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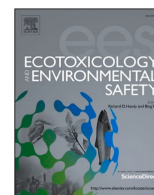

Research paper

Fluoride enhances generation of reactive oxygen and nitrogen species, oxidizes hemoglobin, lowers antioxidant power and inhibits transmembrane electron transport in isolated human red blood cells

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A R T I C L E I N F O

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A B S T R A C T

Fluoride is a widespread environmental pollutant that at high levels exerts numerous deleterious effects on human health. The toxic effects of fluoride are a matter of serious concern since many countries have regions of endemic fluorosis. The main source of fluoride exposure for humans is intake of contaminated groundwater. Fluoride is absorbed from the gastrointestinal tract and enters the circulating blood, where the abundant red blood cells (RBC) are an early and major target of fluoride toxicity. Chronic fluoride exposure generates free radicals, reactive species which leads to redox imbalance, cytotoxicity and hematological damage. This study aimed to determine the effect of sodium fluoride (NaF) on human RBC under in vitro conditions. Isolated RBC were incubated with different concentrations of NaF (10–500 μM) for 8 h at 37 °C. Several biochemical parameters were determined in hemolysates or whole cells. Treatment of RBC with NaF enhanced the generation of reactive oxygen and nitrogen species. This increased the oxidation of hemoglobin to yield methemoglobin and oxoferrylhemoglobin, which are inactive in oxygen transport. NaF treatment increased the degradation of heme causing release of free iron from its porphyrin ring. Cellular antioxidant power was significantly decreased in NaF-treated RBC, lowering the metal reducing and free radical quenching ability of cells. The two pathways of glucose metabolism in RBC i.e. glycolysis and hexose monophosphate shunt, were inhibited. NaF also inhibited the plasma membrane redox system, and its associated ascorbate free radical reductase, to disrupt transmembrane electron transport. These results suggest that fluoride generates reactive species that cause extensive oxidative modifications in human RBC.

1. Introduction

Fluorine is the 13th most abundant element in the earth's crust that is widely dispersed in nature, almost entirely in the form of fluorides. Fluoride is released in the environment through volcanic emissions, weathering of minerals, manufacture of fertilizers, fungicides, rodenticides and coal power production. Fluoride is, therefore, widely recognized as an important natural and industrial environmental pollutant.

Fluoride at low levels promotes healthy bone growth and plays an important role in dental hygiene by preventing dental caries and tooth decay. Fluoride containing compounds are, therefore, routinely added to toothpastes and drinking water (WHO, 2006; Marinho et al., 2016). However, exposure to high levels of fluoride is toxic and deleterious to human health, leading to a condition called fluorosis (Zuo et al., 2018). It results in teeth mottling and crippling skeletal deformities with damage to liver, lung, kidney, blood, nerves, brain and gastrointestinal system (Srivastava and Flora, 2020; Johnston and Strobel, 2020). High levels of serum fluoride damage the kidney (Dharmaratne, 2019) while long term drinking of fluoridated water can be a risk factor in development of type 2 diabetes (Fluegge, 2016). Fluoride also appears to be

Abbreviations: ABTS, 2,2′-Azino-bis(3-ethylbenzothiazoline)−6-sulfonic acid; AFR, ascorbate free radical; AMP, adenosine 5′-monophosphate, AO, antioxidant; ATP, adenosine 5′-triphosphate; CUPRAC, cupric reducing antioxidant capacity; DCFH-DA, 2,7-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant power; G6PD, glucose 6-phosphate dehydrogenase; Hb, hemoglobin; MetHb, methemoglobin; NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotide; $NADP^+$ and NADPH, oxidized and reduced nicotinamide adenine dinucleotide phosphate; NaF, sodium fluoride; oxoferrylHb, oxoferryl hemoglobin; PBS, phosphate buffered saline; PMG, phosphomolybdenum green; PMRS, plasma membrane redox system; RBC, red blood cells; RNS, reactive nitrogen species; ROS, reactive oxygen species.

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mutagenic, genotoxic and causes chromosomal abnormalities (Kharb et al., 2012; Ribeiro et al., 2017).

Fluoride is present in all natural waters at varying levels. WHO recommends fluoride water level at 1.0–1.5 mg/L but much higher concentrations have been reported in groundwater in many countries across the world (Ayoob and Gupta, 2006; Yadav et al., 2019). Millions of people obtain water from groundwater sources that are contaminated with fluoride beyond permissible limits, some reaching concentrations of 30–60 mg/L (Ayoob and Gupta, 2006). Dental products like mouth rinses contain 1000 mg/L while toothpastes have around 1000–1500 ppm fluoride (Marinho et al., 2016). Humans are exposed to fluoride through food products, tea, dental products like toothpastes, vegetation and medicaments. However, the main route of exposure is through consumption of contaminated water. Populations exposed to 4.8–7.3 mg/L NaF through contaminated drinking water suffer from chronic kidney disease (Dharmaratne, 2019). Almost 62 million people in 19 Indian states suffer from dental, skeletal and non-skeletal fluorosis due to high fluoride levels (exceeding 1.5 mg/L) in drinking water (FRRDF, 1999; Susheela and Toteja, 2018). Fluoride toxicity is, therefore, endemic and a cause of worldwide concern.

Oxidative stress is recognized as a major mode of action of fluoride toxicity (Barbier et al., 2010; Chlubek, 2003). Absorbed fluoride causes nuclear DNA damage, mitochondrial dysfunction, alters intracellular redox status and enhances oxidation of membrane lipids and proteins (Miranda et al., 2018). Fluoride exposure beyond 5 mg/L can generate reactive oxygen species (ROS) and cause cellular damage in animal models (Barbier et al., 2010). Fluoride inhibits the activities of several antioxidant (AO) enzymes and lowers glutathione levels. It inhibits ATPase activity leading to ATP depletion and suppresses ROS scavenging ability of cells. Fluoride induces mitochondrial depolarization which increases ROS production and oxidative stress causing apoptotic cell death (Jothiramajayam et al., 2014). Excessive generation of ROS leads to oxidative modification of macromolecules resulting in membrane damage via lipid peroxidation. Treatment with AOs like taurine and quercetin attenuates fluoride induced macromolecular damage and changes in cellular homeostasis (Adedara et al., 2017; Oyagbemi et al., 2018). This suggests that the major mode of fluoride action depends on oxidative and nitrosative mechanisms and alterations in redox homeostasis.

Fluoride is absorbed from the gastrointestinal tract (Kanduti et al., 2016), enters the bloodstream by passive diffusion and is then distributed to various tissues. Blood represents a major target of xenobiotics and toxicants like fluoride that enter by any route and red blood cells (RBC), being the most abundant cells not only in blood but entire human body, are quickly exposed to their action. Thus, in addition to endogenous oxidative stress, due to high content of oxygen and transition metal ions, RBC are also exposed to exogenous oxidants like fluoride. RBC have, therefore, been used as model for investigating molecular and cellular mechanisms of toxicant mediated cell damage (Farag and Alagawany, 2018). Blood is also the biological fluid into which fluoride must pass for its distribution elsewhere in the body as well as its elimination from the body. Blood fluoride is distributed between plasma and the blood cells (Whitford, 1996) and the plasma fluoride concentration depends on the fluoride dose ingested and dose frequency. In humans, fluoride induces hematological abnormalities which include abnormal blood cell counts, lower hemoglobin (Hb) content and anemia (Eren et al., 2005; Susheela et al., 2018). In vitro studies have shown that fluoride causes cell lysis, depletes ATP (Agalakova and Gusev, 2011, 2012) and inhibits membrane bound $Na^{+}K^{+}$ ATPase (Shashi and Meenakshi, 2015). Lower glutathione levels, increased lipid peroxidation and inhibition of catalase and superoxide dismutase have been shown by in vitro and in vivo studies (Gutiérrez-Salinas et al., 2013; Miranda et al., 2018). Despite these reports, the ability of fluoride to cause red cell death, and the underlying molecular mechanism, has not been clearly elucidated. In this study we show that fluoride exposure generates reactive species, oxidizes Hb causing release of free iron, lowers AO power and inhibits the plasma membrane redox system in human RBC. The consequences of fluoride action on RBC and blood functions are discussed.

2. Materials and methods

2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), Trolox, NaF and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Aldrich, USA. Adenosine 5′-triphosphate (ATP), 2,6-dichlorophenolindophenol, 2,4,6-tris(2-pyridyl)-s-triazine, 1,10-phenanthroline, ascorbate oxidase, oxidized and reduced nicotinamide adenine dinucleotide (NAD^{+} and NADH), potassium ferricyanide, ferrozine and sodium chloride were obtained from Sisco Research Laboratory, India. Dihydroethidium (DHE) was from Genetix while neocuproine and folic were from Himedia (Mumbai, India). All other chemicals were of analytical grade.

2.1. Isolation and treatment of human red blood cells

Ethical approval of the study was obtained from the Institutional Ethics Committee of Aligarh Muslim University (Registration number: 714/GO/Re/S/02/CPCSEA). Blood was taken from non-smoking, young and healthy individuals between 20–25 years of age. Blood was collected in heparinized tubes and RBC were prepared as described previously (Qasim and Mahmood, 2015). Briefly, heparinized blood was centrifuged at low speed (210*g*, 10 min) and plasma and buffy coat were removed. The packed RBC in pellet were washed three times with phosphate buffered saline (PBS; 10 mM sodium phosphate buffer, 0.9% sodium chloride, pH 7.4) and suspended again in PBS to give a 10% (v/v) cell suspension (hematocrit) The 10% hematocrit was treated with 10–500 μM NaF for 8 h at 37 °C in a shaking water bath. NaF untreated RBC (10% hematocrit) were similarly incubated for 8 h at 37 °C and served as control. The cell suspensions were then centrifuged at 210*g* for 10 min and absorbance of supernatants taken at 540 nm to calculate percent hemolysis. The RBC pellets were washed thrice with PBS to remove excess reagents. The packed RBC were lysed by adding 10 volumes of hypotonic buffer (5 mM sodium phosphate buffer, pH 7.4) and centrifuged at 630*g* for 10 min to remove any cell debris. The supernatants (hemolysates) were used immediately or kept at −80 °C for later analysis.

The concentration of Hb in hemolysates was determined by measuring the absorbance of the stable cyanomethemoglobin at 540 nm, according to the method of Drabkin and Austin (1935). A commercially available kit (Hemocor D reagent) from Coral Clinical Systems (Goa, India) was used.

2.2. ROS and RNS generation

The formation of superoxide and ROS was assayed fluorometrically by using DHE (Wojtala et al., 2014) and DCFH-DA (Keller et al., 2004), respectively. The 10% hematocrit was separately incubated with l0 μM DHE or DCFH-DA for 1 h at 37 °C. RBC were centrifuged, washed thrice with PBS to remove excess dyes and suspended in PBS to again give 10% hematocrit. RBC were treated with different concentrations of NaF for 60 min (DHE) and 30 min (DCFH-DA) at 37 °C and fluorescence of samples recorded. The excitation and emission wavelengths used were 518 and 605 nm (DHE) and 485 and 530 nm (DCFH-DA), respectively·H_2O_2 concentration was determined spectrophotometrically in hemolysates using ferrous sulphate-xylenol orange as the color reagent and sorbitol as color enhancer (Gay and Gebicki, 2000). Peroxynitrite was determined as a measure of reactive nitrogen species (RNS). Hemolysates from control and NaF treated cells were mixed with 15 μM folic acid for 5 min at room temperature. After removal of proteins by precipitation with barium hydroxide and zinc sulphate and centrifugation, the fluorescence of supernatants was recorded at 470 nm after excitation at 380 nm (Huang et al., 2007).

2.3. Hemoglobin oxidation, heme degradation and release of free iron

Methemoglobin (MetHb) formation was quantified according to Benesch et al. (1973) by recording the absorbance of hemolysates at 540, 576 and 630 nm. MetHb reductase activity was determined from the reduction of 2,6-dichlorophenolindophenol in presence of NADH to give a product that absorbs at 600 nm (Kuma et al., 1972). An extinction coefficient of 21×10^3 M^{-1} cm^{-1} was used to calculate enzyme activity. OxoferrylHb was determined by recording the fluorescence of 20 times diluted (with PBS) hemolysates using 460 nm as excitation and 525 nm as emission wavelengths (Nagababu and Rifkind, 1998).

Heme degradation was determined by recording fluorescence of appropriately diluted hemolysates using 325 nm as excitation and 480 nm as emission wavelengths (Nagababu et al., 2008). The release of ferrous iron from heme was determined by the ferrozine method (Panter, 1994); the absorbance of pink colored ferrozine-ferrous complex was recorded at 550 nm.

2.4. Antioxidant power

The AO power of hemolysates was determined from the metal ion reducing (FRAP, CUPRAC and PMG) and free radical quenching (ABTS, DPPH) methods. Ferric reducing antioxidant power (FRAP) was determined from the blue colored complex formed upon reaction of Fe^{2+} with 2,4,6-tris(2-pyridyl)-s-triazine (Benzie and Strain, 1996). The absorbance of samples was noted at 593 nm. Phosphomolybdenum green (PMG) assay was done according to Prieto et al. (1999) by recording the absorbance of green colored complex at 695 nm. Cupric reducing antioxidant capacity (CUPRAC) method is based on the reduction of the Cu^{2+} to Cu^{+} by sample AOs. The formation of Cu^{+}-neocuproine complex was monitored from its absorbance at 450 nm (Cekic et al., 2012). In the DPPH assay, quenching of purple colored $DPP^{\bullet}$ free radical to light yellow DPPH was followed spectrophotometrically at 517 nm (Mishra et al., 2012). In ABTS assay, the colored $ABTS^{\bullet+}$ radical was decolorized to non-radical ABTS form by accepting electrons from AOs present in samples. The total AO capacity in hemolysates was expressed in μM Trolox equivalents/mg Hb (Re et al., 1999).

2.5. Plasma membrane redox system and ascorbate free radical reductase

Plasma membrane redox system (PMRS) was determined in control and NaF treated packed RBC by suspending them in PBS, containing 5 mM glucose and 1 mM potassium ferricyanide, and incubating at 37 °C for 30 min. Transplasma membrane redox system reduces the extracellular Fe^{3+} ions to Fe^{2+} form (Rizvi and Srivastava, 2010). After centrifugation, Fe^{2+} content in supernatant was quantified spectrophotometrically using 1,10-phenanthroline as the color reagent (Avron and Shavit, 1963). Ascorbate free radical (AFR) reductase activity was determined in hemolysates according to May et al. (2004) by following the oxidation of NADH at 340 nm in presence of ascorbate and ascorbate oxidase.

2.6. Metabolic enzymes

Glyceraldehyde 3-phosphate dehydrogenase activity was monitored by a coupled enzymatic assay by recording the increase in absorbance at 340 nm upon reduction of NAD^{+} to NADH in the presence of glucose 6-phosphate (Heinz and Freimoller, 1982). Glucose 6-phosphate dehydrogenase (G6PD) activity was determined by measuring the increase in absorbance at 340 nm due to conversion of $NADP^{+}$ to NADPH (Shonk and Boxer, 1964). Acid phosphatase was assayed by monitoring the hydrolysis of p-nitrophenyl phosphate to yellow colored p-nitrophenol at 415 nm (Mohrenweiser and Novotny, 1982). To determine glyoxalase-I activity, reduced glutathione and methylglyoxal were incubated at 37 °C to produce hemithioacetal. The addition of sample (hemolysate) converts the hemithioacetal to S-lactoyl glutathione resulting in increase in absorbance of solution at 240 nm. Enzyme activity was calculated using extinction coefficient of 2.86 mM^{-1} cm^{-1} (Arai et al., 2014).

2.7. Statistical analysis

All experiments were performed on six blood samples taken from six different individuals. Statistical significance was calculated by one way ANOVA test using the program Origin Pro 8.0 (USA) and reported as the mean ±standard error. Results were measured by the Bonferroni and Tukey post-hoc test and considered significant when the probability value (P) was <0.05.

3. Results and discussion

Fluoride is a widespread environmental pollutant and toxicant that is associated with hematological disorders. Blood and RBC are early and major targets of fluoride. The present study was, therefore, done to examine the effect of sodium salt of fluoride (NaF) on human RBC. NaF is commonly used as a source of fluoride in experimental studies. It is also used as an anti-helminthic agent, insecticide, rodenticide and in dental care products. Several parameters, especially of oxidative stress and Hb modification, were analyzed. In addition, enzymes of metabolic pathways were also examined. The fluoride concentrations used in this study (10–50 μM NaF corresponding to 0.19–9.5 mg/L fluoride or 0.19–9.5 ppm) correspond to those reported in groundwater at several places (Ayoob and Gupta, 2006). In addition, fluoride concentrations of 18.5 and 40 μM have been reported in the plasma of persons living in fluoride endemic areas in India and Pakistan, respectively (Kumar et al., 2017; Rafique et al., 2015).

3.1. Red blood cell lysis

Hemolysis is an easy way of determining the cytotoxicity of xenobiotics like fluoride (Farag and Alagawany, 2018). It results in release of Hb outside the cell which increases the absorbance of extracellular medium at 540 nm. NaF treatment generates ROS in cells and causes oxidation of membrane proteins and lipids that makes the membrane more fragile (Ribeiro et al., 2017) resulting in release of Hb outside the cell. At 250 and 500 μM NaF, the percent hemolysis was 2.0 and 2.4 times, respectively, of control cells. The lysis is likely due to NaF-induced membrane damage and will result in anemia in fluoride exposed individuals. These results are in agreement with a previous study in which 0.5–16 mM NaF caused hemolysis and phosphatidylserine externalization in rat RBC (Agalakova and Gusev, 2011). Susheela (2001) also found that fluoride exposure leads to membrane degeneration, shortened RBC life span and anemia.

Besides direct RBC lysis, fluoride can cause anemia in other ways also. Fluoride-damaged but unlysed cells are removed from circulation by the reticuloendothelial system lowering their lifespan. Fluoride increases the phagocytic activity of spleenic macrophages that engulf more RBC leading to their removal from circulation (Kumari and Kumar, 2011). Fluoride is also known to inhibit erythropoiesis thereby lowering RBC count and Hb content. Fluoride accumulates not only in bones but also in bone marrow cavities where hematopoiesis occurs. Machalinski et al. (2000) reported that fluoride toxicity causes injury to human hematopoietic (blood forming cells) progenitor cells which will inhibit erythropoiesis. Kahl et al. (1973) found that NaF decreased incorporation of ^{59}Fe into RBC and spleen. They suggested that fluoride interacts with the iron of Hb, thereby inhibiting globin synthesis and retarding the normal process of erythropoiesis leading to anemia.

3.2. ROS and RNS

ROS and RNS are the normal outcome of various metabolic processes and include the superoxide radicals and its downstream consequences like hydroxyl radicals, hydrogen peroxide and peroxynitrite ($ONOO^{-}$).

Their generation is increased under conditions of oxidative stress. The intracellular production of ROS and RNS was, therefore, determined in control and NaF treated RBC.

DCFH-DA method records general ROS level, especially the hydroxyl radicals, while DHE is specific for superoxide radicals only. Fluoride exposure increased the intracellular production of highly reactive molecules. A gradual increase in hydroxyl and superoxide radicals was seen in RBC; at 500 μM NaF their levels were increased to 3 and 1.5 times of the control cells, respectively (Fig. 1). The concentration of non-radical ROS, H_2O_2, in NaF treated cells was increased 2.2 fold relative to the control cells. Increase in superoxide radicals and H_2O_2 has been previously reported in other cell types (Ni et al., 2018; Fina et al., 2014). This was accompanied by a two fold increase in the level of peroxynitrite (Fig. 1), an anion capable of causing oxidative damage to lipids, proteins and DNA (Radi, 2018). This increase in ROS and RNS production can be the major mode of action of fluoride toxicity in RBC. These species will oxidize lipids and proteins resulting in membrane damage and cell lysis. Hence, fluoride-induced oxidative/ nitrosative stress leading to generation of reactive species can be one of the principal causative factors of anemia. The ROS and RNS must have been principally responsible for RBC lipid peroxidation, protein oxidation and inactivation of membrane bound ATPase (Waugh, 2019).

There can be several sources of excess levels of ROS and RNS in NaF treated RBC. The inhibition of AO enzymes catalase, superoxide dismutase, glutathione peroxidase (Gutiérrez-Salinas et al., 2013) and lowering of glutathione levels (Miranda et al., 2018) will result in less quenching and, therefore, higher levels of ROS and RNS. Another reason could be formation of MetHb which releases an electron that is taken up by molecular oxygen to give superoxide radicals. The superoxide radicals, though relatively less reactive, spontaneously react with NO to give peroxynitrite or are converted to H_2O_2. The reaction of H_2O_2 with free iron, released from heme degradation, gives the very reactive and extremely damaging hydroxyl radical (Fenton reaction).

3.3. Oxidative damage to hemoglobin

Hb is the major protein (≥95%) constituent in RBC and should be maintained in reduced form ($Hb.Fe^{2+}$) for blood to have maximum oxygen carrying capacity. ROS target native active form of Hb either by oxidizing iron in heme to ferric form (yielding MetHb), by producing oxoferryHb, degrading heme and releasing iron from the heme porphyrin ring. MetHb formation was increased 1.8 fold while activity of MetHb reductase, an enzyme that converts MetHb back to Hb, was lowered to half of control values at 500 μM NaF (Fig. 2). Inhibition of NADH-dependent MetHb reductase and abnormal increase in MetHb decreases the unloading of oxygen in tissues resulting in hypoxia. Oxidants such as H_2O_2 catalyze a pseudo peroxidase reaction with MetHb (ferric Hb) to produce oxoferrylHb (Kassa et al., 2016), whose level was also increased by NaF. FerrylHb is also related to protein radical formation.

Oxidation of oxyhemoglobin by H_2O_2 produces heme degradation products. Heme degradation and oxoferrylHb formation was enhanced 2 and 2.4 fold in 500 μM NaF treated cells with respect to control cells (Fig. 3). Free heme and its breakdown products, the key degradation byproducts of Hb oxidation, can trigger inflammatory responses. ROS also release iron from Hb in a non-protein bound desferrioxamine-chelatable form (Comporti et al., 2002). NaF treatment led to release of iron in free form, from heme porphyrin ring, which was greatly increased to 2.5 times the control RBC in an NaF-concentration dependent manner (Fig. 3). The released iron is redox active and can cause further oxidation of membrane proteins and lipids upon reaction with H_2O_2. Thus NaF oxidizes Hb to forms that

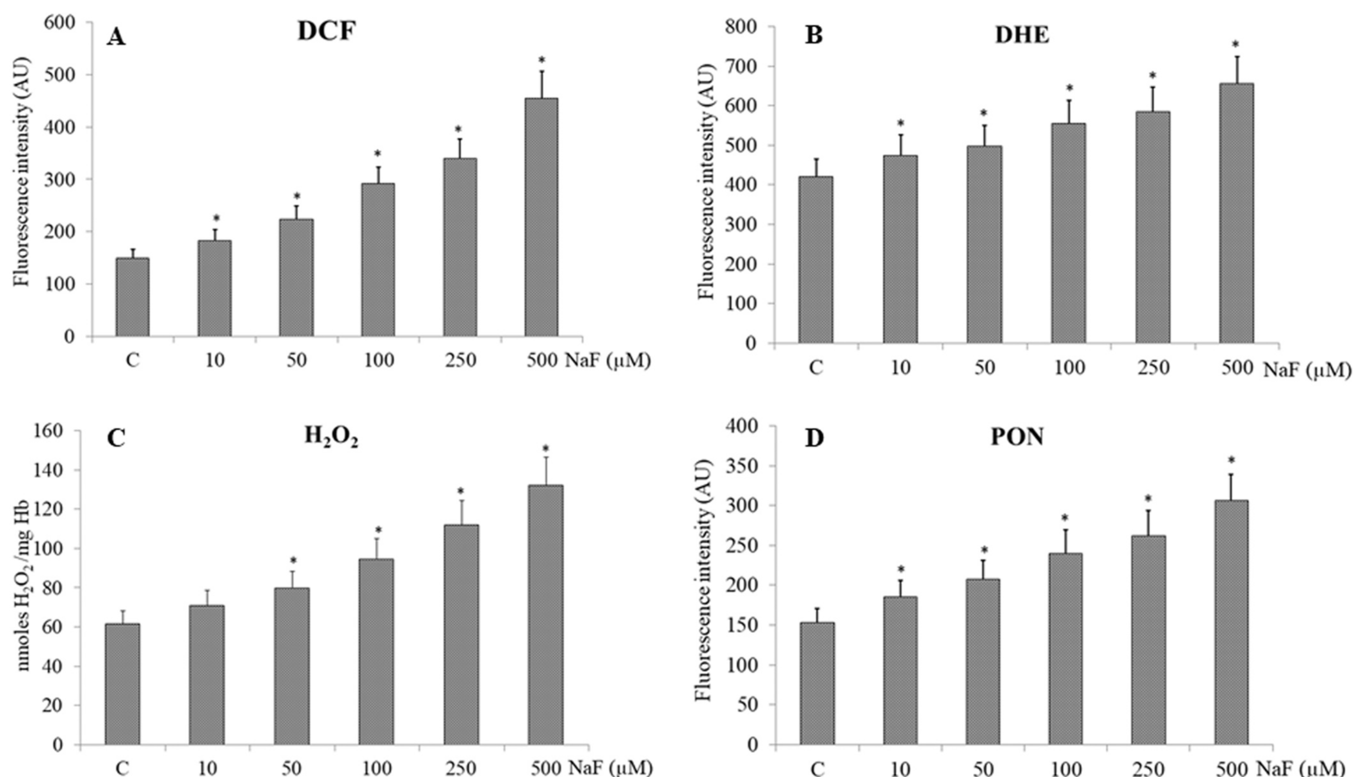

Fig. 1. Effect of NaF on production of ROS and RNS in RBC. (A) ROS were determined by DCFH-DA method and (B) superoxide radicals using DHE. (C) H_2O_2 was determined spectrophotometrically using ferrous sulphate-xylenol orange and (D) peroxynitrite by using folic acid. DCFH-DA, DHE and peroxynitrite are reported in fluorescence intensity (AU, arbitrary fluorescence units) and H_2O_2 concentration is in nmoles H_2O_2/ mg Hb. Results are mean ±standard error of six different samples. The error bar depicts the standard error among the six different samples of that group. Differences from control were considered significant when P-value was <0.05 and are marked with an asterisk (*).

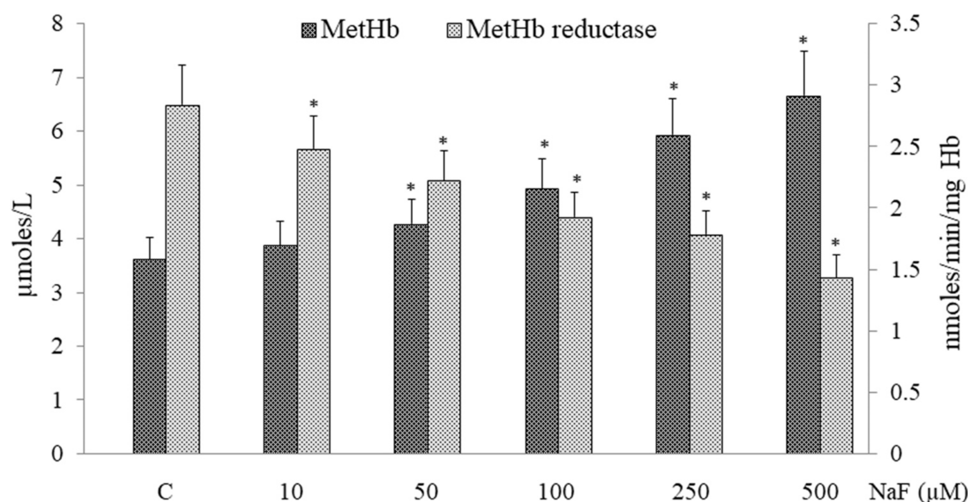

Fig. 2. Effect of NaF on methemoglobin (MetHb) levels and MetHb reductase activity in hemolysates. MetHb is in µmoles/L and MetHb reductase activity is in nmoles/min/mg Hb. Results are mean ±standard error of six different samples. The error bar depicts the standard error among the six different samples of that group. Differences from control were considered significant when P-value was <0.05 and are marked with an asterisk (*).

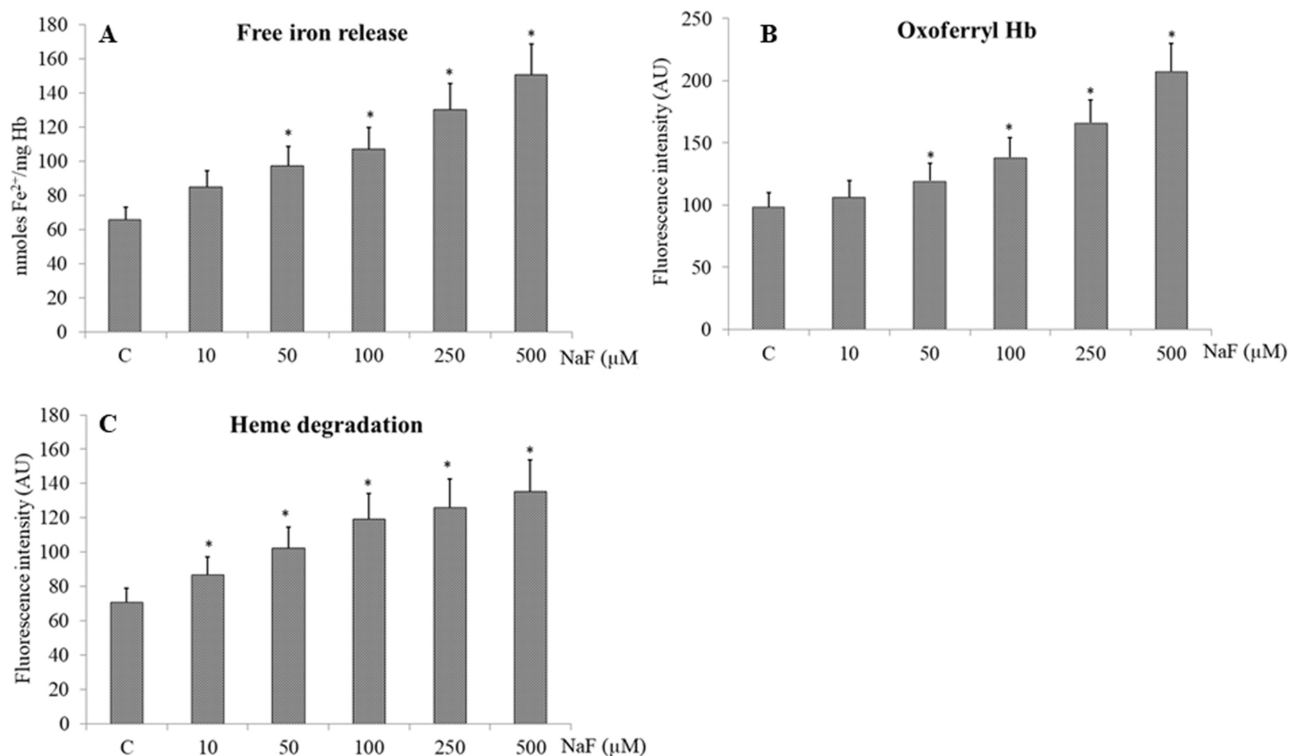

Fig. 3. Effect of NaF on (A) free iron release (B) heme degradation and (C) formation of oxoferrylHb. Free iron release is reported in nmoles/ mg Hb while heme degradation and oxoferryHb are in fluorescence intensity (arbitrary units, AU). Results are mean ±standard error of six different samples. The error bar depicts the standard error among the six different samples of that group. Differences from control were considered significant when P-value was <0.05 and are marked with an asterisk (*).

are inactive in oxygen transport, degrades heme and releases its bound iron in ferrous form.

3.4. Free radical scavenging and metal reducing ability

NaF treatment elevates the oxidative stress in cells that can diminish the reducing ability of RBC. The AO power of hemolysates was determined from their ability to reduce metal ions (Fe^{3+}, Cu^{2+} and Mo^{6+}) or quench free radicals $ABTS^{\bullet+}$ and $DPP^{\bullet}$. The FRAP, CUPRAC and PMG assays showed significant lowering in metal reducing power which was almost half of untreated control cells. In NaF alone treated samples the ability to quench $ABTS^{\bullet+}$ and $DPP^{\bullet}$ radicals was decreased to 43% and 50% of control cells (Table 1). The AO power is lowered probably due to impaired enzymatic and non-enzymatic AO defense systems in NaF treated RBC. The diminished AO power will reduce the ability of RBC to quench ROS and RNS making them more vulnerable to oxidative and nitrosative damage.

Table 1
Effect of sodium fluoride on antioxidant power of RBC.

	NaF (μM)					
	C	10	50	100	250	500
FRAP	163.38 ± 18.12	149.45 ± 16.2	126.16 ± 14.21*	118.57 ± 12..31*	103.24 ± 9.43*	84.483 ± 10.11*
PMG	1.635 ± 0.173	1.512 ± 0.167	1.294 ± 0.144*	1.204 ± 0.137*	1.084 ± 0.112*	0.783 ± 0.105*
CUPRAC	178.02 ± 20.13	165.31 ± 17.84*	149.2 ± 16.31*	137.96 ± 14.72*	120.72 ± 12.64*	88.58 ± 9.11*
DPPH	91.17 ± 10.28	87.38 ± 9.33	80.99 ± 8.06*	73.22 ± 8.14*	60.533 ± 7.66*	45.43 ± 5.18*
ABTS	72.47 ± 9.36	67.40 ± 7.56*	52.26 ± 6.51*	45.95 ± 5.28*	39.54 ± 4.65*	31.69 ± 3.84*

All parameters were assayed in hemolysates. NaF concentration is in micromoles/L in all groups. C is control group (no NaF).
Results are mean ±standard error of six different samples.
FRAP, PMG, CUPRAC are in nmoles/mg Hb, DPPH is reported in % quenching of DPP• radical and ABTS in μmoles Trolox equivalent/mg Hb.
FRAP, ferric reducing antioxidant power; PMG, phosphomolybdenum green; CUPRAC, cupric reducing antioxidant capacity; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline)−6-sulfonic acid.
[*] Differences from control were considered significant when P-value was <0.05 and are marked with an asterisk (*).

3.5. Metabolic enzymes

RBC being the most abundant and sensitive cells, act as an indicator of several metabolic disorders. Since RBC lack cell organelles, glycolysis and hexose monophosphate shunt are the only pathways of glucose metabolism. Due to its strong electronegativity fluoride ions strongly interacts with proteins and enzymes (Miranda et al., 2018). Fluoride acts as an enzyme inhibitor and is a well-known inhibitor of enolase, the penultimate glycolytic enzyme. The activity of another glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase declined to 37% of control value. This enzyme contains an active site sulfhydryl that is easily oxidized by ROS resulting in enzyme inactivation. Inhibition of glycolysis will lower cellular ATP production thereby affecting several metabolic and transport processes. It also renders the cells more prone to oxidative damage. G6PD keeps the RBC healthy and deficient cells tend to lyse easily under oxidative conditions. G6PD inhibition by NaF to half of control activity will deprive the RBC of NADPH, the major cellular reductant (Table 2). NADPH is also used by AO enzymes glutathione peroxidase and glutathione reductase, which will also be affected. This causes premature breakdown of RBC eventually leading to hemolytic anemia.

Due to inhibition of glycolytic pathway and high glucose concentration in RBC, the flux of methylglyoxal metabolized to D-lactic acid via glyoxalase pathway was increased. Elevated cellular concentrations of methylglyoxal, an indicator of carbonyl stress, react with proteins to form advanced glycation end products. AMP-deaminase is highly sensitive to oxidative damage and plays a vital role in purine nucleotide cycle and energy metabolism. It also serves as a potent ROS marker in RBC (Tavazzi et al., 2002). Glyoxylase 1 and AMP-deaminase activities showed an NaF concentration-dependent increase of 2.6 and 2.3 fold (Table 2). Acid phosphatase was also inhibited by NaF. This will affect dephosphorylation of band 3 protein which binds to glycolytic enzymes. Thus NaF inhibits both pathways of glucose metabolism and elevates the activities of marker enzymes of oxidative stress in RBC.

3.6. Plasma membrane redox and associated enzymatic system

Oxidation of membrane proteins and lipids can disturb the functioning of membrane bound enzymatic systems. PMRS and associated AFR reductase act as transmembrane electron transport system by transferring electrons from intracellular donors to extracellular acceptors. They have multiple functions among which are to act as a compensatory operating system in the cell to attenuate oxidative stress, maintain NAD^+/NADH ratio, regenerate extracellular ascorbate etc. PMRS and AFR reductase were inhibited in presence of NaF and at 500 μM NaF were 58% and 27% of control cells, respectively, (Fig. 4). Both PMRS and AFR reductase contain essential thiol residues whose modification by ROS could have led to their inhibition (Rizvi and Srivastava, 2010). Inactivation of PMRS will lower the NAD^+ needed for glycolytic ATP production via transfer of electrons from intracellular reducing equivalents to extracellular acceptors. The regeneration of extracellular ascorbate will also be reduced, thereby lowering the AO power of plasma.

The observed NaF effects on RBC can have several harmful consequences. 1. The increase in MetHb and oxoferrylHb will lower the oxygen transporting ability of blood. 2. The ROS and RNS generated can leak out of damaged RBC and damage other blood cells and also those cells that come in contact with the circulating blood. 3. Blood serves as a mobile AO that quenches ROS and RNS in other cells and tissues. RBC, being by far the most abundant cells in blood, make a major contribution to the AO property of blood. Lower AO power of RBC, in response to NaF exposure, will greatly impair the AO function of blood. 4. Inhibition of PMRS will prevent regeneration of extracellular ascorbate, a major AO of plasma. This will again lower the AO power of blood. 5. ROS and RNS damage the membrane which will affect RBC rheology and their ability to deform and pass through capillaries. This will affect blood flow to tissues and may result in occlusion of blood vessels and hypoxia. 6. Finally, since damaged RBC are quickly cleared from circulation by the reticuloendothelial system, fluoride will reduce RBC lifespan (red cell

Table 2
Effect of sodium fluoride on the activities of some metabolic enzymes.

	NaF (μM)					
	C	10	50	100	250	500
GLO-I	178.83 ± 18.12	216.11 ± 22.25*	282.92 ± 30.34*	364.53 ± 40.12*	425.13 ± 45.39*	476.25 ± 50.11*
ADA	1.946 ± 0.173	2.307 ± 0.245*	2.5353 ± 0.277*	3.284 ± 0.364*	4.238 ± 0.548*	4.516 ± 0.521*
G6PD	210.06 ± 23.31	186.05 ± 20.25*	160.19 ± 17.18*	143.16 ± 15.52*	129.26 ± 14.34*	101.86 ± 11.21*
GAPDH	4.471 ± 0.621	3.645 ± 0.561*	2.8125 ± 0.335*	2.386 ± 0.258*	1.94 ± 0.215*	1.67 ± 0.186*
ACP	15.16 ± 1.63	13.54 ± 1.51	12.26 ± 1.35*	10.61 ± 1.22*	8.66 ± 0.96*	6.253 ± 0.78*

All enzyme activities were determined in hemolysates. NaF concentration is in micromoles/L in all groups. C is control group (no NaF).
Results are mean ± standard error of six different samples.
GLO-I, G6PD, GAPDH and ACP activities are in nmoles/min/mg Hb while ADA is in U/g Hb.
GAPDH, glyceraldehyde 3-phosphate dehydrogenase; G6PD, glucose 6-phosphate dehydrogenase; ADA, AMP deaminase; GLO-I, glyoxylase-I; ACP, acid phosphatase.
[*] Differences from control were considered significant when P-value was <0.05 and are marked with an asterisk (*).

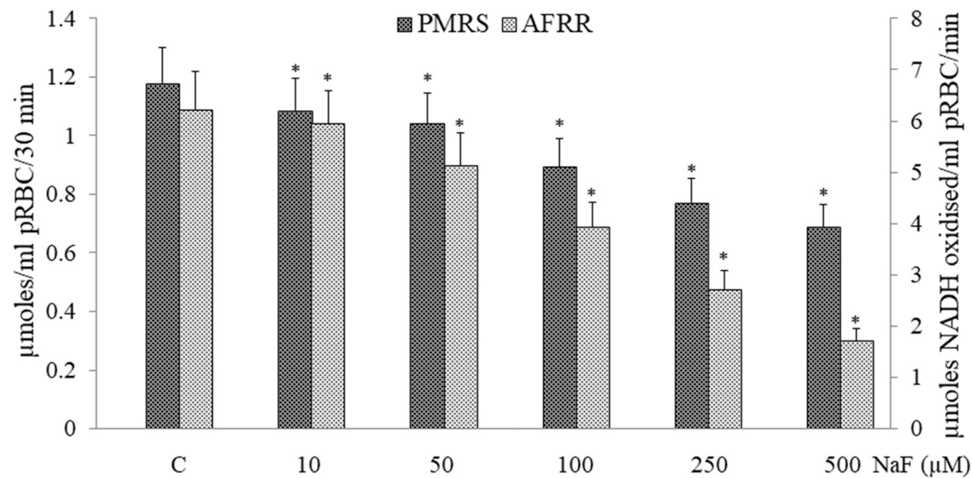

Fig. 4. Effect of NaF on PMRS and AFR reductase activity. PMRS is in nmoles ferrocyanide/30 min/mL pRBC and AFR reductase activity is in µmoles NADH oxidized/min/mL pRBC. Results are mean ±standard error of six different samples. The error bar depicts the standard error among the six different samples of that group. Differences from control were considered significant when P-value was <0.05 and are marked with an asterisk (*). (pRBC, packed RBC).

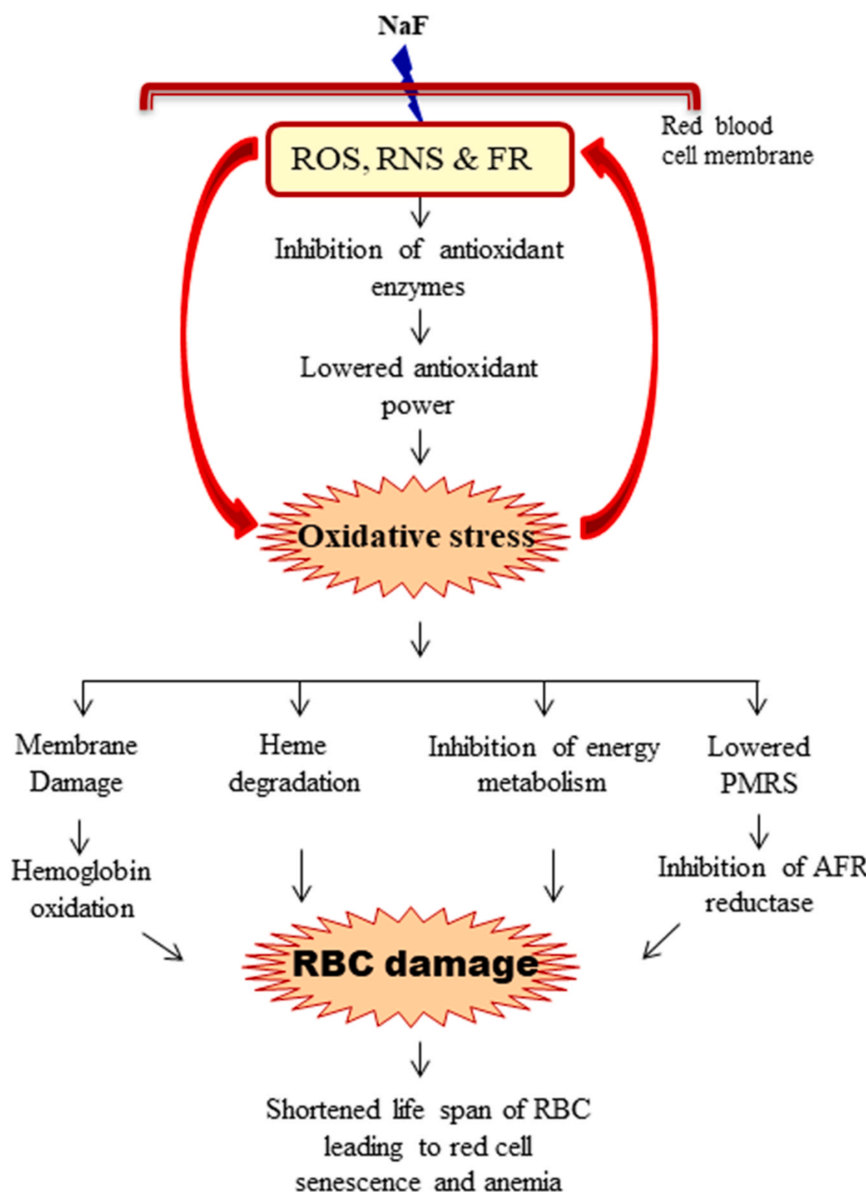

Fig. 5. Schematic representation of NaF-induced oxidative stress and RBC damage. Fluoride enters RBC by passive diffusion where it enhances the generation of ROS and RNS. The AO enzymes are inhibited, GSH level is lowered and AO power is diminished. This results in induction of oxidative stress condition in RBC and leads to oxidative modification of cell components. These include: (i) hemoglobin oxidation which will lower oxygen transport by blood (ii) low ATP and NADPH levels upon inhibition of glycolytic and HMP shunt pathways, making RBC more prone to oxidative damage (iii) membrane damage and inactivation of bound enzymes (iv) inhibition of PMRS and dysregulation of NAD^+/NADH ratio and less regeneration of plasma ascorbate from AFR (v) alterations in cell morphology. All these changes lead to RBC damage. Since damaged RBC are removed from circulation by the reticuloendothelial system, it will shorten their lifespan (red cell senescence) causing anemia, a known consequence of fluoride exposure. FR free radicals; PMRS, plasma membrane redox system; AFR, ascorbate free radical; RBC, red blood cell; ROS, reactive oxygen species; RNS, reactive nitrogen species; NaF, sodium fluoride; AO, antioxidant; PMRS, plasma membrane redox system; NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotide; GSH, glutathione.

senescence), resulting in anemia, a known complication of fluoride toxicity (Abbas et al., 2017; Susheela et al., 2018).

A schematic representation of the results obtained is shown in Fig. 5.

There are, however, certain limitations of these in vitro studies. The two major ones are: (i) difficulty in correlating alterations in parameters observed during these in vitro experiments to observed effects under in vivo condition; these in vitro results should, therefore, be verified and confirmed by animal experiments (ii) difficult to co-relate results obtained with RBC to other cells in the body. This is because RBC are unique non-nucleated atypical cells, specialized for oxygen transport, and do not represent other cells in the body.

4. Conclusions

NaF enhances generation of ROS and RNS in human RBC. This result in oxidation of Hb and heme damage, diminished AO power, inhibition of trans-membrane electron transport and also of glucose metabolism. These results represent a step in an attempt to delineate the molecular mode of fluoride hemotoxicity so that appropriate preventive measures can be designed.

CRediT authorship contribution statement

NM and RM conceived and designed the work and performed data analysis. The experiments were performed by NM and supervised by RM. NM wrote the manuscript. All the authors read and approved the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. There is no declaration of interest.

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