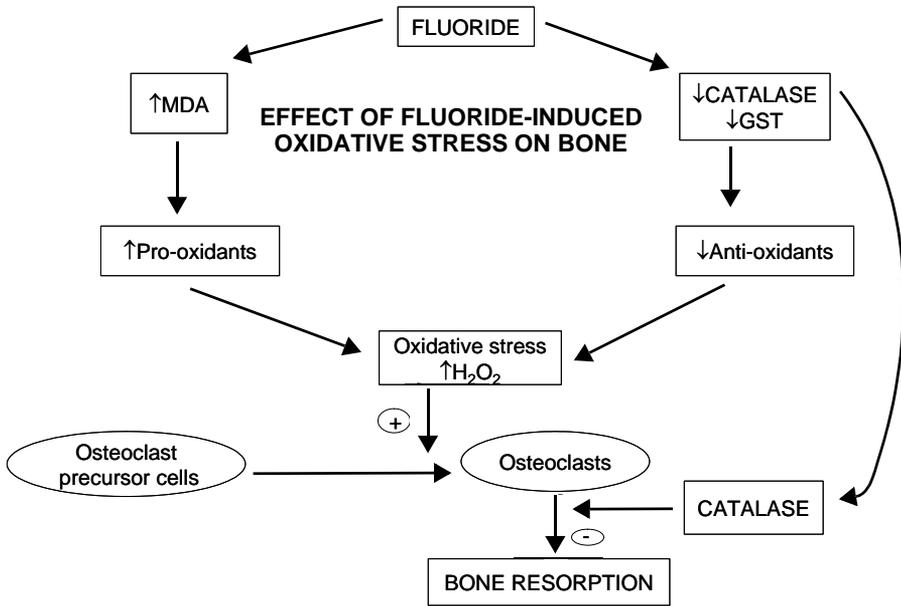


Figure 3. Fluoride-induced oxidative stress increases H_2O_2 and decreases catalase activity promoting bone resorption.

According to Libatini et al.²⁷ and the work of Farley et al.,²⁸ F induces bone formation by increasing osteoblast number. Farley et al. found that a concentration of only 10 μM F/L can enhance osteoblast proliferation and increase alkaline phosphatase (ALP) activity. As a marker of bone formation, ALP is generally elevated in fluorosis.²⁸ In agreement, the ALP activity in our subjects in the fluorotic village was significantly higher than in the controls in the nonfluorotic village.

The affinity of F^- for Ca^{2+} is well known, and biochemical interactions between these two ions are important factors in determining the clinical and metabolic course of skeletal fluorosis. F toxicity also exaggerates the metabolic effects of calcium deficiency on bone.²⁹ Older calcium balance studies by Srikantia and Siddiqui revealed a significantly higher retention of calcium in fluorosis patients.³⁰ As a result, increased retention of calcium induced by F leads to decreased excretion of calcium in urine. In our study, significantly lower levels of urinary calcium were found in the postmenopausal women in the fluorotic village. Although serum calcium and inorganic phosphorus levels and serum ALP activity were within normal limits in the postmenopausal women residing in the fluorosis-

affected village, when compared to age-matched controls in the nonfluorotic village, serum ALP and inorganic phosphorus were significantly higher in the fluorotic postmenopausal women.

In summary, our observations clearly suggest that F induces oxidative stress in postmenopausal women. ROS derived from F-induced oxidative stress appear to stimulate bone resorption and disturb normal PTH function. Thus, even though PTH levels were below normal, serum calcium and serum inorganic phosphorus levels were evidently maintained within normal limits due to ROS-induced bone resorption.

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