*International Journal of Pharmaceutical Science Invention*
*ISSN (Online): 2319 – 6718, ISSN (Print): 2319 – 670X*
*www.ijpsi.org || Volume 3 Issue 11 || November 2014 || PP.51-56*

# Modulation of calcium homeostasis and ATPase activity in erythrocyte membrane of patients afflicted with fluorosis

[1,]Shashi A , [2,]Meenakshi G
[1,2]*Department of Zoology and Environmental Sciences, Punjabi University, Patiala-147002, Punjab, India*

***ABSTRACT :*** *The present study was performed to evaluate effect of consumption of excessive fluoride in drinking water by individuals on erythrocyte membrane activity of $Ca^{2+}$-ATPase and its transport mechanism of $Ca^{2+}$ ions. Areas with different fluoride concentration in drinking water were divided into five study groups viz; Control (0.63-1.00 mg/L), A-I (8.00-10.00 mg/L), A-II (10.01-12.00 mg/L), A-III (12.01-14.00 mg/L) and A-IV (14.01-16.00 mg/L). Erythrocytes were used for the estimation of intra-erythrocyte calcium concentrations. Serum samples were analysed for fluoride content. $Ca^{2+}$-ATPase activity was determined spectrophotometrically from ghost erythrocyte membranes. Results showed a significant decrease in intra-erythrocyte calcium ($F = 50.289$, $P<0.001$) and elevated levels of serum fluoride ($F = 9900.90$, $P<0.001$) in fluorotic patients. Regression analysis showed significant negative ($P<0.001$) relationship of water ($r = -0.972$, $R^2 = 0.945$, $Y = 0.013 + 0.0001 X$) as well as serum fluoride ($r = -0.955$, $R^2 = 0.912$, $Y = 0.007 + 0.072 X$, $P<0.001$) with intracellular calcium concentrations. Membrane bound $Ca^{2+}$-ATPase activity was noted with highly significant reduction ($F = 30819.16$, $P < 0.001$) in patients of fluorosis. Regression analysis exibited significant inverse ($P<0.001$) relationship of water ($r = -0.986$, $R^2 = 0.973$, $Y = 0.33 - 0.01 X$) as well as serum fluoride ($r = -0.997$, $R^2 = 0.995$, $Y = 0.34 - 0.36 X$, $P<0.001$) with erythrocyte membrane $Ca^{2+}$-ATPase activity. These findings suggest that depression of erythrocyte $Ca^{2+}$ ions and $Ca^{2+}$-ATPase pump has been implicated in the pathogenesis of membrane abnormalities observed in fluorotic patients.*

***KEYWORDS:*** *Erythrocyte membrane, Fluoride, Membrane bound $Ca^{2+}$-ATPase, Total intracellular $Ca^{2+}$ content*

## I. INTRODUCTION

Calcium is well known to play a pivotal role in the regulation of cellular physiology. In human red cells, calcium is mainly bound to the inner side of the plasma membrane. A smaller part is present within intracellular calcium storing vesicles, while only a few percent of total red cell calcium is in ionized form (Engelmann, 1991). There is a very large transmembrane electrochemical gradient of $Ca^{2+}$ driving the entry of the ion into cells, yet it is very important that they maintain low concentrations of $Ca^{2+}$ ions for proper cell signalling. Thus, it is necessary for cells to employ ion pumps to remove the $Ca^{2+}$. The plasma membrane calcium ATPases contribute to the maintenance of appropriate cytoplasmic calcium levels by removing calcium from the cell to the extracellular environment (Carafoli, 1994, 2002).

Fluorine is abundant in the environment and exists only in combination with other elements as fluoride compounds. Excessive fluoride intake over a long period of time may result in a serious public health problem called fluorosis. There are number of inhibitors which inhibits the activity of $Ca^{2+}$-ATPase pump. Out of which fluoride is a slow tight-binding inhibitor of $Ca^{2+}$-ATPase pump (Kumari and Rao, 1991; Murphy and Coll, 1992; Daiho et al., 1993; Reis et al., 2001; Han et al., 2006). The activity of erythrocyte membrane bound $Ca^{2+}$-ATPase in fluoride intoxicated rats decreased significantly ($p<0.05$) when compared to control group (Miltonprabu and Thangapandiyana, 2013). Disruptions in membrane pumps has always been suspected in association with other electrolyte imbalances. Reduction in calcium transport across the renal tubule endoplasmic reticulum and plasma membrane, as well as to a reduction of the amount of calcium pump proteins in isolated kidney membranes were reported (Borke and Whitford, 1999). Interruptions caused by fluoride and their consequences in fluorotic subjects are well known but still our knowledge in membrane functions are limited. The pathogenesis and mechanism of fluorosis specifically in case of erythrocyte membrane pump activities remains unresolved. The present study was, therefore, designed to investigate the influence of fluoride on erythrocyte membrane bound $Ca^{2+}$-ATPase pump activity and intracellular $Ca^{2+}$ions in fluorotic subjects.

## II. MATERIALS AND METHODS

**Subjects and experimental design :** 500 patients of both sexes in the age group of 20-50 (mean age 35 ± 12.90 years) affected with dental (Dean, 1934) and skeletal fluorosis (Wang et al., 1994) were proportionate randomly selected from endemic fluorotic areas. 120 healthy individuals with same age and gender matched without

fluorosis were included in the study as control group. The level of fluoride in drinking water varied from 0.63 – 16.00 mg/L. On the basis of fluoride concentration in drinking water, total surveyed areas divided into five study groups, viz; Control (0.63-1.00 mg/L), A-I (8.00-10.00 mg/L), A-II (10.01-12.00 mg/L), A-III (12.01-14.00 mg/L) and A-IV (14.01-16.00 mg/L).

**Ethics :**The study was approved by the Institutional Clinical Ethical Committee (ICEC/31/2012), Punjabi university, Patiala, India. The written informed consent was obtained from all persons included in this study.

**Erythrocyte membrane preparation :**Blood samples of controls and patients were collected in EDTA anticoagulant vacutainers for analysis of intraerythrocyte calcium, serum fluoride and erythrocyte membrane $Ca^{2+}$-ATPase activity. Anticoagulated blood was centrifuged, plasma and buffy coat were discarded. Erythrocytes were lysed, and used for the estimation of intra-erythrocyte calcium ions. Erythrocyte membrane was prepared as described by Weed et al. (1963). Estimation of intraerythrocyte calcium was done with Erba diagnostic kits and serum fluoride by the method of Harwood (1969). The $Ca^{2+}$-ATPase activity was determined by the method of Zaidi and Saleemuddin (1993) and quantity of inorganic phosphate liberated from the hydrolysis of ATP was determined (Fiske and Subbarow, 1925). $Ca^{2+}$-ATPase activity was expressed as micro mole Pi liberated per mg membrane protein/hour.

**Statistical Analysis :** Data are presented as mean ± standard deviation. The significance of differences among the group was assessed using one way analysis of variance followed by post hoc Dunnett's T3 multiple comparison test. The level of significance was set at $P<0{\cdot}05$. Erythrocyte membrane analytes were also correlated with the water and serum fluoride levels by correlation and regression analysis. The statistical program used was SPSS for windows version 16.0.

## III. RESULTS

**Intracellular calcium content and serum fluoride :** The mean erythrocyte membrane calcium level was significantly ($P<0.001$) declined in fluorotic patients compared to controls. One way ANOVA depicted significant ($F = 50.289$, $P<0.001$, Fig. 1) difference in the levels of intracellular calcium. Difference in the erythrocyte membrane calcium concentrations was unequal as determined by levene statistic which was significant (48.785, $P<0.001$). Post hoc Dunnett's T3 multiple comparison test revealed that the levels of intracellular calcium decreased significantly (95% CI = 0.0008 to 0.0041, $P<0.001$) in fluorotic patients among all study groups as well as when compared with control group. The group that displayed the sharpest percent decrease in the level of calcium ions was exposed to 14.01-16.00 mg/L fluoride in drinking water, and this decrease was demonstrated to be 25.6% of the control. The female fluorotic patients exihibited significantly ($P<0.001$) more pronounced lower concentration of erythrocyte membrane calcium ions than males (T = 118.79-30.63). Correlation ($r = -0.972$) and linear regression revealed significant ($P<0.001$) inverse relationship between water fluoride concentration ($Y = 0.013 + 0.0001\ X$, $R^2 = 0.945$, Fig. 2) and intracellular calcium levels. As the water fluoride exposure increased, the levels of fluoride through serum elevated significantly ($r = -0.955$, $R^2 = 0.912$, $Y = 0.007 + 0.072\ X$, $P<0.001$ Fig. 3) which further depleted the levels of calcium ions in erythrocyte membrane. Fluorotic patients with high serum fluoride levels ($F = 9900.90$, $P<0.001$, Fig. 4) had lower concentrations of intracellular calcium ions in erythrocyte membrane.

**$Ca^{2+}$-ATPase assay :** The activity of erythrocyte membrane $Ca^{2+}$-ATPase was significantly ($P<0.001$) declined in fluorotic patients compared to controls. One way ANOVA showed a significant ($F = 30819.16$, $P < 0.001$, Fig. 5) variance in the membrane bound $Ca^{2+}$-ATPase enzyme activity. Variance in the erythrocyte membrane ATPase activity was unequal as determined by levene statistic which was significant (51.431, $P<0.001$). Post hoc Dunnett's T3 multiple comparison test depicted that erythrocyte membrane activity of $Ca^{2+}$-ATPase decreased significantly (95% CI = 0.065 to 0.157, $P< 0.001$) in fluorotic patients among all fluoride exposed groups as well as when compared with control group. 50% decline in the activity of $Ca^{2+}$-ATPase was noted in group A-IV exposed to 14.01-16.00 mg/L fluoride in drinking water. The male fluorotic patients exihibited significantly ($P<0.001$) lowered activity of erythrocyte membrane ATPase than females (T = 8.72- 26.72). Correlation ($r = -0.986$) and linear regression revealed significant ($P<0.001$) negative relationship between water fluoride concentration ($Y = 0.33 - 0.01\ X$, $R^2 = 0.973$, Fig. 6) and $Ca^{2+}$-ATPase enzyme activity. As the water fluoride exposure increased, the levels of fluoride through serum elevated significantly ($r = -0.997$, $R^2 = 0.995$, $Y = 0.34 - 0.36\ X$, $P<0.001$ Fig. 7) which further depleted activity of $Ca^{2+}$-ATPase in erythrocyte membrane.

## IV. DISCUSSION

In this study, human red cells exposed chronically to toxic levels of fluoride through drinking water showed decreased activity of $Ca^{2+}$-ATPase in red cell ghosts of fluorotic patients.

The findings are in aggrement with the study of Kumari and Rao (1991) which reported decreased activity of $Ca^{2+}$-ATPase in human red cell membrane of fluorotic patients. Fluoride exerts multiple effects on erythrocyte physiology and metabolism. The addition of sodium fluoride (10 mmoles/liter) resulted in a rapid decrease in red cell ATP concentration both in mature and immature cells. Fluoride inhibited membrane ATPase 40% at 1 mmole/liter and completely at 5 and 10 mmoles/liter. Fluoride reacts directly with red cell membranes if free calcium is present (Feig et al., 1971). In experimental animals, renal $Ca^{2+}$-ATPase activity was inhibited by a single oral (50 mg/kg) dose of sodium fluoride in rats (Suketa and Mikami, 1977).In contrast, increased cytosolic calcium concentrations have previously been established in several cells/tissues, including proximal tubules (Dominguez et al., 1991) and osteoblasts (Zerwekh et al., 1990). Interestingly, both inhibitory and stimulatory effects of fluoride on the calcium pump have been shown in the cardiac sarcoplasmic reticulum. It was explained that the dissimilar responses were due to differential susceptibility of the conformational state of the calcium pump (Narayanan et al., 1991).

Fluoride is a slow tight-binding inhibitor of the sarcoplasmic reticulum $Ca^{2+}$-ATPase (Murphy and Coll, 1992) reported that the sarcoplasmic reticulum $Ca^{2+}$-ATPase was inactivated by fluoride in the presence of magnesium. Each inhibited enzyme contains two tightly bound fluorides and one tightly bound magnesium. This indicated that fluoride binding causes conversion of the polarity of calcium-binding sites in a fashion similar to that caused by enzyme phosphorylation. The similarity extends to lowering of the sites calcium affinity, indicating that the interaction energies between fluoride and calcium and between phosphate and calcium are in the same range, appears to be simulated well by fluoride binding. A strong inhibitory effect of fluoride on ATP-energized $Ca^{2+}$ uptake and $Ca^{2+}$-ATPase activity of cardiac and fast skeletal muscle sarcoplasmic reticulum was reported by Hawkins et al. (1994).Sarcoplasmic reticulum vesicles treated with 0.65 mM fluoride in the presence of 9 mM magnesium and reported decreased activity of $Ca^{2+}$-ATPase. The extent of the enzyme inactivation and the contents of tightly-bound magnesium, and fluoride give strong support to the conclusion that the enzyme inactivation was due to tight binding of these ligands (Daiho et al., 1993).

During present investigation, the erythrocytes of patients affected with fluorosis showed decreased $Ca^{2+}$-ATPase activity and calcium ion concentrations along with elevated levels of serum fluoride which may be due to altered membrane properties including functional and compositional changes by fluoride. Once overdose of fluorine deposition occurs in erythrocytes, the membrane cholesterol increases, and fluidity of membrane lipid was decreased, transportion of the $Na^{+}$ and $Ca^{2+}$ pump was blocked and, furthermore, endocytic ion concentration was changed (Han et al., 2006).The decreased $Ca^{2+}$-ATPase activity also decreases the membrane bound $Ca^{2+}$ concentrations which indicates that the erythrocyte permeability is altered. The low cellular $Ca^{2+}$ is a major impairment in fluorosis which leads to the loss of membrane integrity and the loss of membrane glycoprotein which was observed to decrease as a result of membrane alteration and increased osmotic fragility.
In conclusion, fluoride induced decrease in the anisotropy of plasma membranes as determined with depressed erythrocyte membrane $Ca^{2+}$-ATPase activity and influx of calcium ions from the extracellular environment leads to disturbance and modulation in $Ca^{2+}$ homeostasis and normal functioning of erythrocyte membrane, which regulates cell death.

## V. ACKNOWLEDMENTS

The work was supported by grants from University Grants Commission, Govt. of India, in the form of Rajiv Gandhi National fellowship (Letter No. F.14-2 (SC)/ 2009 (SA-III). The authors thanks Head, Department of Biochemistry, Post Graduate Institute of Medical Education and Research, Chandigarh, India for laboratory assistance.

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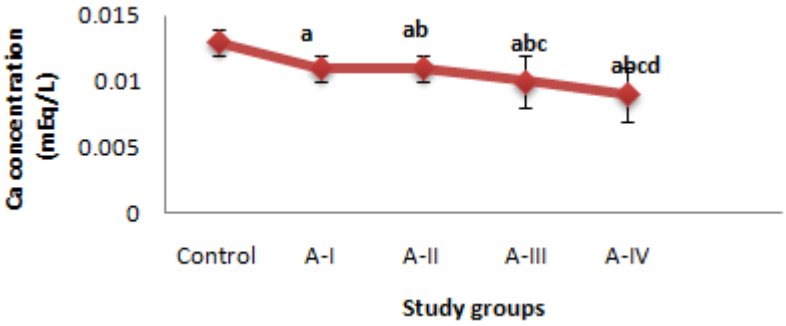


**Fig. 1.** Intracellular calcium (Ca) content in control and patients of fluorosis. [a]P<0.001, A-I to IV versus control group, [b]P<0.001, A-II to IV versus A-I, [c]P<0.001, A-III to IV versus A-II, [d]P<0.001, A-IV versus A-III.

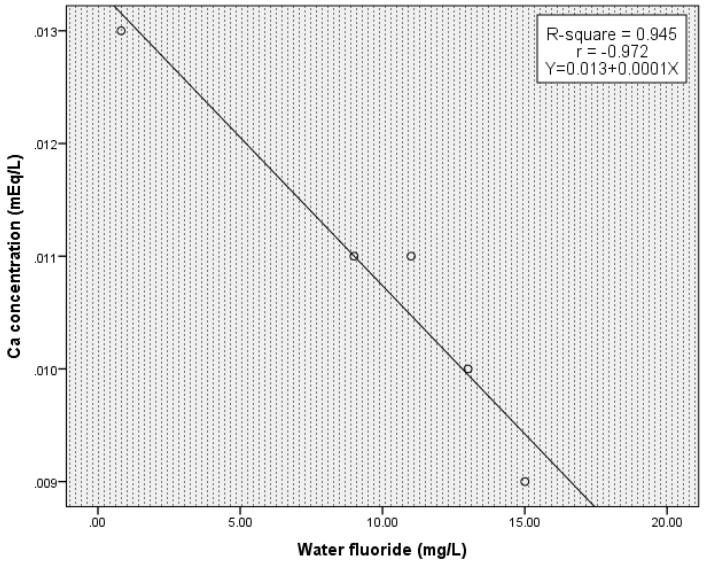


**Fig. 2.** Scatterplot showing correlation between water fluoride and intracellular calcium (Ca) content.

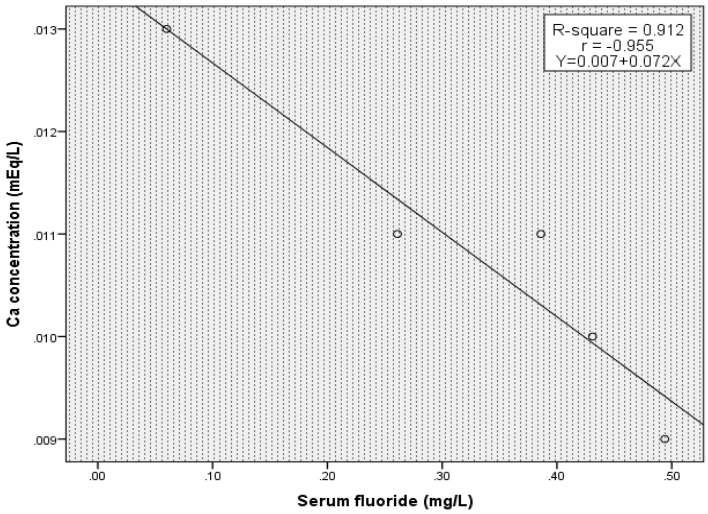


**Fig. 3.** Scatterplot showing correlation between serum fluoride and intracellular calcium (Ca) content.

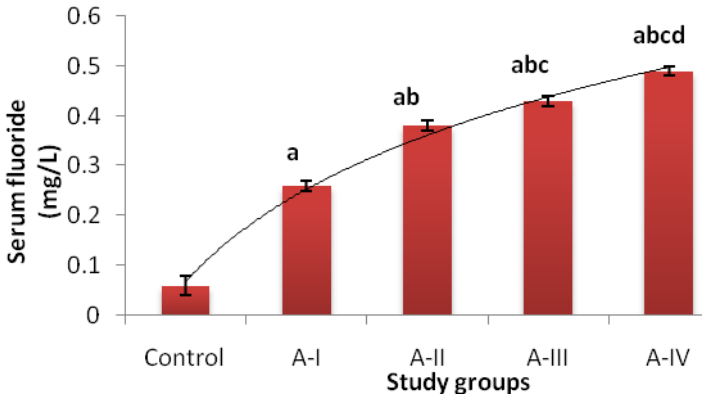


**Fig. 4.** Serum fluoride concentrations in control and patients of fluorosis. [a]P<0.001, A-I to IV versus control group, [b]P<0.001, A-II to IV versus A-I, [c]P<0.001, A-III to IV versus A-II, [d]P<0.001, A-IV versus A-III.

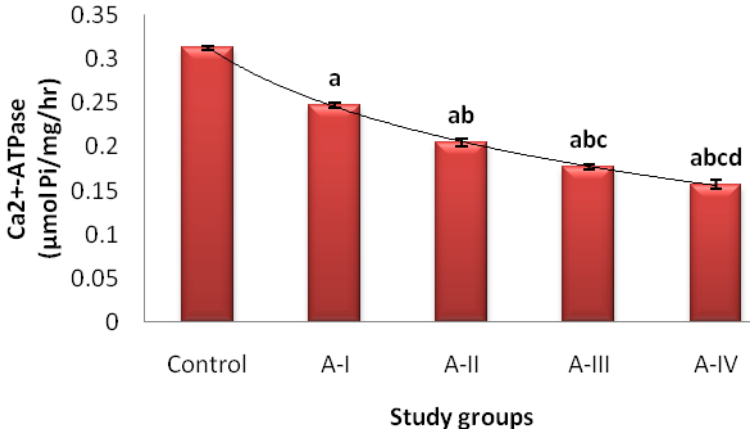


**Fig. 5.** Erythrocyte membrane $Ca^{2+}$-ATPase activity in control and patients of fluorosis. [a]P<0.001, A-I to IV versus control group, [b]P<0.001, A-II to IV versus A-I, [c]P<0.001, A-III to IV versus A-II, [d]P<0.001, A-IV versus A-III.

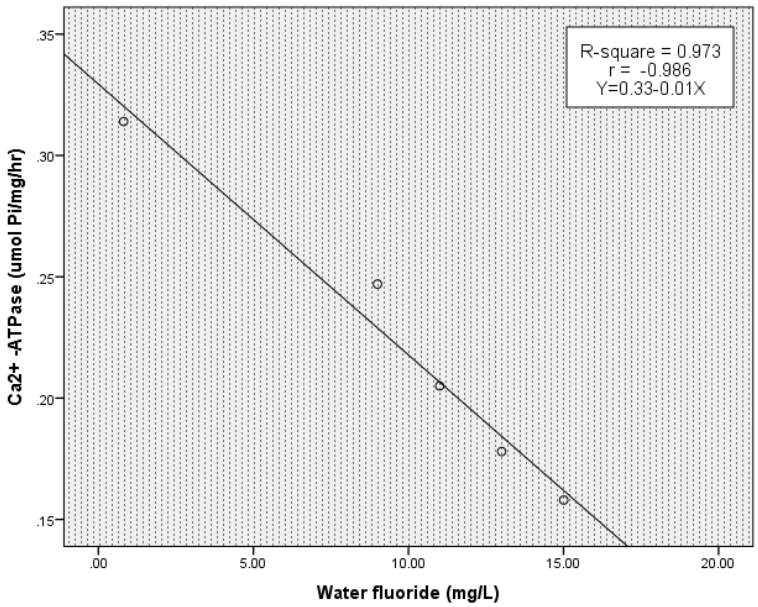


**Fig. 6.** Scatterplot showing correlation between water fluoride and erythrocyte membrane $Ca^{2+}$-ATPase activity.

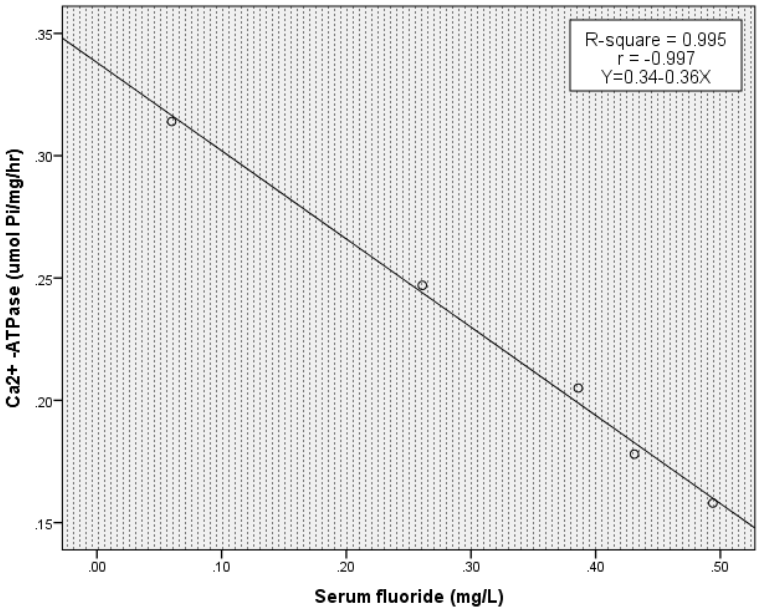


**Fig. 7.** Scatterplot showing correlation between serum fluoride and erythrocyte membrane $Ca^{2+}$ATPase activity.