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Insights into the ameliorative effect of oleic acid in rejuvenating phenylhydrazine induced oxidative stress mediated morpho-functionally dismantled erythrocytes

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ABSTRACT

Phenylhydrazine (PHZ), an intermediate in the synthesis of fine chemicals is toxic for human health and environment. Despite of having severe detrimental effects on different physiological systems, exposure of erythrocytes to PHZ cause destruction of haemoglobin and membrane proteins leading to iron release and complete haemolysis of red blood cells (RBC). Involvement of oxidative stress behind such action triggers the urge for searching a potent antioxidant. The benefits of consuming olive oil is attributed to its 75% oleic acid (OA) content in average. Olive oil is the basic component of Mediterranean diet. Hence, OA has been chosen in our present *in vitro* study to explore its efficacy against PHZ (1 mM) induced alterations in erythrocytes. Four different concentrations of OA (0.01 nM, 0.02 nM, 0.04 nM and 0.06 nM) were primarily experimented with, among which 0.06 nM OA has shown to give maximal protection. This study demonstrates the capability of OA in preserving the morphology, intracellular antioxidant status and the activities of metabolic enzymes of RBCs that have been diminished by PHZ, through its antioxidant mechanisms. The results of the present study firmly establish OA as a promising antioxidant for conserving the health of erythrocyte from PHZ toxicity which indicate toward future possible use of OA either singly or in combination with other dietary components for protection of erythrocytes against PHZ induced toxic cellular changes.

1. Introduction

Phenylhydrazine (PHZ), first characterized by Herman Emil Fisher in 1875, is used worldwide mainly as a chemical intermediate in the pharmaceutical, agrochemical and chemical industries. PHZ has been termed as an active ingredient of organic dye and of selective herbicide also [1]. It is toxic by single exposure via the oral route and is also known to be toxic by inhalation and dermal routes [2]. Large amount of waste water generated from different fine chemical industries using PHZ as source material, is found to be highly lethal and toxic [3]. This toxic environmental pollutant PHZ results in irreversible cellular damage [4] along with its potential skin and eye irritant properties evidenced in humans [5]. Exposure to PHZ may cause damage to red blood cells (RBCs) potentially resulting in anaemia and consequential secondary

Abbreviations: PHZ, Phenylhydrazine; RBC, Red blood Cell; OA, Oleic acid; ROS, Reactive oxygen species; Hb, Haemoglobin; EDTA, Ethylenediaminetetraacetic acid; LPO, Lipid peroxidation; TBARS, Thiobarbituric acid reactive substance; TBA, Thiobarbituric acid; TCA, Tricholoroacetic acid; DTNB, 5 5′- dithio-bis-[2-nitro benzoic acid]; GPx, Glutathione Peroxidase; GR, Glutathione Reductase; GST, Glutathione-S-transferase; SOD, Superoxide dismutase; HK, Hexokinase; PFK, Phosphofructokinase; LDH, Lactate dehydrogenase; PPP, Pentose Phosphate Pathway; G6PDH, Glucose 6 phosphate dehydrogenase; PBS, Phosphate buffered saline; DCFDA, 2′ 7′-Dichlorofluorescin diacetate; DCF, 2′ 7′-Dichlorofluorescin; NBT, Nitro blue tetrazolium chloride; DMSO, Dimethyl sulfoxide; MSA, Methanesulfinic acid; AFM, Atomic force microscope; FSC, Forward scattering; FACS, Fluorescence activated cell sorter; ANOVA, One way analysis of variance; FITC, Fluorescein isothiocyanate; MDA, Malondialdehyde; ATP, Adenosine triphosphate; NADPH, Reduced nicotinamide adenine di-nucleotide phosphate.

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adverse effects to other tissues, such as spleen and liver. PHZ is mutagenic *in vitro* and there exists evidences to indicate that it may express genotoxicity *in vivo*. PHZ is the most carcinogenic compound of hydrazine family [6] and it exhibited carcinogenicity in mice [2]. Many edible mushrooms also have been identified as a source of toxic PHZ [7]. The nucleophilic nature of PHZ [8] makes it an electron donor leading to free radical formation.

Being devoid of nucleus and mitochondria RBCs became unarmed to combat oxidative stress while it became extremely susceptible to reactive oxygen species (ROS) due to the presence of iron (Fe) containing haemoglobin (Hb) in its unique construction [9]. Increased lipid peroxidation [10] promoting membrane damage [11], diminished endogenous antioxidant level [12] inducing increased vulnerability to ROS [13] have become crucial factors to be considered while exploring mechanisms behind detrimental effects of PHZ on erythrocytes [14]. Altered potassium and calcium permeability, band 3 clustering [15], methaemoglobin formation as well as Heinz body [16,17] confirmed disassembly of erythrocytes. Moreover, PHZ treated RBCs show a simulating milieu of β-thalassaemic RBCs, where α-globin chains aggregate with membrane cytoskeletal proteins which decrease the deformability of RBC, thus impeding its function [18].

Applications of several classical antioxidants as well as of different components of our daily intakes to get rid of deleterious effects of haemolytic anaemia and thalassaemia/toxic PHZ have further given conviction to the thought of well built association between ROS and haemolytic conditions [19–22]. Apart from different sources like nuts, fruits and oil seeds, olive oil is the richest source of oleic acid (OA). The benefits of consuming olive oil, known since antiquity, were attributed to its high OA contents. The basic component of Mediterranean diet, olive oil, has been suggested to protect against coronary heart disease, insulin resistance and metabolic syndrome [23,24]. In addition, steady consumption of olive oil exerts beneficial effects in the composition of gut microbiota promoting intestinal health [25] and prevents risk of coronary heart diseases as well as of rheumatoid arthritis by immune and inflammatory modifications [26–28]. Such advantages in consumption of olive oil may be due to the presence of OA which comprises 70–80% of overall fatty acids. Several *in vivo* and *in vitro* studies have indicated that OA plays a crucial role in cancer prevention possibly due to its antioxidant properties [29]. Moreover, it has been reported that hypoglycaemic, hypotensive, cardio-protective [30] and hepato-protective OA participates in blood pressure reduction by improving endothelial function probably by reducing ROS [31] showing its anti-inflammatory and anti-microbial properties [32,33] probably due to the presence of one double bond, which makes it less susceptible to oxidation and thereby contributing to its antioxidant property against excessive oxidative load [34].

Hence, the current study made an attempt to elucidate whether OA is capable of providing protection to PHZ challenged RBCS *in vitro*. The results revealed that OA can provide protection against PHZ induced oxidative stress mediated morpho-functional alteration and disintegration of erythrocytes and antioxidative mechanisms might be associated with such protection indicating usefulness of this mono unsaturated fatty acid (MUFA) in the alleviation of PHZ induced toxic changes.

2. Materials and methods

2.1. Chemicals and reagents

Phenylhydrazine (PHZ) and all other necessary analytical grade chemicals were purchased from Sisco Research Laboratories (SRL), Mumbai, India. Oleic acid (OA) was purchased from Sigma-Aldrich Merck, St. Louis, USA. 2′, 7′-Dichlorofluorescin diacetate (DCFDA) was purchased from Abcam Biotechnology Company, USA.

2.2. Collection and processing of blood to obtain whole RBC

Despite morphometric differences, goat erythrocyte being mammalian class, are biconcave in nature and possess similarities in basic properties to human erythrocytes. In addition, its easy availability has made goat erythrocyte a suitable study model in several studies [35–38]. Hence goat blood was used for our present study.

Anticoagulant buffer was prepared by proportionate dissolution of trisodium citrate, citric acid and dextrose in deionised water. Goat blood was collected immediately after sacrifice in acid-citrate dextrose anticoagulant buffer, [36] from Kolkata Corporation approved slaughter house. Then, in order to obtain packed cells, whole blood was centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant plasma and buffy coat were removed by aspiration and the precipitated whole RBC was washed thrice with 0.9 % NaCl solution.

2.3. In vitro incubation of whole RBC

In order to maintain healthy cell count in reaction mixture, we checked cell number by flow cytometry and after confirming 8.5×10^6 to 9×10^6 cells/μl of 50 % suspension of packed cell we incubated RBCs. PHZ solution was prepared by dissolving appropriate weighed amount in deionised water and oleic acid solution was prepared by dissolving required quantity in phosphate buffered saline (PBS), pH 7.4. The whole RBCs were incubated in several groups for 1 h at 37 °C to identify the best effective concentration of oleic acid. The groups were as follows: Group I: Control (CON), Group II: Treated with 1 mM PHZ (PHZ) [39], Group III– VI: Negative control groups. Each group incubated with oleic acid only at four different concentrations i.e.; 0.01, 0.02, 0.04 and 0.06 nM (OA) respectively, Group VII- X: Each group co-incubated with 1 mM PHZ and four different concentrations of oleic acid i.e.; 0.01, 0.02, 0.04 and 0.06 nM (PHZ + OA) respectively. PHZ and OA were given simultaneously. Another two groups of erythrocytes, one incubated with 0.08 nM of OA and another one, co-incubated with 1 mM PHZ and 0.08 nM of OA was also considered for initial study (Data not shown as 0.08 nM OA didn't found significant with 0.06 nM OA in both negative control and co-incubated groups). Incubation was terminated with 35 mM ethylenediaminetetraacetic acid (EDTA).

2.4. Preparation of hemolysate for assay of antioxidant enzymes

After keeping apart one set of incubated sample other sets were taken to prepare samples for pursuing antioxidant enzyme assay. Washed RBCs were lysed by chloroform-ethanol mixture (2:1, v/v) (for Superoxide Dismutase) to make the samples haemoglobin free [40] and by deionised water (for enzymes other than Superoxide Dismutase) and then centrifuged at 7000 rpm for 25 min at 4 °C. The colourless supernatant, thus obtained were stored in separate microcentrifuge tubes at −20 °C for future use.

2.5. Dose response study of oleic acid

2.5.1. Measurement of biomarkers of oxidative stress

2.5.1.1. Determination of the level of lipid peroxidation. The level of lipid peroxidation (LPO) in RBCs was measured in terms of thiobarbituric acid reactive substance (TBARS) following the method of Buege and Aust [41] with minor modifications [42]. One ml. of TBA-TCA mixture was added to 500 μl of incubated sample and heated at 80 °C for 20 min. The absorbance of the supernatant obtained by centrifugation at 5000 rpm for 5 min was measured at 532 nm spectrophotometrically and expressed as nmoles of TBARS/mg protein.

2.5.1.2. Determination of the status of glutathione (GSH). The reduced glutathione content of different groups of RBCs was estimated

spectrophotometrically at 412 nm using 5, 5′- dithio-bis-[2-nitro benzoic acid] (DTNB) after precipitation of proteins using 10 % ice-cold TCA following the method of Sedlak and Lindsay [43] with minor modifications [42]. The values were expressed as nmoles of GSH/mg protein.

2.5.2. Determination of activities of enzymes regulating glutathione cycle

Glutathione Peroxidase (GPx) activity was measured in different groups of RBCs following the method of Paglia and Valentine [44] where the decrease in absorbance was measured spectrophotometrically at 340 nm and the enzyme activity was expressed as Units/mg protein.

Glutathione Reductase (GR) activity was estimated spectrophotometrically at 340 nm according to the method of Krohne- Ehrich *et al.* [45]. The decrease in absorbance was monitored and the enzyme activity was expressed as Units/mg protein.

Glutathione-S-transferase (GST) activity was determined following the method of Habig*et al.* [46]. The decrease in absorbance at 340 nm was measured using a UV/VIS spectrophotometer and the enzyme activity was expressed as Units/mg protein.

The rest of the experiments were carried out with four groups after determining the best effective concentration of oleic acid.

2.6. Measurement of antioxidant enzyme activity

The method of Marklund and Marklund [47] was employed to determine the activity of Cu- Zn SOD (SOD1) considering the process of pyrogallol autoxidation. The increase in absorbance was measured spectrophotometrically at 420 nm and enzyme activity was expressed in terms of Units/mg protein.

The activity of another important antioxidant enzyme, catalase, was measured in different groups of erythrocytes by the method of Beers and Sizer [48] followed by some modifications [49]. The decrease in absorbance due to gradual breakdown of hydrogen peroxide was monitored spectrophotometrically at 240 nm. The enzyme activity was expressed as μmoles of H_2O_2 consumed/min/mg protein.

2.7. Measurement of activities of glycolytic enzymes and the activity of glucose 6 phosphatedehydrogenase

The activity of hexokinase (HK), that catalyze the first irreversible step of glycolysis, was measured spectrophotometrically at 340 nm following the method of Haritos and Rosemeyer [50]. The increase in absorbance was measured and the values were expressed in terms of Units/mg protein.

Phosphofructokinase (PFK) activity was determined exploiting the method of Layzer *et al.* [51]. A gradual decrease in absorbance was monitored at 340 nm and the values were expressed in terms of Units/mg protein.

The method of Castillo *et al.* [52]. was employed to determine the activity of aldolase spectrophotometrically at 240 nm. An increase in absorbance was observed and the activity was expressed in terms of Units/mg protein.

Lactate dehydrogenase (LDH) activity was measured using UV/VIS spectrophotometer according to the method of Dhanesha *et al.* [53] at 340 nm. A decrease in absorption was monitored and the activity was expressed in terms of Units/mg protein.

Glucose 6 phosphate dehydrogenase (G6PDH), the metabolic enzyme that catalyze the rate limiting step of Pentose Phosphate Pathway (PPP), was spectrophotometrically measured at 340 nm exploiting the method of Beutler [54] where an increase in absorbance was observed which is expressed as Units/mg protein.

2.8. Measurement of total intracellular reactive oxygen species (ROS)

The incubated RBCs of different groups were further diluted using phosphate buffered saline (PBS) and again incubated at room temperature with 2′, 7′-Dichlorofluorescin diacetate (DCFDA) at a final concentration of 0.01 μmole for 30 min strictly in dark. The presence of ROS can be estimated by gradual oxidation of DCFDA to 2′, 7′-Dichlorofluorescin (DCF). The change in fluorescence was measured using FITC- A channel in terms of their side scatter area in Flow cytometer (BD FACS Verse) [55].

2.9. Determination of superoxide anion (O_2^-) content

The content of superoxide anion free radical was measured in different groups of erythrocytes following the method of Serrander *et al.* [56] with some modifications from Zielonka [57]. The incubated samples were further incubated with Nitro blue tetrazolium chloride (NBT) at a final concentration of 1 mg/mL for one h at 37 °C. The formazan precipitate thus formed due to the reduction of NBT, was dissolved in DMSO and KOH and the amount of reduced NBT was measured at 630 nm.

2.10. Determination of erythrocyte hydroxyl radical (·OH) content

The amount of hydroxyl radical generated within different groups of erythrocytes was measured by incubating RBCs in presence of DMSO as a hydroxyl radical scavenger at a final concentration of 500 μmoles for one hour at 37 °C. The methanesulfinic acid (MSA) formed in the final step of series of reactions with ·OH showed a golden yellow colour having absorbance maxima at 425 nm. The values were expressed as nmoles of MSA/mL RBC following the method of Babbs and Steiner [58] as modified by Bandyopadhyay*et al.* [42].

2.11. Morphological study of RBC by atomic force microscopic (AFM) imaging

2.11.1. AFM sample preparation

Incubated erythrocyte samples of different groups were suspended in 2.5 % glutaraldehyde in PBS with a pH of 7.4 as described in Pretorius et al. [59]. After rinsing for three times, erythrocytes were post fixed with osmium tetroxide followed by serial dehydration with series of ethanol and finally was air dried on cover slips.

2.11.2. AFM imaging and measurement

An AFM (Model: Veeco di INNOVA; Make: Bruker, USA) was used in tapping mode with a resonant frequency between 382 and 405 KHz. Each sample was scanned at 10.0 μm × 10.0μm and 5.0μm × 5.0μm size and 3D images of later was made using Nanoscope analysis software. RBC diameter and average roughness were measured from three separate points of each sample using Nanodrive v8 real-time control software.

2.12. RBC morphological analysis by flow cytometry

The incubated RBCs were further diluted with isotonic PBS and the samples were analyzed according to their forward scattering (FSC) in BD FACS Verse flow cytometer. 10,000 events were recorded for 15 s for each sample keeping the flow rate fixed at 10 μl/min so that RBCs can rotate in the flow avoiding deformation due to shear stress as described in Rolfes-Curl *et al.* [60] with some modifications followed by Piagnerelli *et al.* [61]. FSC-A versus FSC-H was measured which have shown two different populations that we have marked as P2 and P3. The %TOTAL of each sample was calculated for each gate of interest.

2.13. Protein estimation

Concentration of protein in different packed cell samples were measured following the method of Lowry *et al.* [62] and Bradford method was employed for determining the protein level in hemolysate [63].

2.14. Statistical analysis

Data have been presented as Mean ± S.E.M. All enzyme activities were calculated using 5 data for each group and presented as bar diagram showing mean value and standard error of mean. These activities have also been presented in scatter plot also showing 5 individual values with marked mean value along with standard error of mean. All free radical measurements and morphological experiments were presented as Mean ± S.E.M, calculated using 3 data sets. One way analysis of variance (ANOVA) followed by post hoc Tukey test was performed to find out the level of significance between groups in different parameters measured. Statistical Package for Social Sciences (SPSS) version 25.0 (IBM) software was used to check the level of significance and results were considered statistically significant at the level of $p < 0.05$. The graphs were plotted using GraphPad Prism 6 software.

3. Results

3.1. Dose response study of oleic acid

3.1.1. Lipid peroxidation (LPO) level

The level of LPO was found to be significantly increased (about 1.43 fold) in RBCs of PHZ (1 mM) treated group in comparison to control (CON) ($p < 0.001$). Oleic acid itself at its four different concentrations of 0.01, 0.02, 0.04 and 0.06 nM (OA) did not show much difference from control level. The co-incubated groups of our experiment (PHZ + OA) showed a significant protection of lipid peroxidation level in presence of oleic acid in a concentration dependent manner. Although groups VIII- X showed significant alterations in LPO level, OA at a concentration of 0.06 nM was found to give optimal protection to PHZ treated RBCs ($p < 0.001$) (Fig. 1A and C), while OA at 0.01 nM concentration was not found to be much effective.

3.1.2. Glutathione (GSH) content

A significant decrease in the level of reduced glutathione (70.93 %) was observed in RBCs incubated with 1 mM PHZ in comparison to control ($p < 0.001$). GSH content of oleic acid treated groups of RBCs (Group III- VI) were well maintained at the level of control. The co-incubated groups of RBCs have exhibited a significant protection in the content of the endogenous antioxidant in a concentration dependent manner. The RBCs treated with PHZ and oleic acid at a concentration of 0.06 nM have shown its maximal efficacy to protect the GSH content from decreasing in comparison to control ($p < 0.001$) (Fig. 1B and D).

3.2. Status of glutathione cycle

Significant elevations in the activities of GPx and GST have been monitored in PHZ treated group (Group II) in comparison to control ($p < 0.05$ and $p < 0.001$ respectively). PHZ induced alteration in GPx activity (1.39 fold in comparison to control) has been found to be successfully ameliorated at the level of control in co-incubated groups in a concentration dependent manner and 0.06 nM OA was monitored as the best effective concentration to drive back the level of GPx from PHZ treated level ($p < 0.05$) (Fig. 2A and D). GST activity also showed the similar trend where PHZ group showed a significant rise (1.58 fold in comparison to control) and all co-incubated groups have shown concentration dependent significant decrease and thus protected activities of the enzyme while OA at concentration of 0.06 nM was found to be best

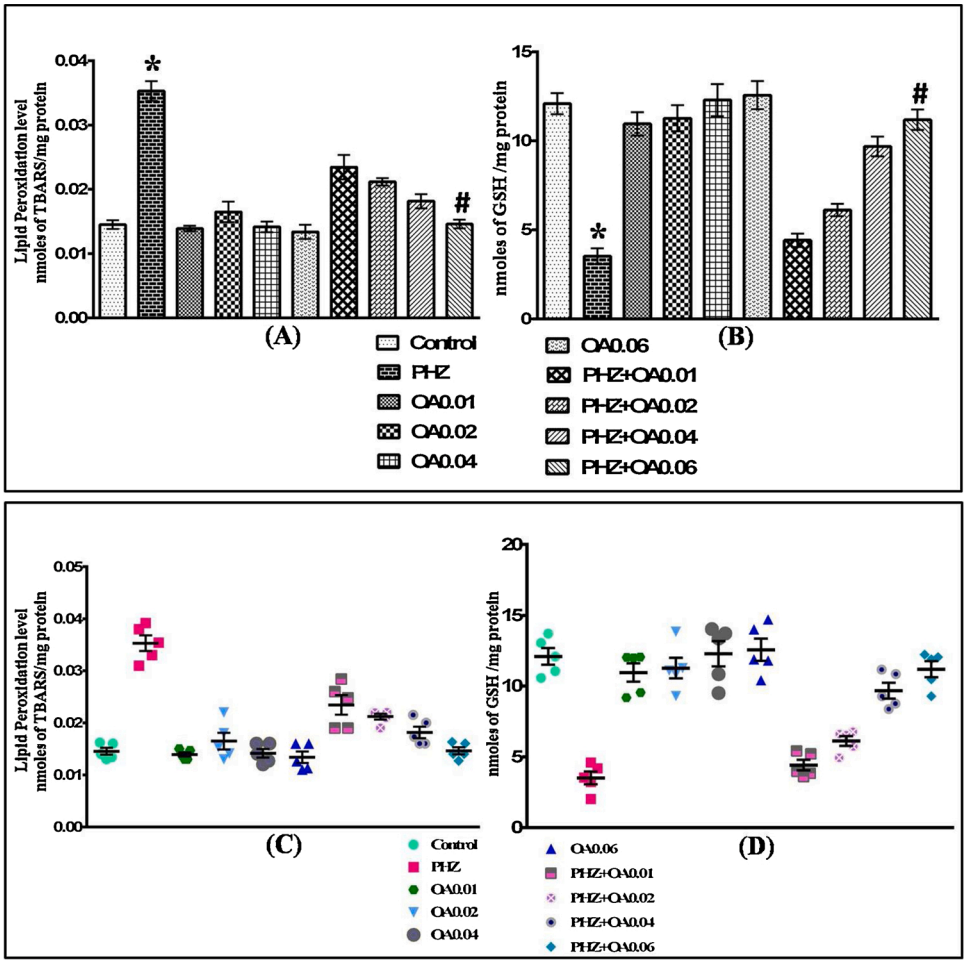


Fig. 1. Ameliorative effect of oleic acid at different concentrations on LPO, GSH level in PHZ treated erythrocytes. Diagrammatic representation of the changes in the levels of (A) LPO, (B) GSH content in erythrocytes treated with different concentrations of OA (0.01, 0.02, 0.04 and 0.06 nM) and/or, PHZ (1 mM). Scatter Plot presentation of the changes in the levels of (C) LPO, (D) GSH content in erythrocytes treated with different concentrations of OA (0.01, 0.02, 0.04 and 0.06 nM) and/or, PHZ (1 mM). The values are expressed as Mean ± S.E.M. (* $p < 0.001$ vs. Control, #$p < 0.001$ vs. PHZ; using one way ANOVA).

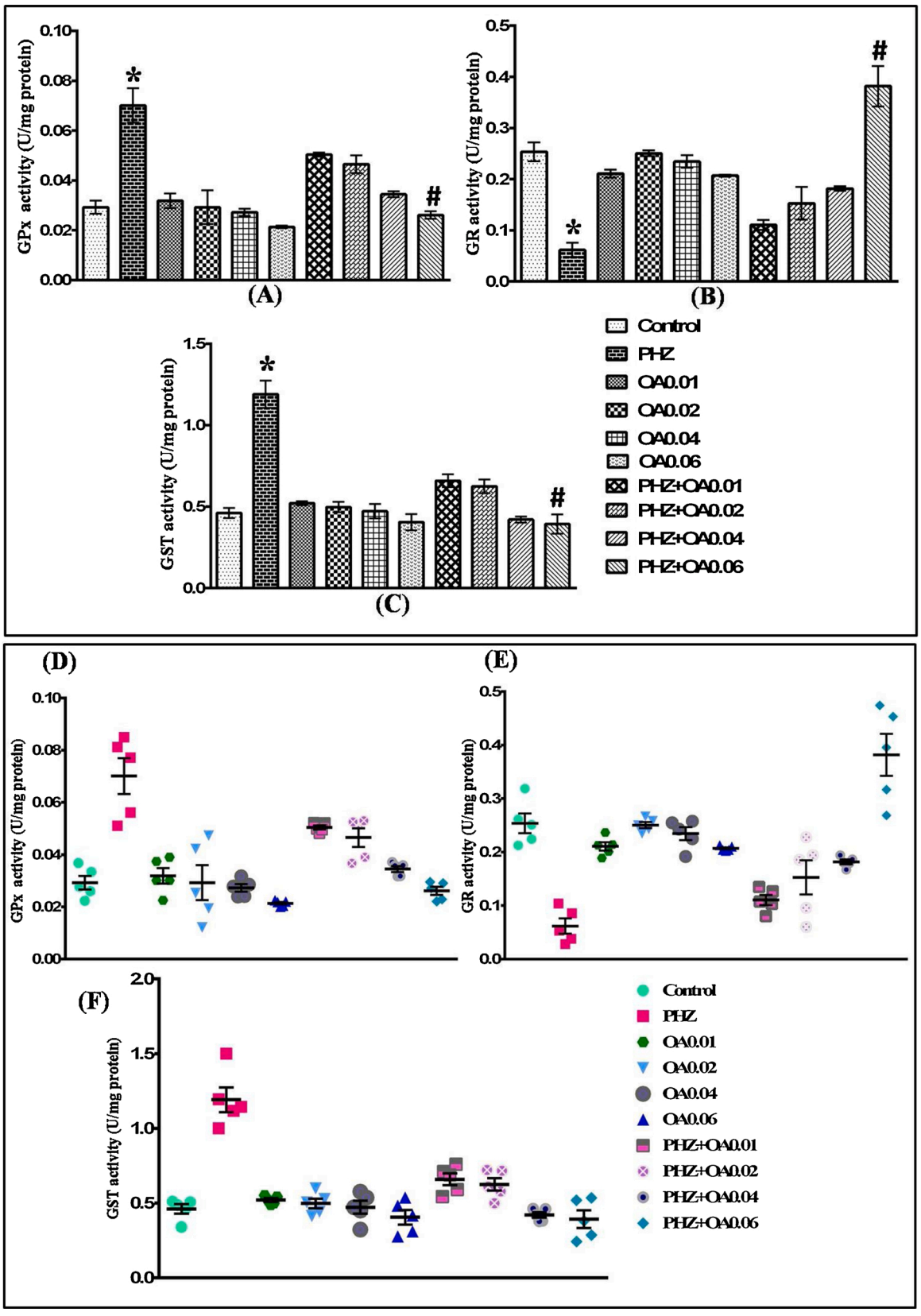


Fig. 2. Attenuation of oxidative stress mediated alterations of status of glutathione cycle (activities of GPx, GR, GST) in PHZ treated erythrocytes by oleic acid administration at different concentrations. Diagrammatic representation of the changes in the activities of (A) GPx, (B) GR and (C) GST in erythrocytes treated with different concentrations of OA (0.01, 0.02, 0.04 and 0.06 nM) and/or, PHZ (1 mM). Scatter Plot presentation of the changes in the activities of (D) GPx, (E) GR and (F) GST in erythrocytes treated with different concentrations of OA (0.01, 0.02, 0.04 and 0.06 nM) and/or, PHZ (1 mM).The values are expressed as Mean ± S.E.M. (* $p < 0.05$ vs. Control, #$p < 0.05$ vs. PHZ; using one way ANOVA).

effective in bringing back the level of GST to near about control ($p < 0.001$) (Fig. 2C and F).

In contrast, the activity of antioxidant GR significantly decreased (75.69 %) in PHZ group ($p < 0.05$) which was fruitfully driven back to control level when co-incubated with different concentrations of oleic acid (Group VII- IX) while group X i.e.; RBCs, co-incubated with 1 mM PHZ and 0.06 nM OA showed a significantly altered GR activity ($p < 0.001$) in comparison to PHZ group even higher than that of control (Fig. 2B and E).

Oleic acid alone in negative control groups i.e.; group III- VI did not significantly influence the activities of GPx, GR and GST in comparison to control.

All of these parameters studied so far have shown that oleic acid at a concentration of 0.06 nM can effectively ameliorate the alterations observed during PHZ exposure. Hence, rest of the experiments were carried out with this best effective concentration of oleic acid.

3.3. Effect of oleic acid on antioxidant enzymes of RBC

Superoxide dismutase activity has been found to be protected nearly to the level of control in PHZ + OA0.06 co-incubated group in a significant manner from PHZ treated group ($p < 0.001$). SOD activity was significantly increased in PHZ treated group (about 88.70 %) in comparison to control ($p < 0.001$). OA alone, however, showed similar trend of the enzyme activity like control (Fig. 3A and C).

In a contrasting manner, activity of another antioxidant enzyme catalase, the hydrogen peroxide (H_2O_2) scavenger, decreased significantly (about 58.87 % compared to control) when RBCs were incubated with PHZ ($p < 0.001$). However, this alteration has been effectively found to be protected from being taken place when co-incubated with OA at 0.06 nM (PHZ + OA) ($p < 0.001$) (Fig. 3B and D). Oleic acid alone, at the concentration of 0.06 nM, did not significantly affect the activities of these antioxidant enzymes.

3.4. Effect of oleic acid on the activities of metabolic enzymes

The activities of glycolytic enzymes HK, PFK and aldolase have shown to be decreased significantly in PHZ group (about 80.16, 69.17 and 75.67 % respectively) in comparison to control ($p < 0.001$). OA group (negative control) did not show any marked changes comparing to control group. However, activities of HK ($p < 0.01$) as well as PFK and aldolase ($p < 0.001$) have been well protected in co-incubated group of PHZ (1 mM) and OA (0.06 nM) (Fig. 4A–C and F–H).

Dissimilarly, PHZ induced significant increase (3.21 fold) in the activity of erythrocyte LDH has been observed when compared to control ($p < 0.01$) which was found to be prevented from increasing in co-incubated group in comparison to PHZ group ($p < 0.01$) (Fig. 4D and I).

Other than glycolytic pathway, PPP also found to be affected by PHZ exposure as the activity of G6PDH enzyme has been monitored to be decreased significantly (74.07 %) comparing to control group ($p < 0.001$). Oleic acid here also shows its efficacy to preserve the activity of the said enzyme at control level in a significant way ($p < 0.001$) (Fig. 4E and J).

3.5. Effect of oleic acid on intracellular ROS level

A significant increase in ROS level has been measured in group of RBCs treated with PHZ (about 16.05 fold) in comparison to control group ($p < 0.001$) which shows negligible amount of ROS generation similarly like the negative control group OA0.06. The increased amount

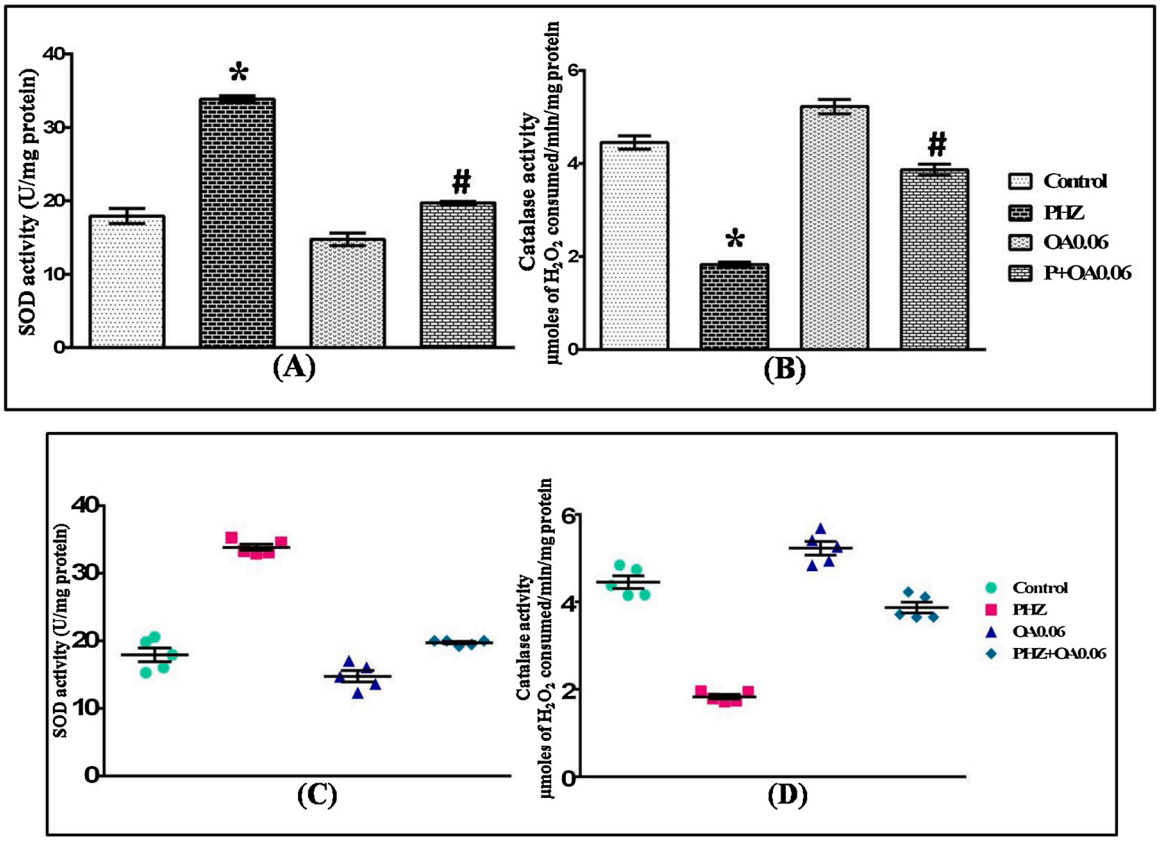


Fig. 3. Efficacy of oleic acid in restoring the status of endogenous antioxidant enzymes (SOD, catalase) in PHZ treated erythrocytes. Diagrammatic representation of the changes in the activities of (A) SOD, (B) Catalsae in erythrocytes treated with OA (0.06 nM) and/or, PHZ (1 mM). Scatter Plot presentation of the changes in the activities of (C) SOD, (D) Catalsae in erythrocytes treated with OA (0.06 nM) and/or, PHZ (1 mM). The values are expressed as Mean ± S.E.M. (* $p < 0.001$ vs. Control, #$p < 0.001$ vs. PHZ; using one way ANOVA).

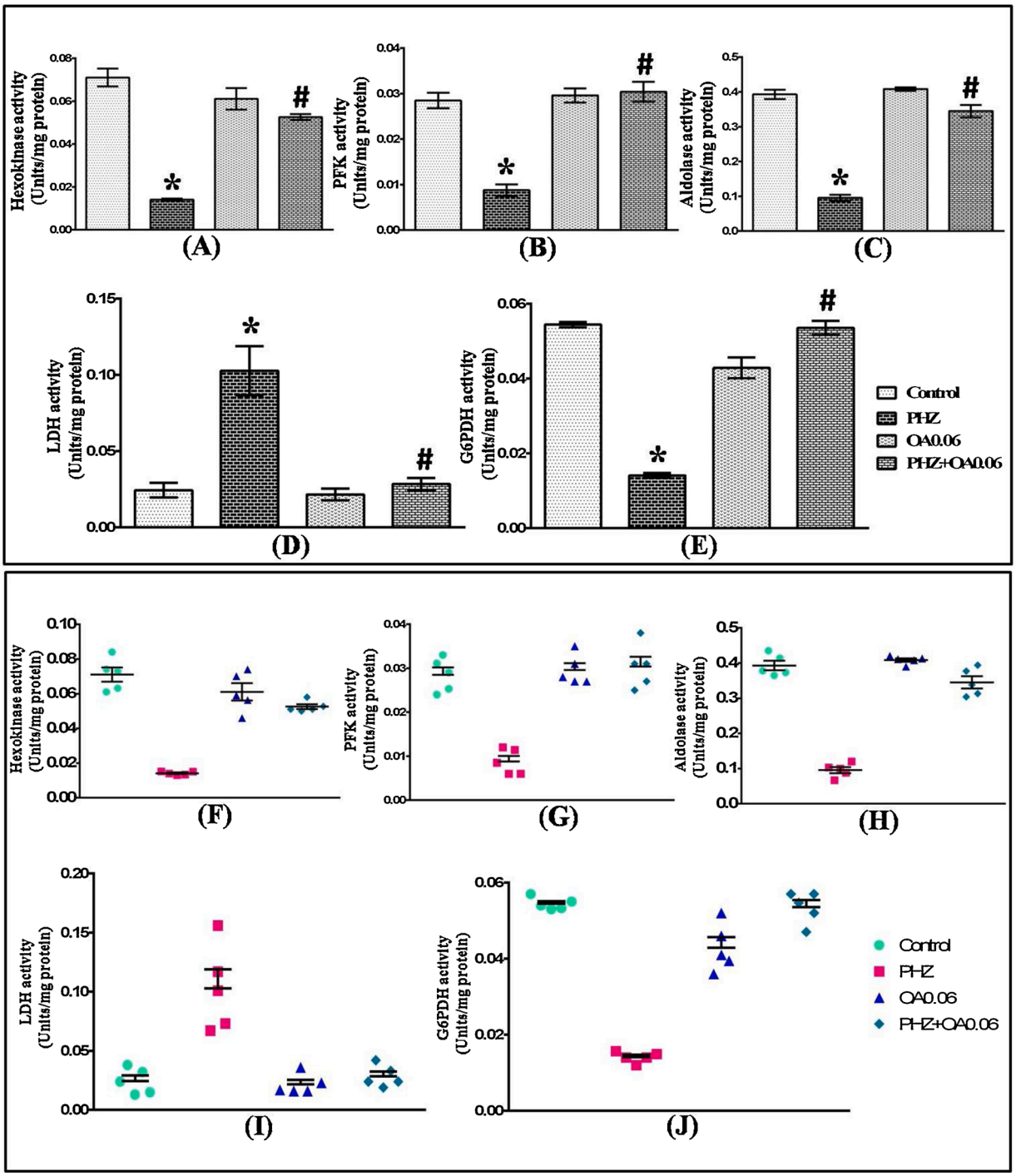


Fig. 4. Protective action of oleic acid on the activities of glycolytic enzymes (HK, PFK, Aldolase, LDH) and G6PDH in PHZ treated erythrocytes. Graphical representation of the changes in the activities of (A) Hexokinase, (B) Phosphofructokinase, (C) Aldolase, (D) Lactate dehydrogenase, (E) Glucose-6-phosphate dehydrogenase in erythrocytes treated with OA (0.06 nM) and/or, PHZ (1 mM). Scatter Plot representation of the changes in the activities of (F) Hexokinase, (G) Phosphofructokinase, (H) Aldolase, (I) Lactate dehydrogenase, (J) Glucose-6-phosphate dehydrogenase in erythrocytes treated with OA (0.06 nM) and/or, PHZ (1 mM). The values are expressed as Mean ± S.E.M. (* $p < 0.01$ vs. Control, #$p < 0.01$ vs. PHZ; using one way ANOVA).

of intracellular ROS in PHZ group reacted with DCFDA to form DCF which gave significantly higher fluorescence to record and have shown more number of cells with increased side scattering in FITC-A channel. However, erythrocytes, when co-incubated with 1 mM PHZ and oleic acid at the concentration of 0.06 nM, ameliorate profound ROS fruitfully either by inhibiting ROS generation or by scavenging excess ROS in a significant manner compared to PHZ group ($p < 0.001$) (Fig. 5).

3.6. Effect of oleic acid on intracellular superoxide anion content

Superoxide anion ($O_2^{\cdot -}$)content of PHZ treated erythrocytes has been found to be increased (about 1.24 fold) significantly ($p < 0.001$) comparing to control group. RBCs incubated with OA did not show any marked alterations in $O_2^{\cdot -}$ content comparing to control. In presence of superoxide anion free radical, NBT reacts with it and leads to its own reduction. The reduced NBT got dissolved in DMSO and KOH mixture which have shown absorbance maxima at 630 nm. Erythrocytes when

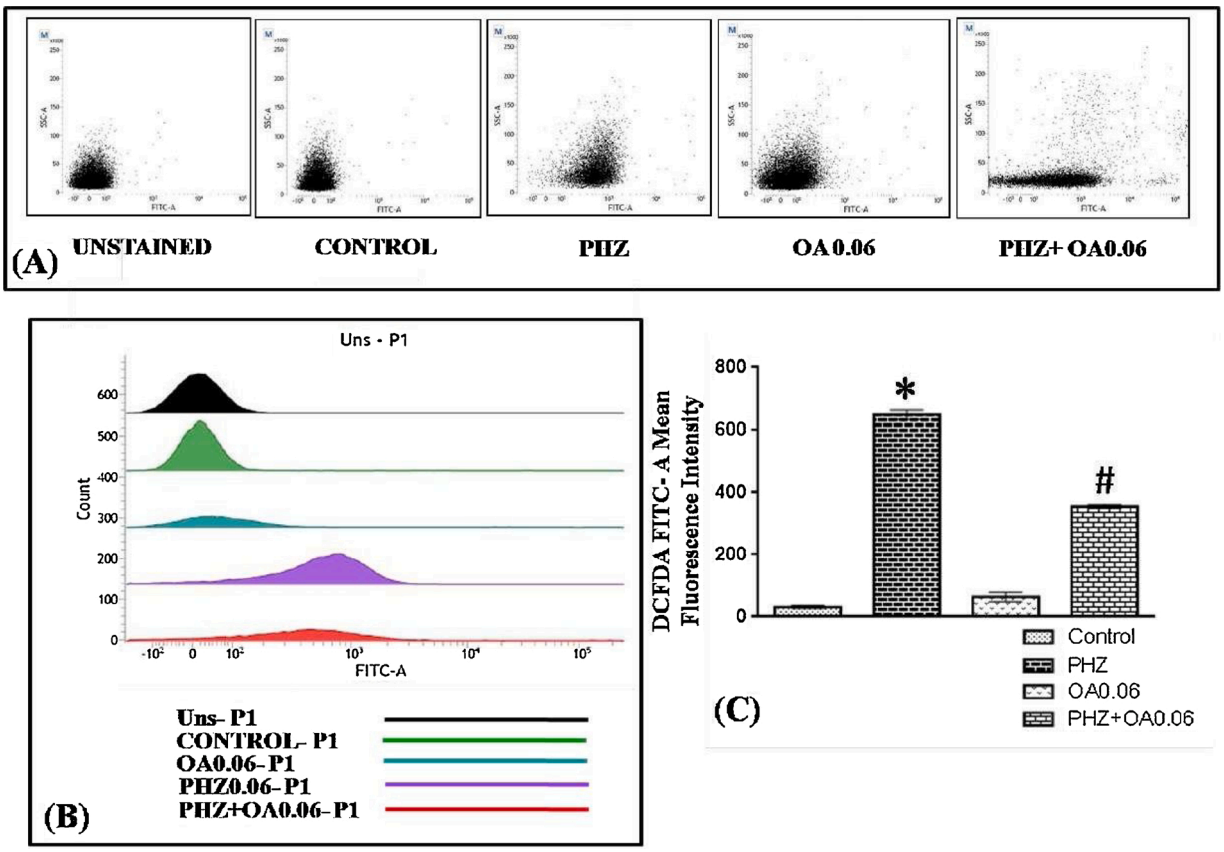


Fig. 5. Amelioration of the level of intracellular total ROS by oleic acid in PHZ treated erythrocytes. (A) Alterations in side scatter area of erythrocytes treated with OA (0.06 nM) and/or, PHZ (1 mM) (B) Offset layout presentation of changes in no. of cell count (in FITC-A channel) of erythrocytes treated with OA (0.06 nM) and/or, PHZ (1 mM) (C) Graphical representation of the changes in the mean fluorescence intensity in erythrocytes treated with OA (0.06 nM) and/or, PHZ (1 mM). The values are expressed as Mean ± S.E.M. (* $p < 0.001$ vs. Control, #$p < 0.001$ vs. PHZ; using one way ANOVA).

co-incubated with PHZ (1 mM) and oleic acid at concentration of 0.06 nM, effectively reduced the content of $O_2^{\cdot-}$ ($p < 0.001$) significantly comparing to PHZ group, as evidenced from the optical density measured (Fig. 6A).

3.7. Effect of oleic acid on intracellular hydroxyl radical content

The hydroxyl radical generated in PHZ treated group has shown a significant rise (about 47.25 %) in comparison to control ($p < 0.001$) that have been successfully scavenged in the group of erythrocytes co-incubated with 1 mM PHZ and 0.06 nM of oleic acid comparing to PHZ group ($p < 0.001$) (Fig. 6B). The nmoles of methanesulfinic acid formed/mL of RBC in four different groups was measured as a reflection of excess ·OH generated after being scavenged by DMSO.

3.8. Effect of oleic acid on morphological status of RBC

The 3D images obtained from atomic force microscopic studies (5.0 × 5.0 μm size) on different incubated groups of erythrocytes have represented marked differences. Spicule formation on the surface of red blood cells was observed in PHZ treated group. However, the surface of RBCs of co-incubated group (PHZ + OA0.06) has became visibly normalized (Fig. 7A). Moreover, PHZ treatment leads to significant decrease in RBC diameter (16.50 %) ($p < 0.001$) (Fig. 7B) and RBC average roughness (21.38 %) ($p < 0.01$) compared to RBCs of the control group (Fig. 7C), measured by software analyzer. Though the alteration in RBC diameter did not exhibit much change in co-incubated group (Fig. 7B), average roughness of erythrocytes were found to be protected well in a significant way ($p < 0.001$) (Fig. 7C) which showed the efficacy of oleic acid in maintaining roughness of RBC, an important criteria for

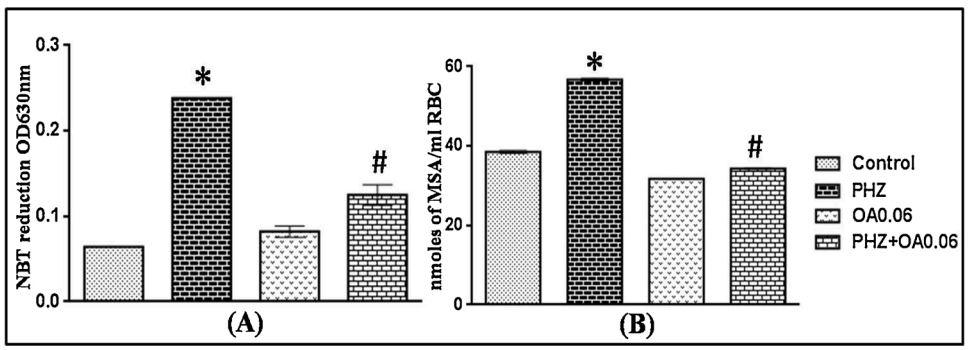


Fig. 6. Superoxide anion free radical and hydroxyl radical scavenging ability of OA in PHZ treated stressed erythrocytes. Graphical representation of the alterations in the levels of (A) Superoxide anion content (expressed as OD at 630 nm) and (B) Hydroxyl radical content (expressed as nmoles of methanesulfinic acid) in erythrocytes treated with OA (0.06 nM) and/or, PHZ (1 mM). The values are expressed as Mean ± S.E.M. (* $p < 0.001$ vs. Control, #$p < 0.001$ vs. PHZ; using one way ANOVA).

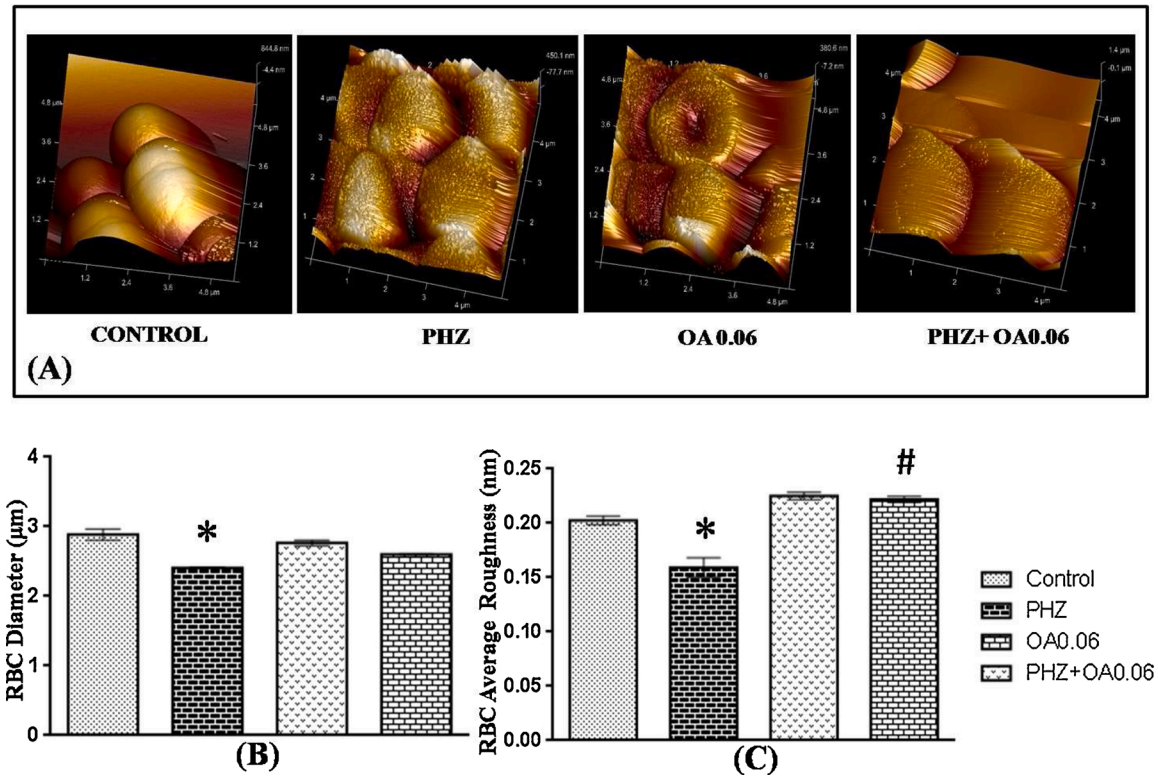


Fig. 7. Preservation of RBC morphology by OA administration along with restoration of surface roughness and diameter in PHZ treated erythrocytes. (A) Alterations in RBC morphology using atomic force microscopic 3D imaging (5 × 5 µm) of erythrocytes treated with OA (0.06 nM) and/or, PHZ (1 mM). Diagrammatic representation of changes in (B) RBC diameter (in µm) and (C) RBC roughness (in µm) of erythrocytes treated with OA (0.06 nM) and/or, PHZ (1 mM). The values are expressed as Mean ± S.E.M. (* $p < 0.01$ vs. Control, #$p < 0.001$ vs. PHZ; using one way ANOVA).

conserving deformability of RBCs.

3.9. Flow cytometric study to find out the effect of oleic acid on structural changes of RBC induced by PHZ

As cell size is the principal component of FSC signal, the flow cytometric result obtained here is a reflection of the cell size. Additionally, two distinct populations of cells were observed in control group of erythrocytes whereas, a marked decrease in a population (P3) has been monitored in PHZ treated erythrocytes which has been found to be preserved well in PHZ + OA0.06 group. OA group of erythrocytes (negative control) also showed two healthy populations of cells in a similar manner compared to control group.

4. Discussion

Several xenobiotics and environmentally abundant chemicals, upon exposure to any individual for a long time, even if in their respective NOAEL levels, are found to alter redox status of exposed individuals along with prominent cytotoxic as well as genotoxic modifications and reconstructions [64,65]. Sometimes, these re-orderings make the endogenous antioxidant system of exposed organism more adapted in combating stress [66]. But, highly toxic substances become vulnerable to cellular system, leading to complete destruction of their structure-function harmony and thus instigate apoptosis. PHZ intoxication resulted into severe damage in cellular system as it was found to bring about RBC hemolysis with supporting view of abated arterial blood pressure and peripheral vascular resistance [22]. But, its toxicity has not been restricted either within cardio-vascular system or within cellular system. PHZ has been found to be accountable for oxidatively damaging different tissue systems as well including liver, kidney and spleen with declined endogenous GSH level [67,68]. Moreover, its toxicity widens into genetic level, when PHZ has been examined to cause liver DNA fragmentation in PHZ subjected rats with concomitant increase in 8-oxo-gunanine and 8-hydroxyguanine in liver and kidney DNA respectively, the signature markers of oxidative DNA damage [69–71].

Despite of having various exogenous threats, our daily intakes in the form of several natural foods cushion our tissue and cellular system from those unfavourable hazards principally by their antioxidant and anti-inflammatory properties. Since, the protective worthiness of olive oil has been elucidated earlier in diverse physiological systems [72], contribution of its different components in such antioxidative function must be elucidated. Rat microsomal lipid peroxidation has been investigated to be hindered by OA rich oils [73], featuring OA as an antioxidant, the major component of olive oil. Beside this, numerous phenolic and hydrophilic compounds of olive oil have proven their antioxidative values in protecting cellular system [74]. But, apart from advantageous actions of those bioactive compounds, many disastrous outcome discourage its prolonged usage at high concentration [75]. This has driven the attention to oxidation stable OA in order to explain antioxidative role of olive oil. Though OA has proven to be effective in different systems [76–78], this study for the first time enlightens its effect on PHZ intoxicated damaged RBC.

The main function of RBCs is the transportation of oxygen into the tissues of the body. Hence, any condition that affects the RBC alters its functions which may be detrimental to the body. Being the carrier of oxygen in every part of the living tissues, erythrocytes are exceedingly exposed to different forms of ROS [79]. A firm association between RBC oxidative stress and haemolytic anaemia [80], sickle cell diseases [81] have also been depicted. Disintegration of RBC structure has also found as an integral part of ROS mediated stress [82,12].

Lipid peroxidation has been identified as an intrinsic part of stressed RBCs [9]. In this study, increased lipid peroxidation has been observed

in PHZ treated RBCs (Fig. 1A), measured in terms of TBARS. TBARS, the product formed in reaction of malondialdehyde (MDA) with thiobarbituric acid is an essential indicator of oxidative damage [83]. Oleic acid treated group of RBCs showed a significant decrease in peroxidation level (Fig. 1A) in a dose-dependent manner confirming the notion of labelling a fatty acid with single double bond as an inhibitor of peroxidation [84]. Oleic acid at 0.06 nM concentration was found to protect RBCs most efficiently from lipid peroxidation (Fig. 1A).

Another oxidative stress biomarker, GSH, showed a significant decrease in its level in PHZ group (Fig. 1B). This is a phenomenon which may outline GSH as highly susceptible to oxidation [85] in RBCs. Interestingly, the trend of GSH cycle monitored in β-thalassaemic RBC [86] is similar to PHZ treated RBC, a simulation model of thalassaemic erythrocyte [18]. Another possible reason behind this GSH depletion may be the decreased activity of GR as OA treated RBC group showed rejuvenated GSH level (Fig. 1B) nearly to the level of control, especially at 0.06 nM concentration along with escalated GR activity (Fig. 2B). Another feasible cause of decreased level of GSH in PHZ group of our study may be the enhanced activity of GPx (Fig. 2A) and GST (Fig. 2C), as these two enzymes utilize GSH as their substrate. The conceivable means by which OA provide protection to erythrocyte GSH cycle status in order to restore intracellular antioxidant level, may be the fact that fatty acid in non- ionic form can simply diffuse through cell membrane [87].

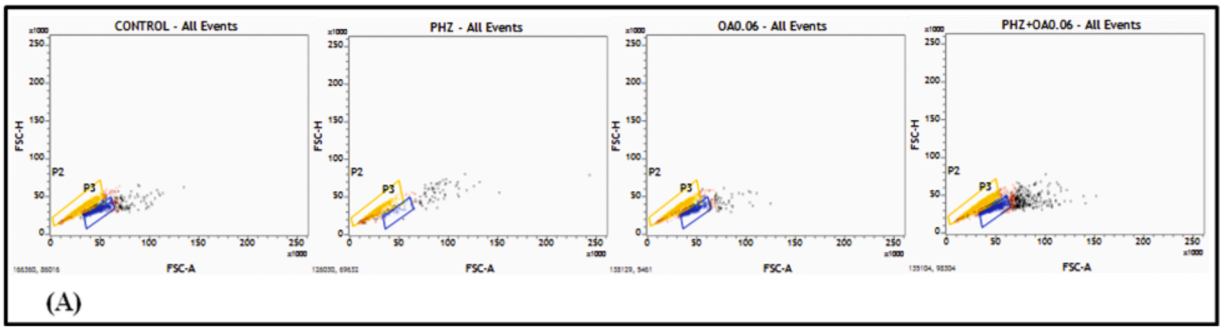
On the other hand, antioxidative enzymes of RBCs exhibit a contrasting nature in their activities. SOD activity has been found to be increased (Fig. 3A), whereas catalase activity was diminished in PHZ treated RBCs (Fig. 3B), resembling the exhibited pattern of sickled erythrocytes [88]. Increased SOD activity, a measure of elevated level of superoxide anion free radical within PHZ treated cells [89] also evidenced from our study (Fig. 6A). Consideration of continuous autoxidation of Hb as a wellspring of superoxide has been depicted in previous studies [90] and OA was found to efficiently amend the level of superoxide anion free radical (Fig. 6A) as well as the activity of SOD1 (Fig. 3A) at a concentration of 0.06 nM in our *in vitro* study. Although OA has established itself as a superoxide radical scavenger, an upsurged level of hydrogen peroxide has become an obvious consequence of this phenomenon [91] even in OA co- treated group also. Here, catalase and GPx came as crucial players to ameliorate the situation. Catalase delayed GSH depletion in accordance with GPx, whose direct binding capability with H_2O_2, [92] especially in the vicinity of RBC membrane, the site of Hb autoxidation, leads to breakdown of intracellular H_2O_2. Our result showed a reduction in catalase activity (Fig. 3B) while a significant elevation in GPx (Fig. 2A) activity appeared to be a wise alternative of RBC to combat deleterious effects of PHZ. Our PHZ and OA co- treated group has driven back the activities of those antioxidants nearly to the level of control portraying abated formation of superoxide anion free radical and H_2O_2 within cells.

Furthermore, a marked elevation in total intracellular ROS in PHZ group (Fig. 5A–C) has been examined in our study which again suggests involvement of oxidative stress behind destructive action of PHZ. The total intracellular ROS level in OA co- incubated group has notably declined (Fig. 5A–C). This result found support with the level of superoxide anion free radical, confirming the scavenging ability of OA at a particular concentration. Moreover, as Hb autoxidation is an identified prime factor behind oxidative stress generation [90], an elevated level of free iron [93,12] within cells is an inevitable outcome. This makes us curious about finding intracellular level of ·OH in different groups of RBCs, as redox active free iron triggers Fenton reaction leading to ·OH generation. A collateral significant increase in ·OH level has observed in PHZ group (Fig. 6B) that can initiate autocatalytic lipid peroxidation [94] giving a conviction to our result of increased LPO while in OA and PHZ co- incubated group, a significant decline in the level of the same (Fig. 6B) was examined in our study. This observation is giving evidence about the potency of OA in hindering Fenton reaction and thus ·OH generation. Hence, estimation of the intracellular levels of total ROS, $O_2^{\cdot -}$ and ·OH is giving a new insight into the restoring antioxidant properties of OA to preserve erythrocytes from PHZ induced stress-mediated effects leading to haemolytic conditions.

Apart from these scavenging activities of OA, this mono unsaturated fatty acid (MUFA) has shown its virtue in maintaining the status of metabolic enzymes, on which erythrocytes rely upon to maintain its shape and flexibility [95]. The RBCs are solely dependent on the glycolytic enzymes to extract its energy in the form of adenosine triphosphate (ATP). Oxidative stress mediated significant decrease in major glycolytic enzymes is evident in our study in PHZ group whereas, OA preserves the activities of those enzymes to maintain normal energy level, which is a solitary requirement of RBCs to traverse through micro capillaries. HK, Aldolase, PFK activities have been diminished in stressed RBCs and OA acts as an ally to those enzymes by maintaining them in health. Fig. 4A–C suggests PHZ induced stress mediated disruption in RBC structure and subsequent disassembly in their function that was ameliorated by OA. In contrast, a downturned LDH activity has been exhibited by our co- treatment group which was significantly augmented in PHZ group comparing to control as well as co-treated group. This rise in LDH activity in PHZ group supports the phenomena of haemolysed RBC [96] which also confirmed about hindered haemolysis in co-treated group of our study, i.e., PHZ + OA (0.06 nM). The significant decline in LDH activity in co-treated group in comparison to PHZ group also gives a speculation about restoration of glycolytic enzyme activity as increased LDH activity in PHZ group leads to escalated lactate production which might give feedback inhibition to other enzymes involved in glycolysis in order to lessen the available substrate for LDH. Moreover, this glycolytic pathway is a prime regulator of redox balance within cells [97]. Hence, disintegration of metabolic enzyme function creates a state of intracellular redox imbalance that trigger more ROS generation. This imbalance became disastrous when the activity of G6PDH has been challenged by PHZ mediated oxidative stress (Fig. 4E) that was observed in our study. G6PDH deficiency has been reported before as an inherited disorder [98] that cause haemolysis of RBCs [99]. Here also, our study depicted a remarkable decrease in G6PDH activity in PHZ group that was prosperously ameliorated by OA (Fig. 4E).

Here, again redox factor NADPH plays a pivotal role as GR utilize NADPH as their cofactor, generated during the action of G6PDH enzyme of Pentose Phosphate Pathway (PPP). In our study, G6PDH exhibited a decline in its activity (Fig. 4E) and thus a marked decrease in intracellular level of redox factor NADPH in PHZ group, proposing itself as major causative factor behind diminished activity of GR (Fig. 2B). However, the activities of both enzymes have found to be well protected in OA co- treated group (Figs. 4E and 2 B) at a concentration of 0.06 nM confirming the conviction of antioxidative properties of OA.

Morphology of red blood cells is a reflection of its function as deformability is an indispensable criterion of circulating erythrocytes [100]. Hence, atomic force microscopic (AFM) imaging and flow cytometric techniques were exploited in our study to investigate alterations of RBC morphology. Atomic force microscopy, which is a suitable method for determining surface smoothness, found worthy to inspect the deformability of red blood cells [101], a necessity for properly functioned erythrocytes. In our study, the AFM images exhibit spicule formation in PHZ treated group of erythrocytes while OA efficiently protect the surface and thus the texture of RBCs (Fig. 7A), showing its ability either to preserve deformability of RBCs or to restore the morphological status of erythrocytes. The RBC average roughness has been found to be decreased significantly in PHZ group which has been preserved in OA co- treated group similar to control group of RBCs (Fig. 7C). In flow cytometric analysis also, no damaging effects of OA has been evidenced while the presence of two distinct populations have been observed in OA and PHZ + OA co- treated group which is a contemplation of bi-concave shape of RBC (Fig. 8A) [61]. A complete destruction of this bi-concave shape was observed in PHZ treated group showing presence of a single population (Fig. 8B) indicating the occurrence of haemolysis of RBCs



NAME	POPULATION	% TOTAL
Control	P2	77.79
Control	P3	6.08
PHZ	P2	77.95
PHZ	P3	0.53
OA0.06	P2	80.89
OA0.06	P3	4.67
PHZ+OA 0.06	P2	77.39
PHZ+OA 0.06	P3	9.11

Fig. 8. Prevention of PHZ induced disassembly of RBC bi-concave shape by OA (A) Flow cytometric analysis of alterations in erythrocyte shape treated with OA (0.06 nM) and/or, PHZ (1 mM). Tabular representation of the % Total in 2 different populations (P2 and P3) of different groups of RBCs studied.

leading to destruction of its bi-concave shape. These morphological alterations hamper the major functions of erythrocytes thus jeopardizing the entire circulatory system.

In vitro study allows a substance to be studied safely without subjecting humans or animals to the possible side effects of toxicity of a new drug. Thus, the current *in vitro* study enlightens the protective ability of OA against PHZ toxicity induced functional and structural alterations of erythrocytes and such protection appears to be afforded through its antioxidative mechanisms. Moreover, the results of this study projects OA as a possible future therapeutic to combat deleterious situations arising due to PHZ toxicity which need further experimented authentication in other affected organs *in vivo* and *in vitro*. The outcome of the current study now prompts us to go in for *in vivo* studies in the coming days for rapid development of therapeutic procedures applicable to human situations.

5. Conclusion

Structural and functional damage to RBCs have been documented along with profound ROS generation following incubation of goat erythrocytes with PHZ. Oleic acid has been shown to provide protection against such deleterious changes. The total ROS, superoxide anion free radical and hydroxyl radical contents have been significantly abated by OA. The current study appears to have the strength of projecting this low molecular weight MUFA singly or the diet enriched in this fatty acid as a possible future therapeutic to combat deleterious situations associated with haemolytic conditions in humans.

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Author's contributions

AB performed the experiments, analyzed all data and contributed in preparation of figures and drafting the manuscript. TD contributed in performing experiments and editing the manuscript. Dr. AKG assisted in carrying out some experiments and SM contributed in pursuing FACS experiment of our study. Dr. DB and Dr. AC contributed in conception, critical review of the data and editing the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

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