Research Article

Melatonin chelates iron and binds directly with phenylhydrazine to provide protection against phenylhydrazine induced oxidative damage in red blood cells along with its antioxidant mechanisms: an *in vitro* study

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ABSTRACT

Oxidative stress is an important causative factor for a number of diseases. Phenylhydrazine (PHZ) is a widely accepted model for studying hemolytic anemia by induction of oxidative stress. In the present study, goat red blood cells (RBCs) were incubated *in vitro* with PHZ (1mM) to generate oxidative stress. To test whether melatonin exhibits protective effects on PHZ induced RBC damage and to explore its potential molecular mechanisms, different concentrations of melatonin (5, 10, 20 and 40 nmoles/ml) were also included. PHZ caused altered profiles on biomarkers of oxidative stress and antioxidative as well as glucose metabolic enzymes in RBCs. These alterations indicated a development of oxidative stress. Melatonin at a concentration of 40 nmoles/ml provided optimal protection against all alterations induced by PHZ. The important cellular membrane proteins, including spectrin and actin, were also damaged by PHZ and this led to RBC deformation similar to that of observed in severe β-thalassaemia; the RBC deformation was also prevented by melatonin. Binding profiles of melatonin with PHZ and ferrous iron indicated favorable binding of melatonin with both of them, respectively. Thus, in addition to the direct antioxidant and free radical scavenging capability, melatonin also inhibited iron overloading by chelating iron and binding with the PHZ. This action of melatonin further reduces free radical generation. Based on the results, melatonin may provide therapeutic relevance to ß-thalassemia and other hemolytic RBC disorders involving oxidative stress.

Keywords: Melatonin, Antioxidant, Oxidative stress, Phenylhydrazine (PHZ), red blood cells (RBCs)

1. INTRODUCTION

Red blood cells are one of the most susceptible biological tissues to oxidative stress due to the presence of both high concentration of polyunsaturated fatty acids (PUFA) in the membrane and the oxygen transport associated redox active haemoglobin (Hb), which are targets of reactive oxygen species (ROS). PHZ, one of the most potent carcinogens belonging to the hydrazine family, is an oxidative agent which usually is used to generate gross hemolysis (1) by triggering oxidation of Hb into hemichromes and to form free radicals (2, 3). PHZ-induced free iron release (4), followed by free radical generation and oxidative stress contribute to PHZ's toxicity (5). Treatment of RBCs with PHZ produces features that are characteristic of phenotypes observed in severe β-thalassaemia (6), i.e., rigidly and mechanically unstable membranes in selective conjunction with oxidized β-globin chains connecting to the membrane skeleton. Thus, PHZ has frequently served as an *in vitro* model for β-thalassemic RBC phenotype (6).

Thalassaemia is a congenital haemoglobinopathy, arising from an imbalanced synthesis of either the alpha (α-thalassaemia) or the beta (β-thalassaemia) globin chain. This disorder is characterized by morphological and functional RBC anomalies, leading to chronic anaemia.

Melatonin, a pineal indoleamine, has extensively been studied for its free radical scavenging and antioxidant properties. Melatonin has also been reported to stimulate cellular antioxidant enzymes and resist the deleterious effects of oxidative stress mediated cell damage (7-11).

The aim of the present study is to elucidate the underlying mechanism of protection provided by melatonin against PHZ induced oxidative damage to RBCs, this knowledge may be of immense therapeutic value for the treatment of β thalassemia. This study clearly demonstrates that melatonin quenches the released iron from PHZ and directly interacts with PHZ thereby preventing further generation of ROS. Apart from melatonin's established role as an antioxidant (7) this property of melatonin is presumed to play a significant role in providing protection to RBCs against PHZ induced oxidative damage.

2. MATERIALS AND METHODS

2.1 Chemicals used

Melatonin and all other chemicals, solvents are of analytical grade and were obtained from Sisco Research Laboratories (SRL), Mumbai, India, Qualigens (India/Germany), SD fine chemicals (India), Merck Limited, Delhi, India.

2.2 RBCs collection from goat blood

Goat blood was carefully collected from slaughter house sanctioned by local Kolkata Corporation in citric acid-dextrose buffer. Packed cells (RBCs) were obtained by centrifugation at 3000 rpm for 10 minutes at 4^{o}C. The plasma (supernatant) and the buffy coat were removed by aspiration and the whole RBCs, the packed cells, were washed thrice with 0.9% NaCl solution.

2.3 *In vitro* incubation of whole RBCs with PHZ and melatonin

Five hundred µl of whole RBCs were incubated with 50 mM sodium phosphate buffer (pH 7.4) containing 1mM PHZ and/or four different concentrations of melatonin (5, 10, 20 and 40 nmoles/ml) at 37^{o}C in a shaking water bath for 1 hour. The incubation was terminated by addition

of 100µl of 16 mM EDTA. PHZ treated RBCs were washed thrice with 0.9% NaCl solution prior to lysis and from that the RBC membrane was prepared.

2.4 Determination of the optimally effective concentration of melatonin against PHZ induced oxidative stress in RBCs *in vitro*

The RBCs were incubated with PHZ (1 mM) and/or different concentrations of melatonin. The treated groups are as follows: Group I: Control (CON) without any treatment; Group II: PHZ treated group (PHZ); Group III: PHZ + Melatonin (5 nmoles/ml) (PM5); Group IV: PHZ + Melatonin (10 nmoles/ml) (PM10); Group V: PHZ + Melatonin (20 nmoles/ml) (PM20); Group VI: PHZ + Melatonin (40 nmoles/ml) (PM40).

After incubation, the biomarkers of oxidative stress, i.e., the lipid peroxidation level (LPO), reduced glutathione content (GSH) and protein carbonyl content (PCO) were detected in RBCs, along with the activities of antioxidant enzymes of superoxide dismutase (SOD) and catalase (CAT).

Based on the study results, the optimal concentration of melatonin against PHZ induced oxidative stress in RBCs was selected for the subsequent studies. Thus, the groups of subsequent studies were as follows: Group I: Control (CON)without any treatment; Group II: treated with the selected melatonin concentration40 nmoles/ml) alone (M40); Groups III: PHZ treated (PHZ); Group IV: PHZ + melatonin (40 nmoles/ml.) (PM40).

2.5 Preparation of hemolysate from incubated whole RBCs

After lysis of the washed RBCs in distilled water, the suspension was centrifuged at 7000 rpm for 25 minutes at 4°C. The supernatants were collected in separate tubes, and stored at –20°C to be used later for determining the enzymatic activities of antioxidant enzymes including SOD, CAT, glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferase (GST).

2.6 Preparation of RBC membrane

Hb-free RBC membrane (either control or treated) was prepared according to the method of Arduini *et al*. (12). The washed RBCs were allowed for hypotonic lysis in 5 mM sodium phosphate buffer (pH 8.0) and centrifuged at 14,000 rpm for 20 min at 4°C. The supernatant was discarded and pellet was re-suspended and washed at least five times in the same buffer until a colorless pellet was obtained. The ghosts of RBC were suspended in the same buffer and stored at –20°C for future use.

2.7 Measurement of biomarkers of oxidative stress in RBCs

2.7.1 LPO measurement.

The level of LPO in RBCs was measured in terms of thiobarbituric acid reactive substances (TBARS) using the method of Buege and Aust (13). Two ml of TBA-TCA-HCl reagent (15% TCA, 0.375% TBA and 0.25N HCl) was added to the incubation mixture and heated for 20 minutes at 80ºC. and the absorbance of the sample was determined at 532nm.

2.7.2 GSH measurement.

The GSH content in RBCs and in their membrane were determined spectrophotometrically at 412nm using 5,5'- dithio-bis-(2-nitro benzoic acid) (DTNB) according to the method of Sedlak and Lindsay (14).

2.7.3 Protein carbonyl content (PCO) measurement.

The PCO content of RBCs was estimated by DNPH assay (15). The sample was incubated with 10 mM DNPH for 45 minutes in the dark. At the end of incubation, 20% TCA was added and the mixture was centrifuged at 7000 rpm for 15 minutes; the supernatant was discarded; the pellets was re-suspended carefully with ethanol : ethyl acetate mixture (1:1) thrice. Then, 6 M guanidine hydrochloride and 0.5 M potassium dihydrogen phosphate (pH 2.5) was added to the washed pellets, mixed thoroughly and centrifuged at 7000 rpm for 15 minutes. The supernatant was collected and the absorbance was determined spectrophotometrically at 375nm.

2.8 Measurement of the levels of GSSG and GSSG:GSH ratio

The GSSG (oxidized glutathione) content of RBCs was measured by the method of Sedlak and Lindsay (14). The reaction mixture contained 0.1 mM sodium phosphate buffer, EDTA (1mM), NADPH (0.3mM) and GR 0.14 units per ml. The absorbance was measured at 340 nm using a UV-VIS spectrophotometer. The values were expressed as GSSG nmoles/mg protein. The GSSG:GSH ratio was calculated using respective standard graph.

2.9 Measurement of the activities of antioxidant enzymes

The activity of Cu-Zn superoxide dismutase (SOD1) was measured by pyrogallol autooxidation method of Marklund and Marklund (16). To extract SOD1, the Hb present in the hemolysate was removed according to the method of McCord and Fridovich (17). The hemolysate was pre-warmed at 37ºC and treated with ethanol-chloroform (2:1, v/v) and mixed thoroughly to obtain a thick precipitate. Then the distilled water was added, mixed and was incubated again at 37ºC for 15 minutes with occasional stirring followed by centrifugation. The colorless supernatant was used for measurement of SOD1 activity. The activity of CAT was measured spectrophotometrically by following the rate of decomposition of H_2O_2 at 240 nm by the method of Beers and Sizer (18).

2.10 Determination of the activities of GPx, GR, GST)

GPx activity was measured according to the method of Paglia and Valentine (19). The assay system contained 0.05 M phosphate buffer with 2 mM EDTA, pH 7.0, 0.025 mM sodium azide, 0.15 mM glutathione and 0.25 mM NADPH (total reaction volume 1 ml). The reaction was started by the addition of 0.36 mM H_2O_2 into the system. The linear decrease in absorbance at 340 nm was recorded using a UV/VIS spectrophotometer and the specific activity was expressed as nmoles of NADPH produced/min/mg protein.

GR activity was measured according to the method of Krohne-Ehrich *et al*. (20). The assay mixture contained 50 mM phosphate buffer, 200 mM KCl, 1 mM EDTA and water (final volume 3 ml). The blank was set with this mixture. Then 0.1 mM NADPH was added along with suitable amount of sample (as the source of enzyme) into the cuvette. The reaction was initiated with 1 mM GSSG application. The decrease in NADPH absorption was monitored

spectrophotometrically at 340 nm and specific activity of the enzyme was expressed as units/min/mg tissue protein.

GST activity of RBCs was measured spectrophotometrically according to Habig *et al*. (21). The enzymatic reaction was measured by observing the conjugation of 1-chloro, 2,4-dinitrobenzene (CDNB) with GSH, i.e. 1 unit of enzyme will conjugate to 10.0 nmol of CDNB with GSH per minute at 25 °C. The rate where the reaction was linear was noted spectrophotometrically at 340 nm. The molar extinction of CDNB was 0.0096 μM^{-1}/cm and the enzyme activity was expressed as units/min/mg of protein.

2.11 Evaluation of oxidative damage of RBCs membrane proteins by SDS-PAGE

Electrophoresis was carried out according to the method of Laemmli (22) by using polyacrylamide gel. Both the treated and untreated membrane preparations containing the same amount of protein were solubilized by boiling them for 5 min in SDS sample buffer containing 1% SDS, 10% (v/v) glycerol, 63 mM Tris-HCl (pH 6.5) and 1% β-mercaptoethanol. Equal amounts of membrane proteins (50 µg) were loaded on the gel lanes and allowed them to run. Then the gels were fixed and stained with 0.1% Coomassie blue R-250. Densitometric analysis was used to detect the levels of the membrane proteins.

2.12 Measurement of the activities of glucose-6-phosphate dehydrogenase (G6PDH), hexokinase, phosphofructokinase, aldolase and lactate dehydrogenase (LDH) in RBCs

G6PDH activity was determined in hemolysate of RBCs by measuring the increase in absorbance at 340 nm. The reaction mixture containing 0.006 M NADP and 0.1 M glucose-6-phosphate in 2.7 ml of 0.055 M Tris HCl buffer, pH7.8 with 0.0033M $MgCl_2$ was individually incubated for 7-8 minutes to reach temperature equilibrium and then hemolysate was added. The change in absorbance was recorded at 340 nm for 5 minutes (23).

The activity of hexokinase was determined from RBC hemolysate by using 0.67 M glucose as the substrate, 0.05 M Tris $MgCl_2$ buffer (pH 8), 16.5mM ATP, 6.8mM NAD and G6PDH (300 IU/ml of Tris $MgCl_2$ buffer). The change in absorbance was observed at 340 nm (24).

The activity of phosphofructokinase was measured at 25° in a coupling system of the oxidation of NADH with a spectrophotometer. The assay medium consisted of 1 ml of 50 mM Tris-HCl, pH 8 along with 1 mM fructose-6-P, 1 mM ATP, 1 mM $MgS0_4$, 7 mm cysteine, 0.25 mM NADH, 0.5 unit of muscle aldolase, 1.2 units of triose phosphate isomerase, and 0.3 unit of a-glycerophosphate dehydrogenase. The change in absorbance was observed at 340 nm (25).

The activity of aldolase was measured in hemolysate based on the fact that 3-phosphoglyceraldehyde reacts with hydrazine to form a hydrazone which absorbs at 420 nm. The reagents used were 0.012 M fructose-bis-phosphate, 0.1 mM EDTA, 0.0035 M hydrazine sulphate, pH 7.5 and the hemolysate as the source of the enzyme. The change in absorbance was recorded at 240 nm for 10 minutes (26).

The activity of lactate dehydrogenase was analyzed by measuring the oxidation of NADH 0.1 mM to NAD^+ at 340 nm using 1.0 mM sodium pyruvate as substrate, according to the method Dhanesha *et al.* (27). The enzyme activity was expressed as IU/L.

To determine whether the PHZ induced alterations in the activities of CAT,GR, G6PDH and hexokinase were reversible or not, Lineweaver Burk Reciprocal Plot was plotted with the concentration kinetics of each of these enzymes from four group (Control, M40, PHZ and PM40) by using different concentrations of the respective substrates namely hydrogen peroxide for CAT (52.63, 25.32, 13.33 and 5.4 µM), oxidized glutathione for GR (0.08, 0.04, 0.02, 0.01 µM),

glucose-6-phosphate for G6PDH (6.66, 3.33, 1.67 and 0.83 µM) and D-glucose for hexokinase (0.14, 0.07, 0.04 and 0.02 µM) respectively.

2.13 Morphological studies of RBCs

2.13.1 Scanning Electron Microscopy

Control and treated groups of RBCs were fixed using 3 % glutaraldehyde and the images were captured using a Scanning Electron Microscope available at CRNN facility of University of Calcutta (28).

2.13.2 Flow cytometric analysis

The data on morphological changes of RBCs were obtained via flow cytometry and a total of 30,000 events were recorded for each sample. The FSC data were collected on a linear scale, whereas for SSC data log scale was used. The height, width and area parameter of the voltage pulse of both FSC and SSC were recorded. On the BD FACS Aria III available at CRNN facility of University of Calcutta, an 85 µm nozzle was used and the custom sheath pressure was set at 14 psi with a minimum sample flow rate of 1ml/minute. Flow cytometry data, in term of (i) area, (ii) height and (iii) width distribution of both FSC and SSC were extracted from the raw experimental data of all events with the help of Flow Diva software (version 6.1.3). Software output also includes basic statistics (mean, median, standard deviation, coefficient of variance, etc.) of the respective distribution (29).

2.14 Estimation of intracellular iron content of RBCs by atomic absorption spectrophotometry (AAS):

The iron content of the RBCs was determined by AAS as per the protocol mentioned in the cook book of the Sophisticated Analytical Instrument Facilities (SAIF) and "Thermo Scientific iCE 3000 Series Atomic Absorption Spectrometer'' available at the Bose Institute, Kolkata. After *in vitro* incubation of the RBCs, the cells were centrifuged at 3000 rpm for 10 minutes. The supernatant was collected in a conical flask. Concentrated nitric acid was then carefully added to it and the conical flask with its contents was placed on the hot plate and heated at 65–70^{o}C for digestion of the RBCs. The contents of the conical flasks were then quantitatively transferred into 25 ml volumetric flasks, and, finally the volume was made up to 25 ml with double distilled water. The iron content of the samples was then measured using AAS (30, 31).

2.15 Binding study between melatonin and PHZ; melatonin and ferrous iron

Whether melatonin binds to PHZ or to ferrous iron was respectively analyzed by isothermal titration calorimetry (ITC) (32) using Microcal ITC-200, Malvern. In this assay, 0.35 ml melatonin (at 40 nmoles/ml concentration) was titrated with 0.06 ml of 1 mM PHZ in the sample cell. Titration was conducted with twenty injections each with 2 µl volume with a 150 sec spacing between two injections for approximately 1 hour.

In a separate assay, 0.35 ml melatonin (at 40 nmoles/ml concentration) was titrated with 0.06 ml of 2 mM ferrous iron in the sample cell. Titration was conducted with twenty injections each with 2 µl volume with a150 sec spacing between two injections for approximately 1 hour.

2.16 Estimation of protein content

Protein content of RBCs, packed cell suspensions and hemolysates were determined according to the method of Lowry (33). The protein content of RBC membranes was determined according to the method of Bradford (34).

2.17 Statistical analysis

Data are presented as means ± SEMs. Significance of mean values among the treatment groups was analyzed using one way ANOVA after ascertaining the homogeneity of variances among the treatments. Comparisons were done by calculating the least significance between groups using t test. Statistical tests were performed using Microcal Origin version 7.0 for Windows.

3. RESULTS

3.1 Effects of melatonin on biomarkers of oxidative stress

3.1.1 LPO level

The level of LPO (Figure 1A) was found to be significantly increased in RBCs with PHZ incubation (39.1% increase vs. control, *: $P \leq 0.001$). However, this PHZ(1mM) induced LPO increase was inhibited when they were co-incubated with melatonin in a concentration dependent manner (*: $P \leq 0.001$), indicating the ability of melatonin to protect the RBCs against oxidative stress-induced changes for lipid peroxidation. Melatonin exhibited optimal protection at the concentration of 40 nmoles/ml which was the highest concentration tested in the current study (27.6% decrease vs. PHZ *: $p < 0.001$) as seen in figure 1A.

3.1.2 GSH content

A significant decrease of GSH content was observed in RBCs incubated with PHZ (1mM) as compared to control (53.1%; decrease vs. control *: $P \leq 0.001$) which is shown in Fig. 1B. However; this decrease was significantly inhibited by melatonin in a concentration dependent manner. Melatonin exhibited optimal protection at a concentration of 40 nmoles/ml (Fig 1B) (114 % increase vs. PHZ *: $P \leq 0.001$).

3.1.3 PCO content

The PCO content showed a significant increase in RBCs incubated with PHZ as compared to control (Figure1C) (42.2 % increase vs. control *: $P \leq 0.001$). However, this increase in PCO content was significantly inhibited when they were co-incubated with melatonin in a concentration dependent manner. The optimal protection of melatonin was found at 40 nmoles/ml (Fig.1C.) (28.8% decrease vs. PHZ *: $P \leq 0.001$).

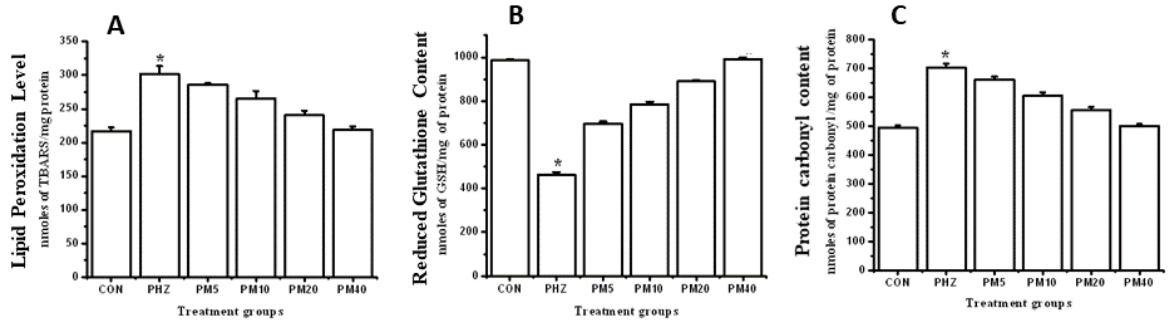

Fig. 1. Effect of melatonin on PHZ induced oxidative stress biomarkers in RBCs

*A: LPO, B: GSH and C: PCO. CON: Control; PHZ: Phenylhydrazine; PM5, PM10, PM20, PM40: PHZ + melatonin 5, 10, 20 and 40 nmoles/ml, respectively. The values are expressed as Means ± SEs (n = 6), *: P ≤ 0.001 compared to other groups.*

3.2 Effects of melatonin on GSH, GSSG and GSSG: GSH ratio

Fig, 2A, B and C showed the significant decrease in GSH content, increases in GSSG level as well as in GSSG:GSH ratio in RBCs treated with 1mM PHZ (54% reduction, 49.5 and 223% increases vs. control group, respectively; *: P ≤ 0.001). Melatonin treatment at 40 nmoles/ml completely reversed these alterations induced by PHZ to the level of control (Fig. 2) However, melatonin alone had no significant effect on the levels of GSH, GSSG and GSSG: GSH ratio in RBCs.

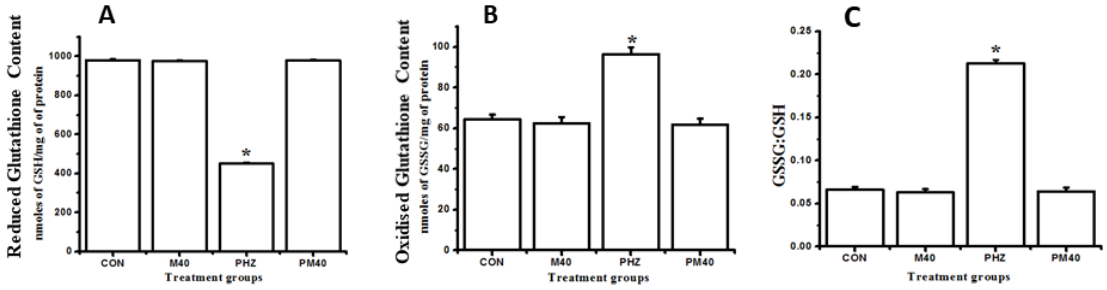

Fig 2. Effect of melatonin on PHZ-induced alteration in the levels of GSH, GSSG and GSSG:GSH ratio in RBCs

*A: GSH, B: GSSG and C: GSSG:GSH ratio in RBCs. CON: Control; PHZ: Phenylhydrazine; M40: Melatonin (40 nmols/ml); PM40: Phenylhydrazine + Melatonin (40 nmols/ml). The values are expressed as Means ± SEMs (n = 6), *: P ≤ 0.001 compared to other groups.*

3.3 Effects of melatonin on the activities and kinetics of antioxidant enzymes of Cu-Zn SOD, CAT, GPx, GR and GST

The activity of Cu-Zn-SOD was significantly increased in RBCs after treatment with PHZ (80.9 % increase vs. control, *:P ≤ 0.001) and was recovered when the RBCs were co-incubated with melatonin at a concentration dependent manner (Fig. 3 A). The optimal protection of

melatonin occurred at a dose of 40nmoles/ml (44.8 % reduction vs PHZ (*: P ≤ 0.001). In contrast, CAT activity was significantly reduced with PHZ(1mM) (91.5% decrease vs. control, *: P ≤ 0.001). Melatonin treatment inhibited this decrease in a concentration dependent manner. Melatonin at the concentration of 40 nmoles/ml exhibited its optimal protection on CAT activity (113% increase vs PHZ, *: P ≤ 0.001) (Fig. 3B).

The Lineweaver Burk Double Reciprocal Plot (LBDR) indicated a decline in V_{max} and an unaltered K_m of the CAT activity in RBCs with PHZ compared to control. However, these alterations were completely reversed by melatonin (40nmoles/ml) (Fig.3C) The significant protective effects of melatonin on impeded activities of GPx, GR and GST induced by PHZ were observed in Fig. 3 D, E, F and G, respectively.

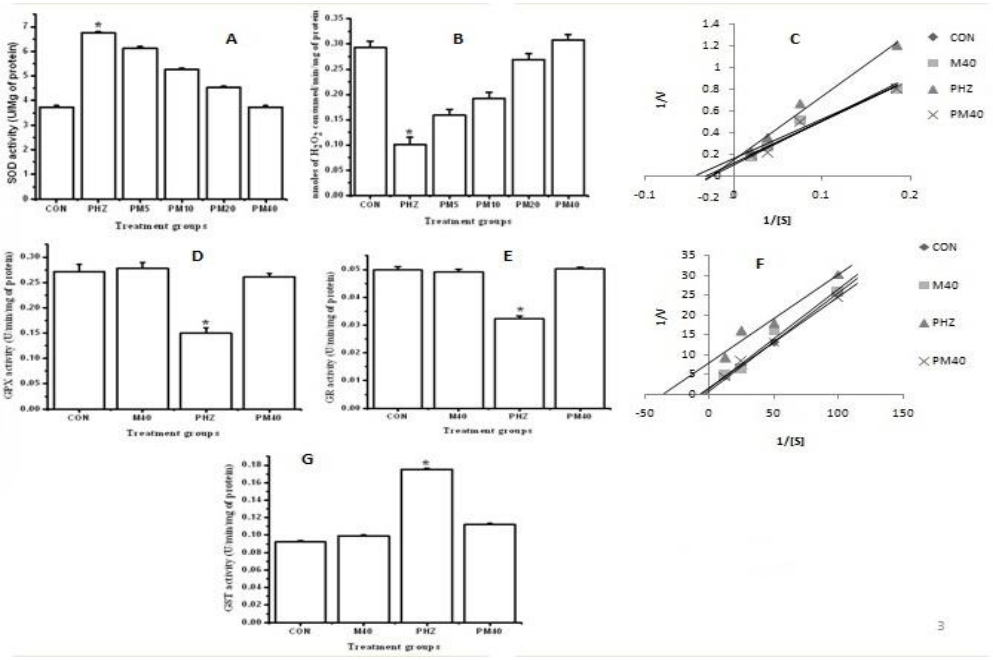

Fig. 3. Effects of melatonin on PHZ induced alterations in the activities and kinetics of SOD, CAT, GPX, GR, GST

*A: SOD activity, B: CAT activity and C: Lineweaver-Burk plot (LBP) of CAT activity. D: GPX activity, E: GR activity, F: LBP of GR activity, G: GST activity. K_m values were calculated from LBP curve using the straight-linee equation. For CAT, y = mx+ c. V_{max} : Con = 9.26 mM/min, M40 = 6.28 mM/min, PHZ = 6.66 mM/min, PM40 = 8 mM/min; K_m : Con = 37.34 mM, M40 = 22.91 mM/min, PHZ = 39.05 mM/min, PM40 = 30.41 mM/min.: For GR, y = mx+c. V_{max} : Con = 0.263 mM/min, M40 = 0.68 mM/min, PHZ = 0.128 mM/min, PM40 = 0.14 mM/min; K_m: Con = 1.076 mM, M40=0.168 mM/min, PHZ = 0.028 mM/min, PM40 = 0.607 mM/min, CON: Control; PHZ: Phenylhydrazine; M40: Melatonin (40 nmoles/ml): PM40: Phenylhydrazine + Melatonin (40 nmoles/ml). The values are expressed as Means ± SEMs (n = 6), *: P ≤ 0.001 compared to other groups.*

3.4 Effects of melatonin on RBC membrane proteins

The intensity of all major cytoskeletal protein bands was significantly reduced by PHZ treatment (lane 3 vs other lanes; Fig. 4A). Melatonin at 40nmoles/ml partially recovered spectrin

band and completely recovered other four protein bands which were reduced by PHZ in the RBCs (Fig. 4A, lane 4). These observations were confirmed when the protein gels were scanned with a Versadoc Ultrascan Laser Densitometer for protein profile (Fig. 4B). The scan showed a reduction of spectrin band along with bands 3, 4.1, 4.2 and actin under PHZ treatment. Most of the bands on the gel corresponding to different membrane proteins also showed different degrees of decrease in intensity with PHZ treatment. The bands 4.1 and 4.2 (Fig. 4B) showed a considerable decrease which indicated their susceptibility to degradation under oxidative damage. However; when the RBCs were co-treated with melatonin (40nmoles/ml) and PHZ (1mM), it conferred almost complete protection to all the protein bands in the gels.

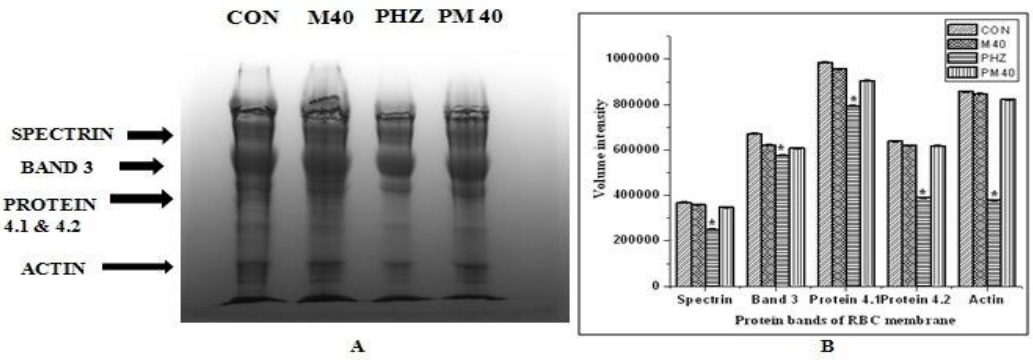

Fig. 4. Effects of melatonin on RBC membrane protein intensity

*The images of the RBC membrane protein intensity were showed in 4A. Identical amounts of protein were applied to each lane (50µg). Lane 1: control (CON); lane 2: Melatonin (40nmoles/ml) (M40); Lane 3: Phenylhydrazine (1mM).(PHZ); Lane4.:Phenylhydrazine + Melatonin (40nmoles/ml) (PM40); 4B: Densitometric scan of membrane proteins of RBCs staining with Coomassie Blue. CON: Control; M40: Melatonin (40 nmoles/ml); PHZ: Phenylhydrazine (1mM); PM40: Phenylhydrazine (1mM) + Melatonin (40 nmoles/ml); the values are expressed as Means ± SEMs (n = 3), *: P ≤ 0.001 compared to other groups.*

3.5 Effects of melatonin on the activities of glucose metabolic enzymes of G6PDH, hexokinase, phosphofructokinase, aldolase and lactate dehydrogenase (LDH)

The activities of G6PDH, hexokinase, phosphofructokinase and aldolase were reduced by 98.09, 48.68, 22.86 and 60.56% respectively, with PHZ treatment compared to the control (*: P ≤ 0.001). In contrast the activity of LDH increased 50% after treatment vs control (*:P ≤ 0.001). These alterations of enzyme activities were completely preserved by melatonin treatment (40nmoles/ml) (Fig. 5A). The kinetics of G6PDH, i.e., V_{max} and K_m, were analyzed using LBDR plot. It indicated that the V_{max} of G6PDH was significantly decreased; however, the K_m was not influenced by PHZ treatment. Melatonin treatment preserved the V_{max} of G6PDH of RBCs (Fig. 5B). The results also showed that both the V_{max} and K_m of hexokinase were significantly reduced by PHZ treatment and again, melatonin treatment completely reversed theses alterations caused by the PHZ (Fig. 5C). Melatonin alone did not modify these parameters (Fig. 5B and C).

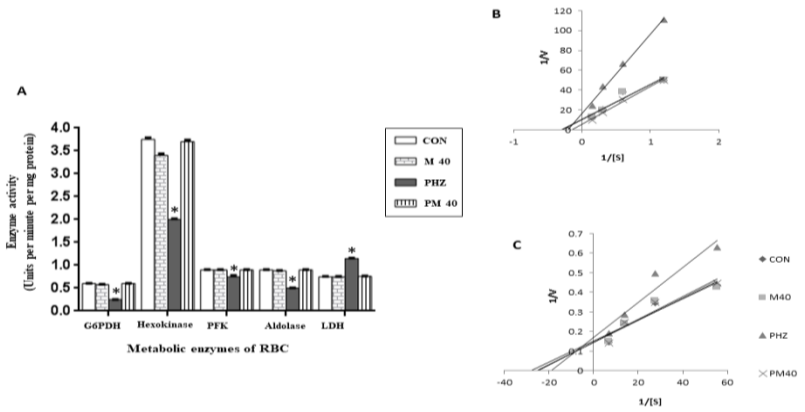

Fig. 5: Effects of melatonin on impeded activities of G6PDH, hexokinase, aldolase, phosphofructokinase (PFK) and LDH (A) as well as the kinetics of G6PDH (B) and hexokinase (C)

*The K_m values were calculated from LBP curves using the straight-line equation. For G6PDH, the y = mx+c. V_{max}: Con= 0.093 mM/min, M40 = 0.1 mM/min, PHZ = 0.061 mM/min, PM40 = 0.182 mM/min; K_m: Con = 3.26 mM, M40=3.57 mM/min, PHZ = 4.81 mM/min, PM40 = 6.82 mM/min. For hexokinase, y = mx+c. V_{max}: Con = 6.9 mM/min, M40 = 6.6 mM/min, PHZ = 5.9 mM/min, PM40 = 6.9 mM/min; K_m: Con = 0.035 mM, M40=0.033 mM/min, PHZ = 0.047 mM/min, PM40 = 0.035 mM/min. CON: Control; M40: Melatonin (40 nmoles/ml); PHZ: Phenylhydrazine (1 mM); PM40: Phenylhydrazine (1 mM) + Melatonin (40 nmoles/ml). The values are expressed as Means ± SEMs (n = 6), *: P ≤ 0.001 compared other groups.*

3.6 Effects of melatonin on the morphology of RBCs:

3.6.1 The results of scanning electron microscopy (SEM) on morphology of RBCs

The morphology of RBCs was assessed using SEM and the results were shown in Fig. 6. Untreated RBCs appeared as typical discocytes while RBCs exposed to PHZ (1mM) exhibited significant change in the cell shape with distinct echinocyte formation. The morphological changes induced by PHZ (1mM) were greatly prevented when the cells were co-incubated with melatonin (40nmoles / ml).

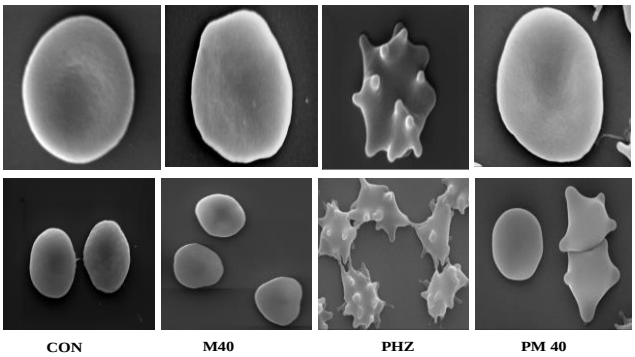

Fig. 6. Effects of melatonin on morphological alterations of RBCs induced by PHZ

Upper panel (60 KX) and lower panel (35 KX). CON: Control; M40: Melatonin (40 nmoles/ml); PHZ: Phenylhydrazine (1 mM); PM40: Phenylhydrazine (1 mM) + Melatonin (40 nmoles/ml).

3.6.2 Effects of melatonin on the results of flow cytometric analysis in RBCs

The results of flow cytometric analysis showed that PHZ treatment significantly decreased rCV of the side scatter area (SSC-A), side scatter height (SSC-H) and side scatter width (SSC-W) by 10.94, 12.73 and 38.04%, respectively compared to the control RBCs (*: $p \leq .0.001$).

Melatonin alone did not influence these parameters. However; when the RBCs were co-incubated with PHZ plus melatonin, the rCV of SSC–A, SSC-H and SSC-W were enhanced by 10.26 ,12.22 and 51.93% respectively; compared to PHZ group (*: $p \leq 0.001$) and there were no significant difference with the control group (Fig. 7). In addition, the substantial declines for the rCV of forward scatter area (FSC-A) and forward scatter height (FSC-H) were recorded in the RBCs treated with PHZ with the 54.69 and 57.67% decreases, respectively, compared to the control (*: $p \leq 0.001$). These declines were completely reversed by melatonin addition (Fig. 7). No significant changes were recorded regarding the rCV of forward scatter width (FSC-W) in the RBCs among the groups.

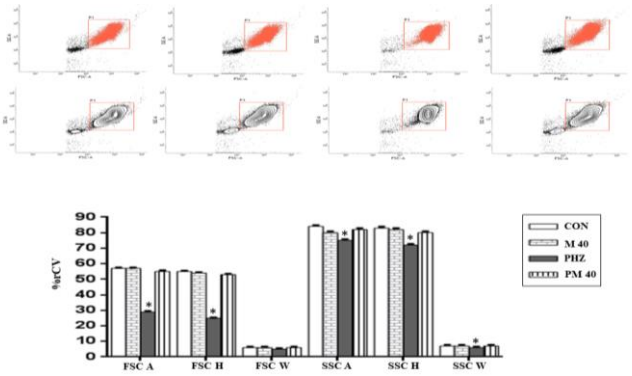

Fig. 7. Effects of melatonin on the parameters of flow cytometric scattering on RBCs .

*CON: Control; M40: Melatonin (40 nmoles/ml); PHZ: Phenylhydrazine (1 mM); PM40: Phenylhydrazine (1 mM) + Melatonin (40 nmoles). The values are expressed as Means ± SEMs (n = 6), *: P ≤ 0.001 compared to other groups.*

3.7 Effects of melatonin on intracellular iron content of RBCs.

The intracellular iron concentration of RBCs was reduced by 25.93% after PHZ treatment compared to level of control (*P≤0.001). Melatonin alone had no significant effect on the intracellular iron level in the RBCs. However; when the RBCs were co-incubated with melatonin (40nmoles/ml) and PHZ (1mM), This co-incubation completely blocked the intracellular iron level decline caused by the PHZ alone (Fig. 8A).

3.8 The interactions of melatonin with PHZ or with ferrous iron

The isothermal titration calorimetry **(**ITC) profile indicated that melatonin directly bound to PHZ at optimized temperature (Fig. 8 B1). The titration profile showed that this binding exhibited a non-saturating behavior. The data were best fitted by a nonlinear approach to the 'sequential binding site' The values obtained from the Chi-Square test indicated that this interaction might have 5 site binding sequential reactions. In each binding site the net energy change was negative. The evidence indicated that these bindings occur spontaneously. From this model it was assumed that PHZ could not saturate all the binding sites of melatonin. For example, once one PHZ

molecule binds with melatonin, it will open another site for binding. It seems that this reaction occurs continuously. Thermodynamic parameters of 40µM melatonin interaction with 2 mM ferrous iron (Fe^{2+}) was also determined by ITC. To minimize the effect of temperature variation that occurs during the injection, melatonin solution was taken in the calorimeter cell and Fe^{2+} was added through the injector syringe. ITC profiles showed that melatonin also bound to the Fe^{2+} at optimized temperature. The titration profile indicated that this binding exhibited a non-saturating behavior. The data were best fitted by a nonlinear approach to the 'sequential binding site' model. The values obtained from the Chi-Square test indicated that this interaction might have 5 site binding sequential reactions. In each binding site the net energy change was negative. The evidence indicated that these bindings occur spontaneously. From this model it was assumed that Fe^{2+}could not saturate all the binding sites of melatonin. For example, once a Fe^{2+} binds with melatonin, it will open another site for binding. It seems that this reaction occurs continuously.

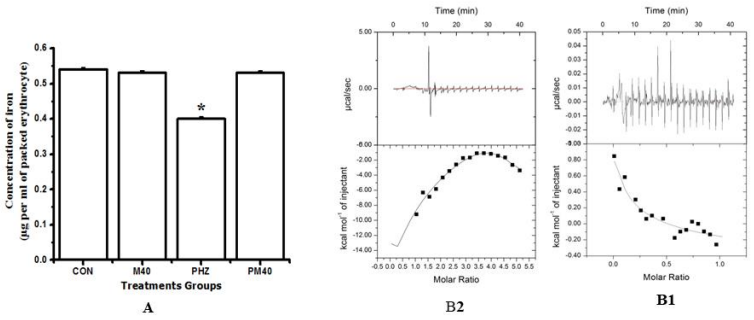

Fig. 8.Interactions of melatonin with PHZ and intracellular Fe^{2+}

*A**:** intracellular iron concentration; B 1: interaction between melatonin (40 nmoles/ml) with PHZ (1 mM); B 2: interaction between melatonin (40 nmoles/ml) with Fe^{2+} (2 mM)**;** CON: Control; PHZ: Phenylhydrazine (1 mM); M40: Melatonin (40 nmoles/ml); PM40: Phenylhydrazine (1 mM0 + Melatonin (40 nmoles/ml). The values are expressed as Means ± SEMs (n = 6), *: P ≤ 0.001 compared to other groups.*

4. DISCUSSION

Oxidative stress is thought to be the primary factor involved in the pathogenesis of a number of diseases with the macromolecular alterations due to the elevated production of ROS (35, 36). PHZ-induced damage to cellular membranes has been reported to strongly associate with oxidative stress (37, 38). Melatonin's remarkable ability to scavenge free radicals such as superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical (·OH) (39, 40) as well as its capability of stimulating various antioxidant enzymes, *in vivo*, including GPx SOD and CAT (41) have acquired great interest in the pharmacological properties of melatonin. In the current study, it was observed that the development of oxidative stress occurred when RBCs was incubated with PHZ. This was reflected by the alterations of biomarkers which are indicators of oxidative stress. A concentration dependent pattern of melatonin protective effects on these biomarkers were also observed an optimal concentration of melatonin on PHZ induced oxidative stress in RBCs was found to be 40nmoles/ml in this study. Previous studies have also documented that melatonin provides protection against lipid peroxidation, DNA and protein damage induced by ROS in various *in vivo* and *in vitro* studies (42, 43). Depletion of GSH content, a major cellular antioxidant, is a primary biomarker of oxidative stress and indicates the impeded cellular metabolic status of RBCs. Melatonin treatment restored the decreased GSH and GSH/GSSG ratio

caused by the PHZ and this indicated the antioxidant capability of melatonin to protect against PHZ toxicity.

It was well documented that, under the influence of PHZ the RBCs exhibited very similar pattern of membrane damage which was observed in G6PDH deficient RBCs under acute or chronic hemolysis, (44-46). Absence of mitochondria in RBCs forces the cell to be highly dependent on pathways related to production of high-energy compounds for example the anaerobic glycolytic (Embden-Meyerhof) pathway, which is also known as the hexose monophosphate shunt or the phosphogluconate pathway. In the present study, the enzymatic activities of glucose metabolic enzymes including G6PDH, hexokinase, PFK and aldolase were all inhibited by PHZ treatment (Fig. 5). The kinetic studies of the glucose metabolic enzymes revealed that treatment with PHZ caused a change in G6PDH turnover number, which was confirmed by an altered V_{max} value along with a significant increase of K_m. In addition, kinetics of another glucose metabolic enzyme, hexokinase, was also inhibited by PHZ and the inhibitory pattern was similar to that of G6PDH. The activities of antioxidant enzymes including CAT, GPx, were inhibited by the PHZ and this inhibition resulted in the increased ROS in RBCs. This is consistent with the previous report that PHZ enhanced the production of hydrogen peroxide (H_2O_2) in RBCs to a level which is beyond the detoxifying capability of GPx and CAT (47)**.** GPx converts H_2O_2 to water and hence plays an important role in cellular detoxification. Inhibition of GPx activity contributes to membrane lipid peroxidation and generation of oxidative stress. GST is a phase 2 detoxifying enzyme for drugs such as polycyclic aromatic hydrocarbons (PAHs) (48)**.** Interestingly, the activities of GST and SOD increased with PHZ treatment. This may be due to a cellular response to counteract the toxic effect of PHZ. The elevated SOD activity with consequential oxidative damage due to accumulating ROS has also been reported in other models of oxidative stress (49-51). Melatonin, at a concentration of 40 nmoles/ml successfully protected the impeded enzymatic activities caused by the PHZ as mentioned above and in most of the case, these enzymatic activities were recovered to nearly normal levels by melatonin (Fig. 3). This is a strong indicator of the free radical scavenging abilities of melatonin. Melatonin has been previously shown to possess a stimulatory effect on antioxidant enzymes such as MnSOD and CuZnSOD, CAT, GPx, GR and G6PDH (52).

The membrane skeletons of RBC in vertebrate are attached to the lipid bilayer through a class of proteins which are in turn bound to the integral membrane proteins. Ankyrin binds to band 3 with one of its domains and to spectrin with the other (53) Protein 4.1 is a member of this linking class and binds both spectrin and actin (54-56). Ankyrin and protein 4.1 interact with spectrin at different sites. Ankyrin binds to the β subunit of spectrin at a site close to the oligomer binding region (57), while protein 4.1 seems to be attached to the opposite end of spectrin, possibly in complex with actin oligomers (54-56). Protein 4.2 stabilizes the link between ankyrin and band 3. Incubation of RBCs with PHZ leads to precipitates of denatured Hb designated Heinz body protein (58). An important mechanism appears to form cross-linked spectrin via disulfide exchange with denatured Hb (58). Spectrin which, lines the intracellular side of the plasma membrane of RBCs, along with the bands 3 and actin, was significantly reduced by PHZ, thereby indicating a correlation between increased lipid peroxidation and degradation of cytoskeletal proteins. Spectrin damage causes the blebbing of cell membrane and ultimately leads to the death of the cell. Membrane proteins 4.1 and 4.2, actin and band 3 are important cytoskeletal proteins that maintain the shape and structure of RBCs were also damaged by PHZ. These resulted in the severe deformation of the RBCs by PHZ and this was visualized by the SEM images (Fig. 6). Oxidative damage to cell membrane leads to alterations in cell rigidity and shape. The oxidative

stress induced by PHZ leads to echinocyte formation and dysfunction; melatonin treatment effectively protected the cellular structures and cell membrane proteins from damage caused by PHZ.

The results from flow cytometric analysis are a strong indicator in morphological changes of RBCs induced by PHZ and the protective effects offered by melatonin. Forward scatter data depicts the cell size and side scatter shows the internal granularity of the particle/cell (i.e. number of cytoplasmic granules, membrane size), and thus cell populations can be characterized on the basis of differences in their size and density. Flow cytometric studies have revealed marked alterations in the morphology of RBCs following PHZ treatment. These changes were prevented by melatonin (40nmoles/ml).

The ITC profiles of melatonin with PHZ and ferrous iron indicated that melatonin could bind to both PHZ and ferrous iron. Binding to PHZ to lower its free level in RBCs limits its toxicity to the cells by melatonin. Interestingly, melatonin also binds with free Fe^{2+} which is released from hemolysis of RBCs. Fe^{2+} participates in ROS producing cascade reactions via Fenton reaction. Binding to free Fe^{2+} by melatonin interrupt this ROS production cascade. Melatonin and its metabolites have been reported to possess metal chelating ability (59) as we observed in the current study.

Based on the results of studies, it can be concluded that melatonin is able to reduce the toxic effects of PHZ, and affords protection to RBCs. This was indicated by that melatonin preserved various metabolic and cellular alterations caused by PHZ. The protective effects of melatonin are mainly attributed to its direct antioxidant abilities along with its quenching the free Fe^{2+}released from the oxidized Hb and binding to PHZ. The potential mechanism(s) by which melatonin protects RBCs against PHZ toxicity are illustrated in the Fig. 9.

PHZ induces similar features as observed in severe β-thalassaemia regarding to the RBC phenotypes and with the similar molecular mechanisms, i.e., the rigidly and mechanically unstable membranes of the RBCs conjugate with oxidized β-globin chains touched to membrane skeletons deforming RBCs. All of these injuries are effectively protected by melatonin. Thus, melatonin may have the therapeutic potential to ß-thalassemia, in particular, and also other hemolytic disorders involving oxidative stress, in general.

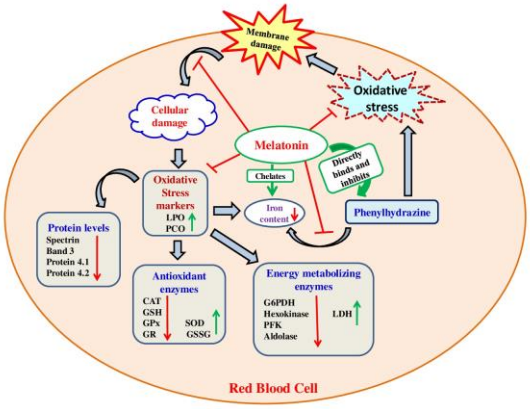

Fig. 9. The potential mechanisms of the protective effects of melatonin on PHZ induced RBC damages.

LPO: lipid peroxidation; PCO: protein carbonyl content; CAT: catalase; GSH: reduced glutathione; GSSG: oxidized glutathione; GPx: glutathione peroxidase; GR: glutathione

reductase; SOD: superoxide dismutase; G6PDH: glucose-6-phosphate dehydrogenase; LDH: lactate dehydrogenase; PFK: Phosphofructokinase.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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