Research Article

Potential anti-haematotoxic and antioxidant effects of Pterostilbene against Fluoride: A biochemical, and histomorphogical changes in the erythrocytes of rats

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Abstract

Objective: The present study has been designed to investigate the toxicity of Fluoride (NaF) on hematological parameters and conceivable approaches to restrain its toxic impact using a blueberry stilbeniod, pterostilbene (PTS) in rats. **Material and Methods:** Male Wistar rats (150-180 grams) were divided into four groups of six rats in each. Group I received normal saline only, Group II was orally administered with NaF (25 mg/kg) and Group III received PTS (40 mg/kg) and NaF (25 mg/kg), while Group IV was administered with PTS (40 mg/kg) alone. **Results:** Our results showed that NaF exposed group exhibited a significant ($P<0.05$) decrease in all the haematological variables when compared to control group. The oxidative stress markers in the RBC were elevated significantly ($P<0.05$) along with the marked decrease in antioxidants and membrane bound ATPases in the NaF treated rats when compared to control rats. NaF administration also caused phenomenal histomorphological alterations in RBC with reference to control. PTS supplementation significantly revert back all the altered biochemical and histomorphological consequences in rats when compared to NaF treated rats. **Conclusion:** The overall findings demonstrated the reversal of haematotoxic effects induced by NaF upon PTS supplementation in rats and the therapeutic efficacy was worthy and significant. Hence PTS could serve as a better antioxidant in the management of fluorosis and its allied complications.

***Keywords*:** Fluoride, Pterostilbene, haematotoxicity, oxidative stress, antioxidant, rats

Introduction

At present, fluoride contamination is an overall issue as the fluoride present in the savoring water throughout the world is frequently at levels over maximum tolerable limits. While we can't expel fluorine and its items from our life, we should avert or alleviate their unfriendly impacts. Recently, the utilization of fluorine and fluorinated items in our way of life wound up normal and broad. Despite the fact that the summed-up utilization of fluorine encourages our life, the long-haul utilization of NaF in high focuses may prompt genuine health complications (Das et al, 2006; Robert, 1980). Among them, NaF-incited haematotoxicity is the most widely recognized illness in people chronically exposed to Fl via drinking water. It is trusted that oxidative insistence assumes an essential role in NaF-prompted haematotoxicity. Sodium fluoride (NaF) is enrolled for use as a bug spray and antihelmintic medicate and is a noteworthy ecological contaminant, entering the biosphere from such sources as iron and steel activities, coal mining, aluminum refining, and phosphate compost fabricating. NaF can be devoured from soil, water, and vegetation. Skeletal and dental Fluorosis, firmness of the joints and weakening often associated with chronic NaF toxicity. Since various logical reports have archived NaF-incited harmfulness in living frameworks, the regularly expanding volume of NaF-dirtied drinking water has been one of the principle wellsprings of open concern (Tylenda, 2011; Viragh et al., 2006).

Multitudes of reports have proposed that NaF inebriation prompts the down-direction of cancer prevention agent catalysts, an expansion in relative oxygen species (ROS), and oxidative stress. The prooxidant/antioxidant lopsidedness

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brought about by NaF inebriation may prompt multiple-organ dysfunctions. Over the top ROS creation and additionally decreased cell reinforcement barriers have been embroiled in malignancy, diabetes, and cardiovascular ailments. It also affects the haemato and lymphopoisis, nervous system and hepatorenal system which results in their dysfunction (Robert et al, 1980; Das Sarkar et al, 2005; Barbier et al, 2010).

The malicious impacts of NaF on blood have been examined well in various exploratory models. NaF influences the development of blood shaping cells i.e., hematopoietic cells in bone marrow and hinders the flow of K^+/Cl^- particles. Additionally, it likewise causes the generation of superoxide radicals (O^{2-}), lipid peroxidation in polymorphonuclear leucocytes and influences the neutrophil alongside diminished phagocytic movement (Eren, 2005; Karadeniz and Altintas, 2008). NaF inebriation of the human causes paleness or untimely erythrocyte passings for example life length of RBCs diminishes due to degeneration that transforms them into echinocytes. In people, hematologic scatters are hypochromic pallor, variety in the size and state of erythrocytes, nearness of Heinz bodies, eosinophilic leukocytosis, lymphopenia, increment in the sum of methemoglobin, and alterations in Hematocrit (Uslu, 1981).

Pterostilbene is a naturally available phytoantioxidant belongs to the stilbene, predominantly found in blueberries, with numerous advantages in the treatment and counteractive action of human ailments dependent on its anti-oxidant, anti-inflammatory, and anti-carcinogenic properties (Chakraborty et al., 2010). The counter oxidative impacts of pterostilbene could be gotten from its one of a kind structure that may rummage extracellular ROS (Perecko et al., 2008). These impacts give the chance to pterostilbene to reduce free radical-activated tissue harm amid constant aggravation. Notwithstanding its ROS rummaging impacts, pterostilbene can likewise manage a inflammation related molecular targets, including inducible nitric oxide synthase (iNOS), cyclooxygenases (COX), NF-κB, TNF-α, interleukins (ILs) and a lot more (Cichocki et al., 2008). These properties of PTS are accepted to remain behind its positive health impacts against maladies. Notwithstanding preclinical proof demonstrating that pterostilbene may have differing pharmacological advantages for the counteractive action and treatment for an immense scope of human ailments, including oxidative stress (Remsberg et al., 2008), little is thought about how or much whether pterostilbene can successfully preclude NaF-prompted haematotoxicity and its conceivable fundamental mechanism of action. Subsequently, the reasons for this investigation/were to approve the role of pterostilbene in securing the RBC against the NaF actuated oxidative affront.

Materials and Methods

Drug and Chemicals

Psterostilbene, Sodium NaF, reduced glutathione, were obtained from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals and solvents were of certified analytical grade and purchased from S. D. Fine Chemicals, Mumbai or Hi media Laboratories Pvt. Ltd., Mumbai, India. Reagent kits were obtained from span Diagnostics, Mumbai, India.

Animals

Male albino rats of Wistar strain with a body weight ranging from 160 to 180 g, were procured from Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College and Hospital, Annamalai University, and were maintained in an air conditioned room (25±2°C) with 42 ± 5% relative humidity and 12 h light/12 h dark cycle. The rats were fed with standard pellet diet (Agro Corporation Private Ltd., Bangalore, India) contained 24.5% protein, 4.4% fat, and 3.7% fiber (by weight; metabolizable energy content, 3.1 kcal/g) and water ad libitum. The study was approved by the Institutional Animal Ethics Committee of Rajah Muthiah Medical College and Hospital (Registration Number: 160/1999/CPCSEA, Proposal number: 1169/2017), Annamalai University, Annamalainagar. Tamilnadu, India.

Experimental design

The animals were randomly divided into four groups of six rats in each group.

Group 1: Control rats treated with normal saline for 28 days.

Group 2: Normal rats orally received PTSB (40mg/kg body weight) (Miltonprabu et al., 2018) dissolved in corn oil for 28 days.

Group 3: Rats received NaF as sodium NaF (25 mg/kg body weight) (Chinoy 1991) in normal saline for 28 days.
Group 4: Rats orally received NaF (25mg/kg body weight) with pre-oral administration of PTS (40 mg/kg body weight) for 28 days.

At the end of the experimental period, animals in different groups were sacrificed by cervical decapitation. Blood samples were collected in heparinized tubes by cardiac puncture from all animals, under light ether anesthesia. Hematological counter (Hemacount, Gesellschaft fur Biochemica, Germany) was used to assess the effect of NaF on hematological parameters i.e., Mean Corpuscular Hemoglobin Concentration (MCHC), Mean Corpuscular Hemoglobin (MCH), Hemoglobin (Hg), Mean Corpuscular Volume (MCV), Platelet Count (PLT), Total Leukocytes Count (TLC), Total Erythrocytes Count (TEC), Hematocrit

(Hct) in experimental and control blood group samples.

Preparation of haemolysates

Blood samples were centrifuged at 1000 g for 15 min. After removing the upper layer, the packed erythrocytes were washed with 0.01 mol/L of phosphate buffer saline (pH 7.4), and lysed by hypotonic phosphate buffer (0.01 mol/L). Cell debris was removed through centrifugation at 3000g for 15 min. Blood haemolysates were collected and immediately used for biochemical analysis (Blackwell and Huang 1965).

Preparation of erythrocytes and erythrocyte membranes

Fresh blood immediately collected in two heparinised tubes was sealed and centrifuged to remove plasma and buffy coat. Erythrocytes from the first tube were washed three times with isotonic buffer. Erythrocyte membranes were prepared by hypotonic haemolysis in Tris-HCl solution (10 mM, pH 7.4) according to Bramley et al. (1971). Membrane protein content was estimated by the method of Lowry et al. (1951). Lipids were extracted from erythrocyte membranes by chloroform/methanol according to Folch et al. (1957).

Oxidative stress study on erythrocyte membrane

Estimation of erythrocyte membrane lipid peroxidation

Lipid peroxidation in the erythrocyte membrane was assayed as malondialdehyde (MDA) according to Ohkawa et al. (1979) and expressed as nanomoles per milligram protein. Increased levels of ROS during oxidative stress attack the polyunsaturated fatty acids of membranes to release cytotoxic aldehydes like MDA, which serves as a good indicator of the oxidative stress status. Packed red cells (0.2 mL) were used for the estimation of MDA as TBARS (Jain et al., 1989). The absorbance at 600 nm was subtracted from absorbance at 532 nm. TBARS was expressed as nanomoles per gram haemoglobin. Haemoglobin concentration was measured by the cyanmethemoglobin method of Tentori and Salvati (1981).

Protein carbonyl (PC) assay

PC levels were measured according to the method described by Reznick and Packer (1994) based on spectrophotometric (Beckman DU 640 B, Switzerland) detection of the reaction of 2,4-dinitrophenylhydrazine with PC to form protein hydrazones. Briefly, after precipitation of protein with an equal volume of 1% TCA, the pellet was resuspended in 10 mM DNPH in 2 N HCl or with 2 N HCl as control blank. After the washing procedure with 1:1 ethanol/ethyl acetate, the final pellet was dissolved in 6 M guanidine hydrochloride. The carbonyl group was determined from the absorbance at 370 nm. The result was expressed as nanomoles of carbonyl groups per milligram of haemoglobin with molar extinction coefficient of 21.5 nmol/L cm.

NO assay

The role of NO synthase (iNOS) was indirectly assessed by estimating the amount of production of NO in both plasma and erythrocytes. NO decomposes rapidly in aerated solution to form stable nitrite/ nitrate products. In our study, nitrite accumulation was estimated by Griess reaction (Giuseppina et al., 1999) and was used as an index of NO production. The amount of nitrite in the sample (micromolar unit) was calculated from a sodium nitrite curve (Mukherjee et al., 2003).

Antioxidant enzymes Assay

Erythrocytes from the second tube were lysed by fourfold dilution with H_2O followed by repeated freezing-thawing cycles. The haemolysates obtained were used for the determination of total GSH contents and antioxidant enzyme activities. Haemoglobin in erythrocytes was determined by the method of Samuel (1989). GSH content was estimated according to the method of Beutler et al. (1963) and expressed as micromoles per gram Hb. Superoxide dismutase (SOD) activity was estimated according to the method described by Misra and Fridovich (1972). Catalase (CAT) activity was determined using the method described by Aebi (1984) by measuring hydrogen peroxide decomposition at 240 nm. Glutathione peroxidase (GPx) activity was assayed using the method described by Flohe and Gunzler (1984) by the subsequent oxidation of NADPH at 240 nm with t-butyl-hydroperoxide as substrate. The values of SOD, CAT, and GPx are expressed in units per gram of haemoglobin (U/g Hb). Glutathione reductase (GR) activity in erythrocytes was assayed by the methods of Goldberg and Spooner (1983). The GR activity in erythrocytes has been expressed as nanomoles NADPH oxidised to NADP per gram of Hb per minute. Glutathione S-transferase (GST) (E.C.2.5.1.18.) was also assayed in haemolysates by the method of Beutler et al. (1963).

Assay of membrane-bound ATPases

Na^+/K^+ ATPases was assessed in erythrocyte membrane preparation according to the method of Quigley and Gotterer (1969) Sodium ion (Na^+)/potassium ion (K^+)-ATPase activity was measured under two conditions: in the presence of magnesium ion (Mg^{2+}), Na^+/K^+ (total ATPase) and in the presence of Mg^{2+}, Na^+/K^+ and ouabain. The Na^+/K^+ ATPases activity was measured as the difference between total ATPase activities and ouabain-insensitive ATPase activities. The inorganic phosphate released by the action of ATPases was estimated by the method of Fiske and Subbarrow (1925). The Ca^{2+}-ATPases activity was measured according to the method of Desaiah et al (1985). The Mg^{2+}-ATPases activity was determined in the presence of 1 mM ethylene glycol tetraacetic acid (which specifically

chelates Ca^{2+} ion), and this was subtracted from the total activity in order to obtain the net Ca^{2+}-ATPases activity.

Morphological examination of red blood cells

Morphological evaluation Blood samples were collected using heparinized tubes. Immediately after mixing with blood by inverting the tubes, blood smears were made by spreading one drop on a slide and then staining with the Giemsa solution. RBC morphology was determined using a Nikon Optiphot-2 microscope with an oil immersion 100/1.25 objective.

Fresh blood samples were drawn from treated and control rats, and a drop of blood was immediately immersion fixed in 2.5% glutaraldehyde made in 0.1 M phosphate buffer (pH 7.4). After 1 h of fixation, cells were centrifuged at 1000–1500 rpm and the pellets were resuspended in triple distilled water. After two to three washings, the final pellet was suspended in triple distilled water. A drop of the sample was smeared on the metallic scanning electron microscopic stubs, which were loaded with a conductive silver tape on the top. The stubs were then coated with gold to a thickness of 100A° using a sputter-ion coater, with a gold source, for 4–5 min and the specimens were finally observed under scanning electron microscope, JSM-6100 (Redwood City, California, USA).

Statistical analysis

The obtained values were analyzed by comparing means using one-way analysis of variance, ANOVA. The p-values < 0.05 were considered to be significant. The values of various hematological parameters were represented by Mean ± S.E.M. The data was analyzed by commercially available software package SPSS (SPSS Inc, Chicago, USA). The values considered significant were represented by different superscript alphabets.

Results

Haematological parameters

Table 1 condenses the dimensions of WBC, RBC, PLT, MCV and HCT and the convergence of HG, MCH and MCHC in blood of control and experimental rodents. In our investigation, a fundamentally ($p < 0.05$) diminishing dimensions of MCV, HCT, WBC, RBC and PLT and diminishing centralization of HGB, MCH and MCHC were found in NaF - treated rodents when contrasted with control rodents. Pretreatment of PTS successfully constricted the NaF - incited modifications in hematological factors. Aside from a noteworthy increment in RBC include in the organization of PTS alone treated rodents and did not demonstrate any modifications in other hematological factors and didn't varies altogether from that of the control rodents.

Erythrocyte membrane oxidative stress markers and enzymatic antioxidants status

Data presented in table 2 show a significant ($p < 0.05$) increase in the level of MDA and HPs in erythrocyte

Table 1. Effect of Pterostilbene (PTS) on Sodium fluoride (NaF) - induced changes on the haematological parameters of control and experimental rats

Groups	Control	PTS (40mg/kg)	NaF (25 mg/kg)	NaF+PTS(40 mg/kg)
RBC, $10^6/mm^3$	9.27 ± 0.41^a	10.82 ± 0.19^d	7.41 ± 0.29^b	9.17 ± 0.21^c
WBC, $10^3/mm^3$	13.08 ± 0.23^a	13.75 ± 0.47^a	9.13 ± 0.53^b	11.21 ± 1.02^c
Hg, g/dl	10.67 ± 0.36^a	12.25 ± 0.41^d	8.13 ± 0.12^b	11.81 ± 0.28^c
Hct, (%)	43.50 ± 0.35^a	43.15 ± 0.25^a	37.00 ± 1.25^b	41.00 ± 0.55^c
MCV(fL)	43.27 ± 0.65^a	43.86 ± 0.71^a	31.05 ± 0.23^b	39.64 ± 0.51^c
MCH(Pg/dl)	21.72 ± 0.47^a	23.15 ± 0.65^d	14.21 ± 0.13^b	19.05 ± 0.28^c
MCHC (g/dl)	31.7 ± 0.39^a	32.1 ± 0.63^a	26.3 ± 0.14^b	29.8 ± 0.32^c
PLT (10^3/dl)	7.69 ± 0.43^a	7.81 ± 0.64^a	4.17 ± 0.21^b	6.02 ± 0.32^c
Neu (%)	20.53 ± 0.74^a	21.20 ± 0.87^a	16.10 ± 0.41^b	19.00 ± 0.53^c
Lym (%)	73.50 ± 2.50^a	75.20 ± 2.10^a	64.25 ± 1.50^b	70.18 ± 1.17^c
Mono (%)	4.76 ± 0.63	4.93 ± 0.75	2.75 ± 0.32	3.88 ± 0.41

Values are mean ± SE; ANOVA followed by Duncan's multiple comparison range test. Values in column with different superscript letters ($^{a-d}$) differs significantly at $P< 0.05$

membrane of NaF treated group when compared with the control group. The level of PC and NO production was also significantly ($p < 0.05$) elevated in rat erythrocyte membrane treated with NaF when compared with the control group. PTS along with NaF effectively ameliorated the toxic effect of NaF significantly by reducing the levels of MDA, HPs, PC content and NO production in the erythrocyte membrane as compared to the NaF alone treated group. Figure 4 shows Further the levels of non-enzymatic antioxidant, namely, GSH was significantly ($p < 0.05$) decreased in the rat erythrocytes treated with NaF when compared with the control group. The depleted level of GSH was significantly ($p < 0.05$) restored with PTS pre-administration in NaF -intoxicated rats.

Effect of GSP on NaF-induced changes in antioxidant enzyme activity of erythrocytes

Table 1 shows that treatment with NaF caused a significant ($p < 0.05$) decrease in the activities of SOD, CAT, GPx, GR, GST and G6PD when compared with the control. A significant ($p < 0.05$) recovery relating to the activities of SOD, CAT, GPx, GR, GST andG6PD was observed in response to the pre-administration of PTS with NaF.

Effect of PTS on NaF-induced alterations in erythrocyte membrane-bound ATPases

Table 2 demonstrates the impact of PTS on NaF inebriated rodent erythrocyte layer bound Na^+/K^+ ATPases, Mg^{2+}-ATPases and Ca^{2+}-ATPases levels in charge and trial rodents. The dimensions of erythrocyte layer bound ATPases were observed to be altogether ($p < 0.05$) diminished in NaF treated rodents when contrasted and that of the ordinary gathering. Pre-organization of PTS alongside NaF had essentially ($p < 0.05$) improved the dimensions of erythrocyte film bound ATPases when contrasted and the NaF - treated gathering. Organization of PTS alone to rodents did not demonstrate any huge modifications in erythrocyte layer bound ATPases when contrasted and the typical rodents.

Defensive impact of PTS on NaF-induced alterations in RBC morphology

On histological examination, RBC in the control gathering (Figure 1A), showed a particular biconcave plate shape. The administration of NaF (Figure 1B) demonstrated a checked increment in echinocyte, discocyte, spherocytes, schizocytes and acanthocytes. Numerous RBCs with slender and long expansions of their layers were found, purported myelinic figures, while the discocyte, acanthocytes and the RBCs with other unusual shapes were the most continuous anomalous RBCs saw in Figure 1B). In Figure 1C, PTS -pretreated NaF intoxicated rats showed an ordinary, recuperated biconcave plate shape, yet little acanthocytes were likewise seen, while the most incessant unusual RBCs were marginally crenated by PTS. The rats got PTS alone (Figure 1D) demonstrated an ordinary morphological picture like that of the control.

Table 2. Effect of Pterostilbene (PTS) on Sodium fluoride (NaF) - induced changes in oxidative/ antioxidant markers level in the erythrocytes of control and experimental rats

Groups	Control	PTS (40mg/kg)	NaF (25 mg/kg)	NaF+PTS(40 mg/kg)
SOD, U/g Hb	2.84 ± 0.09^a	3.27 ± 0.21^d	0.63 ± 0.05^b	1.98 ± 0.16^c
CAT, U/g Hb	8.11 ± 0.27^a	8.79 ± 0.41^d	3.47 ± 0.11^b	6.78 ± 0.35^c
GPx, U/g Hb	34.72 ± 0.24^a	37.56 ± 0.32^d	18.43 ± 0.12^b	31.48 ± 0.35^c
GR, µm/min/g Hb	6.17 ± 0.39^a	7.24 ± 0.48^d	2.62 ± 0.26^b	4.37 ± 0.31^c
GST, U/mg Hb	17.28 ± 1.31^a	19.21 ± 0.81^d	9.45 ± 0.78^b	13.36 ± 1.17^c
G6PD, U/g Hb	4.31 ± 0.19^a	4.72 ± 0.24^d	1.02 ± 0.04^b	3.71 ± 0.11^c
LHs, nmol/mg Hb	4.11 ± 0.79^a	3.94 ± 0.68^d	8.67 ± 1.23^b	6.38 ± 0.46^c
PC, nmol/mg Hb	6.78 ± 0.37^a	6.12 ± 0.21^d	15.63 ± 0.82^b	8.17 ± 0.28^c
NO, µmol/mg Hb	8.75 ± 0.31^a	8.97 ± 0.27^a	18.47 ± 2.78^b	10.14 ± 0.28^c
GSH, µmol/g Hb	20.57 ± 1.24^a	22.85 ± 2.49^d	9.27 ± 0.71^b	18.45 ± 1.16^c
MDA, nmol/mg Hb	3.58 ± 0.08^a	3.06 ± 0.11^d	9.67 ± 1.12^b	4.71 ± 0.17^c

Values are mean ± SE; ANOVA followed by Duncan's multiple comparison range test. Values in column with different superscript letters ($^{a-d}$) differ significantly at $P< 0.05$.

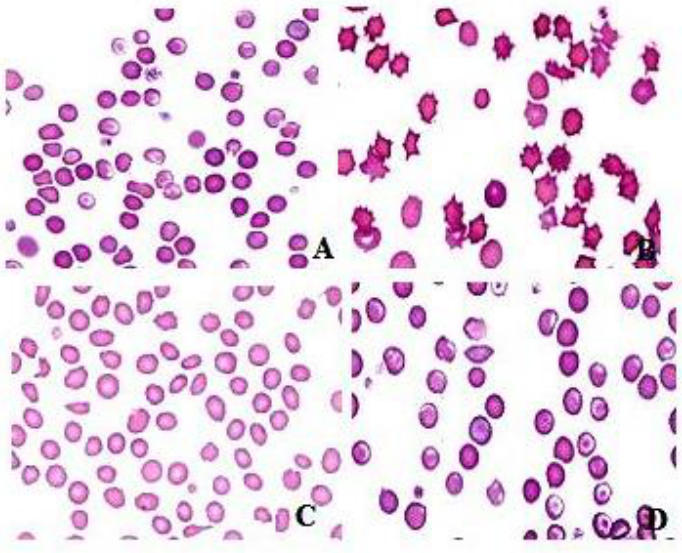

Figure 1. Effect of Pterostilbene (PTS) on Sodium fluoride (NaF) induced erythrocyte histomorphological changes in control and experimental rats. (A) shows normal RBCs with central pallor. (B) NaF-treated group showed the presence of acanthocytes, spherocytes (arrow), echinocytes (arrow head), stomatocytes (S) and schistocytes (star), fragmented and faded RBC were seen in the majority. PTS pretreated rats (C) showed the recovered architecture of RBC as compared to NaF-treated group, but some faded cells with no resurgence were also seen. (D) PTS-treated group showed normal RBC as seen in the control group. x1100. Bars 5 µm. PTS: Pterostilbene; RBC: red blood cell; NaF: Sodium fluoride.

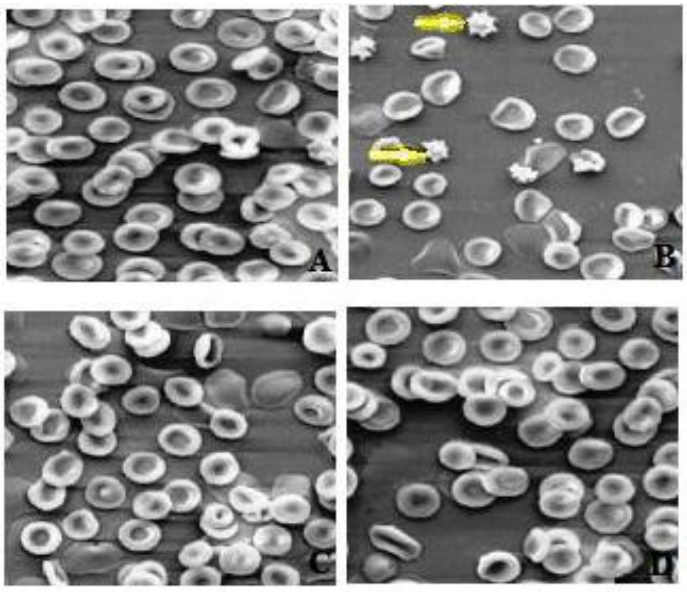

Figure 2. Scanning electron micrograph images from the erythrocytes of control and treated rats. Control (a) and PTS (b) showed normal micrographs of RBC; (c and d) NaF group show the presence of acanthocytes (A), fragmented RBC-schistocytes (SZ), spherocytes (D), discocyte (D) and echinocytes (E). Most of the erythrocytes showed a biconcave shape. PTS pretreated rats (e) showed the recovered RBCs. x3000. Bars 10–20 nm. PTS:Pterostilbene; RBC: red blood cell; NaF: Sodium fluoride.

Effect of PTS and NaF on Rat Erythrocyte Morphology

To examine whether morphological change of rodent erythrocytes had happened due to NaF introduction, 10 ml aliquots of control and exploratory erythrocyte suspensions were expelled following a 2-h brooding and arranged for checking electron microscopy. As appeared in figure 2A, control red cells hatched for 2 h at 37°C displayed the biconcave appearance of ordinary discocytes. Echinocytic cells were seen once in a while, yet spoke to, 3% of the all-out cells. Conversely, over half of the RBC presented to NaF treated gathering had lost their discocytic morphology and displayed moderate to serious degrees of echinocytosis (Figure 2B). The cells were portrayed by a few tolerably estimated projections that were lopsidedly dispersed on the cell surface, and in Giemsa-stained smears observed under light microscopic dimension, which showed that the progressions observed in NaF-treated red cells were not a

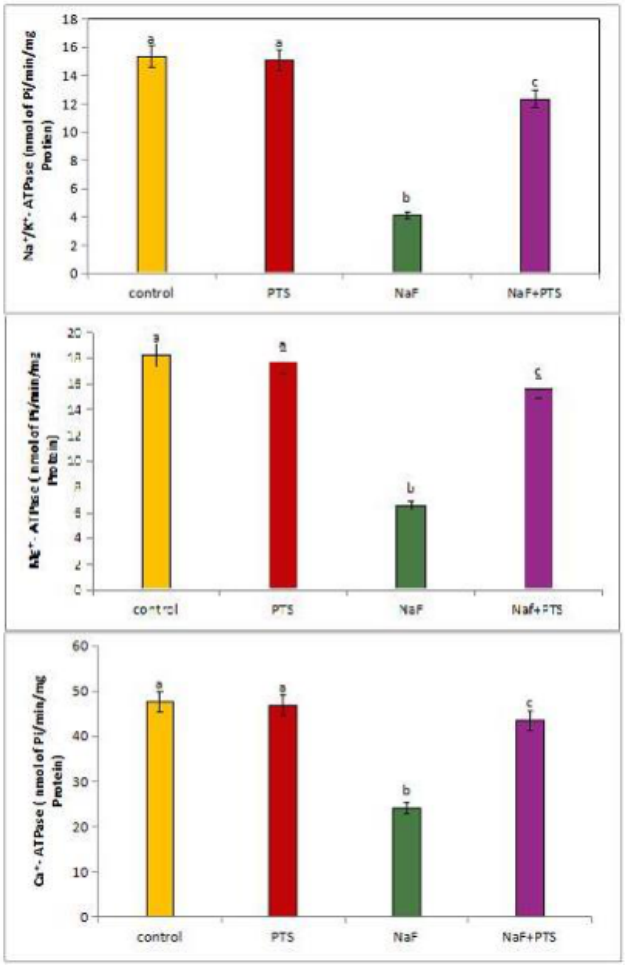

Figure 3. Effect of Pterostilbene (PTS) on Sodium fluoride (NaF) - induced changes in membrane bound ATPases levels in the erythrocytes of control and experimental rats. Values are mean ± SE; ANOVA followed by Duncan's multiple comparison range test. Values in column with different superscript letters (a-c) differ significantly at P< 0.05.

curio of the way toward setting up the cells for checking electron microscopy. The PTS along with NaF treated group RBC's exhibited almost the regular morphology (2C) without any major alterations in their membrane morphology when compared with NaF treated rats. The PTS alone treated rats RBC's showed the normal appearance (2D) without any alterations in their membranous structure.

Discussion

NaF being the most harming natural pollutant bothers the typical metabolic pathways of a life form at altered dimensions. Considering the current controversies in reports with respect to the impact of NaF on hematological parameters, this investigation was worked to examine the haematological, antioxidant and morphological status of RBC in rats following NaF intoxication in about a month of test period. So as to get further understanding into the ameliorative capability of PTS, the present investigation was intended to assess the *in vivo* antioxidant potential of this compound by utilizing a rodent model of NaF-inebriation. The outcomes have affirmed that our working speculation that PTS is fit of securing rodent erythrocytes against NaF-prompted oxidative harm.

Viable metabolism and functioning of erythrocytes required an adjusting exhibit of catalysts, proteins, sugars, lipid anions and cations in the cells. Degenerative changes in hemoglobin, cell films and compounds required for ordinary erythrocyte capacities are a direct result of the unevenness between the erythrocyte segments because of the deficient capacity of erythrocytes to adapt the oxidative pressure instigated by NaF (Nabavi et al., 2012). From aversion of the oxidative responses, there is a lot of enthusiasm for the utilization of PTS as a compelling toxic preventive agent in the ameliorative strategies against NaF haematotoxicity.

The blood is a fundamental fluid, which contains a homeostatic adjusted grouping of RBCs, WBCs and PLTs. ROS have been ensnared in the component of harm of the RBCs in NaF-treated rodents with the aftereffect of hematological entanglements, which primarily comprise of variations from the norm in the capacity, morphology and metabolism of hematological variables (Bhaskara rao and Vidyunmala, 2010). In our investigation, a significant lessening in the dimensions of WBC, RBC, PLT and HCT and diminishing convergence of HGB, MCH, MCV and MCHC was found in NaF-treated rodents implies that the area of RBCs was diminished, showing microcytic sickliness because of iron deficiency.

Due to NaF-incited oxidative stress, a lot of reactive radicals are freed from the neutrophils into flow, which prompts the deluge of ROS in erythrocytes. Introduction to NaF incites weakness related with a reduction in RBCs count and enlistment of oxidative harm and lipid peroxidation in blood and RBCs. Additionally, NaF harms the amalgamation of erythropoietin, a hormone used to advance the development of RBCs. Decrease in WBC, MCHC, MCV and MCH may propose the abatement in resistance in battling against xenobiotics, appearing further capacity of NaF inebriation to stifle the action of haematopoietic tissues, weaken erythropoiesis and quicken erythroclasia on account of the changed RBCs layer penetrability, expanded RBCs mechanical delicacy as well as inadequate Fe metabolism (Mikstacka et al., 2010). In our experiment, the pretreatment of PTS in NaF-treated rodents demonstrated a huge reestablishment of these hematological parameters by taking them back to close normal dimensions because of the position of the 3-and 5-methoxy groups combined with 4-hydroxy group works as electron benefactors to frame securities with electrophilic particles, in this manner it help in the recoupment of the antioxidants, guard framework and shielding heme from NaF-initiated oxidative insult. The methoxy groups on pterostilbene appear to allow a greater antioxidative capacity (Remsberg et al., 2008).

Peroxidation of membrane lipids is said to be the result of free radical-intervened chain oxidative response of membrane polyunsaturated unsaturated fats. It is a fundamental cell crumbling process instigated by oxidative pressure and happens quickly in tissues rich in profoundly oxidizable polyunsaturated fatty acids. NaF organization in the trial rodents brought about a significant increment in the level of TBARS, MDA and PC, in erythrocyte layers through the spread of lipid peroxidation and the erosion of antioxidant defense system that matches with the findings of consequences of (Miltonprabu and Thangapandiyan, 2013) The oxidative pressure incited by NaF yields different essential and auxiliary items, which eventually results in the basic interruption of RBC lipid bilayer and produce injurious impacts on the characteristic procedures of layer bound proteins (Shanthakumari et al ., 2004; Chinoy, 2003; Sato et al., 1998). Administration of PTS especially constricted the rise of TBARS and other lipid peroxidative subsidiaries in erythrocyte layers as PTS searches the dangerous free radicals and other receptive intermediates by authoritative with NaF cations that successfully diminishes their retention and the degree of their oxidative harm and consequently restricting the spread of lipid peroxidation via its antioxidant activity.

NO is perceived as a physiological emissary atom created from L-arginine by NOS. It is constitutively created in endothelial cells to keep up the expansion of veins and to hinder PLT collection and adhesion (Huk et al., 1998) In our examination, administration of NaF was found to cause a noteworthy increment in NO generation of erythrocytes,

which results in erythrocyte membrane harm and fiery changes because of ROS which represses NO synthetase (eNOS) that at last causes extreme NO production. PTS on the opposite side rummages NO production in plasma and erythrocyte membrane in NaF-treated rodents because of its antioxidative capacity. PTS prompts the enactment of Nrf2/HO-1 pathway and eNOS phosphorylation in endothelial cells and thus reestablishes the NO level in control condition (Xue et al., 2017).

In the present investigation, there was a huge lessening in the dimensions of GSH, in erythrocyte of NaF-inebriated rodents. Our outcomes are in accordance with the findings of Nabavi et al. (2012). During NaF intoxication, free radicals are produced in excess and GSH acts to balance these overabundance oxyradicals, bringing about the over consumption of GSH level as it frames buildings with GSH through the free sulphydryl group and exhausts the intracellular sulphydryl groups, in this way diminishing its levels in the RBC's of rats. Our result showed that pretreatment of PTS expanded the dimension of thiol aggregates by the assurance of SH bunches in GSH from oxidative harms by means of its free radicals extinguishing activity that limits the utilization of antioxidants, accordingly reestablishing their dimensions.

SOD, CAT and GPx partake an indispensable job in the antioxidative pressure guards of the cells by securing against ROS toxic impacts and lipid peroxidation. SOD catalyzes the transformation of superoxide radicals to H_2O_2, while CAT changes over H_2O_2 into H_2O. In this manner, these cell reinforcement proteins can reduce the dangerous impacts of ROS. In this investigation, erythrocyte SOD, CAT and GPx levels were diminished in the NaF-treated rats, which might be appointed to the immersion of SOD amid the way toward changing over O_2 to H_2O_2. The CAT reduction may include CAT immersion or hindrance amid the breakdown of free radicals and H_2O_2. Inhibition of GPx exercises is joined by an exhaustion of GSH, which may result in oxidative pressure. Diminished GSH, related to GPx and GST, is in charge of the GSH redox cycles that keep up the redox status of tissues and ensure basic and administrative proteins against ROS-prompted damage. G6PD is a critical chemical of HMP shunt pathway. It changes over one particle of glucose-6-phosphate into 6-phosphogluconolactone within the sight of Mg^{2+}, Mn^{2+} and Ca^{2+} particles, and along these lines, $NADP^+$ is decreased to NADPH. A consequent decrease of the G6PD movement in NaF-intoxicated rodents demonstrated a weakening of NADPH, which is required for the decrease of GSSG to GSH. The development of NaF – SH complex with SH gatherings of the chemical prompts a diminishing in the exercises of GR and G6PD and exhaustion of GSH level (Miltonprabu and Thangapandiyan, 2013). PTS pretreatment expanded the dimension of these enzymatic antioxidant enzymes, most likely because of the security of SH bunches in GSH from oxidative harms because of the presence of two that 3,5-meta-methoxyl groups increment entire blood antioxidative status via its Nrf2/HO-1 pathway activation ability (Xue et al., 2017).

In the present examination, a critical lessening in the levels of membrane bound ATPases in the erythrocyte was seen in NaF-treated rats. Diminished level of Na^+/K^+-ATPase could be because of upgraded lipid peroxidation by free radicals on NaF exposure. Moreover Na^+/K^+-ATPase is a SH group bearing compound and is lipid subordinate. Diminished level of Na^+/K^+-ATPase can prompt a reduction in sodium efflux, subsequently modifying membrane penetrability (Agalakova and Gusev, 2008; Waugh, 2019). The interruption of membrane porousness or discontinuity of the layer prompts the spillage of Ca^{2+} particles into cells subsequently potentiating irreversible cell annihilation. The Ca^{2+} over-burden caused by NaF likewise diminished the Ca^{2+} ATPase action in cell membrane. It is commonly acknowledged that because of high proclivity for SH gatherings, NaF ties energetically to different chemical proteins and inactivates them. Mg^{2+} ATPase action is engaged with other vitality requiring forms in the cell and its action is susceptible to altering levels of lipid peroxidation. PTS supplementation in NaF-inebriated rats essentially decreased the lipid peroxidation in erythrocytes and maintained the normal exercises of membrane bound compounds. This might be because of the capacity of PTS to shield the SH bunches from oxidative harm through the hindrance of peroxidation of membrane lipids and stabilized the membrane.

The morphological and ultrastructural examination uncovered numerous alterations in RBC of NaF-treated rats. NaF instigated oxidative pressure harms cell membrane prompting the modifications in cell inflexibility and structure. This oxidative pressure prompts echinocyte arrangement as a result of membrane damage, which in turn contributes to the adjustment of entire RBC auxiliary compliance and its functioning. In the present investigation, divided RBCs (schistocytes) were seen in NaF treated rats because of adjustments in the cytoskeleton (layer proteins or potentially lipids) of RBC, consequently influencing the surface region of the cell. Echinocytes and discocyte are formed when the compound supplements into the inward monolayer, acanthocytes and spherocytes are delivered when it situates into the external moiety. NaF harms the layer structure, particle penetrability and the metabolism of erythrocytes, accordingly it may cause morphological harm in erythrocyte development. Overall, these progressions instigated by NaF were viably weakened by the pretreatment with PTS through the nearness of flavan-3-ol

particle, which contains both hydrophobic and hydrophilic deposits and permit these mixes to associate with phospholipid head gatherings and be adsorbed onto the outside of membrane. These associations can prompt the adjustment of membrane properties, prompting changes in the guideline of layer bound chemicals and receptors (Perecko et al., 2008).

Conclusion

This study has shown that PTS administration mitigates Fluoride-induced oxidative stress in rat erythrocytes, which could be due to its antioxidant, free radical scavenging, and membrane stabilizing properties.

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Ethics approval

The study was approved by the Institutional Animal Ethics Committee of Rajah Muthiah Medical College and Hospital (Registration Number: 160/1999/CPCSEA, Proposal number: 1169/2017), Annamalai University, Annamalainagar. Tamilnadu, India. Institutional guidelines for the care and use of animals were followed. All procedures performed in the study involving animals were in accordance with the ethical standards of the institution.

Conflict of Interest

The authors declare that there is no conflict of interest.

References

Aebi H. 1984. Catalase in vitro. Methods Enzymol (Bergneyer HU), 105:121–6.

Agalakova NI, Gusev GP. 2008. Diverse effects of fluoride on Na^+ and K^+ transport across the rat erythrocyte membrane. Fluoride, 41: 28–39.

Barbier O, Arreola-Mendoza L, Del Razo LM. 2010. Molecular mechanisms of fluoride toxicity. Chemico Biological Interactions, 188:319–333.

Beutler E, Dixon O, Kelly BM. 1963. Improved method for the determination of blood glutathione. Journal of Laboratory and Clinical Medicine, 61:882–90.

Bhaskara rao AV, Vidyunmala S. 2010. Cumulative effect of fluoride on hematological indices of mice Mus Norvegicus albinus. American-Eurasian Journal of Toxicological Sciences, 2:93–95.

Blackwell QR, Huang JTH. 1965. Simplified Preparation of Blood Hemolysates for Hemoglobin Electrophoresis. Clinical Chemistry, 11(6): 628-632.

Bramley TA, Coleman L, Finean JB. 1971. Chemical, enzymological and permeability properties of human erythrocyte ghosts prepared by hypotonoic lysis in media of different osmolarities. Biochim Biophys Acta, 241:752–69.

Chakraborty A, Gupta N, Ghosh K, Roy P. 2010. In vitro evaluation of the cytotoxic, anti-proliferative and anti-oxidant properties of pterostilbene isolated from Pterocarpus marsupium. Toxicology in Vitro, 24(4):1215-28.

Chinoy NJ. 2003. Fluoride stress on antioxidant defense systems. Fluoride, 36:138–141.

Cichocki M, Paluszczak J, Szaefer H, Piechowiak A, Rimando AM, Baer-Dubowska W. 2008. Pterostilbene is equally potent as resveratrol in inhibiting 12-O-tetradecanoylphorbol -13-acetate activated NFkB, AP-1, COX-2, and iNOS in mouse epidermis. Molecular Nutrition & Food Research, 52 Suppl 1:S62-70.

Das Sarkar S, Maiti R, Ghosh D. 2005. Induction of oxidative stress on reproductive and metabolic organs in sodium fluoride-treated male albino rats: Protective effect of testosterone and vitamin E co-administration. Toxicology Mechanisms and Methods, 15:271–277.

Das Sarkar S, Maiti R, Ghosh D. 2006. Fluoride-Induced Immunotoxicity in Adult Male Albino Rat: A Correlative Approach to Oxidative Stress, Journal of Immunotoxicology, 3(2): 49-55.

Desaiah D, Chetty CS, Rao KS. 1985. Chlordecone inhibition of calmodulin activated calcium ATPase in rat brain synaptosomes. Journal of Toxicology and Environmental Health, 16:189–95.

Eren E. 2005. Fluorosis and its hematological effects. Toxicology and Industrial Health, 21 (9): 255–258.

Fiske CH, Subbarrow Y. 1925. The colorimetric determination of phosphorous. J Biol Chem, 66:375–81.

Flohe L, Gunzler WA. 1984. Assays of glutathione peroxidase. Methods in Enzymology, 105:114–21.

Folch J, Less M, Slone-Stanley GH. 1957. A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry, 226:466–8.

Giuseppina MR, Rosario M, Oreste G. 1999. Prolactin induction of nitric oxide synthase in rat C6 glioma cells. Journal of Neurochemistry, 73: 2272–2277.

Goldberg DM, Spooner RJ. 1983. Glutathione reductase. In: Bergmayer HU, editor. Methods of enzymatic analysis. Dearfield Beach: Verlag Chemie, 258–65.

Huk I, Brovkovych V, Nanobash Vili J. 1998. Bioflavonoid quercetin scavenges superoxide and increases nitric oxide concentration in ischemia reperfusion injury: an experimental study. British Journal of Surgery, 85:

1080–1085.

Jain SK, Vie RM, Duett J, Herbest JJ. 1989. Erythrocyte membrane lipid peroxidation and glycosylated hemoglobin in diabetes. Diabetes, 38:1539–43.

Karadeniz A, Altintas L. 2008. Effects of panax ginseng on fluoride-induced haematological pattern changes in mice. Fluoride 41 (1): 67.

Lowry OH, Rosenbrough NJ, Randall R. 1951. Protein measurement with the folin phenol reagent. Journal of Biological Chemistry, 193:265–75.

Mikstacka R, Rimando AM, Ignatowicz E. 2010. Antioxidant effect of trans-resveratrol, pterostilbene, quercetin and their combinations in human erythrocytes in vitro. Plant Foods for Human Nutrition, 65(1):57–63.

Miltonprabu S, Thangapandiyan S. 2013. Protective Effect of Epigallocatechin Gallate on Fluoride-Induced Oxidative Stress Related Haematotoxicity in Rats. Research and Reviews: Journal of Pharmacology and Toxicological Studies. 1 (2): 1–12.

Misra HP, Fridovich I. 1972. The role of superoxide anion in the autooxidation of epinephrine and a simple assay for superoxide dismutase. Journal of Biological Chemistry, 247:3170–3175.

Mukherjee S, Das D, Darbar S. 2003. Dietary intervention affects Cd-generated nitric oxide and reactive oxygen intermediate toxicity in islet cells of rats. Current Science, 85:786–793.

Nabavi SF, Nabavi SM, Abolhasani F, Moghaddam AH, Eslami S. 2012. Cytoprotective Effects of Curcumin on Sodium Fluoride- Induced Intoxication in Rat Erythrocytes. Bulletin of Environmental Contamination and Toxicology, 88:486–490.

Ohkawa H, Ohishi N, Yagi K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry, 95:351–8.

Perecko T, Jancinova V, Drabikova K, Nosal R, Harmatha J. 2008. Structure-efficiency relationship in derivatives of stilbene. Comparison of resveratrol, pinosylvin and pterostilbene. Neuro Enocrinology Letters, 29 (5): 802–805.

Quigley JP, Gotterer GS. 1969. Distribution of Na^+K^+-stimulated ATPase activity in rat intestinal mucosa. Biochem Biophys Acta, 73:456–68.

Remsberg CM, Yáñez JA, Ohgami Y, Vega-Villa KR, Rimando AM, Davies NM. 2008. Pharmacometrics of pterostilbene: preclinical pharmacokinetics and metabolism, anticancer, antiinflammatory, antioxidant and analgesic activity. Phytotherapy Research, 22(2):169–79.

Reznick AZ, Packer L. 1994. Oxidative damage to proteins: spectrophotometric methods for carbonyl assay. Methods in Enzymology, 233:357–63.

Robert Jr. CH. 1980. Agents affecting calcification: Calcium, parathyroid hormone, calcitonin, vitaminD and other compounds. In: Goodman Gillman's the Pharmacological Basis of Therapeutics, 7th Edition, (Goodman, L. S., Rall, T. W., and Murad, F. Eds.), Macmillan Publishing Co., New York, pp. 1496–1522.

Samuel KM. 1989. Fundamentals of Clinical Chemistry. Philadelphia: WB Saunders and Company, 602–603.

Sato Y, Kanazawa S, Sato K, Suzuki Y. 1998. Mechanism of free radical induced hemolysis of human erythrocytes: II. Hemolysis by lipid soluble radical initiator. Biological and Pharmaceutical Bulletin, 21:250–256.

Shanthakumari D, Srinivasalu S, Subramanian S. 2004. Effect of fluoride intoxication on lipid peroxidation and antioxidant status in experimental rats. Toxicology, 204: 219–228.

Tentori L, Salvati AM. Hemoglobinometry in human blood. 1981. Methods in Enzymology, 76:707–15.

Tylenda CA. 2011. Toxicological profile for fluorides, hydrogen fluoride, and fluorine (update), 96. Collingdale: DIANE Publishing.

Uslu B. 1981. Effect of fluoride on hemoglobin and hematocrit, Fluoride 14 (1) (1981) 38–41.

Viragh EH, Laczka VJ, Coldea V. 2006. Health effects of occupational exposure to fluoride and its compounds in a small-scale enterprise. Industrial Health 44: 64–68.

Waugh DT. 2019. Fluoride Exposure Induces Inhibition of Sodium-and Potassium-Activated Adenosine Triphosphatase (Na^+, K^+-ATPase) Enzyme Activity: Molecular Mechanisms and Implications for Public Health. International Journal of Environmental Research and Public Health, 16(8):1427.

Xue EX, Lin JP, Zhang Y, Sheng SR, Liu HX, Zhou YL, Xu H. 2017. Pterostilbene inhibits inflammation and ROS production in chondrocytes by activating Nrf2 pathway. Oncotarget, 8:41988–42000.