

STUDY OF OXIDATIVE STRESS, BONE MINERAL STATUS AND FLUORIDE LEVELS IN POSTMENOPAUSAL WOMEN IN ENDEMIC FLUOROTIC AREA OF ANDHRA PRADESH, INDIA

Parinita Kataraki¹, Pragna Rao², Dileep Rathi¹

¹ Department of Biochemistry, BLDEU'S Shri B. M. Patil Medical College, Bijapur-586103 Karnataka, India,

² Department of Biochemistry, Kasturba Medical College, Manipal, Karnataka, India.

*Corresponding Author Email: drparinita.s@gmail.com

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ABSTRACT

Osteoporosis is a major health problem of old age. This silently progressing metabolic bone disease is widely prevalent in India, the prevalence of osteoporosis increases with age especially involving long bones. It has been observed that females suffer from osteoporosis, after the menopause, accelerated process of osteoporosis occur and it is observed in India that about 50% suffer from osteoporosis and estimated number is 30 million. Fluorosis is a serious public health problem in many parts of the world where drinking water contains more than 1 ppm of fluoride. Higher intake of fluoride will result in dental and skeletal fluorosis and affect collagen synthesis and bone mineralization. Approximately 99% of the body burden of fluoride is associated with calcified tissues. The fluoride concentration in bone is not uniform. Fluoride concentration in bone is not uniform and higher fluoride levels in the body are associated with calcified tissues. The study was conducted on 100 subjects with their consent which consisted of control group of 50 non-pregnant women in their reproductive age group. Study group consisted of 50 women who had attained menopause either naturally or surgically, all residing in the endemic fluorotic area of Nalgonda district, Andhra Pradesh, India. 5 ml of venous blood was collected from both the groups. Serum fluoride levels (0.68 ± 0.39 ; $p < 0.005$) was statically significant when compared to the controls (0.45 ± 0.28). The biochemical parameters of bone mineral status, serum calcium (9.19 ± 0.620) $p = 0.488$ and serum phosphorus (4.27 ± 0.71 ; $p = 0.799$) was statistically insignificant whereas total alkaline phosphatase (132 ± 37.60 ; $p < 0.001$) and bone specific alkaline phosphates (97.41 ± 38.74 ; $p < 0.001$) were statistically significant indicating that there is more osteoblastic activity in patients of osteoporosis. Malondialdehyde was measured as an indicator of oxidative stress which was statistically significant (287.86 ± 49.79 ; $p < 0.001$). Bone turnover markers, serum total ALP and BSALP have a role in assessment of fluorosis in postmenopausal women and serum malondialdehyde, a lipid peroxidation product, is used as an indicator of oxidative stress which is induced by fluorosis.

KEYWORDS: Fluorosis, post menopausal women, malondialdehyde, oxidative stress, osteoporosis.

INTRODUCTION

Fluoride is a trace element that is ubiquitously distributed throughout the environment in a wide range of concentrations especially in soil and water resources¹. Fluoride is a potent anion and the most electronegative element as well as being a cumulative toxin². The recommended concentration range in the drinking water is 0.7 – 1.2 ppm, depending on the average regional temperature. Lower levels are recommended for warmer regions where water intake tends to be higher.³ Fluoride metabolism is pH dependent and that the transmembrane migration of the ion occurs in the form of hydrogen fluoride in

response to differences in the pH of adjacent body fluid compartments. About 80 – 90% of the ingested amount is absorbed from the gastrointestinal tract in presence of sufficient concentrations of cations such as calcium and aluminium form insoluble fluoride compounds which decrease its absorption. The half time for absorption is about 30 minutes³. Being highly soluble in water, environmental fluoride is absorbed easily in the stomach and gut. Although high amount of fluoride in plasma is in bound form, a small fraction of it is in ionic form. Fluoride passes easily through cell membranes in its ionic form. Therefore primary involvement of bone in chronic

fluorosis is attributed to the ability of these tissues to accept fluoride ion in exchange for other ions². After about 50% of an ingested fluoride dose has been absorbed, plasma concentrations decline rapidly. This is due to renal excretion and uptake by calcified tissues. Fluoride is freely filtered through the glomerular capillaries and then undergoes a variable degree of tubular reabsorption. Among the halogens, the renal clearance of fluoride is unusually high; about 35 ml/min. Approximately 99% of fluoride in the body fluids is associated with calcified tissues. The fluoride concentration in bone is not uniform. In long bones, the concentrations are highest in the periosteal region. They decline sharply within a few millimetres of the periosteal surface and increase slightly as the endosteal region is approached. Cancellous bone has higher fluoride concentrations than compact bone. The clearance of fluoride from plasma by the skeleton proceeds at a high rate that exceeds even that for calcium. Approximately 50% of the fluoride absorbed each day by healthy young or middle aged adults becomes associated with calcified tissues within 24 hours, while virtually all of the remainder is excreted in the urine.

Uptake of fluoride by bone occurs in stages. The first stage involves fluoride migration into the hydration shells of bone crystallites. These ion-rich aqueous shells are continuous with, or at least available to, the extracellular fluids. Presumably, fluoride in this pool is rapidly exchangeable so that it can undergo net migration in either direction, depending on the relative concentrations in the extracellular fluid and the hydration shells. Later stages involve fluoride association with or incorporation into precursors of hydroxyfluoroapatite and finally into the apatitic lattice itself. Apatitic fluoride re-enters the circulating body fluids as a result of the long term process of bone resorption.³ Fluorosis is a slow and progressive process causing symptoms related to several systems particularly related to musculo-skeletal system.²

Fluoride causes lipid peroxidation, DNA damage and apoptosis, and there is a positive relationship among these changes. Fluoride induces apoptosis by oxidative stress induced lipid peroxidation, and there by releasing cytochrome C into the cytosol and further triggering caspase cascade leading to apoptotic cell death⁴. Hence this study was planned to assess the importance of biochemical parameters in the diagnosis of osteoporosis, bone turnover marker status and relation between fluoride and oxidative stress in the pathogenicity of postmenopausal osteoporosis in postmenopausal women residing in endemic fluorotic area.

MATERIALS AND METHODS

The study was conducted on 100 subjects with their consent which consisted of control group of 50 non-pregnant women in their reproductive age group. Case group consisted of 50 women who had attained menopause either naturally or surgically, all residing in the endemic fluorotic area of Nalgonda district, Andhra Pradesh, India. Women with liver disorders, alcoholism hyper / hypothyroidism and women on medication with vitamin D, calcium and hormone replacement therapy were excluded from the study. 5 ml of random venous blood was drawn from the out patients, with a sterile disposable syringe which was transferred into centrifuge tubes. The sample was centrifuged at 3000 rotations per minute for 10 minutes and the serum was collected from the centrifuge tubes. Serum calcium was estimated by Q- Cresolphthalein complex kit method Calcium in an alkaline medium combined with o-cresolphthalein complex to form a purple complex. Intensity of colour formed is directly proportional to the amount and calcium present in the sample^{19,20}. Serum phosphorus was measured by Molybdate UV method, here phosphate ions in an acidic medium react with ammonium molybdate to form a phosphomolybdate complex. This complex has an absorbance in the ultraviolet region and is measured at 340 nm. Intensity of complex formed is directly

proportional to amount of inorganic phosphorus present in the sample^{21, 22}. Total alkaline phosphatase by pNPP – AMP kinetic method, At 10.3 pH alkaline phosphatase catalyses the hydrolysis of colourless p-nitrophenyl phosphate (pNPP) to yellow colored p-nitrophenol and phosphate, change in absorbance due to yellow colour formation is measured kinetically at 405 nm which is proportional to the alkaline phosphatase activity in the sample and bone specific alkaline phosphatase was estimated by heat inactivation analysis of total alkaline phosphatase²³. Serum and water fluoride estimation was done using Ion selective electrode (Eutech fluoride electrode). Serum malondialdehyde, an indicator of oxidative stress was estimated by Thiobarbituric acid reactive assay method. One molecule of

malondialdehyde in the serum reacts with two molecules of thiobarbituric acid in acidic medium and gives rise to color complex (pink) which is measured at 532 nm against distilled water in a spectrophotometer²⁴.

RESULTS

The present study was undertaken in the Department of Biochemistry, Kamineni Institute of Medical Sciences, Narketpally, Andra Pradesh, India. The study group included the postmenopausal women aged between 50 to 70 years. The control group women were in their reproductive age group, non-pregnant, healthy individuals aged between 20 to 40 years. The mean age for the patient and control group is shown in the **figure 1**.

Figure 1: Age distribution among patients and controls:

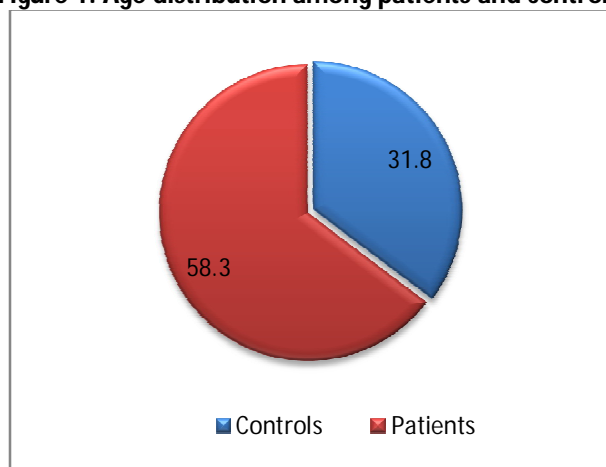


Table: Mean and SD values of serum F, Ca, P, ALP, BSALP and MDA in patients and controls

Biochemical parameters	Controls (n=50)	Patients (n=50)	p value
Serum fluoride (ppm)	0.45 ± 0.28	0.68 ± 0.39	<0.005
Serum calcium (mg/dl)	9.26 ± 0.46	9.19 ± 0.62	NS
Serum phosphorus (mg/dl)	4.32 ± 0.86	4.27 ± 0.71	NS
Serum total alkaline phosphatase (U/L)	87.5 ± 11.05	132 ± 37.6	< 0.001
Bone specific alkaline phosphatase (U/L)	47.51 ± 14.98	97.41 ± 38.74	< 0.001
Serum malondialdehyde (nmol/dL)	127.36 ± 47.01	287.86 ± 49.79	< 0.001

The biochemical parameters, serum calcium and phosphorus were measured to know the bone mineral status in patients and controls. The mean values of these parameters in both the groups are shown in the table which do not show a significant difference between the two groups. The p value for serum calcium ($p=0.488$) and serum phosphorus ($p=0.799$) are not statistically significant.

Serum total alkaline phosphatase and bone specific alkaline phosphatase were estimated as the indicators of bone turnover in both patient and control groups. The mean values of total alkaline phosphatase and bone specific alkaline phosphatase are shown which show a significant difference in both the groups. The mean values for both the parameters were two fold higher when compared to that of control group. The p value of total alkaline phosphatase ($p < 0.001$) and bone specific alkaline phosphatase ($p < 0.001$) are statistically significant and indicate that there is more osteoblastic activity in patients of osteoporosis. Serum malondialdehyde levels were estimated by method of TBARS as an indicator of oxidative stress in both the groups. The mean values for both the groups are shown in table 4, which show the increased values in the patients when compared to that of control group. The mean values of serum malondialdehyde between patient and control groups were statistically significant. ($p < 0.001$). Water fluoride from the surrounding villages of Nalgonda district ($n=10$) was estimated using Ion selective electrode, which showed the mean of 4.35 ± 2.28 ppm. The subjects included in the study were the local residents of Nalgonda district who were exposed to the fluoride consumption for >10 years. Serum fluoride levels were measured to know the fluorotic state in both case and control groups. The mean values for both the groups are shown which shows the increased levels in the patient group. Statistical analysis shows ($p < 0.005$) significant difference in patient and control group. This indicates that although both patients and controls are residing in the area of endemic fluorosis,

patients had higher levels of fluoride. This could be due to difference in age.

DISCUSSION

Fluoride is toxic to osteoblasts (bone forming cells). A first response to cell injury is to initiate repair and when that fails the cell dies. In fluoride intoxication the repair mechanism fails, and the result is an initial increase in both bone formation and resorption. This response to cell injury by fluoride leads to pathological bone formation, and such increased formation or decreased resorption of bone results in increased bone mass. When injured osteoclasts die, new osteoclasts are formed from monocytes. The secondary injury of osteoclasts does not result in a paucity of osteoclasts on the surface of fluorotic bone. Fluoride injures cells involved in bone formation, and a poorer quality bone accumulates in patients with increased intake of fluoride.⁵ The serum fluoride levels of both case and control groups are low compared to that of the water fluoride levels estimated. This is because, the clearance of fluoride from plasma by the skeleton proceeds at a high rate that exceeds even for the calcium. Approximately 50% of the fluoride absorbed each day becomes associated with calcified tissues within 24 hours, while virtually all of the remainder is excreted in the urine.³ Arjun L Khandare et al⁵ and M Yildiz et al¹⁸ showed that there is significant increase in the serum fluoride level in the patients with high fluoride intake. Our study shows significant increase in the serum fluoride in the patient group.

In this study, which includes 50 patients and 50 controls, serum calcium, phosphorus are studied as the indicators of bone mineral status. Serum total alkaline phosphatase and bone specific alkaline phosphatase are estimated as the indicators of bone turnover. Serum malondialdehyde was assayed as the indicator of oxidative stress. A study conducted by Boink AB et al⁶ showed that fluoride induces hypocalcemia. The mechanism is based on the formation of fluorapatite which is responsible for the

hypocalcemia. In our study, both patients and controls had normal serum calcium levels. The normal serum calcium levels can be due to the various dietary sources containing calcium which contribute to the distribution of calcium in the body. The mean values of phosphorus in the two groups did not reveal any statistically significant difference. This finding is in accordance with study of S. G. Srikantia et al⁷ who suggested that serum phosphorus levels were within normal limits in subjects suffering from various degrees of skeletal fluorosis. Serum total alkaline phosphatase and bone specific alkaline phosphatase which were estimated as the indicators of the bone turnover showed a statistically significant difference in the mean values when compared between patient and control groups, with two fold higher levels of alkaline phosphates and bone specific alkaline phosphatase in patient group. Though there is increase bone turn over in postmenopausal women, the alkaline phosphatase levels can either be increased or normal. A study by K. S. Leung et al⁸ and Lennart Krook et al⁹ have shown that in patients with fluorosis there is significant increase in the total ALP and BSALP. Patrick Garner et al¹⁰ has shown that there is highly significant increase in the total ALP and BSALP levels in the patients exposed to high intake of fluoride. Fluoride induced cell injury in both osteoblasts and osteocytes initiates a repair response and results in increased serum alkaline phosphatase production in both of these cell population. The repair response in osteoblasts results in increased proliferation, matrix production and serum alkaline phosphatase production. When the repair process in osteoblasts fails, the osteoblast undergoes either apoptosis or necrosis, and is replaced by proliferation of osteoprogenitor cells. These new osteoblasts will then be injured and cycle of increased repair response in osteoblasts would contribute to increased serum alkaline phosphatase⁹. The increase in plasma alkaline phosphatase may reflect a fluoride induced regeneration of new bone. Bone specific alkaline phosphatase is a

sensitive marker of increased bone turnover. It is found in the plasma membrane of osteoblasts which is released into the circulation by an unknown mechanism. Fluoride increases the levels of bone specific alkaline phosphatase by causing injury to osteoblasts initiating a repair response.¹¹ Lipid peroxidation products, serum malondialdehyde were estimated in both the case and control group indicating the oxidative stress in group II. Aysel Guven et al¹² conducted a study which included the assessment of the effect of chronic fluoride intoxication on lipid peroxidation by estimating serum malondialdehyde. They found that malondialdehyde levels were increased which could be associated with peroxidation of membrane phospholipids and the accumulation of malondialdehyde. Our study showed a statistically significant difference in malondialdehyde levels in both case and control group. Oxidative stress is an independent risk factor for osteoporosis¹³. The role of reactive oxygen species in bone metabolism is unique and dual considering their effect under physiological and pathological conditions. Under physiological conditions, the production of reactive oxygen species by osteoclasts assists in accelerating destruction of calcified tissue and hence assists in bone remodelling¹⁴. Oxidative stress is able to inhibit bone cell differentiation of a preosteoblastic cell line and a marrow stromal cell line that undergoes osteoblastic differentiation¹⁵. Enhanced osteoclastic and depressed osteoblastic activity is attributable to primary estrogen deficiency characteristic of osteoporosis¹⁴. Oxidative stresses may contribute to the development or progress of osteoporosis may indicate an underlying, or accompanying state of inflammation¹⁶. A study by Hui Xu, Chun-hong Wang et al, has shown that, both low and high doses of fluoride caused active state of oxidative stress in osteoblasts, suggesting that oxidative stress may be excited by the active osteoblasts viability induced by low dose of fluoride.¹⁷ Our study showed the normal bone mineral status

with increased serum total alkaline phosphatase and bone specific alkaline phosphatase enzymes indicating the increased bone turnover. Serum malondialdehyde levels also increased in case group when compared to control group, indicating oxidative stress.

CONCLUSION

- Biochemical markers are non-invasive, inexpensive and can be repeated often.
- Biochemical markers have a role in the diagnosis of postmenopausal osteoporosis in endemic fluorotic area.
- Bone mineral status measured using serum total calcium and phosphorus have less significant role in fluorotic patients.
- Bone turnover markers, serum total ALP and BSALP have a role in assessment of fluorosis in postmenopausal women.
- Fluorosis induces oxidative stress where serum malondialdehyde, a lipid peroxidation product, is used as an indicator of oxidative stress.

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***Corresponding Author:**

Dr Parinita Kataraki

Assistant Proessor, Dept of Biochemistry

Shri B M Patil Medical College

Smt Bangaramma Sajjan Campus

Bijapur 586103,

Karnataka