Journal of Trace Elements in Medicine and Biology 69 (2022) 126888

Contents lists available at ScienceDirect

Journal of Trace Elements in Medicine and Biology

journal homepage: www.elsevier.com/locate/jtemb

3,4-Dihydroxybenzaldehyde mitigates fluoride-induced cytotoxicity and oxidative damage in human RBC

Ruhi Anjum [1], Nikhil Maheshwari [1], Riaz Mahmood *

Department of Biochemistry, Faculty of Life Sciences, Aligarh Muslim University, Aligarh 202002, U.P., India

ARTICLE INFO

Keywords:
3,4-Dihydroxybenzaldehyde
Sodium fluoride
Oxidative stress
Red blood cells
Cytotoxicity

ABSTRACT

Background: Fluoride is an essential micronutrient that is needed for mineralization of bones and formation of dental enamel. It is a widely dispersed environmental pollutant and chronic exposure to it is toxic, resulting in malignancies and hematological damage in humans. Blood is a major and early target of environmental pollutants and toxicants like fluoride. Fluoride generates reactive oxygen species and free radicals which induce oxidative stress in target cells and mediate its toxic effects. The aim of this study was to determine the mitigating effect of plant antioxidant 3,4-dihydroxybenzaldehyde (DHB) on sodium fluoride (NaF) induced oxidative damage and cytotoxicity in isolated human red blood cells (RBC)

Method: Isolated human RBC were treated with 0.5 mM NaF, in absence or presence of different concentrations of DHB (0.1–2.5 mM). Several biochemical parameters were analyzed in cell lysates and whole cells.

Results: Treatment of RBC with NaF increased the formation of reactive oxygen and nitrogen species. It oxidized thiols, proteins and lipids and generated their peroxidative products. Methemoglobin level, heme degradation and lipid peroxidation were increased but cellular antioxidant status declined significantly in NaF alone treated RBC, compared to the control. NaF inhibited antioxidant, membrane bound and glycolytic enzymes in RBC. However, prior incubation of RBC with DHB significantly attenuated the NaF-induced alterations in all these parameters in a DHB concentration-dependent manner.

Conclusion: These results show that DHB mitigates NaF-induced oxidative damage in human RBC, probably because of its antioxidant character.

1. Introduction

Fluorine is the 13th most abundant element in the earth's crust. It is widespread in nature, almost entirely in the form of fluoride. The sodium salt, sodium fluoride (NaF), is used as a wood preservative, insecticide, anti-helminthic drug, corrosion inhibitor, cleaning agent, in metallurgy and glass industries [2]. Low concentrations of fluoride are beneficial to bone growth and dental health. Fluoride is, therefore, used in toothpastes as fluorinating agent to prevent dental caries, in pharmaceuticals to prevent oral cavities and also added to municipal water and food products to maintain dental health [1]. However, chronic exposure to fluoride is toxic resulting in malignancies and tissue damage. Being toxic and highly corrosive, fluoride alters cellular physiology and metabolism resulting in apoptosis [3]. It causes vomiting, diarrhea [4] and even death when swallowed [5]. Human exposure to excess fluoride can take place, mainly through direct intake of contaminated water and also from food, air, medications and cosmetics. Fluoride contamination and toxicity affects 62 million people in more than 20 Indian states who suffer from dental/enamel fluorosis, skeletal fluorosis and osteosclerosis [6,7].

Numerous studies show that fluoride enhances generation of reactive oxygen and nitrogen species in vital organs which increase the oxidation of proteins and lipids and inhibit antioxidant (AO) enzymes. This leads to induction of oxidative stress condition in cells and tissues [2]. Many of these effects ultimately lead to apoptotic cell death [8].

Antioxidants (AOs) prevent oxidative damage of cellular components, either by giving electrons to free radicals or neutralizing them. 3,4-Dihydroxybenzaldehyde (DHB) is a natural plant polyphenolic AO that exhibits antimicrobial property and is commonly used to treat diarrhea [9]. DHB minimizes hydrogen peroxide (H_2O_2), superoxide and peroxyl radical mediated oxidative damage and apoptosis [10–12]. Since fluoride is thought to exert toxicity by inducing oxidative stress we

* Corresponding author.
E-mail addresses: ruhishaz8@gmail.com (R. Anjum), nikhil.5795@gmail.com (N. Maheshwari), riazmahmood2002@yahoo.co.in (R. Mahmood).
[1] First two authors contributed equally to this work.

https://doi.org/10.1016/j.jtemb.2021.126888
Received 4 July 2021; Received in revised form 7 October 2021; Accepted 28 October 2021
Available online 30 October 2021

examined the possibility that DHB could protect against it due to its AO character. In this study we show that DHB attenuates the cytotoxic effects of NaF using human RBC as the cellular model.

2. Materials and methods

NaF and DHB were purchased from Sigma-Aldrich, USA. All other chemicals were obtained from either Sigma-Aldrich, Sisco Research Laboratory (Mumbai, India) or Himedia Laboratories (Mumbai, India) and were of analytical grade.

2.1. Isolation and treatment of human red blood cells

Blood donors were healthy, non-smoking, 20−30 year old persons; informed and written consent was taken from all donors. Venous blood was taken in heparinized tubes and RBC suspension (10 % hematocrit) was prepared as described previously [13].

The RBC (10 % hematocrit) were divided in four groups: untreated control, DHB alone, NaF alone and DHB + NaF. Untreated control and 2.5 mM DHB alone treated RBC were incubated for 10 h and 0.5 mM NaF alone (9.5 ppm fluoride) for 8 h at 37 °C in a water bath with gentle shaking. In the DHB + NaF group, the RBC were first treated with different concentrations of DHB (0.1, 0.5, 1.0 and 2.5 mM) for 2 h, then 0.5 mM NaF was added followed by incubation for 8 h at 37 °C. After these treatments, the cell suspensions were centrifuged for 10 min at 210×g and supernatants used to determine percent hemolysis. The RBC in pellets were rinsed three times with phosphate buffered saline (PBS), pH 7.4, and lysed with 10 volumes of 5 mM sodium phosphate buffer, pH 7.4. The lysed cells were centrifuged at 630×g for 12 min to pellet any cell debris and supernatants (hemolysates) were saved for further analysis. Hemoglobin (Hb) in hemolysates was quantified by the method of Drabkin and Austin [14] using a reagent kit from Coral Clinical System (Goa, India).

2.2. Oxoferryl hemoglobin formation, heme degradation and free iron release

Oxoferryl hemoglobin formation was measured by recording fluorescence of 20x diluted (with PBS) hemolysates using 460 nm and 525 nm as the excitation and emission wavelengths [15]. Heme degradation was determined from fluorescence of 50x diluted hemolysates using excitation wavelength of 325 nm and following emission at 480 nm [16]. The release of free iron (Fe^{2+}) from heme proteins was measured using ferrozine, which forms a colored complex with ferrous ions that absorbs at 550 nm [17].

2.3. Hemoglobin oxidation and methemoglobin reductase activity

Methemoglobin (MetHb) formation was measured from the absorbance of hemolysates at 540, 576 and 630 nm [18]. MetHb reductase was assayed using 2,6-dichlorophenolindophenol as the electron acceptor and reduced nicotinamide adenine dinucleotide (NADH) as reductant. The change in absorbance at 600 nm was followed and the activity calculated using molar extinction coefficient of $21 \times 10^3\ M^{-1}\ cm^{-1}$ [19].

2.4. Reactive oxygen species (ROS) and reactive nitrogen species (RNS)

ROS generation was assayed by the dichlorodihydrofluorescein diacetate (DCFH-DA) method [20]. The 10 % cell suspension was incubated with l0 μM DCFH-DA for 60 min at 37 °C. The cell suspension was centrifuged, washed three times with PBS, suspended in PBS to again give 10 % hematocrit, and then treated with 0.5 mM NaF for 15 min. In the DHB + NaF sample, the hematocrit was first treated with varying concentrations of DHB for 30 min followed by 0.5 mM NaF for 15 min while DHB alone sample was treated with 2.5 mM DHB for 30 min. The fluorescence of 50x diluted (with PBS) sample was noted at 530 nm using 485 nm as the excitation wavelength. H_2O_2 levels were quantified spectrophotometrically in hemolysates using the FOX reagent with 0.1 M sorbitol added to enhance sensitivity [21].

The RNS determined were nitric oxide (NO) and peroxynitrite ($ONOO^-$). NO was measured in protein free hemolysates using Griess reagent [22]. Peroxynitrite was determined in hemolysates from its reaction with folic acid which yields a highly fluorescent adduct [23].

2.5. Oxidative stress markers

Reduced glutathione (GSH) was quantified using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) in protein free hemolysates. Sulfhydryl group of GSH reduces DTNB to yellow thionitrobenzoate anion (TNB) which absorbs at 412 nm [24]. Malondialdehyde (MDA), a marker of lipid peroxidation, was determined using thiobarbituric acid which reacts with MDA to form a pink adduct whose absorbance was read at 535 nm [25].

Carbonyl groups, marker of protein oxidation, were determined from their reaction with 2,4-dinitrophenylhydrazine to form hydrazone adducts [26]. To determine advanced oxidation protein products (AOPP), 10 μL hemolysate was mixed with 0.8 mL of 0.2 M citric acid and 0.16 mL of distilled water. After 15 min at room temperature, absorbance was read at 340 nm [27]. A calibration curve of chloramine-T was also prepared. Free amino groups were quantified from their reaction with 2, 4,6-trinitrobenzenesulfonate [28]. Total sulfhydryl groups (T—SH) were quantified from their reaction with DTNB and absorbance recorded at 412 nm [29].

2.6. Antioxidant enzymes and antioxidant power

Specific activities of all AO enzymes were determined in hemolysates. Catalase activity was followed from the dissociation of H_2O_2 to water and oxygen at 240 nm [30]. Superoxide dismutase (SOD) activity was monitored from the inhibition of autoxidation of pyrogallol [31]. The activity of glutathione peroxidase (GPx) was measured from the conversion of nicotinamide adenine dinucleotide phosphate reduced (NADPH) to its oxidized form ($NADP^+$) in presence of oxidized glutathione (GSSG) and glutathione reductase (GR) [32]. GR was assayed from the enzymatic cleavage of GSSG to GSH. The resulting increase in absorbance due to conversion of NADPH to $NADP^+$ was noted at 340 nm [33]. Thioredoxin reductase (TR) activity was determined by following the increase in absorbance at 412 nm upon reduction of DTNB to yellow TNB in presence of NADPH [34].

The antioxidant power of hemolysates was determined by cupric reducing antioxidant capacity (CUPRAC), ferric reducing antioxidant power (FRAP) and phosphomolybdenum green (PMG) methods. These assays involve the reduction of Cu^{2+}, Fe^{3+}, Mo^{6+} to their lower oxidation states by sample AOs. CUPRAC is based on the formation of highly colored copper(I)-neocuproine chelate that absorbs at 450 nm [35]. FRAP was determined by the method of Benzie and Strain [36] using 2, 4,6-tris(2-pyridyl)-s-triazine,. PMG assay involves the reduction of Mo^{6+} to Mo^{5+} by AOs in the sample [37]. DPPH and ABTS assays are based on the quenching of colored $DPP^{\bullet}$ and $ABTS^{\bullet+}$ free radicals to their non-radical form by taking electrons from sample AOs. The light yellow DPPH formed was followed from decrease in absorbance of solution at 517 nm [38]. The decolorization of $ABTS^{\bullet+}$ to ABTS was followed at 734 nm and expressed in μmoles Trolox equivalents per mg Hb [39].

2.7. Membrane bound enzymes

Plasma membrane redox system (PMRS) was determined by suspending packed RBC in PBS that contained 5 mM glucose and 1 mM potassium ferricyanide and incubating them for 30 min at 37 °C. PMRS activity converts the extracellular ferricyanide to ferrocyanide [40]; the ferrocyanide concentration was determined using 1,10-phenanthroline

[41]. Ascorbate free radical (AFR) reductase was assayed from the formation of AFR upon incubation with ascorbate and ascorbate oxidase [42].

Total ATPase and Na^+K^+-ATPase were measured from the inorganic phosphate liberated upon ATP hydrolysis in absence and presence of 1 mM ouabain. The inorganic phosphate reacts with Taussky-Shorr reagent [50 g ferrous sulfate and 100 mL ammonium molybdate (10 % in 10 M sulfuric acid] to give blue complex which absorbs at 660 nm [43]. Acetylcholinesterase (AChE) was assayed in hemolysates using S-thiocholine iodide and DTNB as substrates [44].

2.8. Metabolic enzymes

Hexokinase (HK) activity was measured from the lowering of free glucose levels when the enzyme was present [45]. Pyruvate kinase (PK) was assayed by the method of Bergmeyer [46]. Lactate dehydrogenase (LDH) activity was followed from the oxidation of NADH to NAD^+, in the presence of sodium pyruvate, at 340 nm [47]. Glucose 6-phosphate dehydrogenase (G6PD) was determined by monitoring the reduction of $NADP^+$ to NADPH at 340 nm, in presence of glucose 6-phosphate [48]. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity was determined by a coupled enzymatic assay. The change in absorbance due to oxidation of NADH to NAD^+ was recorded at 340 nm [49].

2.9. Statistical analysis

All experiments were done on six blood samples obtained from six different donors. To calculate statistical significance one way ANOVA was employed using the program Origin Pro 8.0 (USA). Results are given as the mean ± standard error. Results were measured by the Bonferroni and Tukey post-hoc test and were considered significant when probability value (P) was < 0.05.

3. Results

The mitigating effect of DHB on NaF-induced oxidative damage in isolated RBC was studied. RBC were incubated with NaF in absence and presence of DHB; control (untreated) and DHB alone treated cells were also used. RBC were then freed of excess reagents, lysed with hypotonic buffer and hemolysates were prepared. These cell lysates, and also whole cells, were assayed for various parameters.

3.1. Oxoferryl hemoglobin formation, heme degradation and free iron release

Incubation of RBC with 0.5 mM NaF alone caused 2 % hemolysis which, though low, was 2.16 fold higher when compared to the control value. However, prior treatment with increasing concentrations of DHB protected RBC from NaF-induced cytotoxic damage (Fig. 1A). Oxoferryl Hb content was increased to 4.7 fold in NaF alone treated RBC (Fig. 1B). A 3.3 fold increase in heme degradation (Fig. 1C) and 2.2 fold enhanced free iron level (Fig.1D) was seen in NaF alone treated RBC. The presence of DHB prevented this damage and at 2.5 mM DHB the changes were insignificantly different from control values.

3.2. Hemoglobin oxidation and methemoglobin reductase activity

Incubation of RBC with NaF alone significantly enhanced MetHb content by 1.6 fold compared to the control value. However, in presence of DHB the MetHb level was restored and in 2.5 mM DHB + 0.5 mM NaF group it was almost the same as in control RBC (Fig. 2). Surprisingly, the MetHb level in the DHB alone sample was even lower than the control value (53 % of control). NaF treatment inhibited the MetHb reductase activity to 58 %, but in presence of DHB it was completely restored to the control value (Fig. 2).

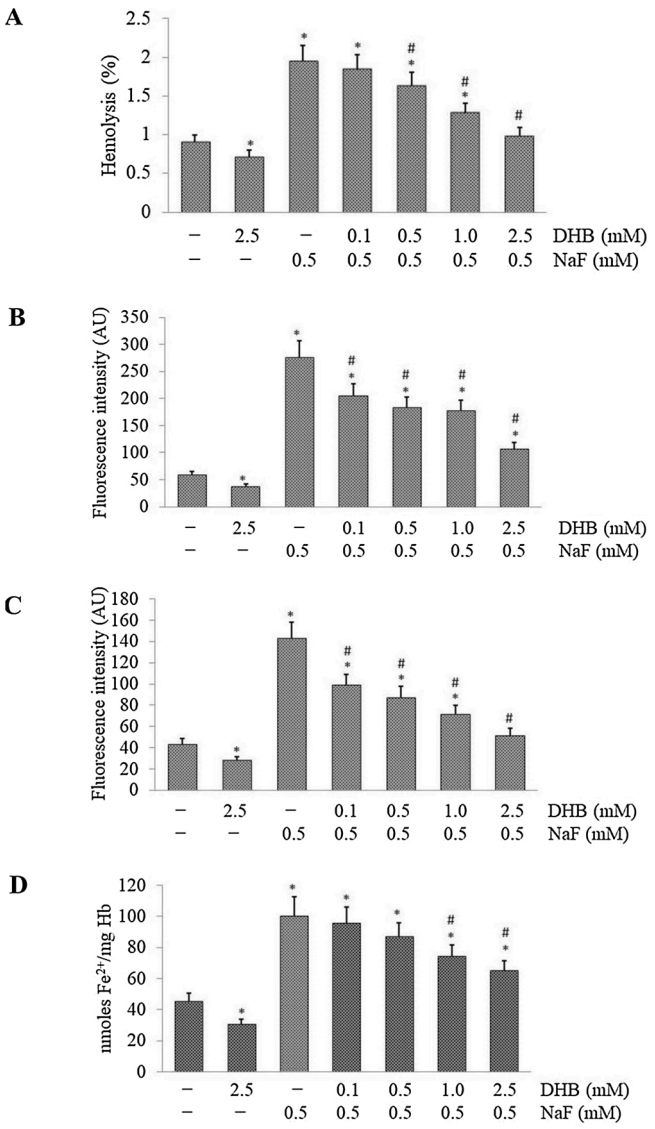

Fig. 1. Protective effect of DHB on NaF-induced (A) hemolysis of RBC (B) formation of oxoferryl hemoglobin (C) heme degradation and (D) release of free iron from hemoglobin.
Oxoferryl hemoglobin and heme degradation are reported in fluorescence arbitrary unit (AU) and free iron release is in nmoles/mg Hb.
Results are mean ± standard error of six different samples by one way ANOVA. *Significantly different from control and # significantly different from NaF alone sample at $P < 0.05$

3.3. Reactive oxygen species and reactive nitrogen species

In the DCFH-DA assay, the fluorescence intensity in NaF alone samples was significantly increased and was 50 % above control. This shows increased ROS generation in NaF treated RBC. The level of H_2O_2, a non-radical ROS, was also higher in NaF alone treated RBC being 1.7 fold the control value (Table 1). Peroxynitrite ($ONOO^-$) formation was enhanced and was 1.9 fold in NaF alone treated cells, compared to control (Fig. 3). The NO level was also increased in NaF alone treated RBC and was 1.7 times the control value. In presence of DHB all these changes were mitigated in a DHB-concentration dependent manner.

3.4. Oxidative stress markers

A significant reduction of 54 % in GSH content was seen upon treatment of RBC with 0.5 mM NaF. Free SH groups and amino groups

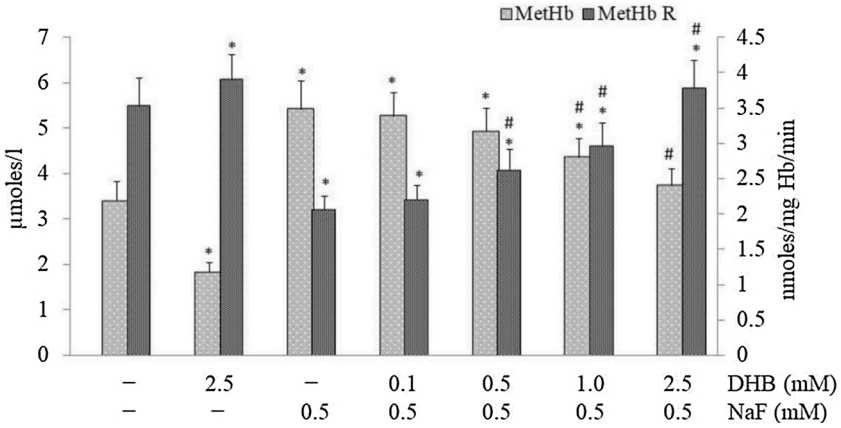

Fig. 2. Ameliorating effect of DHB on NaF-induced methemoglobin formation and methemoglobin reductase activity. Methemoglobin level is in µmoles/l and methemoglobin reductase activity in nmoles/mg Hb/min. Both were determined in hemolysates.
Results are mean ± standard error of six different samples by one way ANOVA.
*Significantly different from control and # significantly different from NaF alone sample at $P < 0.05$

Table 1
Effect of DHB on sodium fluoride-induced oxidation of proteins and lipids.

	GSH	Total-SH	PC	Amino groups	AOPP	MDA	NO	H_2O_2
Control	6.03 ± 0.68	0.65 ± 0.074	42.25 ± 4.36	228.5 ± 28.4	182.5 ± 21.2	3.04 ± 0.287	18.64 ± 1.08	11.86 ± 1.45
DHB 2.5	6.74 ± 0.71	0.69 ± 0.076	33.60 ± 3.51*	273.5 ± 27.6	136.5 ± 13.8*	2.07 ± 0.254*	18.08 ± 1.96	10.56 ± 1.12
NaF	2.83 ± 0.31*	0.30 ± 0.033*	172.5 ± 15.7*	104.1 ± 10.7*	350.5 ± 41.1*	5.58 ± 0.593*	35.33 ± 4.12*	20.05 ± 2.67
DHB 0.1+ NaF	3.23 ± 0.33*	0.33 ± 0.037*	160.1 ± 18.6*#	121.5 ± 12.6*#	309.5 ± 31.2*#	4.87 ± 0.536*	33.95 ± 3.61	16.97 ± 1.83*
DHB 0.5+ NaF	3.93 ± 0.44*#	0.39 ± 0.051*#	134.5 ± 12.5*#	139.2 ± 15.6*#	295.5 ± 27.0*#	4.19 ± 0.442*#	32.15 ± 2.96*	15.13 ± 1.74*#
DHB 1.0+ NaF	4.66 ± 0.52*#	0.48 ± 0.055*#	103.5 ± 10.8*#	159.5 ± 13.7*#	266.1 ± 23.3*#	3.72 ± 0.315*#	29.19 ± 2.72*#	14.07 ± 1.64*#
DHB 2.5+ NaF	5.62 ± 0.63#	0.58 ± 0.063#	71.01 ± 8.32*#	201.2 ± 20.6*#	216.1 ± 20.6*#	3.28 ± 0.340#	25.83 ± 2.69*#	12.64 ± 1.35#

All parameters were determined in hemolysates. DHB concentration is in millimoles/l while NaF concentration is 0.5 mM in all groups. GSH, PC, amino groups, AOPP, MDA, NO and H_2O_2 are reported in nmoles/mg Hb while total-SH in µmoles/mgHb.
Results are mean ± standard error of six different samples by one way ANOVA.
*Significantly different from control and # significantly different from NaF alone sample at $P < 0.05$.
DHB, 3,4-dihydroxybenzaldehyde; NaF, sodium fluoride; GSH, reduced glutathione; -SH, sulfhydryl groups; PC, protein carbonyls; AOPP, advanced oxidation protein products; MDA, malondialdehyde; NO, nitric oxide.

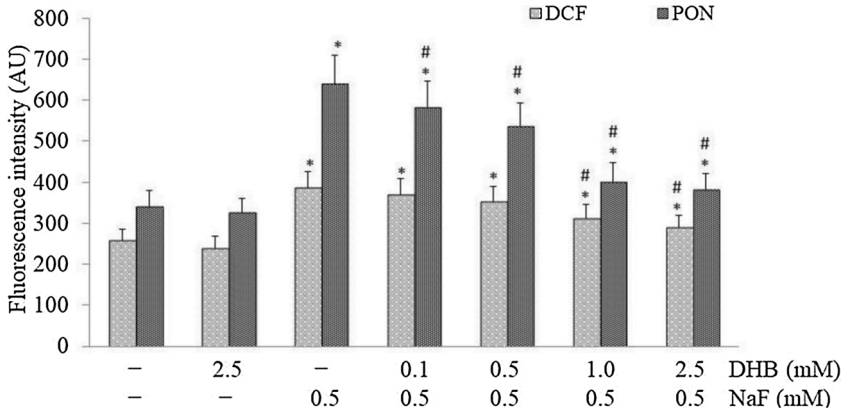

Fig. 3. Effect of DHB on NaF-induced ROS and RNS generation in human RBC. Both DCF and peroxynitrite are reported in fluorescence arbitrary unit (AU).
Results are mean ± standard error of six different samples by one way ANOVA.
*Significantly different from control and # significantly different from NaF alone sample at $P < 0.05$

were lowered by 55 % in NaF alone treated RBC. This decrease was attenuated by DHB in a concentrated-dependent fashion (Table 1). Protein oxidation increases in an environment of oxidative stress. Treatment of RBC with 0.5 mM NaF dramatically enhanced protein oxidation to 4 fold of control cells. This was significantly reduced to 1.7 fold when DHB was also present in the samples (Table 1).

Incubation of RBC with 0.5 mM NaF enhanced AOPP level by 90 % while lipid peroxidation was increased to 1.84 fold of control value (Table 1) However, addition of DHB, diminished AOPP levels and lipid peroxidation in a concentration-dependent manner. In all the above parameters, maximum protection was seen in presence of 2.5 mM DHB (Table 1).

3.5. Antioxidant enzymes and antioxidant power

Incubation of RBC with NaF only led to inhibition of the major AO enzymes catalase and SOD, and their activities were only 57 % and 15 % of control cells, respectively. Similarly, GPx, GR and TR were also inhibited by more than 50 %. All enzyme activities, except SOD, were almost completely restored and were insignificantly different from control values in presence of 2.5 mM DHB (Table 2).

The AO power assays (CUPRAC, PMG, FRAP and K_3FeCN_6 reduction)

Table 2
Effect of DHB on sodium fluoride-induced alterations in the activity of antioxidant enzymes.

	CAT	SOD	GPx	GR	TR
Control	7.08 ± 0.812	1.95 ± 0.206	55.71 ± 5.31	51.62 ± 6.63	74.70 ± 8.78
DHB 2.5	7.10 ± 0.725	1.99 ± 0.187	57.96 ± 6.10	49.04 ± 4.36	80.96 ± 8.67
NaF	4.04 ± 0.516*	0.346 ± 0.038*	17.99 ± 1.67*	19.74 ± 2.18*	22.74 ± 2.24*
DHB 0.1+ NaF	4.54 ± 0.481*	0.399 ± 0.042*	24.78 ± 2.46*#	29.22 ± 3.22*#	38.75 ± 4.18*#
DHB 0.5+ NaF	5.05 ± 0.612*#	0.482 ± 0.051*#	28.04 ± 2.62*#	33.70 ± 4.02*#	50.53 ± 5.61*#
DHB 1.0+ NaF	5.72 ± 0.527*#	0.854 ± 0.076*#	38.83 ± 4.32*#	38.46 ± 3.86*#	55.40 ± 6.07*#
DHB 2.5+ NaF	6.31 ± 0.634#	1.255 ± 0.134 *#	51.22 ± 4.85*#	47.22 ± 3.97*#	66.39 ± 6.77*#

All enzymes were assayed in hemolysates. DHB concentration is in millimoles/l while NaF concentration is 0.5 mM in all groups. Catalase activity is in μmoles/min/mg Hb and SOD in U/mg Hb while GPx GR and TR are in nmoles/min/mg Hb.
Results are mean ± standard error of six different samples by one way ANOVA. *Significantly different from control and # significantly different from NaF alone sample at $P < 0.05$.
DHB, 3,4-dihydroxybenzaldehyde; NaF, sodium fluoride; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase; TR, thioredoxin reductase.

showed that treatment of RBC with NaF reduced the metal (Cu^{2+}, Mo^{6+} and Fe^{3+}) reducing ability which were 30 %, 50.6 %, 43 % and 53 % of control values. The $DPP^{\bullet}$ and $ABTS^{\bullet+}$ free radical quenching ability was also lowered to 69 % and 51 % in NaF alone treated RBC while in control it was 83 % (Table 3). However, prior treatment with DHB restored AO potential of RBC in a concentration-dependent manner.

3.6. Membrane bound enzymes

Total and Na^+K^+-ATPase activities were decreased to 46 % and 44 % respectively, in NaF alone treated cells, when compared to untreated control. Incubation of RBC with NaF alone decreased the AChE activity to 41 % of control cells (Table 4). However, this decrease in enzyme activities was mitigated by prior incubation of RBC with varying concentrations of DHB (Table 4).

Incubation of RBC with NaF lowered the PMRS to 65 %, while in presence of DHB it was restored to 92 % in a concentration-dependent manner. RBC incubated with 0.05 mM NaF alone have tremendously lowered AFR-reductase activity while presence of DHB enhanced its activity from 20 % to 84 %, when compared to untreated control cells (Table 4).

Table 3
Effect of DHB on sodium fluoride induced changes in antioxidant power of RBC.

	FRAP	PMG	CUPRAC	DPPH	ABTS
Control	86.65 ± 9.21	486.07 ± 52.12	5.11 ± 0.614	81.5 ± 9.21	69.86 ± 8.56
DHB 2.5	93.25 ± 10.11*	502.66 ± 51.83*	5.04 ± 0.522*	85.0 ± 10.02	75.28 ± 8.07
NaF	37.60 ± 4.05*	246.18 ± 26.18*	1.02 ± 0.126*	56.25 ± 5.88*	35.87 ± 4.14*
DHB 0.1+ NaF	43.45 ± 4.86*#	256.99 ± 27.21*	1.39 ± 0.151*	58.25 ± 6.07	59.40 ± 6.28*#
DHB 0.5+ NaF	52.35 ± 5.65*#	281.44 ± 29.0*#	1.92 ± 0.204*#	61.5 ± 6.23*	45.65 ± 5.53*#
DHB 1.0+ NaF	65.05 ± 6.07*#	352.58 ± 33.33*#	2.72 ± 0.302*#	68.25 ± 7.11*#	51.22 ± 6.92*#
DHB 2.5+ NaF	80.10 ± 8.48*#	404.82 ± 42.65*#	4.34 ± 0.458#	75.25 ± 8.06*#	56.86 ± 6.57*#

All parameters were assayed in hemolysates. DHB concentration is in millimoles/l while NaF concentration is 0.5 mM in all groups. FRAP and PMG are in nmoles/mg Hb and CUPRAC in μmoles/mg Hb. DPPH is reported in % quenching of $DPP^{\bullet}$ radical and $ABTS^{\bullet+}$ in μmoles Trolox equivalent/mg Hb.
Results are mean ± standard error of six different samples by one way ANOVA. *Significantly different from control and # significantly different from NaF alone sample at $P < 0.05$.
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.

3.7. Metabolic enzymes

The activity of HK, the first enzyme of glycolysis, was inhibited to 34 % upon treatment of RBC with NaF. PK catalyzes the last reaction of glycolysis and it was inhibited by NaF to 41 % of control value (Table 5). G6PD and GAPDH activities declined to 46 % and 50 % of control value, respectively. There was a three-fold increase in LDH activity. All alterations in enzyme ativities were normalized in presence of DHB (Table 5).

4. Discussion

Endemic fluorosis has become a major health risk in several parts of the world, including India. Toxicants like fluoride that enter the body via any route primarily target RBC and blood. It has been shown in earlier reports that NaF oxidatively damages human RBC [13,50]. Since cytotoxicity of fluoride in living systems is associated with the generation of free radicals and ROS, the protective effect of DHB, a plant AO, on NaF-induced damage in human RBC was examined in this study.

Hemoglobin represents a major and important target of toxicants and ROS in these cells. The ferrous iron of Hb undergoes slow but spontaneous oxidation to ferric ion yielding MetHb which is inactive as oxygen transporter. MetHb formation is accompanied by generation of superoxide radicals and is a marker of oxidative stress in RBC [51,52]. The MetHb level was increased upon NaF treatment, probably due to ROS-induced oxidation of ferrous ion of Hb to ferric form. This will

Table 4
Effect of DHB on sodium fluoride-induced alterations in activity of membrane bound enzymes.

	Na^+K^+ATPase	Total ATPase	AFRR	PMRS	AChE
Control	189.0 ± 7.02	329.39 ± 41.23	0.963 ± 0.104	1.65 ± 0.174	78.29 ± 9.21
DHB 2.5	265.5 ± 7.12*	345.43 ± 37.20	1.053 ± 0.112	1.93 ± 0.212	32.36 ± 4.27*
NaF	65.0 ± 2.95*	152.72 ± 16.36*	0.196 ± 0.017*	1.08 ± 0.097*	80.56 ± 9.44
DHB 0.1 + NaF	82.5 ± 3.91*#	161.56 ± 15.94*	0.261 ± 0.029*	1.16 ± 0.101*	39.79 ± 4.61*#
DHB 0.5 + NaF	105.0 ± 5.21*#	179.69 ± 18.22*#	0.364 ± 0.046*#	1.23 ± 0.146*	49.06 ± 5.76*#
DHB 1.0 + NaF	135.1 ± 6.11*#	213.72 ± 21.06*#	0.587 ± 0.057*#	1.34 ± 0.157*#	59.31 ± 6.84*#
DHB 2.5 + NaF	173.0 ± 6.55*#	288.65 ± 27.63*#	0.815 ± 0.102*#	1.53 ± 0.162*#	68.28 ± 7.34*#

All parameters were determined in hemolysates. DHB concentration is in millimoles/l while NaF concentration is 0.5 mM in all groups. Na^+ K^+-ATPase and total ATPase are in nmoles Pi/h /mg Hb. The PMRS values are in μmoles ferrocyanide/30 min/mL pRBC while AFR reductase (AFRR) activity is in μmoles NADH oxidized/min/mL pRBC. AChE is reported in nmoles/min/mg Hb.
Results are mean ± standard error of six different samples by one way ANOVA. *Significantly different from control and # significantly different from NaF alone sample at $P < 0.05$.
DHB, 3,4-dihydroxybenzaldehyde; NaF, sodium fluoride; pRBC, packed red blood cells; PMRS, plasma membrane redox system; AFR, ascorbate free radical; AChE, acetylcholinesterase.

Table 5
Effect of DHB on sodium fluoride-induced changes in the activity of some enzymes of glucose metabolism.

	HK	LDH	G6PD	PK	GAPDH
Control	193.40 ± 20.49	1.437 ± 0.192	124.93 ± 12.91	36.97 ± 4.33	12.94 ± 1.47
DHB 2.5	204.09 ± 22.94	1.258 ± 0.156	121.40 ± 12.02	35.35 ± 4.07	12.09 ± 1.32
NaF	67.242 ± 6.41*	4.346 ± 0.514*	58.025 ± 5.81*	15.26 ± 1.97*	6.45 ± 7.44*
DHB 0.1+ NaF	76.323 ± 7.19*#	3.345 ± 0.381*#	62.285 ± 7.12*	16.88 ± 1.88*	6.82 ± 7.17*
DHB 0.5+ NaF	87.323 ± 9.12*#	2.676 ± 0.305*#	67.255 ± 8.04*#	20.19 ± 2.25*#	7.57 ± 8.16*
DHB 1.0+ NaF	122.28 ± 14.22*#	1.927 ± 0.217*#	97.701 ± 10.11*#	25.12 ± 2.86*#	8.46 ± 9.05*#
DHB 2.5+ NaF	165.85 ± 13.23*#	1.640 ± 0.186*#	109.16 ± 12.32*#	32.54 ± 3.64*#	10.72 ± 11.47*#

Enzymes were assayed in hemolysates. DHB concentration is in millimoles/l while NaF concentration is 0.5 mM in all groups. HK, G6PD, PK and GAPDH activities are in nmoles/min/mg Hb while LDH in μmoles/min/mg Hb.
Results are mean ± standard error of six different samples by one way ANOVA. *Significantly different from control and # significantly different from NaF alone sample at $P < 0.05$.
DHB, 3,4-dihydroxybenzaldehyde; NaF, sodium fluoride; HK, hexokinase; LDH, lactate dehydrogenase; G6PD, glucose 6-phosphate dehydrogenase; PK, pyruvate kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

reduce the oxygen carrying capacity of RBC. DHB normalized the MetHb formation in a concentration-dependent manner in RBC. Cells normally have low MetHb levels due to presence of MetHb reductase, an enzyme which converts MetHb to Hb using mainly NADH as the reductant. MetHb reductase activity was decreased which must have been responsible for elevated MetHb levels seen in NaF alone treated samples. Pre-treatment with DHB restored the MetHb reductase activity which will maintain low MetHb level in RBC.

H_2O_2 initiates a pseudo-peroxidase reaction with Fe^{2+}-Hb to form oxoferryl Hb. This ferryl species autoreduces to ferric ion and in presence of excessive H_2O_2, the ferryl iron (Fe^{4+}) is regenerated [53]. H_2O_2 also reacts with Hb to initiate a cascade resulting in heme degradation. This can increase membrane damage and shorten red cell life span. Simultaneous treatment with increasing concentrations of DHB protected hemeproteins from NaF-induced damage. Heme degradation also results in leaching of iron from the porphyrin ring of heme that accumulates in membranes and appears to be responsible for membrane damage [16]. Treatment of RBC with DHB, prior to incubation with NaF, lowered both heme degradation and free iron release. As also seen with MetHb, the DHB alone samples had significantly lower heme degradation products and free iron level than the control cells.

ROS generation was monitored using DCFH-DA which enters the cell and is hydrolysed by intracellular esterases to DCFH and diacetate. DCFH is then oxidized by intracellular ROS to the highly fluorescent DCF; enhanced fluorescence, therefore, indicates higher formation of ROS. Superoxide radical, generated during MetHb formation, is easily converted to H_2O_2 whose level was increased in NaF-treated RBC. The H_2O_2 can react with the redox active free iron released on heme degradation to give the extremely reactive and damaging hydroxyl radical. The enhanced generation of ROS and H_2O_2 in presence of NaF alone was greatly reduced in DHB + NaF samples and was insignificantly different from control values. One reason could be that DHB enhanced the activity of SOD, which scavenges superoxide radicals which will also lower MetHb formation. Two RNS, peroxynitrite and NO, were also determined and were increased in NaF treated cells. Excessive NO reacts spontaneously with superoxide radical to produce peroxynitrite [54] whose level was also increased. Both NO and peroxynitrite levels showed dramatic reduction and were insignificantly different from control when DHB was also present along with NaF. DHB exerts its protective effect through various modes either by direct scavenging of ROS or the involvement of vicinal hydroxyl groups in its AO activity like several polyphenolics as catechin, quercetin, etc [55]. These results show that DHB eliminates NaF-induced ROS and RNS generation in RBC which will lower cellular damage.

Several enzymatic and non-enzymatic markers of oxidative stress were analyzed. GSH is a major non-enzymatic AO that maintains the reducing environment of cells by protecting them against ROS and free radicals. NaF-induced ROS effectively modified thiol (-SH) groups consequently depleting total SH content and also GSH [8]. Pre-treatment of RBC with DHB restores the thiol status of cells. Induction of oxidative stress leads to an increase in oxidation of proteins and unsaturated lipids. Malondialdehyde production was significantly increased upon treatment of RBC with 0.5 mM NaF alone [8]. ROS oxidize side chains amino groups of lysine and arginine residues to carbonyl groups and also generate various advanced oxidation protein products (AOPP) including dityrosine and cross-linked proteins. The generation of AOPP increases under oxidative stress conditions [56]. Thus NaF-induced ROS resulted in oxidative modification of cellular thiols, proteins and lipids. In the DHB + NaF samples, these oxidative modifications were greatly decreased, in a DHB concentration- dependent manner. These findings strongly suggest that the production of ROS, induction of oxidative stress and consequent oxidative modification of cellular components was increased by NaF, but was significantly overcome in presence of DHB.

Treatment of RBC with NaF inhibited major AO enzymes but this was attenuated in presence of DHB. The AO power of cells was determined next using several assays. These are based on metal reduction and free radical quenching by AOs in sample. NaF lowered AO power of RBC which must be due to inhibition of AO enzymes and lower GSH content [8]. However, pre-incubation of RBC with DHB restored the AO power in a DHB concentration-dependent manner. In DHB alone treated RBC the reduction of metal ions was more than that of control cells (Table 3). This is because DHB being an AO can donate electron(s) and thus increase the metal reducing power of cells. The DHB induced restoration in AO power must be due to improvement in the activities of endogenous AO enzymes and also to higher levels of GSH.

NaF damages the RBC, increases membrane fragility resulting in cell lysis, and consequent release of Hb in the solution. Hemolysis of RBC by NaF suggested plasma membrane damage. This was supported by lower activity of bound enzymes, total and Na^+K^+-ATPase [57]. Similarly, the activity of acetylcholinesterase, another membrane bound enzyme, was lowered by NaF but the inhibition was overcome in presence of DHB. PMRS is found in all cell types and maintains redox signaling by generating more NAD^+ for ATP production by glycolysis. PMRS transfers electrons from intracellular reducing equivalents to extracellular acceptors across the plasma membrane. PMRS was lowered by NaF which will disrupt membrane homeostasis, increase ROS generation and lead to cell death. Prior treatment with DHB up-regulates the PMRS activity, probably by recycling oxidized AOs to their reduced active form thus increasing the trans-membrane electron transport. AFR reductase recycles AFR to ascorbate by its oxidoreductase activity [42]. NaF-induced inhibition of AFR reductase was restored by DHB. These enzymatic and hemolysis results strongly suggest that DHB mitigates NaF-induced membrane damage in RBC.

The activities of key enzymes of glucose metabolic pathways were determined. Inhibition of HK and PK will deprive the cells of energy by suppressing glycolysis. In PK deficient cells, the Ca^{2+} concentration increases due to loss of K^+ and water which renders the membrane more rigid and affects normal function, leading to hemolytic anemia [58]. GAPDH inhibition can be due to ROS mediated oxidation of sulfhydryl in its active site. This will slow down or inhibit glycolysis and deprive the RBC of ATP that may lead to cell death. Lower ATP production in cells

also affects several metabolic and transport processes. ATP depleted RBC are also more prone to oxidative damage [59]. A 3 fold increase in activity of lactate dehydrogenase, an enzyme of anaerobic glycolysis was seen. This could be an adaptive response to allow regeneration of NAD^+, needed for glycolysis to continue, or release of the enzyme form the inner side of plasma membrane due to membrane damage. The activity of cytosolic G6PD, the first enzyme of HMP shunt, was decreased. Inhibition of G6PD will lower the production of NADPH, a major cellular reductant utilized by some AO enzymes (GR and TR) to combat ROS. In presence of DHB all these enzyme activities were restored to control values which will increase cellular ATP and NADPH levels.

Since NaF enhanced the generation of ROS and RNS which was decreased by DHB, the protective effect of DHB must have involved its AO property. There are several ways by which DHB could have alleviated NaF-induced oxidative damage. First, it can directly react with and quench free radicals and ROS, thereby sparing the cell components from oxidative modification. Second, it can chelate free iron and prevent it from reacting with H_2O_2, eliminating production of hydroxyl radical. Third, it can act indirectly by increasing the activities of AO enzymes and level of GSH, the major non-enzymatic AO in RBC. However, the exact biochemical mode by which DHB protects RBC from fluoride cytotoxicity needs to be investigated further.

A schematic representation of the results of this study is given in Fig. 4.

5. Conclusions

NaF increases the production of ROS and RNS in RBC which target various cell components resulting in oxidative damage. DHB significantly and dose dependently mitigates these NaF-induced oxidative modifications. Since NaF enhanced ROS and RNS, the protective effect of DHB must be due to its AO character. These results show that DHB can be possibly used as a chemoprotectant against fluoride induced toxicity.

Compliance with ethical standards

The experiments were conducted according to the guidelines approved by Institutional Ethics Committee (IEC) of Aligarh Muslim University with Registration Number- 714/GO/Re/S/02/CPCSEA.

Author contributions

RM designed and supervised the study. RA and NM performed the experiments and analyzed the data. NM and RM wrote the manuscript. RM, RA and NM read an approved the final manuscript.

Funding information

Financial support to the Department of Biochemistry from the UGC-SAP-DRS III, DST-FIST-II, and DBT-PURSE schemes is gratefully

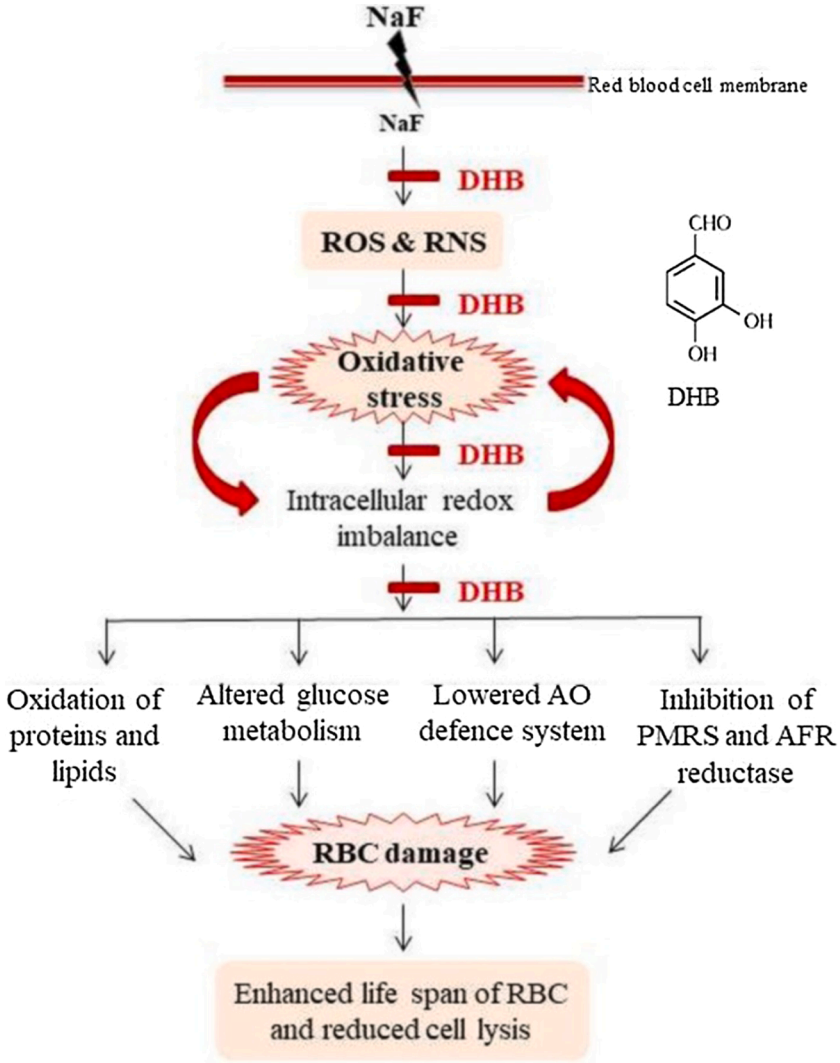

Fig. 4. Schematic depiction of DHB-mediated attenuation of NaF-induced toxicity in human RBC. NaF enters the cells where it enhanced the generation of free radicals, ROS and RNS. These oxidative species compromise the enzymatic and non-enzymatic AO defenses of RBC thereby lowering their AO power. This impairs the ROS/RNS quenching ability of the RBC resulting in oxidative modification of cellular components. Since damaged RBC are quickly removed from circulation it will lower their lifespan (red cell senescence). DHB, a plant polyphenolic AO, can mitigate NaF-induced toxicity at several levels. It can lower the intracellular production of ROS and RNS or can directly quench the reactive species. DHB treatment reduces oxidative damage in RBC and can, therefore, enhance their lifespan.
AO, antioxidant; AFRR, ascorbate free radical reductase; DHB, 3,4-dihydroxybenzaldehyde; NaF, sodium fluoride; PMRS, plasma membrane redox system ROS, reactive oxygen species; RNS, reactive nitrogen species. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

acknowledged.

Availability of data and material

Data and material related to this study are available in the form of figures and tables in 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.

Acknowledgements

NM is thankful to Council of Scientific and Industrial Research, New Delhi, for the award of Senior Research Fellowship (File No. 09/112 (0624)2K19-EMR-I).

References

[1] C. Tascioglu, K. Umemura, S.S. Kusuma, T. Yoshimura, Potential utilization of sodium fluoride (NaF) as a biocide in particleboard production, J. Korean Wood Sci. Technol. 63 (2017) 652–657.
[2] H. Zuo, L. Chen, M. Kong, et al., Toxic effects of fluoride on organisms, Life Sci. 198 (2018) 18–24.
[3] J. Zhang, Z. Li, M. Qie, R. Zheng, J. Shetty, J. Wang, Sodium fluoride and sulfur dioxide affected male reproduction by disturbing blood-testis barrier in mice, Food Chem. Toxicol. 94 (2016) 103–111.
[4] R. Ullah, M.S. Zafar, N. Shahani, Potential fluoride toxicity from oral medicaments: a review, Iran. J. Basic Med. Sci. 20 (8) (2017) 841–848.
[5] WHO, Preventing Disease Through Healthy Environments: Inadequate or Excess Fluoride: a Major Public Health Concern, World Health Organization, 2019, pp. 1–8.
[6] N. Arlappa, A.I. Qureshi, R. Srinivas, Fluorosis in India: an overview, Int. J. Res. Dev. Health. 1 (2) (2013) 97–102.
[7] I. Mukherjee, U.K. Singh, P.K. Patra, Exploring a multi-exposure-pathway approach to assess human health risk associated with groundwater fluoride exposure in the semi-arid region of east India, Chemosphere 233 (2019) 164–173.
[8] G.H.N. Miranda, B.A.Q. Gomes, L.O. Bittencourt, et al., Chronic exposure to sodium fluoride triggers oxidative biochemistry misbalance in mice: effects on peripheral blood circulation, Oxid. Med. Cell. Longev. (2018) 8379123, https://doi.org/10.1155/2018/8379123.
[9] N. Maheshwari, F.H. Khan, R. Mahmood, 3,4-Dihydroxybenzaldehyde lowers ROS generation and protects human red blood cells from arsenic(III) induced oxidative damage, Environ. Toxicol. (2018), https://doi.org/10.1002/tox.22572.
[10] I.K. Lee, J.Y. Jung, S.J. Seok, W.G. Kim, B.S. Yun, Free radical scavengers from the medicinal mushroom Inonotusxeranticus and their proposed biogenesis, Bioorg. Med. Chem. Lett. 16 (21) (2006) 5621–5624.
[11] H. Etoh, K. Murakami, T. Yogoh, et al., Anti-oxidative compounds in barley tea, Biosci. Biotechnol. Biochem. 68 (12) (2004) 2616–2618.
[12] N. Maheshwari, R. Mahmood, 3,4-Dihydroxybenzaldehyde attenuates pentachlorophenol-induced cytotoxicity, DNA damage and collapse of mitochondrial membrane potential in isolated human blood cells, Drug Chem. Toxicol. (2020) 1–18, https://doi.org/10.1080/01480545.2020.1811722.
[13] N. Maheshwari, N. Qasim, R. Anjum, R. Mahmood, 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, Ecotoxicol. Environ. Saf. 208 (2021) 111611, https://doi.org/10.1016/j.ecoenv.2020.111611.
[14] D.L. Drabkin, J.H. Austin, Spectrophotometric studies II. Preparation from washed blood cells; nitric oxide and sulf-hemoglobin, J. Boil. Chem. 112 (1935) 51–66.
[15] E. Nagababu, J.M. Rifkind, Formation of fluorescent heme degradation products during the oxidation of hemoglobin by hydrogen peroxide, Biochem. Biophys. Res. Commun. 47 (3) (1998) 592–596.
[16] E. Nagababu, M.E. Fabry, R.L. Nagel, J.M. Rifkind, Heme degradation and oxidative stress in murine models for hemoglobinopathies: thalassemia, sickle cell disease and hemoglobin C disease, Blood Cells Mol. Dis. 41 (1) (2008) 60–66.
[17] S.S. Panter, Release of iron from hemoglobin, Meth. Enzymol. 231 (1994) 502–514.
[18] R.E. Benesch, R. Benesch, S. Yung, Equations for the spectrophotometric analysis of hemoglobin mixtures, Anal. Biochem. 55 (1973) 245–248.
[19] F. Kuma, S. Ishizawa, K. Hirayama, H. Nakajima, Studies on methemoglobin reductase. I. Comparative studies of diaphorases from normal and methemoglobinemic erythrocytes, J. Biol. Chem. 247 (2) (1972) 550–555.
[20] A. Keller, A. Mohamed, S. Drose, U. Brandt, et al., Analysis of dichlorodihydrofluorescein and dihydrocalcein as probes for the detection of intracellular reactive oxygen species, Free Radic. Res. 38 (2004) 1257–1267.
[21] C. Gay, J.M. Gebicki, A critical evaluation of the effect of sorbitol on the ferric-xylenol orange hydroperoxide assay, Anal. Biochem. 284 (2000) 217–220.
[22] K.M. Miranda, M.G. Espey, D.A. Wink, A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite, Nitric Oxide 5 (2001) 62–71.
[23] J.C. Huang, D.J. Li, J.C. Diao, et al., A novel fluorescent method for determination of peroxynitrite using folic acid as a probe, Talanta. 72 (4) (2007) 1283–1287.
[24] E. Beutler, Red Cell Metabolism: a Manual of Biochemical Methods, 3rd edition, Grune and Stratton, New York, USA, 1984.
[25] J.A. Buege, S.D. Aust, Microsomal lipid peroxidation, Meth. Enzymol. 52 (1978) 302–310.
[26] R.L. Levine, D. Garland, C.N. Oliver, et al., Determination of carbonyl content in oxidatively modified proteins, Meth. Enzymol. 186 (1990) 464–478.
[27] M. Hanasand, R. Omdal, K.B. Norheim, L.G. Goransson, et al., Improved detection of advanced oxidation protein products in plasma, Clin. Chim. Acta 413 (2012) 901–906.
[28] S.L. Snyder, P.Z. Sobocinski, An improved 2,4,6-trinitrobenzenesulfonic acid method for the determination of amines, Anal. Biochem. 64 (1975) 284–288.
[29] J. Sedlak, R.H. Lindsay, Estimation of total, protein bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent, Anal. Biochem. 25 (1968) 192–205.
[30] H. Aebi, Catalase in vitro, Meth. Enzymol. 105 (1984) 121–126.
[31] S. Marklund, G. Marklund, Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase, Eur. J. Biochem. 47 (1974) 469–474.
[32] L. Flohe, W.A. Gunzler, Assays of glutathione peroxidase, Meth. Enzymol. 105 (1984) 114–121.
[33] I. Carlberg, B. Mannervik, Glutathione reductase, Meth. Enzymol. 113 (1985) 484–490.
[34] T. Tamura, T.C. Stadtman, A new selenoprotein from human lung adenocarcinoma cells: purification, properties, and thioredoxin reductase activity, Proc. Natl. Acad. Sci. 93 (1996) 1006–1011.
[35] S.D. Cekic, N. Kara, E. Tutem, R. Apak, Protein-incorporated serum total antioxidant capacity measurement by a modified CUPRAC (cupric reducing antioxidant capacity) method, Anal. Lett. 45 (2012) 754–763.
[36] I.F.F. Benzie, J.J. Strain, The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay, Anal. Biochem. 239 (1996) 70–76.
[37] P. Prieto, M. Pinda, M. Aguilar, Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E, Anal. Biochem. 269 (1999) 337–347.
[38] K. Mishra, H. Ojha, N.K. Chaudhury, Estimation of antiradical properties of antioxidants using DPPH assay: a critical review and results, Food Chem. 130 (2012) 1036–1043.
[39] R. Re, N. Pellegrini, A. Proteggente, et al., Antioxidant activity applying an improved ABTS radical cation decolorization assay, Free Radic. Biol. Med. 26 (1999) 1231–1237.
[40] S.I. Rizvi, N. Srivastava, Erythrocyte plasma membrane redox system in first degree relatives of type 2 diabetic patients, Int. J. Diabetes Mellit. 2 (2010) 119–121.
[41] M. Avron, N. Shavit, A sensitive and simple method for determination of ferrocyanide, Anal. Biochem. 6 (1963) 549–554.
[42] J.M. May, Z.C. Qu, C.E. Cobb, Human erythrocyte recycling of ascorbic acid: relative contributions from the ascorbate free radical and dehydroascorbic acid, J. Biol. Chem. 279 (2004) 14975–14982.
[43] S.L. Bonting, K.A. Simon, N.M. Hawkins, Studies on sodiumpotassium-activated adenosine triphosphatase: I. Quantitative distribution in several tissues of the cat, Arch. Biochem. Biophys. 95 (1961) 416–423.
[44] G.L. Ellman, D.K. Countney, V.J. Andres, et al., A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem. Pharmacol. 7 (1961) 88–95.
[45] H.U. Bergmeyer, K. Gawehn, M. Grassl, Pyruvate kinase, in: 2nd ed., in: H. U. Bergmeyer (Ed.), Methods of Enzymatic Analysis, Volume I, Academic Pres Inc, New York, NY, 1974, pp. 509–510.
[46] H.U. Bergmeyer, M. Grassl, H.E. Walter, in: 3rd ed., in: H.U. Bergmeyer (Ed.), Methods of Enzymatic Analysis, Volume II, Verlag Chemie, Deerfield Beach (FL), 1983, pp. 222–223.
[47] S.J. Khundmiri, M. Asghar, F. Khan, S. Salim, A.N. Yusufi, Effect of ischemia and reperfusion on enzymes of carbohydrate metabolism in rat kidney, J. Nephrol. 17 (2004) 377–383.
[48] C.C. Shonk, G.E. Boxer, Enzyme patterns in human tissues I. Methods for the determination of glycolytic enzymes, Cancer Res. 24 (1964) 709–721.
[49] F. Heinz, B. Freimoller, Glyceraldehyde 3-phosphate dehydrogenase from human tissues, Meth. Enzym. 89 (1982) 301–302.
[50] J. Gutierrez-Salinas, et al., In vitro effect of sodium fluoride on malondialdehyde concentration and on superoxide dismutase, catalase, and glutathione peroxidase in human erythrocytes, Transfus. Apher. Sci. (2013) 864718, https://doi.org/10.1155/2013/864718.
[51] C.C. Winterbourn, Free-radical production and oxidative reactions of hemoglobin, Environ. Health Perspect. 64 (1985) 321–330.
[52] I. Tsamesidis, P. Perio, A. Pantaleo, K. Reybier, Oxidation of erythrocytes enhance the production of reactive species in the presence of artemisinins, Int. J. Mol. Sci. 21 (13) (2020) 4799, 54.
[53] T. Kassa, S. Jana, F. Meng, A.I. Alayash, Differential heme release from various hemoglobin redox states and the upregulation of cellular heme oxygenase-1, FEBS Open Bio 6 (9) (2016) 876–884.
[54] P. Pacher, J.S. Beckman, L. Liaudet, Nitric oxide and peroxynitrite in health and disease, Physiol. Rev. 87 (1) (2007) 315–424.

[55] S. Hatia, S.A. Malaterre, F. Le Sage, et al., Evaluation of antioxidant properties of major dietary polyphenols and their protective effect on 3T3-L1 pre adipocytes and red blood cells exposed to oxidative stress, Free Radic. Res. 48 (4) (2014) 387–401.
[56] V. Witko-Sarsat, M. Friedlander, C. Capeillere-Blandin, et al., Advanced oxidation protein products as a novel marker of oxidative stress in uremia, Kidney Int. 49 (5) (1996) 1304–1313.
[57] D.T. Waugh, Fluoride exposure induces inhibition of sodium-and potassium-activated adenosine triphosphatase (Na^+K^+ATPase) enzyme activity: molecular mechanisms and implications for public health, Int. J. Environ. Res. Public Health 16 (8) (2019) 1427, https://doi.org/10.3390/ijerph16081427.
[58] F.A. Oski, L.K. Diamond, Erythrocyte pyruvate kinase deficiency resulting in congenital nonspherocytic hemolytic anemia, N. Engl. J. Med. 269 (1963) 763–770.
[59] F. Schutt, S. Aretz, G.U. Auffarth, J. Kopitz, Moderately reduced ATP levels promote oxidative stress and debilitate autophagic and phagocytic capacities in human RPE cells, Invest. Ophthalmol. Vis. Sci. 53 (9) (2012) 5354–5361.