In Vitro Effect of Cypermethrin on the Perturbations of Rat Erythrocytes: The Ameliorative Role of Carvone

Nadia A. Hamed [1], Eman M. Mosallam[1] and Reda K. Abdel-Razik [1]

ABSTRACT

Erythrocytes are a suitable model to study the membrane oxidative injury resulted by different xenobiotic. An attempt was made to study the antioxidant property of carvone (CAR), a monoterpene presents in herbs to mitigate toxicity of cypermethrin (CYP), a type II pyrethroid, in male rat erythrocytes *in vitro*. Erythrocytes were prepared and exposed to CYP (25-800 µM) alone and together with CAR (40 and 320 µM). Results showed superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities were significantly decreaseed. Lipid peroxidation (LPO) level, reduced glutathione (GSH) content, both of glutathione s-transfarase (GST), and glutathione reductase (GR) activities were significantly increased in comparison of control value. Scanning electron microscope (SEM) supported the toxic effect of CYP which presented various degrees of alteration in shape and ruptured membranes. Pre-treatment of erythrocytes with CAR induced alleviation in all the tested parameters. It can be recommended that CYP induced oxidative stress *in vitro* in rat erythrocytes and supplementation with CAR ameliorated these effects.

Key words: Cypermethrin, Carvone, Erythrocytes, Oxidative damage, antioxidant status, Scanning electron microscope.

INTRODUCTION

The use of pesticides in agriculture is bidirectional; lead to higher crop yields and also could have toxic effects on animals and humans as a non-target organisms of pesticide application. Synthetic pyrethroids are used in agricultural, veterinary and home formulations and account for almost 25% of the worldwide insecticide market (Shafer *et al.*, 2005). Although pyrethroids are rapidly replacing other insecticides due to relatively lower toxicity for mammals, the extensive and uncontrolled use have resulted in harmful effects (Arif *et al.*, 2020). Many studies (El-Demerdash, 2007; Sadowska-Woda *et al.*, 2010; Das *et al.*, 2016; Arif *et al*., 2020) reported the induction of oxidative stress by pyrethroids such as lambda-cyhalothrin, bifenthrin, cypermethrin, and bioallethrin. Cypermethrin (CYP) a type II pyrethroid pesticide is used widely for pest control in agriculture, household, and garden either alone or in combination (AL-Eed, 2007). It results in a protracted opening of the sodium channels in the central nervous system, which promotes hyper-depolarization and excessive nerve cell excitability. (Narahashi *et al.*, 1992). It also causes neurotoxicity by alteration gamma-aminobutyric acid level (Manna *et al.*, 2005).

Oxidative stress, an imbalance between the production of free radicals or reactive oxygen species (ROS) and the antioxidant defense system. Clemens and Waller (1987) reported red blood cells (RBCs) or erythrocytes, most abundant cells in the human body, are highly sensitive to oxidative damage because of their high membrane polyunsaturated fatty acids (PUFA) content and high cellular content of oxygen and hemoglobin . The principal function of RBCs is to carry oxygen to tissues and because of their structure and function, RBCs are increasingly used as a cell model for study the *in vitro* toxicity of different xenobiotics (Farag and Alagawany, 2018). The intactness of erythrocyte membrane is essential and important for their function, which is the target for various xenobiotic. Although their well-developed antioxidant defense system, RBCs can be oxidatively injured, as a result of exposure to toxic substances and environmental pollutants. Many studies have determined various structural changes in erythrocytes accompanied with biochemical changes as a result of exposure to different pyrethroids as fenvalerate, cypermethrin, lambda-cyhalothrin, bifenthrin, and bioallethrin (Prasanthi and Rajini, 2005; Sadowska-Woda *et al.,* 2010; Das *et al.,* 2016; Deeba *et al.*, 2017; Arif *et al.,* 2020). Oxidative stress influences and alters RBC shape (Bolotta *et al.*, 2018), whose effective antioxidant mechanism, in the form of antioxidative enzymes such as CAT, SOD, and GST scavenges ROS to sustain membrane integrity (Quintana *et al.*, 2018).

Natural antioxidants have the ability to decrease the intensity of oxidative processes, such viable compounds are *β*-carotene, resveratrol, quercetin, caffeic, Zinc, vitamins E and C, etc. (Ben Abdallah *et al.*, 2012; Eroglu *et al.*, 2013; Das *et al.,* 2016; Revin *et al.*, 2019). Terpenes that contain only carbon, hydrogen and oxygen atoms are volatile components of the essential

oils of citrus fruits, cherries, mints and herbs. A number of dietary monoterpenes have demonstrated multiple pharmacological activities as antibacterial, antifungal, antioxidant, anti-inflammatory, and anticancer (Bouyahya *et al.*, 2021). Carvone (CAR) is a monoterpene presents in a high yield in essential oils of caraway, dill, and spearmint. These plants are among the oldest herbs aware and used by herbalists. CAR is now used in both pharmaceutical and cosmetic products and extensively used in foods, including the chewing gum industry. It is used as a fragrance and flavor, potato sprouting inhibitor, antimicrobial agent, and many other medical applications such as veterinary medicine (Bouyahya *et al.*, 2021). A few studies documented the antioxidant activity of CAR; when comparing with standard antioxidant, a-tocopherol, Elmastaş *et al.* (2006) found that S-carvone possess high antioxidant activity. Another study was carried by Sabir *et al.* (2015) who evaluated the inhibitory activity against lipid peroxidation induced by some pro-oxidants and found that CAR exhibited antioxidant activity in a dose dependent manner (25-200 µg/ml) when examined in rat liver and brain homogenates. As far as we know, there wasn`t available data about using CAR as a natural antioxidant to reduce the toxicity effect of pesticides. Therefore, our study was aimed to evaluate the antioxidant role of CAR on the cypermethrin toxicity in rat erythrocytes as an important and suitable tool *in vitro,* by determining the changes in the antioxidant status and the morphological structure.

MATERIAL AND METHODS

Chemicals

Cypermethrin (96 %) was purchased from Zhejiang Ray full Chemicals Co., China. Carvone (R)-(-)-carvone, (98%) was supplied from Aesar GmbH & Co KG, Zeppelinstr.7. D-76185 Karlsruhe. Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Analytical reagent grade of all other reagents were used.

Animals and care

Male albino rats weigh up, 240 ± 5g were purchased from the animal house, Faculty of Agriculture, University of Alexandria. Rats were housed under laboratory conditions; 25 ± 1 ℃, 12/12-h light/dark cycle, and 65–75% humidity. Animals were fed on a well-balanced rodent diet supplied from Animals Food Manufactory of Ministry of Agriculture, Embaba, Giza, Egypt that composed of 60% maize, 20% soybean, 3% mollas, 1.5% brown dust, 0.5% salt, and 0.2% vitamins and water *ad libitum* for two weeks before being tested.

Isolation of erythrocytes

Under a light anaesthetic with diethyl ether, blood was taken by cardiac puncture into ethylenediaminetetraacetic acid (EDTA) coated tubes as an anticoagulant and centrifuged at 210 xg for 10 min at 4 °C using Sigma K30 bench refrigerated centrifuge, Germany. Supernatant was carefully discarded and the erythrocyte pellet was washed for three times by centrifugation at 1500 xg for 10 min at 4 °C in ice-cold phosphate-buffered saline (PBS; 10 mM sodium phosphate buffer, 0.9% NaCl, pH 7.4). After the final wash, the packed erythrocytes were suspended in phosphate buffer (0.1 M, pH 7.4) to get a 10% hematocrit and divided into portions used for treatments (Arif *et al*., 2020).

Erythrocytes Treatment:

Erythrocytes were treated with various concentrations of CAR (0, 2.5, 5, 10, 20, 40, 80, 160 and 320 µM) for 30 min at 37 °C to detect the effect of CAR on blood cells hemolysis. The low and high concentrations of CAR (40 and 320 µM) were chosen to study the amelioration of CAR on oxidative damage in blood cells induced by CYP. Three portions of erythrocytes were taken and classified as follow; group (I) was incubated with a series of concentrations from CYP (0, 25, 50, 100, 200, 400 and 800 µM) for 4 h at 37 °C; groups (II and III) were pre-incubated with 40 µM or 320 µM of CAR, respectively for 30 min at 37 °C followed by CYP (25, 50, 100, 200, 400 and 800 µM) for 4 h at 37 °C. Also, the influence of CAR alone (40 and 320 µM) on erythrocytes was checked. The erythrocytes groups were centrifuged at 1500 ×g for 5 min at 4 °C, the supernatants kept (to determine hemolysis) and erythrocyte pellets washed three times with PBS. An aliquot (50 µl) from each treatment was kept for scanning electron microscope (SEM) study. Then, the rest of erythrocytes were lysed by suspending in 10 volumes of hypotonic buffer (5 mM sodium phosphate buffer, pH 7.4) and kept at 4 °C for 1 h. The lysates were centrifuged at 1500 ×g at 4 °C for 10 min, to precipitate any cell debris, and then the supernatants (hemolysates) were frozen at −80 °C for later use.

Hemolysis determination:

The release of hemoglobin (Hb) outside the cell (supernatant) was used to determine the extent of hemolysis in different samples. Absorbance of supernatants from all erythrocytes groups was measured at 540 nm using T-80+UV/VIS spectrometer PG Instruments Ltd. (Leicestershire, UK). The hemolysis percentage was calculated using the following equation:

Hemolysis % = (OD $_{(treatment)}$ − OD $_{(control)}$) / (OD $_{(control)}$ ×100%.

Hemoglobin determination:

Hb is the major portion (>95%) of total protein content in erythrocytes whose concentration in hemolysates was determined following the method of

Drabkin and Austin (1935) expressed as gm/dl . Hb concentration in the samples was used to calculate various enzymatic as well as non-enzymatic parameters in terms of per mg Hb.

Oxidative stress biomarkers

All oxidative stress biomarkers were measured according to the instructions included in the Biodiagnostic kits.

Non enzymatic oxidative stress biomarkers

Lipid peroxidation (LPO)

The level of LPO was determined in erythrocyte hemolysate spectrophotometrically according to the method of Kei (1978) using the Biodiagnostic kits. Measuring of the thiobarbituric acid reactive substance (TBARS), as a marker for LPO, was based on its formation as a pink compound from the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA). Briefly, 0.2 ml of hemolysate sample was added to 1 ml of (25 mM) TBA solution and mixed well. The mixture was heated for 30 min in a boiling water bath. The absorbance was recorded at 534 nm after cooling. The TBARS levels were expressed in terms of nmol MDA/mg Hb.

Reduced glutathione (GSH)

Determination of GSH relies on the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) by glutathione to yield a yellow compound, which directly proportional to GSH content (Beutler, 1963). The equal volume of erythrocyte lysate and (0.5 M) trichloroacetic acid (TCA) was mixed and kept for 5 min. After centrifugation at 3400 xg for 15 min, 0.5 mL of the supernatant and 1 ml of 0.1 M phosphate buffer, pH 7.4 were added to 0.1 ml of DTNB reagent (1 mM) and mixed well. Absorbance was measured at 405 nm after incubation for 5-10 min. The GSH level was calculated as µmol/mg Hb.

Enzymatic oxidative stress biomarkers

Superoxide dismutase (SOD)

Activity of SOD was measured in erythrocyte lysate spectrophotometrically following the method of Nishikimi *et al.* (1972). The process is depending on the ability of SOD to decrease the phenazine methosulphate (PMS)-mediated reduction of nitroblue tetrazolium (NBT) dye. In brief, 0.1 ml lysate was mixed with 1.0 ml of working reagent (50 mM phosphate buffer pH 8.5, 1mM NBT and 1mM nicotinamide adenine dinucleotide dehydrogenase (NADH) with ratio 10:1:1). The reaction was initiated by adding 0.1 ml of (0.1 mM) PMS, and then absorbance was recorded at 560 nm for 5 min for control and sample. SOD activity was expressed as U/mg Hb.

Catalase (CAT)

CAT activity was determined spectrophotometrically using the method of Aebi (1984). 50 µl of erythrocyte lysate was added to 0.5 ml of 0.1M phosphate buffer, pH 7.0. The reaction was initiated by adding 0.1 ml of (0.5 mM) H_2O_2 and then incubated for 1 min at 25 °C. Then, 0.2 ml of catalase inhibitor was added to stop the reaction. The amount of H_2O_2 residual in the reaction mixture is then estimated by the oxidative coupling reaction of 0.5 ml of 2 mM 4-aminoantipyrene (AAP) and 3,5-dichloro-2-hydroxybenzenesulfonic acid (DHBS) in the presence of horseradish peroxidase (HRP). The mixture was incubated for 10 min at 37 °C. The resulting quinoneimine dye is measured at 510 nm and the color intensity inversely related to the content of catalase in the lysate sample. Activity of CAT was expressed in U/mg Hb.

Glutathione S-transferase (GST)

The measurement of the GST activity was depend on the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with GSH in a reaction catalyzed by GST (Habig *et al.*, 1974). Briefly, 50 µl of the erythrocyte lysate was incubated with 1 ml phosphate buffer, (0.1 M, pH 7.4) and 0.1 ml of (5 mM) GSH at 37 °C for 5 min. Then, 0.1 ml of (1 mM) CDNB was mixed well and incubated for 5 min at 37 °C. The reaction was stopped by adding TCA and centrifuged at 3400 xg for 5 min. Absorbance readings are performed at a wavelength of 340 nm versus the blank. GST activity was expressed in terms of U/mg Hb.

Glutathione peroxidase (GPx)

The estimation of GPx activity was determined according to (Paglia and Valentine, 1967). The method is based on the oxidation of GSH to oxidized glutathione (GSSG) by organic peroxide using GPx, therefore oxidation of β-nicotinamide adenine dinucleotide phosphate (NADPH) and reduction of GSSG using glutathione reductase to reproduce the reducing equivalents (GSH). The reaction mixture contained 1 ml of 50 mM phosphate buffer, pH 7.0 with 0.1% Triton X-100, 0.1 ml of NADPH reagent (24 µM GSH, 4.8 µM NADPH and 12 units glutathione reductase) and 0.01 ml of sample. Reaction was started by adding 0.1 ml of H_2O_2 as substrate. Activity of GPx was measured by the decrease in absorbance at 340 nm over a period of three minutes. The activity of the enzyme was expressed as mU/mg Hb.

Glutathione reductase (GR)

The activity of GR was measured following the method developed by Goldberg (1984). The assay estimate the ability of glutathione reductase to catalyze the oxidation of NADPH to NADP during the reduction

of GSSG and the decrease in absorbance was monitored at 340 nm. In the assay cuvette, 50 µl of sample was mixed with 1 ml of 100 mM potassium phosphate buffer, pH 7.5 containing (1 mM) EDTA, 0.1 ml of (50 mM) GSSG and 0.1 ml of (2 mM) NADPH. The change in absorbance was recorded every 1 min for 5 min. The activity of GR enzyme was expressed as U/mg Hb.

Scanning electron microscopy (SEM):

Erythrocyte aliquots from all groups were fixed immediately in 2.5% glutaraldehyde solution (v/v) for 3 h at 4 °C. The specimens were post fixed in 2% Osmium tetroxide (OsO$_4$) for 2 h at 4 °C. Then, they were washed with phosphate buffer, pH 7.4 and dehydrated through gradient ethanol of 30%, 50%, 70%, 90%, 95% and 100% (v/v) at 4 °C. The samples were dried by using a critical point method, mounted using carbon paste on an AL-stub and covered with gold in a sputter–coating unit (JFC-1100 E). The cell morphology was achieved by a scanning electron microscope (Jeol JSM-IT 200) and images were recorded at 5000 and 10000 x magnification (Fischer *et al.*, 2012).

Statistical analysis

All the results were stated as mean ± standard error (SE). Data were analyzed using One-way variance analysis (ANOVA), after that the Student-Newman-Keuls Test to determine significance between different groups. The criterion for statistical significance was set at $p \leq 0.01$. These tests were done using a computer CoStat program, version 6.400 (CoHort Software: Tucson, 1998-2008).

RESULTS AND DISCUSSION

RESULTS

Hemolysis

The hemolytic activity of the CAR alone was increased at concentrations 80 and 160 µM, while the concentrations of CAR at 2.5, 5, 10, 20, 40 and 320 µM had no hemolytic effect almost as the control (Fig. 1). On the grounds of the obtained results, it may be stated that, 40 and 320 µM of CAR concentrations were selected for subsequent experiment. Incubation of erythrocytes with CYP was induced a significant (p <

0.01) hemolysis at 400 and 800 µM by 4% and 45%, respectively (Fig. 2A). The protective effect of CAR (40 and 320 µM) was also investigated. Pre-incubation with 40 or 320 µM CAR was protected erythrocytes completely from hemolysis, only pre-treatment with 320 µM CAR was reduced the induction of hemolysis at 800 µM CYP to about 14%.
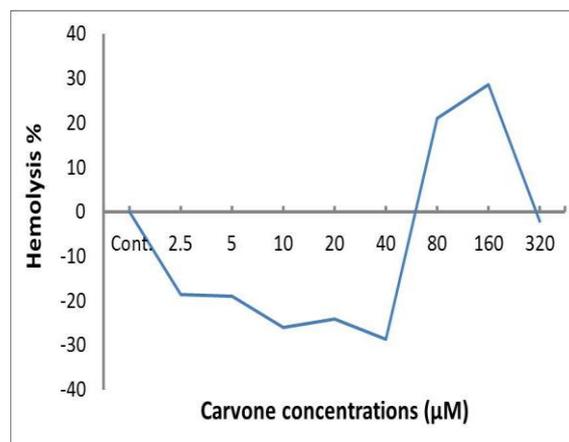
Fig. 1. The hemolysis percentage induced after incubating 10% hematocrit with various concentrations of CAR for 30 min at 37 °C

Non enzymatic oxidative stress biomarkers

MDA content in cell lysate was significantly increased (p < 0.01) as a result of CYP treatment until reach about 9 folds in a concentration dependent pattern (Fig. 2B). Pre-treatment of erythrocytes with CAR at concentrations of 40 and 320 µM showed a significantly lower level of MDA (p < 0.01) compared to the control. Also, the results indicated that 320 µM (group III) had more mitigate effect than 40 µM (group II) compared to control. GSH content was significantly increased (p < 0.01) in CYP-treated erythrocytes at all concentrations by 3.2-3.7 folds in comparison to control erythrocytes. Pre-treatment with CAR at 320 µM afforded more protection than at 40 µM of GSH levels in erythrocytes treated with CYP (Fig. 2C).
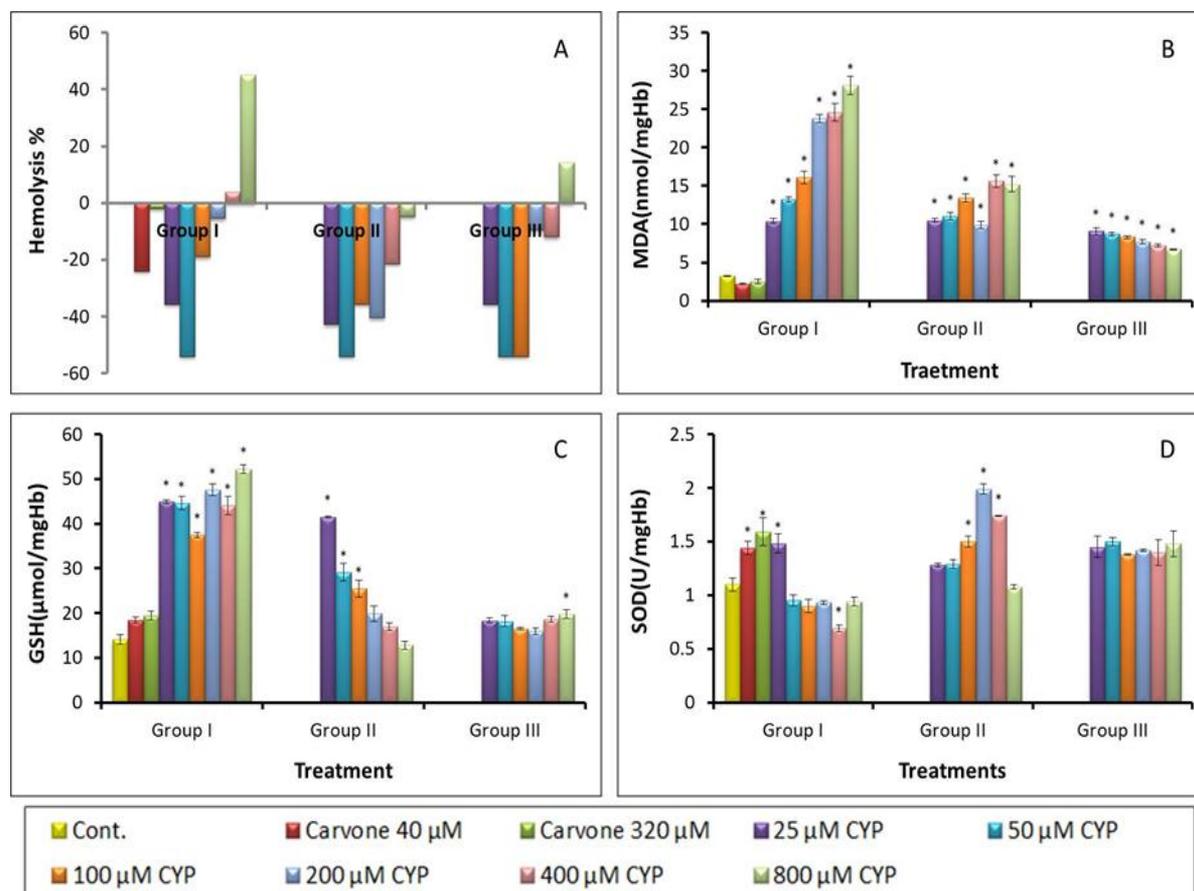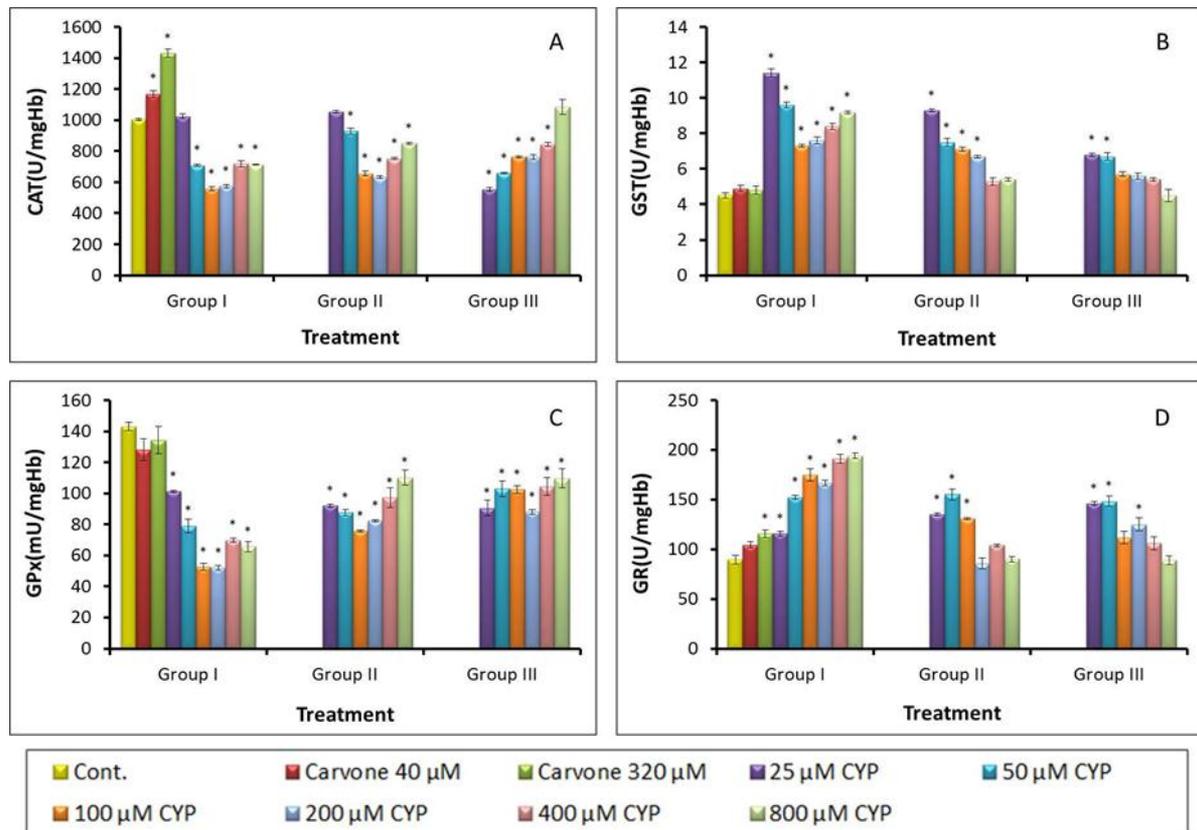
Fig. 2. Changes in hemolysis percentage, MDA and GSH contents, and SOD activity of rat erythrocytes treated with series concentrations of CYP (group I). Pre-treated with CAR (40 and 320 μM) as group II and III, respectively and followed by CYP. The values are expressed as means ± SE. Asterisk (*) indicates significant (p < 0.01) effect of different treatments

Enzymatic oxidative stress biomarkers

The data in Fig. 2D showed that the two concentrations of CAR (40 and 320 μM) caused a significant increase (p < 0.01) in the activity of SOD related to control. Four hours of incubation of rat erythrocytes with CYP at 50, 100, 200, 400 and 800 μM (Group I) induced a significant decrease (p < 0.01) in SOD activity compared to the control. The most reduction of SOD activity about 38% was at 400 μM CYP treatment. It was observed that the treatment with 25 μM CYP was significantly (p < 0.01) increased in SOD activity compared with control. While, the erythrocyte pre-treatment with CAR at 40 or 320 μM caused elevation in SOD activity compared with control; pre-treatment with 40 μM CAR caused 80% and 58% rising in SOD activity at 200 and 400 μM CYP, respectively.

Treatment of CAR alone significantly (p < 0.01) increased the activity of CAT in both concentrations (40 and 320 μM) and such rise was significantly more in the 320 μM concentration. CAT activity was found to significantly decrease (p < 0.01) in all erythrocytes incubated with CYP concentrations except at 25 μM compared to control. Pre-incubation of erythrocytes with CAR at 40 or 320 μM caused initiation in CAT activity, except the pre-treated cells with 320 μM CAR and low concentrations of CYP (25 or 50 μM) were lower when compared to control (Fig. 3A).

Fig. 3. Changes in CAT, GST, GPx and GR activities of rat erythrocytes treated with series concentrations of CYP (group I). Pre-treated with CAR (40 and 320 µM) as group II and III, respectively and followed by CYP. The values are expressed as means ± SE. Asterisk (*) indicates significant (p < 0.01) effect of different treatments

Erythrocytes exposure to different concentrations of CYP resulted in a significant (p < 0.01) increase in the activity of GST ranging from 1.6-2.5 folds (Fig. 3B). Maximum elevation in GST activity was observed at 25 µM of CYP treatment. A significant reduction in the activity of GST was observed when CAR (40 or 320 µM) was incubated before CYP treatment (p < 0.01). Non-significant modification was revealed in GST activity between CAR alone (40 and 320 µM) and control.

Activity of GPx was found to be significantly decreasing (p < 0.01) in erythrocytes incubated with CYP in comparison to the control erythrocytes (Fig. 3C). Pre-treatment with CAR at both the concentrations mitigate the decline in GPx activities that were induced by CYP in erythrocytes.

The results in Fig. 3D revealed a significant increase (p < 0.01) in GR activity as a result of CYP treatment and that effect was in a concentration-dependent pattern. Erythrocytes pre-treated with 40 or 320 µM CAR presented a significant decrease in GR levels at the higher concentrations of CYP (100, 200, 400 and 800 µM).
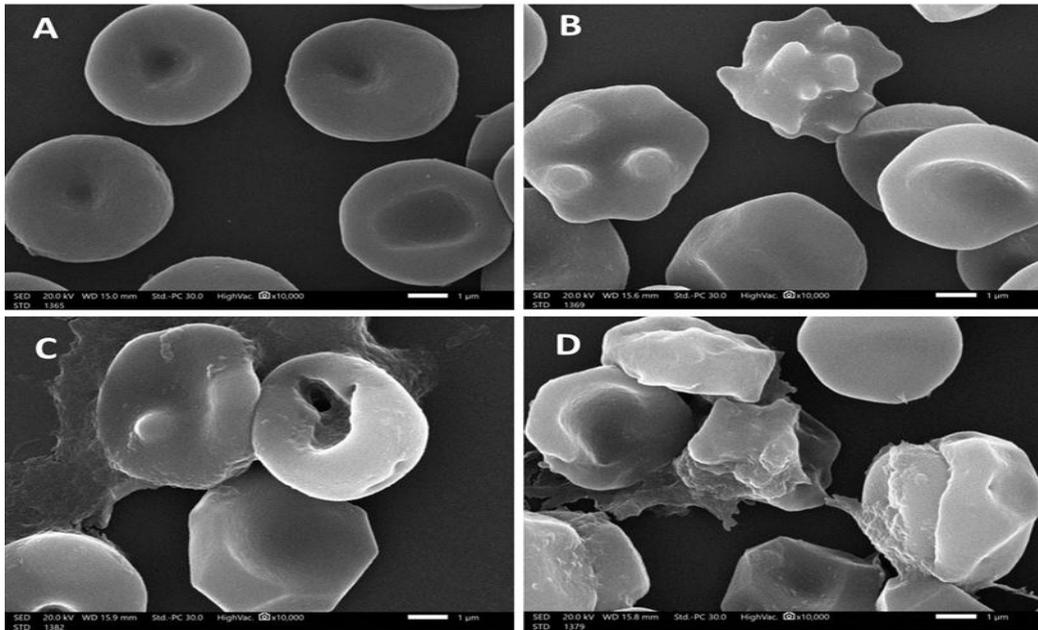
Scanning electron micrographs

Control and CAR-treated erythrocytes (40 and 320 µM) showed the normal biconcave and discoidal shape (Fig. 4 A, B, and C). Morphological changes with varying degree were significantly evident among CYP-treated erythrocytes in a concentration-dependent manner (Fig.4 D-G).
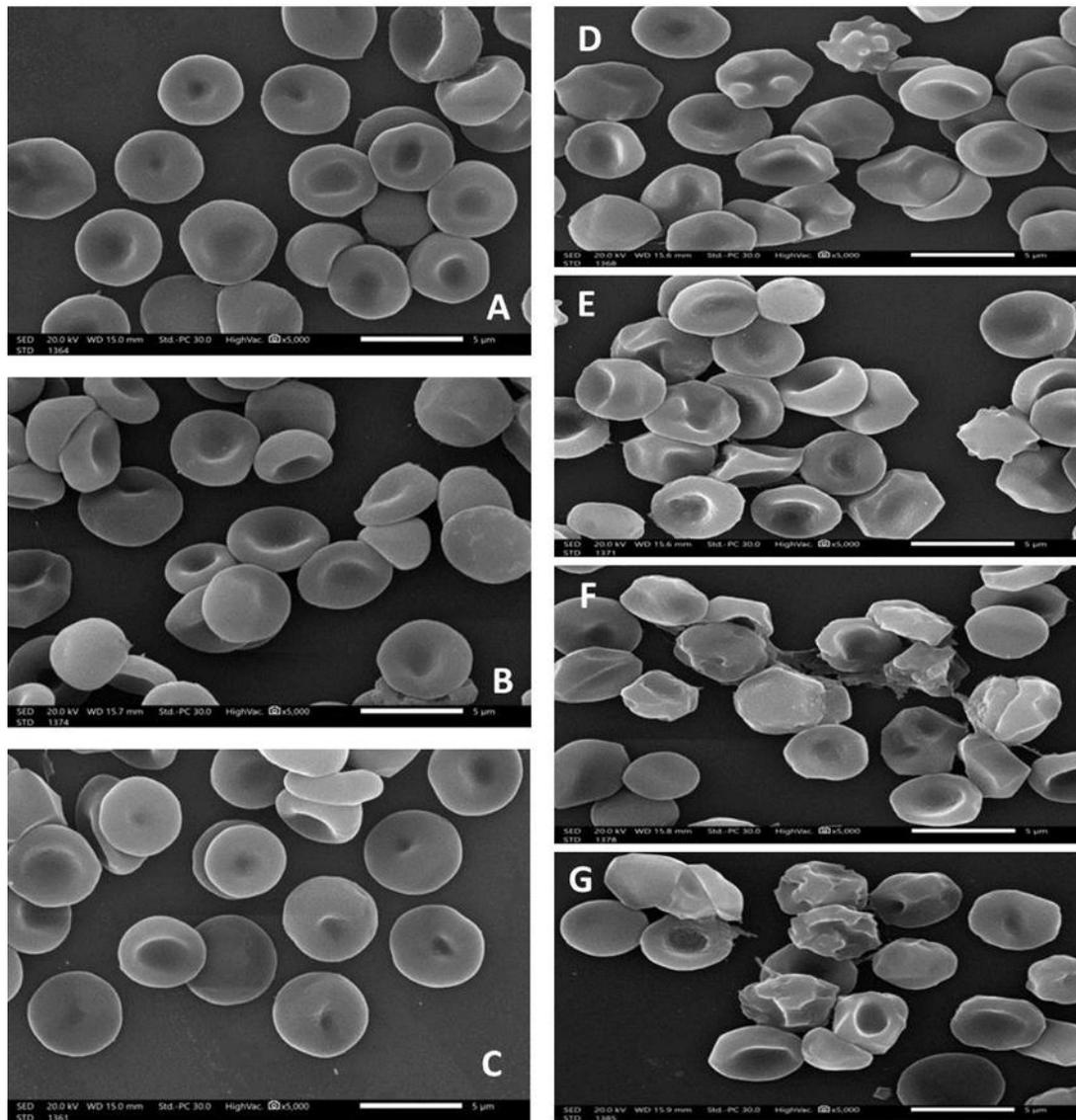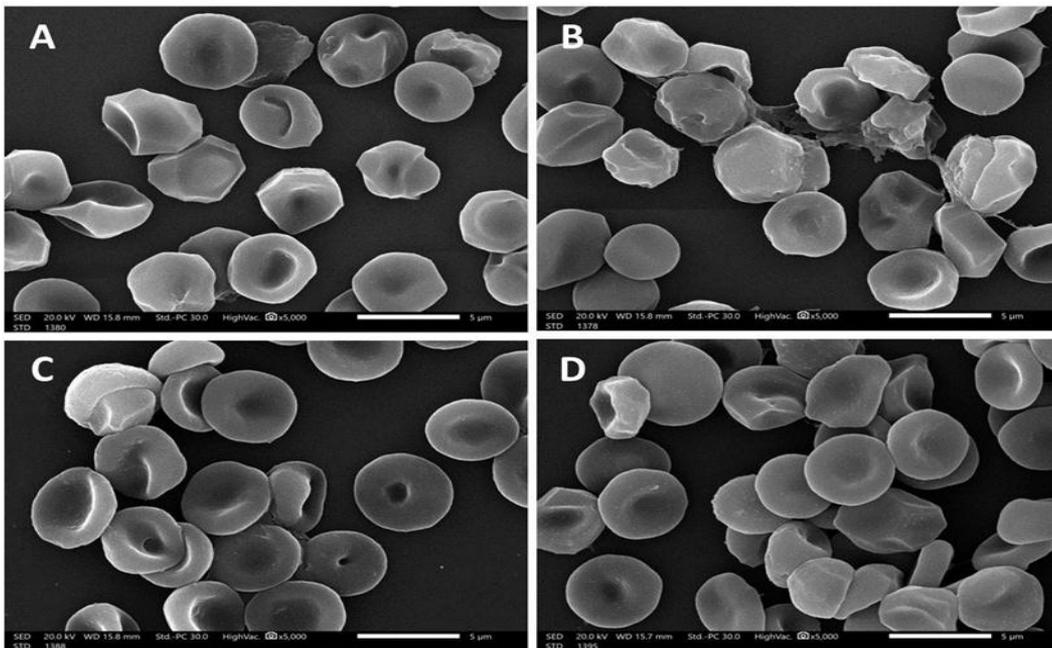
Fig. 4. Scanning electron micrograph of rat erythrocytes; (A) Control (untreated) erythrocytes shows normal shape; (B) and (C) cells treated with 40 and 320 μM CAR, respectively, also show normal and healthy RBCs shape; (D) and (E) treated cells with 25 μM CYP notify echinocytes and spherocytes formation; (F) and (G) treated cells with 800 μM CYP. Magnification is 5000 fold

The red cells biconcave shape (discocytes) was altered to spiked cells (echinocytes) at lower concentrations that are the most frequent pathological erythrocytes and spherocytes with unusual swollen shape was also appeared (Fig. 5 B). The major damage was appeared at higher concentrations with distortion in shape, a ruptured hole in cell, stomatocytes formation and cells had a hemispherical protrusion (Fig.5 C and D). Also, the erythrocyte membranes were significant ruptured resulting in morphological alterations in erythrocytes that may influence their functioning or result in cell death. Since deteriorated RBCs are quickly getting rid of with the reticulo-endothelial system, this will lead to senescence of red cell and anemia. Obviously, the pre-treatment with CAR for 30 min at 37 °C was decreased the damaged cells (Fig. 6). Also, the protective effect of the pre-treatment with 320 μM CAR against CYP is better than 40 μM CAR.

Fig. 5. Scanning electron micrograph of rat erythrocytes; (A) shows RBCs with normal biconcave shape as a control; (B) represents spherocytosis and gradual alteration into echinocytes as a result of treatment with 25 μM CYP; (C) demonstrates a sign of hemolysis, punctate (tiny) holes in the membrane of RBCs; and (D) shows distortion in shape and significant ruptured membranes resulted from 800 μM CYP treatment. Magnification is 10000 fold

Fig. 6. Scanning electron micrograph of rat erythrocytes; (A) and (B) represent the CYP-treated erythrocytes at 800 μM for 4 h at 37 ºC and show the damaged and ruptured cells; (C) represents the pre-treated cells with 40 μM CAR and (D) shows the pre-incubated cells with 320 μM CAR for 30 min before treatment with 800 μM CYP for 4 h at 37 ºC. Magnification is 5000 fold

DISCUSSION

Blood is the main way to transport xenobiotics such as drugs and pesticides in the human body. So, blood cells are considered as the first target for any xenobiotics that enter human body, therefore the erythrocytes were chosen in our study as a cellular model for toxicity evaluation due to their simple structure, abundance, simplicity of isolation and handling (Farag and Alagawany, 2018). Pyrethroids, as widely used insecticides, have been proved to cause oxidative damage in different animal species (Das *et al.*, 2016; Afolabi *et al.*, 2019; Rajawat *et al.*, 2019). RBCs are extremely sensitive to oxidative damage due to their great membrane PUFA content and high cellular concentrations of Hb and oxygen (Clemens and Waller, 1987). Therefore, they are endowed with well-developed enzymatic as well as non-enzymatic antioxidant mechanisms.

Hemolytic assay as an easy biological toxicity testing method, is convenient to obtain initial information about the xenobiotics toxicity (Farag and Alagawany, 2018). The extent of hemolysis was estimated from the amount of the extracellular Hb. The interaction of xenobiotics with Hb is a source of radical generation in RBCs (French *et al.*, 1978), giving rise to ROS resulting in membrane lipid peroxidation and hemolysis (Clemens *et al.*, 1984). Despite CYP inducing oxidative stress in red cells, hemolysis was observed at higher concentrations (400 and 800 μM). Increment in pesticide concentration may results in direct or indirect damage to the cells and enhanced hemolysis as a consequence, particularly the plasma membrane. Moreover, the released Hb during hemolysis in intravascular space is toxic and induces numerous pathophysiologic effects (Schaer and Buehler, 2013). Anemia was resulted by increased hemolysis, as mentioned by (Arif *et al.*, 2020; Salam *et al.*, 2020) in human erythrocytes treated with bioallethrin and thiram, respectively.

ROS produced during oxidative stress damage alter the components of cells mainly unsaturated lipids, thiols and proteins. PUFA oxidation results in the MDA formation whose level was elevated in CYP-treated erythrocytes. This result is agree with a previous report showing increased lipid peroxidation in rat and human erythrocytes (*in vitro*) on exposure to cypermethrin, lambda-cyhalothrin and bioallethrin (Das *et al.*, 2016; Deeba *et al.*, 2017; Arif *et al.*, 2020). GSH is the major non-enzymatic antioxidant present in millimolar concentration in erythrocytes. GSH functions as free radical scavenger and neutralizes radicals involved in biological damage (Schmitt *et al.*, 2015). It eliminates reactive intermediates by reducing of hydroperoxides in the presence of GPx. A significant increase (about 3.5 folds) in the content of GSH in rats erythrocytes after CYP treatment showed pro-oxidant conditions in the RBCs, which may be an initial adaptive response to increased oxidative stress in CYP-treated erythrocytes (Kale *et al.*, 1999). Our results in the same way with Liu *et al.* (2020) who found that treating rats with pyrethroid insecticides tetramethrin and prallethrin induced elevation of GSH in erythrocytes.

Erythrocytes have a well-developed defense system to contend oxidative stress, of which antioxidant enzymes are the most potent due to their efficiency. The major enzymatic antioxidant system includes SOD, CAT, GST, GPx, and GR were assayed (Arif *et al.*, 2020). SOD provides the first line of defense against ROS and converts superoxide radicals to less reactive H_2O_2 that may in turn be decomposes by CAT into water and oxygen. The decrease in the SOD and CAT activities in response to CYP exposed erythrocytes may be attributed to the consumption of SOD and CAT during the free radicals breakdown and consequent high levels of H_2O_2 formation or the enzyme inhibition by these ROS as a direct effect of SOD and CAT by CYP treatment (Eraslan *et al.*, 2007). Supporting our results, some authors found that fenvalerate and lambda-cyhalothrin induced a significantly reduced SOD and CAT activities in rat (Prasanthi and Rajini, 2005; Ben Abdallah *et al.*, 2012). Similar changes in the SOD and CAT activities in rat RBCs have been documented as a result of *in vivo* exposures to cypermethrin (Das *et al.*, 2016). Also the same decrease in SOD and CAT activities were detected in rabbit erythrocytes treated *in vitro* with lambda-cyhalothrin at various concentrations (El-Demerdash, 2007). Previous studies also suggested similar observations after rat and human erythrocytes were *in vitro* incubated with different pesticides, such as bifenthrin (Sadowska-Woda *et al.*, 2010; Tatipamula and Kukavica, 2022), chlorpyrifos and lambda-cyhalothrin (Deeba *et al.*, 2017) and thiram (Salam *et al.*, 2020).

GST, considered as detoxifying enzyme that catalyzes the conjugation of various xenobiotic substances to the thiol group of GSH producing less toxic forms (Nicotera and Orrenius, 1986). The significant increase of GST activity subsequently exposure to CYP may point to sufficient detoxification of pesticide in the rat RBCs. Similar trends of significant elevation in GST activity have been noticed in human and rat erythrocytes exposed to bioallethrin and bifenthrin, respectively (Arif *et al.*, 2020; Tatipamula and Kukavica, 2022) This could be understood considering that pesticides are mainly

detoxified through GST catalyzed reaction, utilizing GSH which increased by almost the same trend. Pesticides were expected to stimulate the GST activity as an efficient protective mechanism of the organism.

GPx is involved in protecting cell membrane and protein from oxidative injury by reducing H_2O_2 in the presence of GSH-forming GSSG (Jacob, 1995). Activities of GPx were reduced in CYP-treated erythrocytes (group I). These results are in line with the observations of Das *et al*. (2016). The decrease of GPx activity induced by CYP could be attributed to a direct inhibitory oxidative effect on the enzyme. Inhibition of GPx by CYP can lead to the increase of H_2O_2 with subsequent oxidation of the lipids. GPx, considered to be the major antioxidant enzyme for the detoxification of H_2O_2 especially in human RBCs, as CAT has much lower affinity for H_2O_2 than GPx (Izawa *et al*., 1996).

GR is responsible for maintaining the supply of GSH by reducing GSSG in the presence of NADPH (Couto *et al.*, 2016). Thus increased activity of GR was due to induction of natural defense mechanism of erythrocytes against the toxicity of CYP. These results are in harmony with the idea that exposure to some organophosphates *in vitro* causes elevation in activity of GR in rat RBCs (Singh *et al.*, 2006). On the contrary, earlier reports showed that *in vitro* exposure to fenvalerate and bioallethrin results in decrease in the activity of GR (Prasanthi and Rajini, 2005; Arif *et al.,* 2020).

Oxidative stress is often implicated in several severe and chronic illnesses such as cancers, cardiovascular diseases, diabetes, and many others. Since antioxidant compounds can mitigate oxidative stress due to of their antiradical ability and/or reducing power, the search for new effective and safe antioxidants from plants has intensified in recent years (Benali *et al.*, 2020). Many studies were conducted to evaluate the antioxidant role of natural antioxidants to reduce the oxidative stress induced by cypermethrin or other pyrethroides (El-Demerdash, 2007; Sadowska-Woda *et al.,* 2010; Ben Abdallah *et al.,* 2012; Das *et al.,* 2016; Tatipamula and Kukavica, 2022). As far as we know, the capability of CAR to protect erythrocytes against CYP-induced oxidative stress has been investigated for the first time. The results revealed that pre-treatment of erythrocytes with CAR at 40 μM was completely protecting the cells from the CYP induced hemolysis. The antioxidant effects biochemical levels in rat erythrocytes treated with CAR alone compared to control, suggesting that CYP affects as a catalyst in the oxidative damage of biological macromolecules and this effect might be reduced by antioxidants treatment. Our results indicated that erythrocytes treated with CAR before CYP incubation had a significant amelioration effect in the SOD, CAT, GST, GPx, GR activities, and the contents of MDA and GSH as compared to CYP-treated erythrocytes (Elmastaş *et al.,* 2006; Sabir *et al.,* 2015). Also the present results were in line with (Zhao and Du, 2020) who found that pre-treatment of D-carvone significantly provided anti-inflammatory and defensive effect by amelioration of oxidative stress in lipopolysaccharide-exposed mice, where the levels of MDA were decreased along with increase in SOD and CAT activities.

The above biochemical results were confirmed by SEM of all the erythrocytes groups. Gross deformation in erythrocyte morphology was seen in CYP-treated cells, ranging from formation of spherocytes and echinocytes at the low CYP concentration treatment, to ruptured tinny holes, which resulted in hemolysis, stomatocytes formation, significant ruptured membranes, and cells, had a hemispherical protrusion at the high CYP concentration. These changes in RBCs will modify their rheological properties and minimize the cells facility to distort and pass via microcapillaries, some of whom have lesser diameter than the RBCs. This will lead to the erythrocytes coagulation, occlusion of blood vessels and result in renal failure (Salam *et al.*, 2020). Various degrees of alteration in shape and ruptured membranes were also noticed in rat and human erythrocytes following exposure to fenvalerate and bioallethrin (Prasanthi and Rajini, 2005; Arif *et al.,* 2020). Since the changes in erythrocytes shape induced by external factors, e.g., pesticides and drugs resulted in damaged cells which are quickly eliminated from blood, this will result in anemia (Stasiuk *et al.*, 2009). From the obtained results, it was observed that pre-treated with CAR could offer protection to the erythrocyte morphology against CYP-induced abnormalities.

CONCLUSION

In conclusion, CYP was found to have cytotoxic effects on rat erythrocytes (*in vitro*) inducing oxidative stress injury as evidenced in terms of increment level of hemolysis, lipid peroxidation, and altered the antioxidant defense system of erythrocytes. Also it was found to damage plasma membrane and changes cell morphology. However, pre-treatment with CAR reduced the extent of hemolysis, lipid peroxidation and the significant changes in the oxidative status of the blood cells induced by CYP. Therefore, the current study demonstrates that the supplementation of CAR can restore the alterations in tested biomarkers induced by CYP.

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الملخص العربي

تاثير السيبرمثرين فى المختبر على اضطرابات كرات الدم الحمراء من الجرذان: الدور التحسيني للكارفون

نادية على حامد ، إيمان محمد مسلم ، رضا خميس عبد الرازق

تعتبر كرات الدم الحمراء نموذجًا مناسبًا لفهم الضرر التأكسدي للغشاء الناجم عن المركبات الغريبة المختلفة. فى هذا البحث تم دراسة خاصية مضادات الأكسدة للكارفون (CAR)، وهو مركب أحادي التربين موجود في الأعشاب للتخفيف من سمية مبيد السيبرمثرين (CYP)، وهو النوع الثاني من البيروثريدات، في كريات الدم الحمراء في ذكور الجرذان فى المختبر. تم تحضير كرات الدم الحمراء وتعريضها لتركيزات من السيبرمثرين تتراوح بين ٢٥ إلى ٨٠٠ ميكرومولر بمفردها أو مختلطة مع الكارفون (تركيز ٤٠ & ٣٢٠ ميكرومولر). أظهرت الدراسة انخفاضًا معنويًا في نشاط إنزيمات سوبروكسيد ديسميوتيز، كتاليز، جلوتاثيون بيروكسيديز. كما حدث زيادة فى مستوى اكسدة الدهون، ومحتوى الجلوتاثيون المختزل، ونشاط كلا من إنزيمى

الجلوتاثيون س ترنسفريز والجلوتاثيون ريدكتاز بشكل ملحوظ مقارنة بقيمة الكنترول. ولقد دعم الميكروسكوب الإلكتروني الماسح نتائج التأثير الضار لمبيد السيبرمثرين مع إظهار درجات مختلفة من التشوه في الشكل وتمزق الأغشية. أدت المعالجة المسبقة لخلايا الدم الحمراء بالكارفون الى تخفيف التأثيرالضار للمبيد في جميع المؤشرات المختبرة. يمكن الاستنتاج من هذه الدراسة أن مبيد السيبرمثرين تسبب في الإجهاد التأكسدي في كرات الدم الحمراء في الفئران وأن تناول الكارفون خفف من هذه التأثيرات.

الكلمات المفتاحية: سيبرمثرين، كارفون، كرات الدم الحمراء، الإجهاد التأكسدي، الحالة التأكسدية، ميكروسكوب المسح الإلكتروني.