International Journal of Basic and Applied Medical Sciences ISSN: 2277-2103 (Online)
An Open Access, Online International Journal Available at http://www.cibtech.org/jms.htm
2014 Vol. 4 (3) September-December, pp. 152-162/Shashi and Meenakshi
Research Article

CLINICAL STUDY OF LIPID DOMAINS IN ERYTHROCYTE MEMBRANE IN CHRONIC FLUOROSIS

***Shashi A and Meenakshi G**
Department of Zoology and Environmental Sciences, Punjabi University, Patiala-147002, Punjab, India
**Author for Correspondence*

ABSTRACT
The quantification of cholesterol, phospholipid, and proteins in erythrocyte membrane of patients exposed to varying concentrations of fluoride in drinking water was assessed to understand the mechanism of relationship between chronic fluorosis and erythrocyte membrane lipid bilayer. Cholesterol in erythrocyte membrane was determined in dry lipid extract of erythrocyte membrane by the enzymatic manual CHOD-PAP method in blood samples of 500 patients of male and female patients of fluorosis as well as in age and sex-matched 120 controls. The extract was used for estimation of erythrocyte membrane phospholipids. Aliquots of erythrocyte membrane were used to determine the protein content. The relationship of water as well as serum fluoride with cholesterol, phospholipid, cholesterol/phospholipid ratio, and proteins in erythrocyte membrane were assessed by correlation and linear regression analysis. The mean values for cholesterol in erythrocyte membrane in fluorotic patients of both sexes were elevated significantly ($F = 1357.07$, $P<0.001$) in all study groups in comparison to control. There was significant ($F = 222.40$, $P<0.001$) accumulation of phospholipids in erythrocyte membrane in the examined groups. The cholesterol/phospholipid ratio was significantly ($F = 108.04$, $P<0.001$) higher in fluorotic patients. The level of membrane protein was declined significantly ($F = 1378770.14$, $P<0.001$) in erythrocyte membrane in all study groups. Correlation and linear regression analysis showed a positive correlations ($P<0.001$) of water as well as serum fluoride with cholesterol, phospholipid and cholesterol/phospholipid ratio. Erythrocyte membrane proteins revealed negative correlations ($P<0.001$) with water and serum fluoride, that indicate water fluoride being the strong predictor of involvement in depletion of protein content and increased levels of serum fluoride. These pronounced alterations in erythrocyte membrane metabolites of fluorotic patients are responsible for the changes in erythrocyte membrane fluidity that may play a direct role in destabilizing the plasma membrane.

Keywords: *Fluoride, Erythrocyte Membrane, Membrane Cholesterol, Membrane Phospholipid, Membrane Cholesterol/Phospholipid Ratio, Membrane Protein*

INTRODUCTION
Fluorosis is a worldwide health problem and is endemic in areas where the fluoride content of drinking waters is higher than permissible limits of 1.0 mg/L (WHO 2008). In India, it is the foremost problem in different parts of the country. Its primary manifestations in humans and mammals are mottling of teeth and osteosclerosis of the skeleton. Skeletal fluorosis causes crippling and severe pain and stiffness of the backbone and joints (Jaganmohan *et al.*, 2010).

Approximately one half of the mass of the human erythrocyte membrane consists of lipid, largely arrange as a bilayer (Balls and Krasnow 1980). Cholesterol is intercalated between the phospholipid molecules. The relative amounts of phospholipids and cholesterol are responsible for the fluid properties of the erythrocyte membrane (Schmit-Schonbein and Volger 1976). It is also responsible for the biconcave shape and basic structural integrity of the erythrocyte.

Reports concerning effects of fluoride on human biological membranes are scanty. Very little information is available regarding alterations of membrane lipid bilayer and protein due to fluoride (Kumari and Rao 1991; Suwalsky *et al.*, 2004; Han *et al.*, 2005).

Despite decades of intensive research, fluoride toxicity also remains one of the most studied subjects of all within the fields of environmental health. So, the present study was undertaken to study the effects of fluoride at variable exposure on human erythrocyte membrane lipid bilayer under in vivo conditions.

MATERIALS AND METHODS

Study Areas

The present study was conducted in high fluoride endemic areas selected from Bathinda district, Punjab, India on the basis of fluoride content in drinking water. The total surveyed areas were divided into five study groups, viz; Control (0.63-1.00 mg fluoride/L), A-I (8.00-10.00 mg fluoride/L), A-II (10.01-12.00 mg fluoride/L), A-III (12.01-14.00 mg fluoride/L) and A-IV (14.01-16.00 mg fluoride/L).

Selection of Subjects

500 fluorotic patients and 120 control subjects from selected fluoride endemic areas in the age groups of 20-50 years (mean age 35 ± 12.90 years) were randomly selected. Details of physical manifestations, age, sex, economic conditions, duration of the disease, symptoms were collected from them through questionnaires.

Ethics

The study was pre-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.

Membrane Studies

500 venous blood samples from fluorotic patients as well as 120 control age and sex-matched individuals were collected in anticoagulated vacutainers coated with ethylenediaminetetraacidic acid. Erythrocytes were separated by centrifugation at 3000 rpm for 10 minutes and plasma and buffy coats were separated and used for membrane studies. Erythrocyte membrane isolation was carried out by hypotonic hemolysis of saline-washed erythrocytes (Weed *et al.,* 1963). The resulting erythrocyte membranes were suspended in 0.05 M Tris-HCl (pH 7.0). Lipid extraction from erythrocyte membrane suspensions were performed according to the method of Bligh and Dyer (1959). Aliquots of lipid extracts were assayed for cholesterol and phospholipid with the use of commercially available Erba diagnostic kits. The cholesterol/phospholipid ratio of erythrocyte membrane was calculated by dividing the cholesterol value by the phospholipid value. The protein content in erythrocyte membrane suspensions was measured according to the method of Lowry *et al.,* (1951).

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 characteristics were also correlated with the water and serum fluoride levels by linear regression analysis. The statistical program used was SPSS for windows version 16.0.

RESULTS AND DISCUSSION

Results

Membrane Cholesterol

One way ANOVA showed significant ($F = 1357.07$, $P<0.001$, Figure 1) variance in the erythrocyte membrane cholesterol. Variance in the membrane cholesterol levels was unequal determined by levene statistic which was significant (12.564, $P<0.001$). Post hoc Dunnett's T3 multiple comparison test demonstrated that erythrocyte membrane concentrations of cholesterol increased significantly (95% CI = 1.23 to 2.11, $P<0.001$) in fluorotic patients among all fluoride exposed groups as well as when compared with control group. The patients in study groups A-III and A-IV exposed to 12.01-16.00 mg/L of fluoride displayed the maximum elevation of 128 to 154% in the levels of membrane cholesterol. The all female fluorotic patients revealed significantly ($P<0.001$) higher concentration of membrane cholesterol as compared to males (T = 119.56-142.57). Correlation ($r = 0.996$) and linear regression analysis ($Y = 1.258 + 0.153\ X$, $R^2 = 0.992$, Figure 2) revealed that there was significant ($P<0.001$) positive correlation between water fluoride levels and erythrocyte membrane cholesterol. As the concentration of fluoride increases in drinking water, the level of serum fluoride increased ($Y = 1.193 + 4.771\ X$, $R^2 = 0.866$, Figure 3) and the level of erythrocyte membrane cholesterol was also elevated.

Membrane Phospholipids

One way ANOVA depicted significant (F = 222.40, P<0.001, Figure 4) difference in the erythrocyte membrane phospholipids. Variance in the membrane phospholipid levels was unequal determined by levene statistic which was significant (38.086, P<0.001). Post hoc Dunnett's T3 multiple comparison test depicted that erythrocyte membrane concentration of phospholipids increased significantly (95% CI = 0.55 to 1.14, P<0.001) in fluorotic patients among all fluoride exposed study group as well as compared with control. The groups that displayed the sharpest percent elevation in the membrane phospholipids in fluorotic patients was exposed to 12.01-16.00 mg/L fluoride in drinking water, and this elevation was demonstrated to be 38 to 47% of the control. However, the patients exposed to 8.0-12.00 mg/L fluoride in drinking water revealed 16-31% percent increase in the levels of membrane phospholipids. The male fluorotic patients exihibited significantly (P<0.001) higher concentration of membrane phospholipid as compared to females (T= 119.41-257.406). Correlation (r = 0.969) and regression equation indicates that increased water fluoride contributed significantly for high levels of membrane phospholipids (Y = 2.533 + 0.088 X, R^2 = 0.939, Figure 5). Further regression analysis revealed significant (P<0.001) relationship of serum fluoride with erythrocyte membrane phospholipid content (Y = 2.469 + 2.835 X, R^2 = 0.568, Figure 6) It showed that serum fluoride contributed significantly to the variance of levels of phospholipid.

Membrane Cholesterol/Phospholipid Ratio

One way ANOVA revealed significant (F= 108.04, P<0.001, Figure 7) difference in the erythrocyte cholesterol to phospholipid ratio. Variance in the erythrocyte cholesterol to phospholipid ratio was unequal determined by levene statistic which was significant (51.36, P<0.001). Post hoc Dunnett's T3 multiple comparison test depicted that the cholesterol to phospholipid ratio increased significantly (95% CI = 0.33 to 0.32, P<0.001) in fluorotic patients among all study groups as well as when compared with control group. Maximum percent elevation of 70% was recorded in study group A-IV exposed to 14.01-16.00 mg/L of fluoride. The female fluorotic patients showed significantly (P<0.001) higher values of cholesterol/phospholipid ratio in erythrocyte membrane than males (T= 132.42-211.63). Correlation (r = 0.985) and regression analysis (Y = 0.540 + 0.027 X, R^2 = 0.970, Figure 8) indicated highly significant (P<0.001) positive relationship between water fluoride and cholesterol/phospholipid ratio in fluorotic patients. Further analysis revealed highly significant (Y = 0.536 + 0.819 X, R^2 = 0.369, P<0.001, Figure 9) positive relationship between serum fluoride levels and cholesterol/phospholipid ratio.

Membrane Proteins

One way ANOVA demonstrated significant (F = 1378770.14, P<0.001, Figure 10) variance in the levels of membrane proteins. Variance in the erythrocyte membrane proteins was unequal determined by levene statistic which was significant (46.725, P<0.001). Post hoc Dunnett's T3 multiple comparison test revealed that the levels of membrane proteins decreased significantly (95% CI = 51.78 to 85.14, 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 hypoproteinemia in fluorotic patients was exposed to 14.01-16.00 mg/L fluoride in drinking water, and this decrease was demonstrated to be 35% of the control. However, the patients exposed to 8.0- 14.00 mg/L fluoride in drinking revealed 21 to 33% percent decrease in the membrane protein content. The male fluorotic patients as well as females exhibited significantly (P<0.001) lower concentration of erythrocyte membrane proteins (T = 3.826-18.275). Correlation (r = 0.993) and linear regression (Y = 244.938 - 6.243 X, R^2 = 0.985, Figure 11) revealed significant (P<0.001) inverse relationship between water fluoride concentration and protein levels. As the water fluoride exposure increased, the levels of fluoride through serum elevated significantly (Y = 247.561 - 194.927 X, R^2= 0.961, P<0.001 Figure 12) which further depleted the levels of erythrocyte membrane proteins.

Discussion

This study demonstrated that sodium fluoride exposure to erythrocyte membrane cholesterol, phospholipid, cholesterol/phospholipid ratio showed a significant (P<0.001) increase while decrease in membrane proteins. The results are in consonance with the study of Kumari and Rao (1991) which reported significant increase in human red cell membrane cholesterol and phospholipids under chronic fluoride toxicity.

It has been shown that erythrocyte membrane fluidity as well as deformability may be related to membrane lipid composition (Mcmillian *et al.,* 1978). The membrane lipids specifically phospholipids played a significant role in maintenance of cell shape, cell permeability as well as movement of various compounds across the membrane. It is well established that the phospholipid distribution across the erythrocyte membrane bilayer is asymmetrical (Verklij *et al.,* 1973). Protein–lipid interactions have crucial effects on the function of membranes. The changes in erythrocyte membrane lipid and phospholipid composition observed in the present study may induce changes in the physico-chemical properties of red blood cell membrane as well as in fluidity / rigidity.

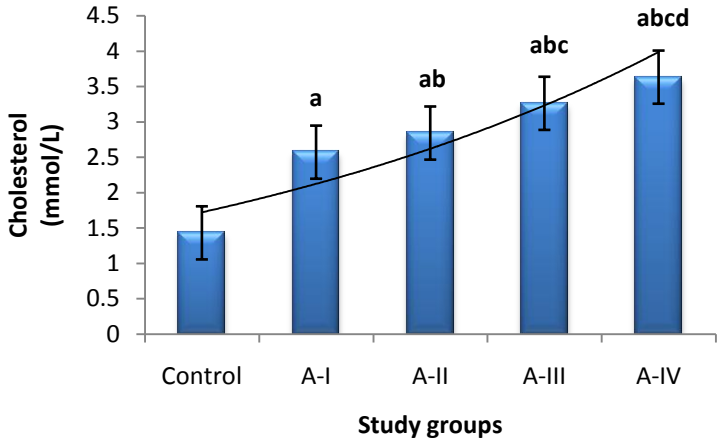

Figure 1: Mean erythrocyte membrane cholesterol levels in control and patients of fluorosis

[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

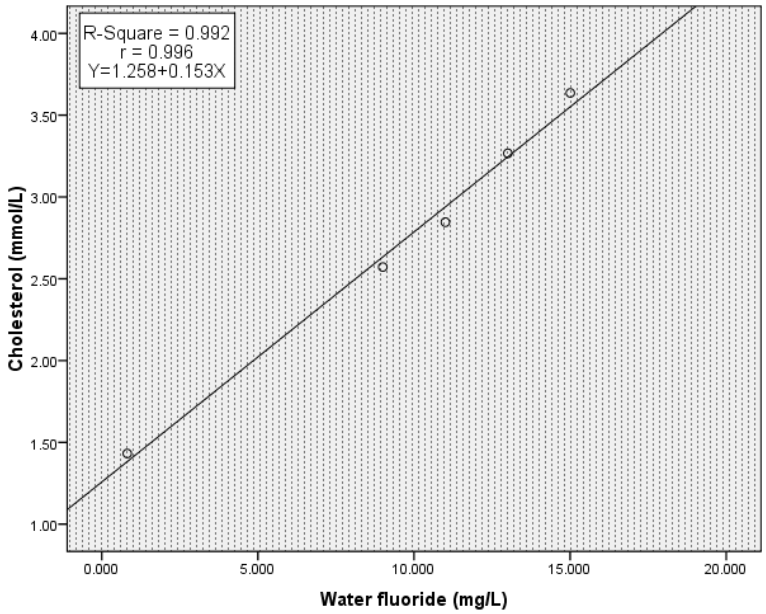

Figure 2: Scatterplot showing linear regression between fluoride and erythrocyte membrane cholesterol

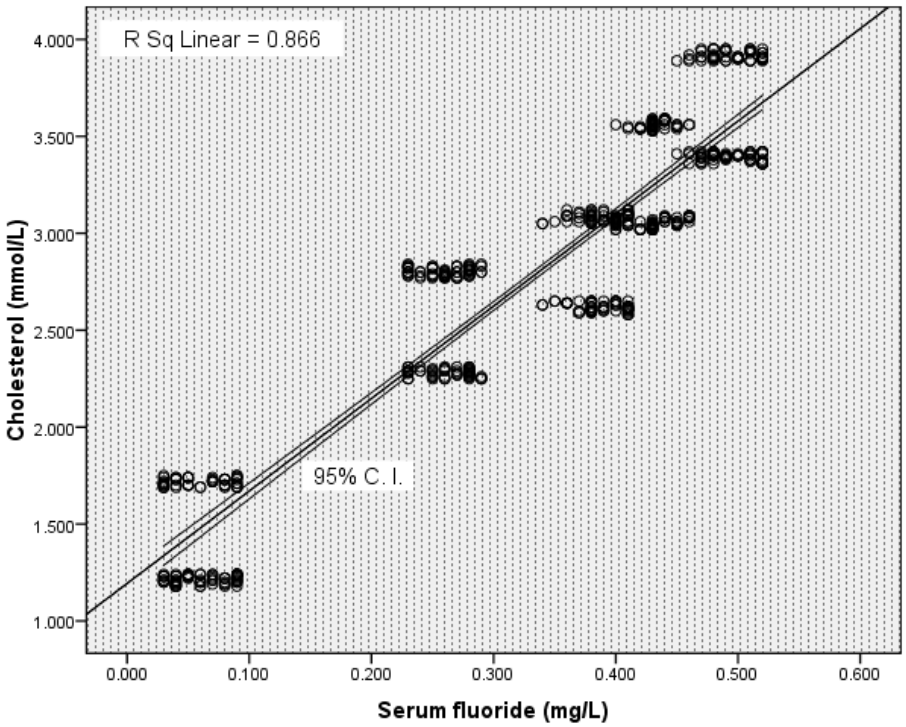

Figure 3: Scatterplot showing linear regression between serum fluoride and erythrocyte membrane cholesterol

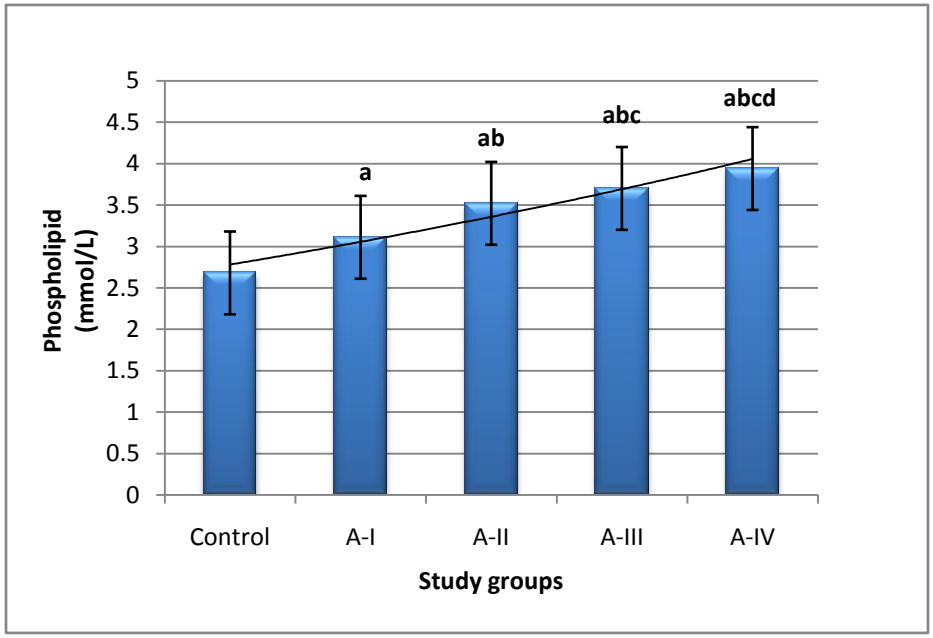

Figure 4: Mean erythrocyte membrane phospholipid contents in control and patients of fluorosis

[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

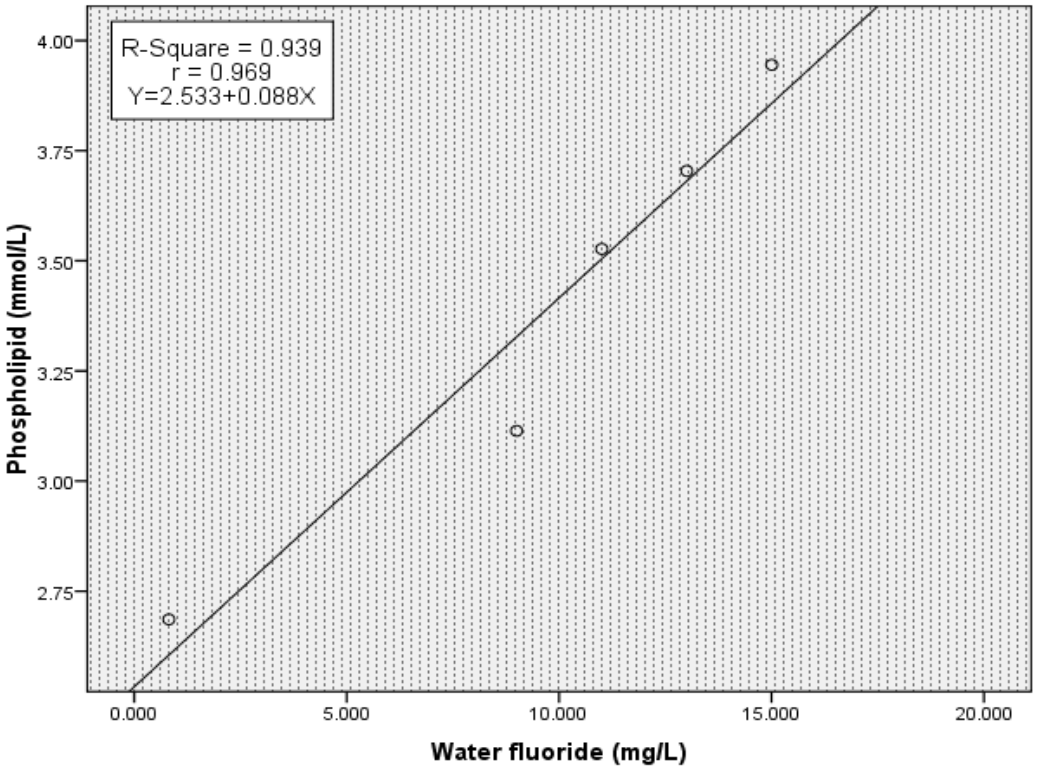

Figure 5: Scatterplot showing linear regression between fluoride and erythrocyte membrane phospholipid

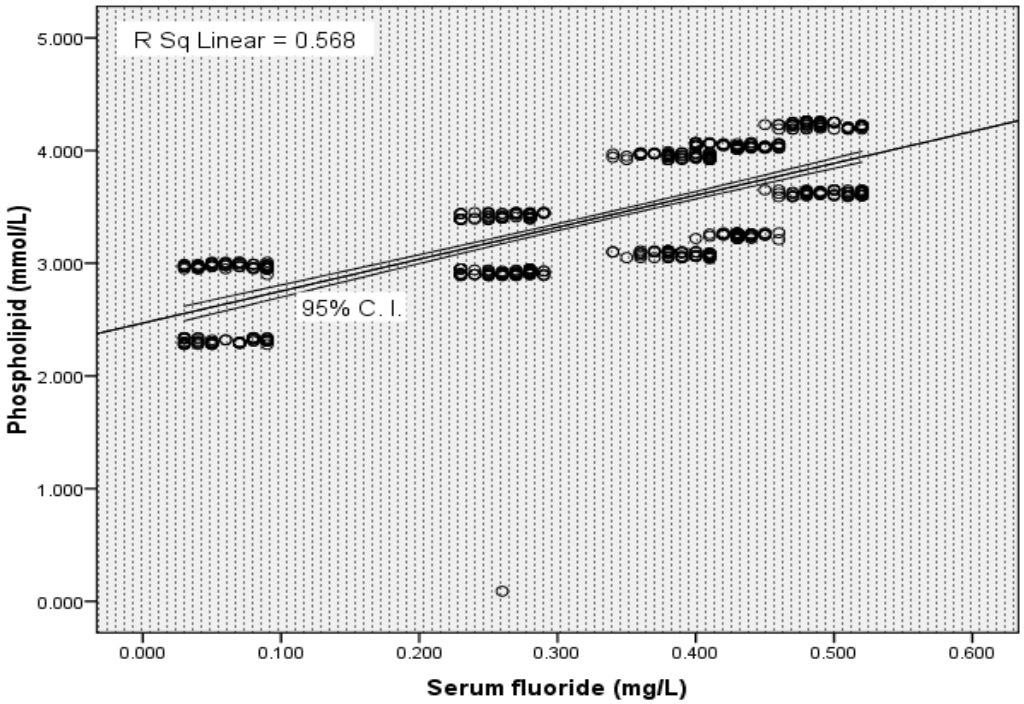

Figure 6: Scatterplot showing linear regression between serum fluoride and erythrocyte membrane phospholipid

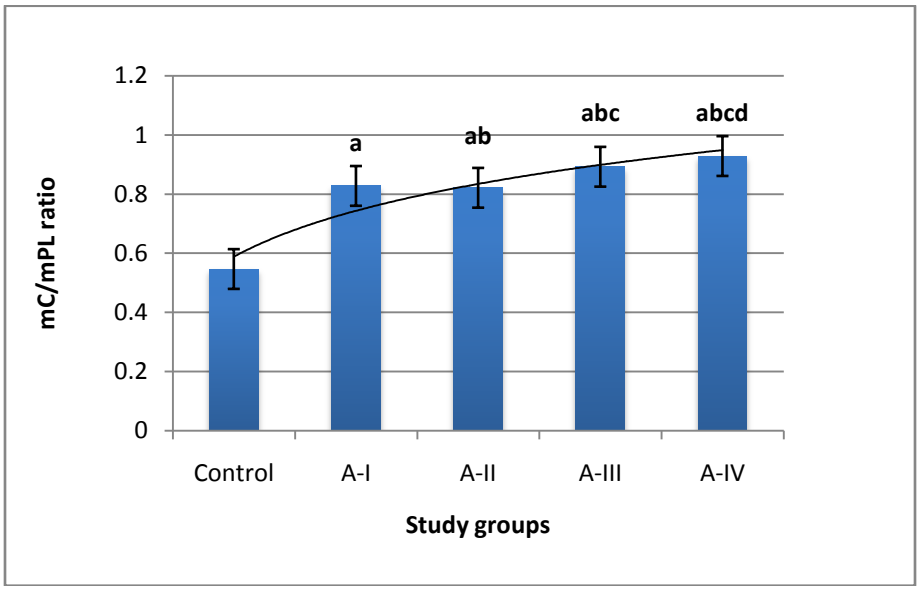

Figure 7: Calculated ratio of erythrocyte membrane cholesterol to phospholipid (mC/mPL) in control and patients of fluorosis

[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

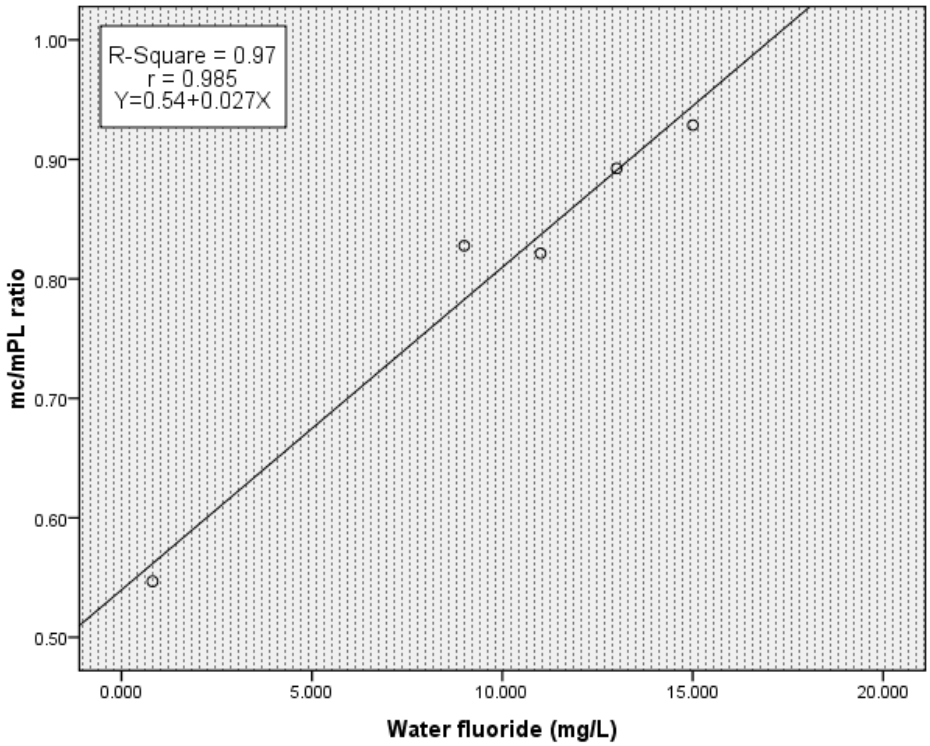

Figure 8: Scatterplot showing linear regression between fluoride and erythrocyte membrane cholesterol to phospholipid (mC/mPL) ratio

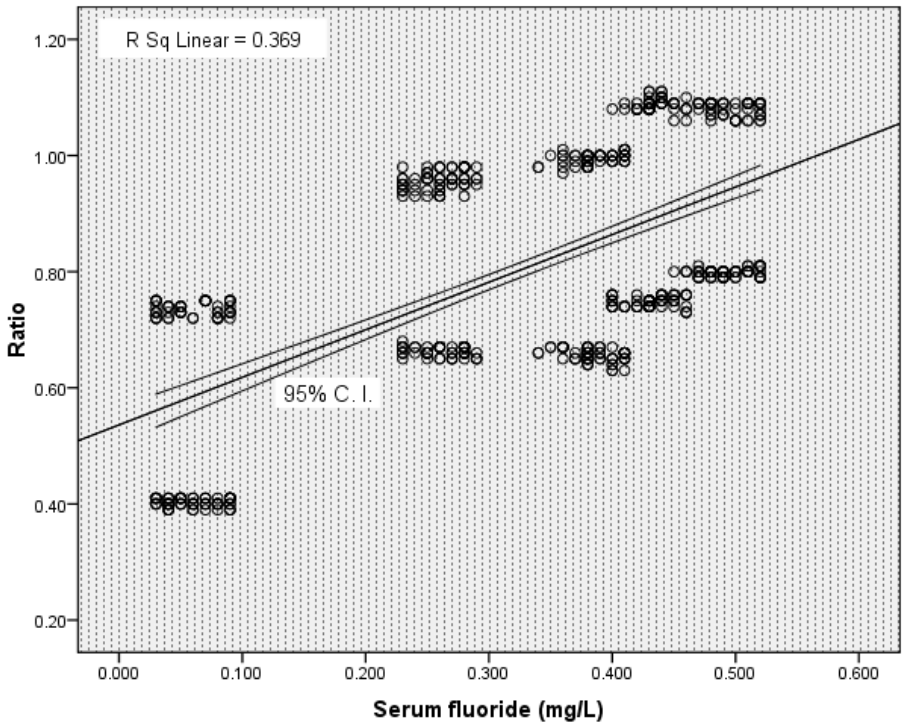

Figure 9: Scatterplot showing linear regression between serum fluoride and erythrocyte membrane cholesterol to phospholipid (mC/mPL) ratio

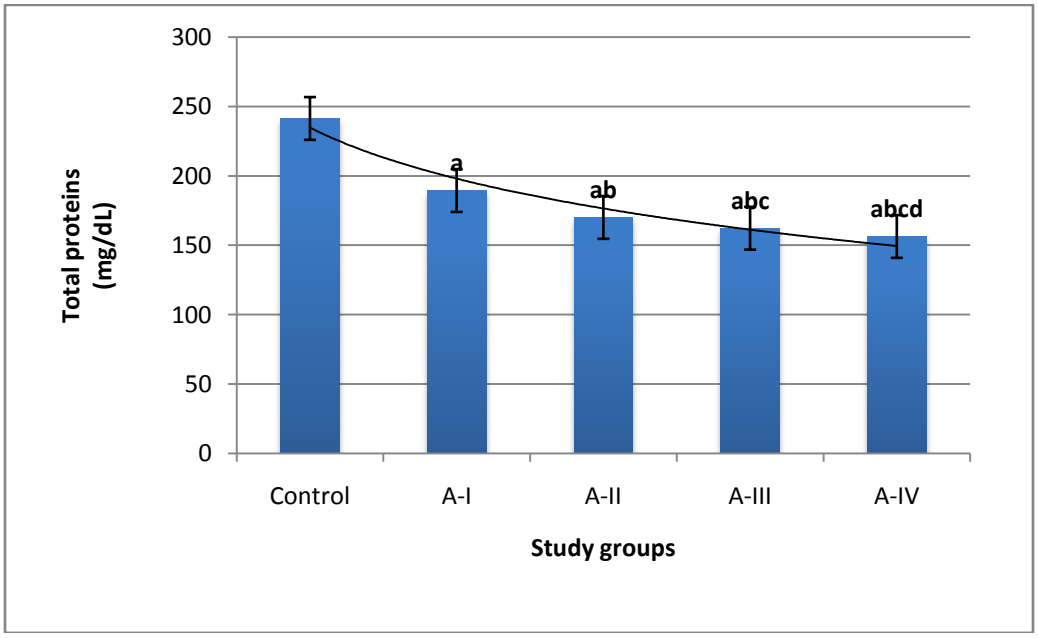

Figure 10: Mean erythrocyte membrane protein content in control and patients of fluorosis

[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

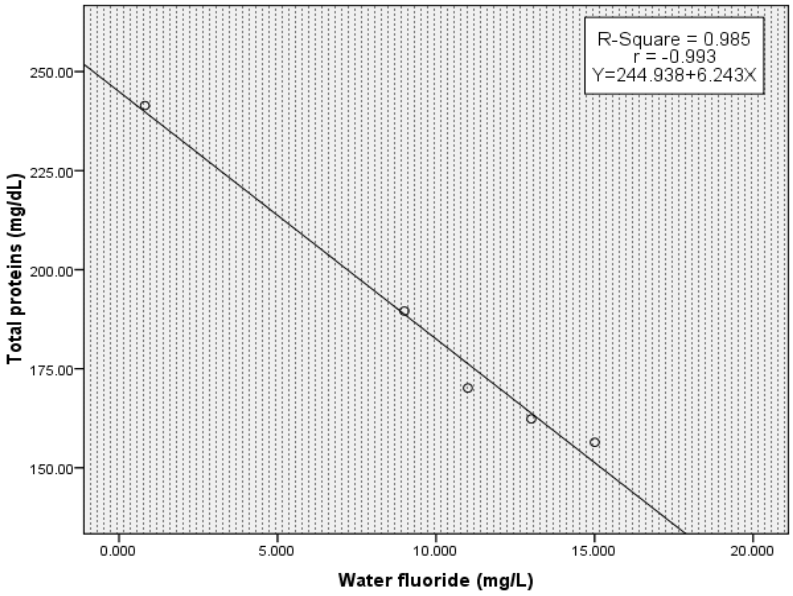

Figure 11: Scatterplot showing linear regression between fluoride and erythrocyte membrane protein content

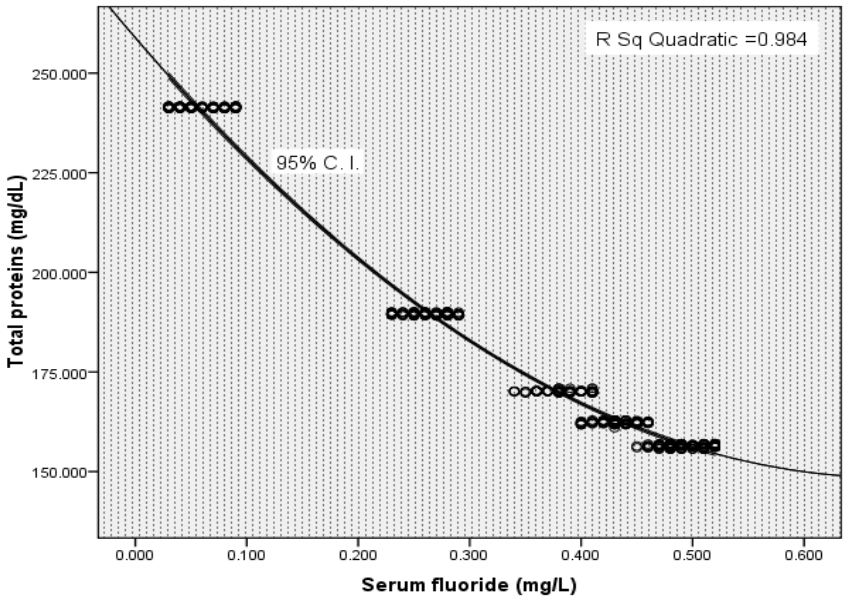

Figure 12: Scatterplot showing linear regression between serum fluoride and erythrocyte membrane protein content

Fluoride affects the structure and functions of human cell membranes and interacts with lipid bilayers; as a consequence, it altered the shape of erythrocytes inducing the formation of echinocytes. There is a evidence that hypercholesterolaemia influences deposition of cholesterol in the extracellular part of the erythrocyte membrane and sometimes even changes the shape of normal erythrocytes to acantocytes (Memon *et al.*, 2003). Accumulation of cholesterol in the membrane causes the rigidity of the membrane in acantocytes. So fluoride alters the composition, structure and function of plasma membranes, as observed in the present investigation.

The effect of fluoride on the lipid fluidity of biomembranes probably results from either the direct interaction with biomembrane surface structures, or interference with lipid metabolism of biomembrane. In the present study, the alterations of membrane activity by fluoride may be due to disruption of the lipid bilayer, changes in lipid structure and protein dysfunction, direct interaction with proteins forming ion channels, receptors or enzymes and damage to channels or enzymes facing the cytoplasm. X-ray diffraction study revealed that aluminium fluoride perturbed the structure of dimyristoyl phosphatidylcholine, class of lipids located in the outer monolayer of the erythrocyte membrane (Suwalsky *et al.*, 2004).

Han *et al.*, (2005) assessed the role of selenium in alteration of erythrocyte parameters in bovine fluorosis and reported that once overdose of fluorine deposition occurs in erythrocytes, the membrane cholesterol increases, and fluidity of membrane lipid is decreased, transportion of the Na^+ and Ca^{2+} pump is blocked and, furthermore, endocytic ion concentration is changed.

The modifications of membrane lipids might be induced by oxidative stress, which might be an important factor in the pathogenesis of chronic fluorosis. A possible insertion and aggregation of fluoride in the lipid bilayer of model membranes creating special domains with a consequent increase in membrane instability and also fluoride induced fluidizing effect, so fluoride modified bilayer order (Wang *et al.*, 2000).

The present study thus compared the biochemical composition of erythrocyte membranes of subjects consuming high fluoride, revealed significant ($P<0.001$) positive correlation of water as well as serum fluoride with cholesterol, phospholipid and cholesterol/phospholipid ratio. The inverse relationship between water fluoride concentration and protein levels were found. As the water fluoride exposure increased, the levels of fluoride through serum elevated significantly which showed that water fluoride contributed significantly to the variance of erythrocyte membrane lipid bilayer and proteins in fluorotic patients.

Thus, erythrocyte membrane proteins of fluorotic patients seem to be of major importance in the impairment of red blood cell functions.

It is concluded from these interactions that, a rapid change in cell membrane composition, particularly with regard to cholesterol, phospholipid, and protein content, leading to alterations in membrane lipid bilayer and enzyme activities appear to be major determinants in erythrocyte integrity, elasticity and deformability (structure and function) and adds a new dimension to the relationship between fluoride membrane composition and pathogenesis of fluoride.

ACKNOWLEDGEMENT

This work was supported by grants from University Grants Commission, Govt. of India, in the form of Rajiv Gandhi National fellowship. The authors wish to thanks Head, Department of Biochemistry, Post Graduate Institute of Medical Education and Research, Chandigarh, India for laboratory assistance.

Conflict of Interest

The authors declare that they have no conflicts of interest.

REFERENCES

Balls SK and Krasnow SH (1980). Structure of erythrocyte membrane and its transport function. *Annals of Clinical & Laboratory Science* **10** 209-219.

Bligh EG and Dyer WJ (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**(8) 911-917.

Han B, Yoon SS, Wu PF, Han HR and Liang LC (2005). Role of selenium in alteration of erythrocyte parameters in bovine fluorosis. *Asian-Australasian Journal of Animal Sciences* **19**(6) 865-871.

Jaganmohan P, Narayana Rao SVL and Sambasiva Rao KRS (2010). Biochemical and haematological investigations on fluorosis threaten patients at Nellore district, Andhra Pradesh, India. *World Journal of Medical Sciences* **5**(3) 54-58.

Kumari DS and Rao PR (1991). Red cell membrane alterations in human chronic fluoride toxicity. *Biochemistry Research International* **23**(4) 639-648.

Lowry OH, Rosenbrough NJ, Farr AL and Randall RJ (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193** 265-275.
McMillan DE, Netterbach NG and Lapuma J (1978). Reduced erythrocyte deformity in diabetes. *Diabetes* **27** 895-901.
Memon L, Kalimanovska VS and Jovi P (2003). Determination of cholesterol in erythrocyte membrane. *Jugoslovenska Medicinska Biohemija* **22**(3) 213-219.
Schmit-Schonbein H and Volger E (1976). Red cell aggregation and red cell deformability in diabetes. *Diabetes* **25**(Suppl.2) 897-902.
Suwalsky M, Norris B, Villena F, Cuevas F, Sotomayor P and Zatta P (2004). Aluminum fluoride affects the structure and functions of cell membranes. *Food and Chemical Toxicology* **42** 925-933.
Verklij AJ and Zwaal RF *et al.*, (1973). The asymmetric distribution of phospholipids in the human red cell membrane. *Biochimica et Biophysica Acta* **323** 178- 193.
Wang YN, Xiao KQ, Liu JL, Dallner G and Guan ZZ (2000). Effect of long term fluoride exposure on lipid composition in rat liver. *Toxicology* **146**(2-3) 161-169.
Weed RI, Reed CF and Berg G (1963). Is haemoglobin an essential component of erythrocyte membrane? *J & C Investigations* **42** 581.
World health Organisation (2008). *Fluoride in Drinking Water* (IWA Publishing) London, Geneva 1-131.