Research Journal of
Environmental Sciences

**Academic
Journals Inc.**

Effect of Fluoride Intoxication on Bone Tissue of Experimental Rats

D. Shanthakumari and S. Subramanian
Department of Biochemistry and Molecular Biology, University of Madras,
Guindy Campus, Chennai-600 025, Tamil Nadu, India

Abstract: The purpose of this prospective study was to investigate the toxic effects of fluoride on bone tissues by assessing the serum and bone biochemical parameters in the experimental rats orally treated with 25 mg L^{-1} fluoride/rat/day for 8 and 16 weeks, respectively. The electron microscopic studies of bone in fluoride intoxicated rats shows flattened bone cells with damaged nucleus, endoplasmic reticulum and enlarged lacunae. An increase in serum fluoride, alkaline and acid phosphatase, sodium, potassium, phosphorous, urea, creatinine, uric acid, blood glucose levels and decrease in the levels of protein and calcium were observed in experimental groups of rats. The levels of fluoride, tartrate resistance acid phosphatase and hyaluronic acid were increased and the levels of alkaline phosphatase, collagen, chondroitin sulphate and heparan sulphate were decreased in the bone tissue of experimental groups of rats. The altered serum and bone biochemical parameters may be attributed to bone damage which in turn may leads to skeletal fluorosis.

Key words: Fluoride, alkaline phosphatase, acid phosphatase, collagen, blood glucose, chondroitin sulphate, hyaluronic acid, heparan sulphate, creatinine

INTRODUCTION

Fluorides are naturally occurring contaminants in the environment (Choubisa, 1999). It is a ubiquitous element present in both soil and water in low concentrations. Occurrence of fluorine in groundwater has drawn worldwide attention due to its considerable impact on human physiology. Extensive contamination of ground water by fluoride has been reported in China (Wang and Huang, 1995) and India (Susheela, 1999), where endemic fluorosis continues to be prevalent. The assimilation of fluorine by the human body from potable water at the level of 1 mg L^{-1} enhances bone development and prevents dental caries (Kundu *et al.*, 2001). The maximum tolerance limit of fluoride in drinking water specified by the World Health Organization (WHO, 1984) is 1.5 mg L^{-1}.

Fluoride is often called a double edged sword because deficiency of fluoride intake leads to dental caries while excess consumption leads to dental and skeletal fluorosis. Dental fluorosis characterized initially by opaque white patches, staining, mottling and pitting of teeth (Kundu *et al.*, 2001). Skeletal fluorosis may occur when fluoride concentrations in drinking water exceeds 4 to 8 mg L^{-1}, which leads to an increase in bone density, calcification of ligaments, rheumatic or arthritic pain in joints and muscles along with stiffness and rigidity of the joints, bending of the vertebral column and so on. The disease may be present in an individual at subclinical, chronic or acute levels of manifestation (Teotia and Teotia, 1988). During skeletal fluorosis, both cortical and cancellous bones undergo structural and biochemical changes and, in advanced stages of the disease, the changes are irreversible (Krishnamachari, 1986; Teotia and Teotia, 1991).

Corresponding Author: Dr. S. Subramanian, Department of Biochemistry and Molecular Biology,
University of Madras, Guindy Campus Chennai-600 025, India
Tel: +91 44 22300488 Fax: +91 44 22300488

According to a recent report, 3555 habitations have been identified as fluoride affected settlements spreading over the Vellore, Dharmapuri, Trichy, Karur, Salem, Namakkal, Erode, Coimbatore and Virudhunagar Districts of Tamil Nadu, India (Mariappan *et al.*, 1999). Seventy ground water samples covering the entire area of Poongulam and Sowedakuppam Panchayats of Tirupattur block and Marimanikuppam and Narasingapuram Panchayats of Alangayam blocks of Vellore district, Tamilnadu, India were collected and analysed for fluoride content. Analytical results of all the samples from Narasingapuram panchayt revealed the presence of fluoride over and above the permissible limits.

The people living in Narasingapuram Panchayat of Alangayam block of Vellore district, Tamil Nadu were selected for the present study, since they have a relatively high fluoride concentration (2.35 to 4.59 mg L^{-1}) in their drinking water. A cross-sectional survey was done and a total of 391 children and adults were examined as per norms of the World Health Organization (1970) for dental and skeletal fluorosis. For the evidence of dental fluorosis, teeth of children and adults of both sexes were examined in day time and recorded and graded the degree of dental fluorosis using Dean's index (Dean, 1934). Adults residing in these villages for more than 15 years were examined clinically for skeletal fluorosis.

The incidence and severity of dental fluorosis among different age groups and sex groups of Narasingapuram Panchayat revealed that between the two sexes, 72.0% males were affected with dental fluorosis as compared to only about 50.8%, females which showed that males are more vulnerable as compared to females. The incidence and severity of skeletal fluorosis among different age groups and sex groups of Narasingapuram panchayat revealed that between the two sexes, 4.4% males were affected with skeletal fluorosis as compared to only about 3.2% females which showed that males are more vulnerable as compared to females.

In the light of the above findings, the present study was aimed to evaluate the effect of oral administration of sodium fluoride at a concentration of 25 mg L^{-1} /rat/day/single dose for 8 and 16 weeks, respectively on serum and transmission electron microscopic studies of femoral bone were carried out to assess the toxic effects of fluoride on skeletal system during long term exposure.

MATERIALS AND METHODS

Chemicals

Sodium fluoride (AR, BDH) was used as the source of fluoride. Distilled water was always used to prepare the fluoride solutions. A stock solution of fluoride was prepared by dissolving 2.21 g of sodium fluoride and the solution was made up to 1 L in a standard flask (Bellack and Schouboe, 1968).

Collection of Water Samples

The ground water sampling was carried out systematically from dug wells, shallow hand pumps and over head tanks. Ground water samples covering the entire area of Poongulam and Sowedakuppam panchayats of Tirupattur block and Marimanikuppam and Narasingapuram panchayats of Alangayam blocks of Vellore district, Tamil Nadu were collected. The water samples were collected in pre cleaned 500 mL polythene bottles with air tight lids and stored under sterile conditions.

Fluoride Assay

To determine the fluoride concentration in the water samples, Orion ion analyser (Orion, 720-ise-fluorimeter, USA) with fluoride ion selective electrode was used. By constructing the cell using the fluoride ion-selective electrode and calomel reference electrode in a solution of fluoride at pH 5.35, adjusted with total ionic strength adjusting buffer (TISAB), the cell potential can be determined.

Experimental Animals

Male albino rats of Wistar strain weighing around 120-160 g obtained from Tamil Nadu University of Veterinary and Animal Sciences (TANUVAS), Madhavaram, Chennai were used for the study. The animals were housed in solid bottomed polypropylene cages, acclimatized to animal house conditions. The rats were fed with commercial rat diet (Hindustan Lever Limited, Mumbai, India) and water *ad libitum*. Autoclaved paddy husk was used as the bedding material. The experiments were designed and conducted in accordance with the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines (IAEC No. 01/035/04).

Experimental Setup

The animals were divided into three groups with a minimum of six rats in each group.

Group I: Control rats receiving unfluoridated water.
Group II :   Experimental rats orally treated with 0.5 mL water containing 25 mg L^{-1} fluoride/rat/day for 8 weeks.
Group III: Experimental rats orally treated with 0.5 mL water containing 25 mg L^{-1} fluoride/rat/day for 16 weeks.

During the course of treatment, daily water consumption, body weight gain and feed consumption were recorded periodically. At the end of the experimental period, the control and experimental animals were fasted overnight and sacrificed by cervical dislocation. The blood samples were collected without the anticoagulant for serum preparation.

Femoral bone was dissected out and the muscles around the bones were selectively removed. Approximately 100 mg of bone samples was taken and homogenized with 2 mL of 0.1 M Tris HCl buffer (pH 7.2) using mortar and pestle and the homogenates were centrifuged at 10,000 xg for 30 min at 4°C and the supernatant was used for biochemical estimations (Gopala Krishnan *et al.*, 2003). A portion of the supernatant was used for the estimation of fluoride using fluoride ion selective electrode. Another portion of the supernatant was used to estimate the biochemical parameters.

Biochemical Parameters

Hemoglobin was estimated by the cyanmethemoglobin method of Drabkin and Austin (1932). The total erythrocyte count was determined by the method of Huxtable (1990). WBC count was determined using Raghuramulu *et al.* (1983). Platelets was determined using the method of Brecher and Cronkite (1964).

Serum biochemical parameters: Serum was used for the estimation of sodium and potassium by a flame photomeric method (Varley, 1988), calcium and phosphorous levels were estimated by following the metallochromogen arsenazo method (Bauer, 1981) and modified Daly and Erlinghausen (1972) method, respectively. Blood glucose was estimated by the method of Sasaki *et al.* (1972). Protein was estimated by the method of Lowry *et al.* (1951). Urea was estimated by the method of Natelson *et al.* (1951). Creatinine was estimated by the method of Broad and Sirota (1948) using Jaffe's reaction, uric acid was estimated by the method of Caraway (1963), alkaline phosphtatase and acid phosphatase were estimated by the method of King (1965). Serum fluoride was measured with a fluoride ion selective electrode (Orion, 720-ise-fluorimeter, USA) in 1.0 mL of serum with an equal volume of TISAB buffer, which adjusted the pH to 5.0.

Bone Biochemical Parameters

Alkaline phosphatase activity in bone was determined by the method of Andersch and Szecypinski (1947) using p-nitrophenyl phosphate as the substrate. Tartrate resistance acid phosphatase was estimated by the method of Tenniswood *et al.* (1976). Hydroxyproline is exclusively

present in collagen. Estimation of hydroxyproline gives the exact amount of collagen present in the tissue. It was measured according to the procedure of Stegemann (1958). The GAGs were extracted following the method of Homer *et al.* (1993). Bone fluoride was measured with a fluoride ion selective electrode (Orion, 720-ise-fluorimeter, USA) in 1.0 mL of supernatant with an equal volume of TISAB buffer, which adjusted the pH to 5.0.

Electron Microscopy Studies

For electron microscopic examination of bone, decalcification was done with 10% HCl for 1 h, primer fixation was made in 3% glutaraldehyde in sodium phosphate buffer (200 milli Molar (mM), pH 7.4) for 3 h at 4°C. Materials were washed with same buffer and postfixed in 1% osmium tetroxide and in sodium phosphate buffer (pH 7.4) for 1 h at 4°C. Tissue samples were washed with same buffer for 3 h at 4°C and were dehydrated in graded ethanol series and were embedded in Araldite. 60-90 nanometer (nm) sections (60-90 nm) were cut on an LKBUM4 ultramicrotome using a diamond knife and sections were mounted on a copper grid and stained with uranyl acetate and Reynolds lead citrate. The grids were examined under a Phillips EM201C transmission electron microscope.

RESULTS

From the results of Fig. 1 obtained it is evident that fluoride administration decrease the levels of RBC, WBC platelet and hemoglobin in a dose dependent manner and time duration in fluoride treated groups of rats (Group II and III). There was a significant increase in the platelet count in the experimental groups of rats.

Table 1 shows that there was a significant increase in sodium, potassium and phosphorous levels in experimental groups of rats and a concomitant decrease in the level of calcium was observed in the experimental groups of rats (Groups II and III).

Table 2 shows that there was an increase in the levels of blood glucose, creatinine, urea and uric acid in experimental groups of rats. There was a significant decrease in the level of protein in the experimental groups of rats when compared to control group.
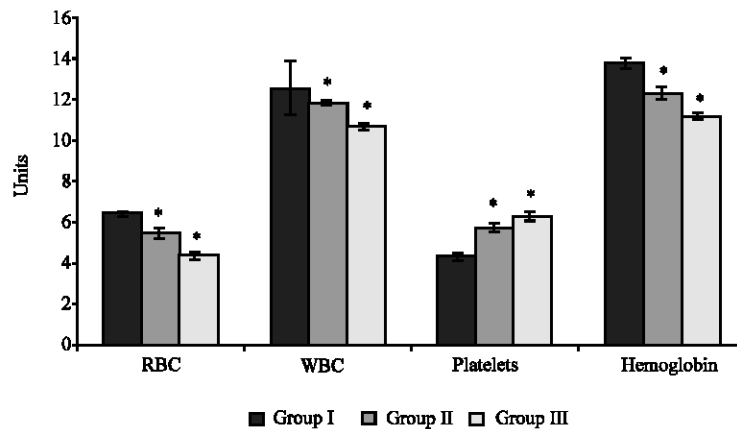

Fig. 1: Levels of RBC, WBC, platelets and hemoglobin in control and experimental groups of rats. Values are expressed as mean±SD for six animals in each group. One way ANOVA followed by post hoc test LSD. *Values are statistically significant at (p<0.05). Groups II, III compared with group I. Units: RBC×10^6 mm^{-3}, WBC×10^6 mm^{-3}, Platelets×10^6 mm^{-3}, Hemoglobin g/d

A significant increase in the levels of fluoride, alkaline phosphatase and acid phosphatase in serum were observed in the experimental groups of rats (Table 3).

Table 4 shows that levels of alkaline phosphatase was significantly decreased in the bone of experimental groups of rats. There was a significant increase in tartrate resistance acid phosphatase in bone of the experimental groups of rats. A significant increase in the level of fluoride in bone of experimental groups of rats were also observed.

Table 5 shows the GAG fractions in bone of control and experimental groups of rats. The level of hyaluronic acid was significantly increased and a concomitant decrease in the levels of chondroitin sulphate and heparan sulphate were observed in the experimental groups of rats.

Figure 2 shows the level of collagen in bone of control and experimental groups of rats. The collagen content in the experimental groups of rats was significantly decreased when compared to control group.

Table 1: Levels of serum cations in control and experimental groups of rats

Parameters	Group I	Group II	Group III
Sodium	146.1±1.5	150.2±1.9*	156.1±2.3*
Potassium	4.15±0.08	4.81±0.08*	5.11±0.09*
Calcium	9.05±0.30	7.95±0.18*	7.05±0.18*
Phosphorous	5.08±0.28	6.71±0.23*	8.05±0.18*

Values are expressed as mean±SD for six animals. One way ANOVA followed by post hoc test LSD. *Values are statistically significant at ($p < 0.05$). Groups II, III compared with group I. Units: Sodium, Potassium-Meq L^{-1} calcium, Phosphorous-mg 100 dL^{-1}

Table 2: Levels of blood glucose, protein, creatinine, urea and uric acid in control and experimental groups of rats

Parameters	Group I	Group II	Group III
Blood glucose	82.59±2.21	85.55±0.78*	87.71±1.14*
protein	8.11±0.05	5.32±0.03	2.56±0.01
Creatinine	0.97±0.03	1.03±0.09*	1.87±0.21*
Urea	18.56±0.38	20.50±0.47*	21.45±0.26*
Uric acid	2.37±0.19	3.82±0.15*	4.78±0.14*

Values are expressed as mean±SD for six animals. One way ANOVA followed by post hoc test LSD. *Values are statistically significant at ($p < 0.05$). Groups II, III compared with group I. Units: Blood glucose, protein, creatinine, urea and uric acid-mg dL^{-1}

Table 3: Levels of serum fluoride, alkaline phosphatase and acid phosphatase in control and experimental groups of rats

Parameters	Group I	Group II	Group III
Fluoride	3.61±0.72	6.52±1.01*	8.73±1.2*
Alkaline phosphatase	86.7±9.21	106.6±11.63*	127.7±13.01*
Acid phosphatase	29.34±1.01	30.21±2.22*	32.86±3.29

Values are expressed as mean±SD for six animals. One way ANOVA followed by post hoc test LSD. * Values are statistically significant at ($p < 0.05$). Groups II, III compared with Group I. Units: Fluoride - μmol L^{-1}; Alkaline and Acid phosphatase IU L^{-1}

Table 4: Levels of bone fluoride, alkaline phosphatase and tartrate resistance acid phosphatase in control and experimental groups of rats

Parameters	Group I	Group II	Group III
Fluoride	172.3±7.7	354.6±6.9*	410.5±6.1*
Alkaline phosphatase	1.04±0.30	0.58±0.23*	0.21±0.10*
Tartrate resistance acid phosphatase	0.03±0.63	0.39±0.51*	0.50±0.34*

Values are expressed as mean±SD for six animals. One way ANOVA followed by post hoc test LSD. *Values are statistically significant at ($p < 0.05$). Groups II, III compared with Group I. Units: Fluoride μ g^{-1}, Alkaline phosphatase and tartrate resistance acid phosphatase-μmoles of p-nitrophenol formed/hour/mg protein

Table 5: GAG fractions in bone of control and experimental groups of rats

Parameters	Group I	Group II	Group III
Chondroitin sulphate	0.55±0.18	0.27±0.16*	0.15±0.11*
Heparan sulphate	12.95±0.59	7.03±0.29*	5.81±0.23*
Hyaluronic acid	44.13±2.03	62.01±2.20*	73.26±2.46*

Values are expressed as mean±SD for six animals. One way ANOVA followed by post hoc test LSD. *Values are statistically significant at ($p < 0.05$). Groups II, III compared with group I. Units: Chondroitin sulphate, heparan sulphate, hyaluronic acid-μg of GAG/100 mg bone
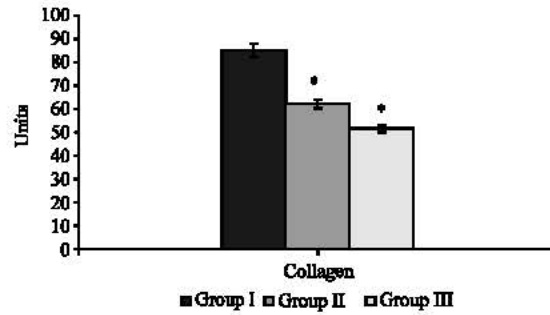
Fig. 2: Level of bone collagen in control and experimental groups of rats. Values are expressed as mean±SD for six animals in each group. One way ANOVA followed by post hoc test LSD.* Values are statistically significant at ($p<0.05$). Groups II, III compared with group I. Units: Hydroxyproline/g defatted tissue
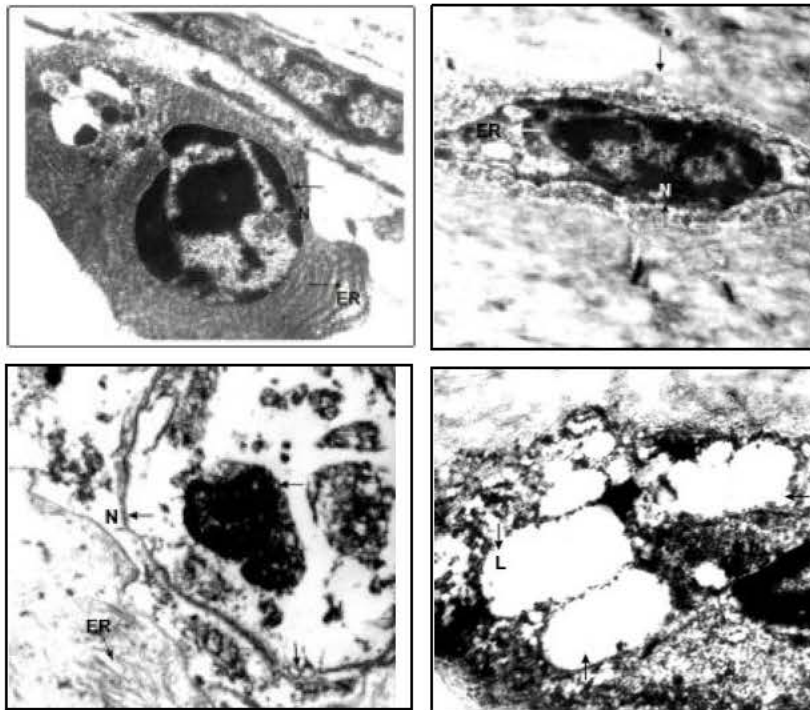

Fig. 3: Electron microscopic studies made on the bone tissue of control and experimental groups of rats

3a. Bone cell of control rat showing normal nucleus (N), with prominent nucleolus and endoplasmic reticulum (ER). Magnification: X×20,000

3b. Bone cell of experimental group of rats orally treated with fluoride (25 mg L^{-1}) for 8 weeks showing mild flattening of bone cells with damaged in nucleus (N) and endoplasmic reticulum (ER). Magnification: X×20,000

3c. Bone cell of experimental groups of rats orally treated with fluoride (25 mg L^{-1}) for 16 weeks showing severally damaged nucleus (N) and nucleolus with large distention of endoplasmic reticulum (ER). Magnification: X×20,000

3d. Bone cell of experimental group of rats orally treated with fluoride (25 mg L^{-1}) for 16 weeks showing enlarged lacunae (L). Magnification: X×20,000

Electron Microscopic Studies

The electron microscopic studies revealed extensive alterations in the bone cells of fluoride treated rats (Fig. 3a-d). The bone cells of control rats showed normal architecture (Fig. 3a). Experimental group of rats orally treated with fluoride (25 mg L^{-1}) for 8 weeks showed mild flattening of bone cells with damage in nucleus and endoplasmic reticulum (Fig. 3b). Experimental groups of rats orally treated with fluoride (25 mg L^{-1}) for 16 weeks showing severally damaged nucleus and nucleolus with large distention of endoplasmic reticulum (Fig. 3c). Experimental group of rats orally treated with fluoride (25 mg L^{-1}) for 16 weeks showed enlarged lacunae (Fig. 3d).

DISCUSSION

Fluoride being a highly electronegative element has extraordinary tendency to get attracted by positively charged ions like calcium. Hence the effect of fluoride on mineralized tissues like bone and teeth leading to developmental alterations is of clinical significance as they have highest amount of calcium and thus attract maximum amount of fluoride that gets deposited as calcium-fluorapatite crystals and this forms the basis for skeletal fluorosis. Skeletal fluorosis affects children as well as adults. Fluoride mainly gets deposited in the joints of neck, knee, pelvic and shoulder bones and makes the movement difficult. The early symptoms of skeletal fluorosis include sporadic pain, back stiffness, burning like sensation, pricking and tingling in the limbs, muscle weakness, chronic fatigue, abnormal calcium deposits in bones and ligaments. The advanced stage is osteoporosis in long bones and in some cases bony outgrowths may occur.

Alkaline phosphatase (ALP) is the marker enzyme of fluoride toxicosis and bone pathology. An increase in serum alkaline phosphatase activity in animals treated with fluoride has been reported (Blood *et al.*, 1983). The observed increase in the activity of alkaline phosphatase may be due to the effect of fluorine intoxication on bone tissues, causing protuberances. Similar observations were made by various authors (Farley *et al.*, 1983; Teotia and Teotia, 1991). The level of acid phosphatase is increased during fluoride intoxication which is also an early marker of tissue damage because of its specificity and catalytic activity. The elevated levels of the enzymes are as a result of bone disorders which are characterised by osteoblastic activity. This concept is proved in a study with fluoride on human osteoporosis patients (Marie and Hott, 1986). Upon treatment with sodium fluoride, it led to an increase in trabecular bone density which in turn lead to an increase in serum alkaline phosphatase (Haber and Willvonseder, 1979). Fluoride induced cell injury in both osteoblasts and osteocytes initiates a repair response and results in increased alkaline phosphatase production in both of these cell populations. The repair response in osteoblasts results in increased proliferation, matrix production and alkaline phosphatase production (Farley *et al.*, 1983; Marie and Hott, 1986). When the repair process in osteoblasts fails, the osteoblast undergoes either apoptosis or necrosis (Tomkinson *et al.*, 1997; Noble *et al.*, 1997) and is replaced by proliferation of osteoprogenitor cells. These new osteoblasts will then be injured and the cycle of increased repair and cell death would be repeated. This activation of a repair response in osteoblasts would contribute to increased serum alkaline phosphatase.

The hypocalcaemia observed in the present study might be due to decreased calcium absorption from the gut, increased calcium accumulation in tissues and/or parathyroid gland stimulation (secondary hyperparathyroidism). With a high fluoride intake, insoluble calcium fluoride is formed in the intestine and excreted in faeces, increasing the likelihood of a low blood calcium if there is an insufficient dietry intake. In turn, hypocalcaemia may lead to parathyroid stimulation with a secondary hyperparathyroidism, bone matrix resorption/osteoporosis and osteomalacia (Zang *et al.*, 1996). There was an increase in the level of phosphorous which may be due to parathyroid stimulation.

Uric acid is the major catabolic product from the purine nucleotides by xanthine oxidase enzymatic system. The enzyme xanthine oxidase catalyses the oxidation of xanthine to hypoxanthine and finally to uric acid, the excess of which initiates the symptomatology of gout. Increased uric acid level is a risk factor for cardiovascular disease (Maxwell and Bruinsma, 2001). Fluoride intoxication induced elevation of urea and creatinine which are considered as significant markers for renal dysfunction.

In the present study, the levels of sodium and potassium concentrations were significantly increased in the fluoride treated rats. This could be due to fluoride-induced potassium efflux from cells (McIvor *et al.*, 1985).

Fluoride damages erythrocytes and induces echinocyte formation (Jain and Susheela, 1986). These damaged erythrocytes are eliminated through the process of phagocytosis. Reports have shown that fluoride decreases RBC and hemoglobin (Pillai and Mane, 1985). Hlynczak and Ubranska (1987) reported a significant decrease in blood haemoglobin and haematocrit of catfish, cows and rats exposed to fluoride, caused by inhibition of haemopoeisis.

Serum fluoride concentration is recognized as a good indicator of fluoride exposure and provides an important basis for endemic fluorosis control and prevention (Xiang *et al.*, 2005).

Hyperglycemia have been demonstrated in experimental rats exposed to fluoride and may be due to an increase in hepatic glucose-6-phosphatase activity (Suketa *et al.*, 1985). After acute administration of fluoride in rats insulin secretion is inhibited, which led to hyperglycemia (Rigalli *et al.*, 1990).

Collagen is the major protein of connective tissues. As a tissue, bone is rich in collagen, when fluoride enters bone structure in toxic amounts, it modifies not only the mineral metabolism but also the collagen component of bone matrix (Krishnamachari, 1986). In the present study fluoride treated group, collagen synthesis was found to be defective, while it was normal in the controls. Along with potentially altering/damaging the interface between the bone mineral and collagen matrix, fluoride may also directly damage the quantity/quality of the collagen itself. Collagen synthesized and laid down during fluoride exposure is under hydroxylated and inadequately cross-linked. As a consequence, this collagen is rapidly catabolized and collagen content of the bone is decreased.

Fluoride is known to increase bone remodeling rate (Riggs *et al.*, 1990). Bone in a state of high turn over may have reduced mechanical properties (Einhorn, 1992). Fluoride could shift the mineralization profile of bone towards hypermineralization by stabilizing the apatite lattice and therefore decreasing bone mineral solubility (Grynpas, 1993). This shift in mineralization would make the bone more brittle despite an increased mass. Reduced bone strength might be expected as fluoride changes the quality of bone collagen by impairing the cross-linking, changing the glycosaminoglycans, reducing interfacial bonding between mineral and organic components of bone (Walsh *et al.*, 1994).

In the present study there was an increase in the activity of tartrate resistance acid phosphatase. The osteoblastic tartrate-resistance acid phosphatase protein tyrosine phosphorylation is known to be inhibited by micro molar concentrations of fluoride (Lau *et al.*, 1989). In addition, fluoride at micro molar concentrations has been shown to be a potent osteogenic agent and stimulates osteoblast proliferation and bone formation *in vivo* and *in vitro* (Lau and Baylink, 2001).

CONCLUSIONS

Hence, it may be concluded that prolonged ingestion of drinking water containing high fluoride may lead to extensive bone damage which may readily accounts for the prevalence of skeletal fluorosis in endemic areas where fluoride content is over and above the permissible limits in drinking water.

REFERENCES

Andersch, M.A. and A.J. Szecypinski, 1947. Use of p-nitrophenyl phosphate as the substrate for determination of serum acid phosphatase. Am. J. Clin. Pathol., 17: 571-574.

Bauer, P.J., 1981. Affinity and stoichiometry of calcium binding by arsenazo III. Analyt. Biochemi., 110: 61-72.

Bellack, E. and P.J. Schouboe, 1968. Rapid photometric determination of fluoride with SPADNS-zirconium lake. Anal. Chem., 30: 2032-2034.

Blood, D.C., O.M. Radostits, J.A. Henderson, J.H. Arundel and C.C. Gay, 1983. Veterinary Medicine. 6th Edn., Bailliere Tindal and Cassel Ltd., London.

Brecher, g. and E.P. Cronkite, 1964. Estimation of the Number of Platelets by Phase Microscopy. In: Blood Coagulation, Hemorrhage and Thrombosis. Tocantins, L.M. and L.A. Kazal (Eds.), Grune and Strallon Inc Ed., New York.

Broad, J. and J.H. Sirota, 1948. Renal clearance of endogenous creatinine in man. J. Clin. Invest., 27: 645-654.

Caraway, W.I., 1963. Uric acid. In: Standard Methods of Clinical Chemistry. D. Seligson, Vol.4, Academic Press, New York, pp: 239-247.

Choubisa, S.L., 1999. Some observations on endemic fluorosis in domestic animals in Southern Rajasthan (India). Vet. Res. Commun., 23: 457-465.

Daly, J.A. and g. Erlinghausen, 1972. Determination of inorganic phosphate in human blood serum. Clin. Chem., 18: 263-265.

Dean, H.T., 1934. Classification of mottled enamel diagnosis. J. Am. Dental Assoc., 21: 1421-1426.

Drabkin, D.L. and J.M. Austin, 1932. Spectrophotometric constants for common hemoglobin derivatives in human, dog and rabbit blood. J. Biol. Chem., 98: 719-733.

Einhorn, T.A., 1992. Bone strength: The bottom line. Calcif Tissue Int., 51: 333-339.

Farley, J.R., J.E. Wergedal and D.J. Baylink, 1983. Fluoride directly stimulates proliferation and alkaline phosphatase activity of bone-forming cells. Sciences, 222: 330-332.

Gopala Krishnan, V., J. Arunakaran, P. Govindarajulu and N. Srinivasan, 2003. Effects of estradiol and progesterone on vertebral collagen, glycosaminoglycans and phosphatases in ovariectomized adult rats. Horm. Metab. Res., 35: 137-141.

Grynpas, M., 1993. Age and disease-related changes in the mineral of bone. Calcif. Tissue Int., 53: 57-64.

Haber, P. and R. Willvonseder, 1979. Therapy of senile osteoporosis using sodium fluoride continuous and intermittent long term therapy. Schweiz-Med-Wochenschr, 109: 688-692.

Hlynczak, A.J. and A. Urbanska, 1987. The biochemical parameters in the blood of breeding animals exposed to fluoride compounds in the environment. Foliamed. Cracov., 28: 89-96.

Homer, K.A., L. Denbow and D. Beighton, 1993. Spectrophotometric method for assay of glycosaminoglycan depolymerizing enzymes. Anal. Biochem., 79: 431-437.

Huxtable, R.J., 1990. Activation and pulmonary toxicity of Pyrrolizidine alkaloids. Pharmacol. Therap., 47: 371-389.

Jain, S.K. and A.K. Susheela, 1986. Fluoride Toxicity: Erythrocyte Membrane Abnormally and Echinocyte Formation. In: Fluoride Research. Tsunoda, H. and M.H. Yu (Eds.), Studies in Environmental Sciences, Vol. 27, Amsterdam: Elsevier Science BV.

King, J., 1965. The hydrolases-acid and alkaline phosphatases. In: Practical clinical enzymology, Van D. Nostrand Co., London, pp: 199-208.

Krishnamachari, K.A., 1986. Skeletal fluorosis in humans: A review of recent progress in the understanding of the disease. Prog. Food Nutr. Sci., 10: 279-314.

Kundu, N., M.K. Panigrahi, S. Tripathy, S. Munshi, M.A. Powell and B.R. Hart, 2001. Geochemical appraisal of fluoride contamination of groundwater in the Nayagarh District of Orissa. Indian Environ. Geol., 41: 451-460.

Lau, K-HW., J.R. Farley, T.K. Freeman and D.J. Baylink, 1989. A proposed mechanism of the mitogenic action of fluoride on bone cells: Inhibition of the activity of an osteoblastic acid phosphatase. Metabolism, 38: 858-868.

Lau, K-H.W. and D.J. Baylink, 2001. Fluoride Therapy for Osteoporosis. In: Osteoporosis. Marcus R., D. Feldman and J. Kelsey (Eds.), 2nd Edn., Academic Press, San Diego, CA, USA., pp: 675-697.

Lowry, O.H., N.J. Rosenbrough, A.L. Farr and R.J. Randal, 1951. Protein determination using Folin-Phenol Reagent. J. Biol. Chem., 193: 265-275.

Mariappan, P., T. Vasudevan and V. Yegnanaraman, 1999. Behaviour of fluoride with respect to static water level in Salem Distrct, Tamil Nadu. In: Proceedings of the Int. Sem. on Applied Geochem., Annamalai Nagar, pp: 31-46.

Marie, P.J. and M. Hott, 1986. Short-term effects of fluoride and strontium on bone formation and resorption in the mouse. Metabolism, 35: 547-551.

Maxwell, A.J. and K.A. Bruinsma, 2001. Uric acid is closely related to vascular nitric oxide activity. J. Am. Coll. Cardiol., 38: 1850-1858.

McIvor, M.E., C.C. Cummings, M.M. Mower, R.F. Balttazer, R.E. Wenk, J.A. Lustgarten and J. Solomon, 1985. The manipulation of potassium efflux during fluoride information: Implication for therapy. Toxicology, 37: 233-239.

Natelson, S., M.L. Scott and C. Beffo, 1951. A rapid method for the estimation of urea in biological fluids by means of the reaction between diacetyl and urea. Am. J. Clin. Pathol., 21: 275-281.

Noble, B.S., H. Stevens, N. Loveridge and J. Reeve, 1997. Identification of apoptotic changes in osteocytes in normal and pathologic human bone. Bone, 20: 273-282.

Pillai, K.S. and U.H. Mane, 1985. Effect of airborne fluoride on some haematological parameters of chick. Bull. Environ. Contam. Toxicol., 33: 510-516.

Raghuramulu, N., K. Madhavan and S. Kalyana Sundram, 1983. Hematological Techniques, In: A Manual of Laboratory Techniques, Silver Prints, India, pp: 254-258.

Rigalli, A., J.C. Ballina, E. Roveri and R.C. Puche, 1990. Inhibitory effect of fluoride on insulin secretion. Calcif. Tissue Intl., 46: 333-338.

Riggs, B.L., S.F. Hodgson, W.M. O'Fallon, E.Y.S. Chao, H.W. Wahner, J.M. Muhs, S.L. Cedel and L.J. Melton III, 1990. Effect of fluoride treatment on the fracture rate in postmenopausal women with osteoporosis. New Eng. J. Med., 322: 802-809.

Sasaki, T., S. Matsy and A. Sonae, 1972. Effect of acetic acid concentration on the colour reaction in the O-toluidine boric acid method for blood glucose estimation. Rinsh Kagaku, 1: 346-353.

Stegemann, H., 1958. Microdetermination of hydroxyproline with chloramines-T and p-dimethylamino benzaldehyde. Hoppe. Seylers. Z. Physiol. Chem., 311: 41-45.

Suketa, Y., Y. Asao, Y. Kanamoto, T. Shakashita and S. Okada, 1985. Changes in adrenal function as a possible mechanism for elevation of serum glucose by single large dose of fluoride. Toxicol. Applied. Pharmacol., 80: 199-205.

Susheela, A.K., 1999. Fluorosis management programme in India. Curr. Sci., 77: 1250-1256.

Tenniswood, M., C.E. Bird and A.F. Clake, 1976. Acid phosphatases: Androgen dependent markers of rat prostate. Can. J. Biochem., 54: 350-357.

Teotia, S.P.S. and M. Teotia, 1988. Endemic skeletal fluorosis: Clinical and radiological variants. Fluoride, 21: 39-44.

Teotia, S.P.S. and M. Teotia, 1991. Endemic fluoride: Bone and teeth-update. Ind. J. Environ. Toxicol., 1: 1-6.

Tomkinson, A., J. Reeve, R.W. Shaw and B.S. Noble, 1997. The death of osteocytes via apoptosis accompanies estrogen withdrawal in human bone. J. Clin. Endocrin. Metab., 83: 3128-3135.

Varley, H., 1988. Practical Clinical Biochemistry. CBS Publishers, Delhi, India.

Walsh, W.R., D.P. Labrador, H.D. Kim and N. Guzelsu, 1994. The effect of *in vitro* fluoride ion treatment on the ultrasonic properties of cortical bone. Ann. Biomed. Eng., 22: 404-415.

Wang, L-F. and J-Z. Huang, 1995. Outline of control practice of endemic fluorosis in China. Soc. Sci. Med., 41: 1191-1195.

WHO., 1970. Fluoride and Human Health. W.H.O. Monograph, Serin No. 59, Geneva 273.

WHO Guidelines for Drinking Water Quality, 1984. Vol. 1, World Health Organization, Geneva, Switzerland.

Xiang, Q.Y., L.S. Chen, X.D. Chen, C.S. Wang and Y.X. Liang *et al.*, 2005. Serum fluoride and skeletal fluorosis in two villages in Jiangsu Province, China, Fluoride, 38: 178-184.

Zang, Z.Y., J.Y. Fan, W. Yen, J.Y. Tian, J.G. Wong, X.X. Li and E.L. Wang, 1996. The effect of nutrition on the development of endemic osteomalacia in patients with skeletal fluorosis. Fluoride, 29: 20-24.