Stimulation of Erythrocyte Membrane Blebbing by Bifenthrin Induced Oxidative Stress

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Abstract

Background: Bifenthrin is an insecticide and anti-estrogenic compound primarily used to control residential pests by depolarizing sodium gated voltage channels in the nervous system. Eryptosis, the suicidal death of erythrocytes, featured by PS exposure, membrane blebbing and cell shrinkage. Anemia is an outcome of uncontrolled eryptosis.

Research Design: In this study, erythrocytes were treated with different concentrations (.5-1-1.5 μM) of bifenthrin over a period of 48 hours. In order to investigate the oxidative stress induced by bifenthrin, catalase, superoxide dismutase, and glutathione peroxidase activities were investigated.

Results: Obtained data indicated the decrease in the enzymes (superoxide dismutase, glutathione peroxidase, and catalase) activities in bifenthrin treated cells at 1 μM concentration. In addition, measurement of cell size and confirmation of the role of calcium in the stimulation of the eryptotic activity of bifenthrin were performed. A significant increase in mean cell volume was found in the presence of bifenthrin and a decrease in mean cell volume in the presence of calcium channel blocker was observed. Similarly, there was also a significant increase in the percentage of hemolysis indicating the necrotic activity of bifenthrin.

Conclusions: It is concluded that the indicated doses of bifenthrin triggered oxidative stress which may lead to early cell death by eryptosis and hemolysis.

Keywords
bifenthrin, oxidative stress, catalase, superoxide dismutase, glutathione peroxidase, eryptosis

Introduction

Bifenthrin (2-methyl-3-phenyl phenyl methyl (1S, 3S)-3-(Z)-2-chloro-3,3,3-trifluoroprop-1-enyl 2,2-dimethylocyclopropane-1-carboxylate) is a third generation pyrethroid insecticide with more photostability and insecticidal activity.1 World Health Organization (WHO) classifies it under toxicity class II and considered as moderate hazardous pesticide.2 Bifenthrin is a type I synthetic pyrethroid having wide agricultural and public health applications.3 It also finds extensive applications in the control of domestic pests such as termites in urban areas.1 Type I pyrethroids normally characterized by aggressive sparring, high sensitivity to external stimuli, tremors, and prostration referred to as T syndrome.4 Pyrethroids are established neurotoxins and their primary site of action is the voltage-gated sodium channels in the central nervous system.5 It is documented that pyrethroids induce neurological and behavioral effects in various animal species including humans. A number of environmental pollutants, including pesticides, are known to

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Received 2 November 2021; accepted 11 January 2022

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cause imbalance between the formation and removal of free radicals, thus leading to oxidative stress. The mode of action of most pesticides involves induction of oxidative stress. Pyrethroids are known to induce the formation of reactive oxygen species.

Oxidative stress is referred to as a shift in the balance of oxidants/antioxidants in favor of oxidants. Pathological conditions like cancer, neurological problems, atherosclerosis, and hypertension are caused by oxidative stress. Oxidative stress can be caused either by an internal mechanism (e.g., ETC reactions and NOx pathway) or by external factors (e.g., xenobiotics) involved in increasing the number of ROS. Furthermore, bifenthrin incubation decreased antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) activities in primary microglial cells.

Eryptosis is a suicidal death of erythrocytes, mainly triggered by oxidative stress. Characteristics of eryptosis include phosphatidylserine-exposure from the inner to the outer side of the cell membrane, cell shrinkage, and membrane blebbing. Oxidative stress triggers various factors that lead to different events of suicidal death of erythrocytes including triggering of Ca2+ channels, activation of caspases, and the release of phospholipase A2-mediated platelet activation factor which promotes ceramide formation by sphingomyelinase activation. High calcium and ceramide levels promote membrane scrambling with phosphatidylserine exposure on outer face of membrane. Erythrocytes with phosphatidylserine on outer side are rapidly removed from circulating blood as such cells are primarily bound to the phosphatidylserine receptors of splenic macrophages and CD207+ dendritic cells that are responsible for engulfment and destruction of apoptotic erythrocytes. Similarly, Ca2+ influx activates the enzyme calpain which degrades cytoskeletal proteins leading to the blebbing of the cell membrane. It is reported that different xenobiotic compound are involved in the stimulation of eryptosis. Uncontrolled and uncompensated removal of erythrocytes contributes to the pathophysiology of several clinical disorders mainly by inducing anemia.

Very few studies have been conducted to investigate the adverse impact of bifenthrin on human health. Oxidative stress and anemia induced by bifenthrin are reported in literature. The aim of this research work was to investigate bifenthrin induced oxidative stress and its involvement in suicidal cell death of erythrocytes by monitoring its key marker membrane blebbing.

**Material and Method**

The research work was conducted after its approval from the Institutional Bioethics Committee (IBC) and Directorate Graduate Studies (DGS), University of Agriculture, Faisalabad, Pakistan. Freshly collected screened blood samples were acquired from various blood banks of Faisalabad, Pakistan. Leukocyte free erythrocytes were obtained by using a protocol described by Fink et al. Blood samples were centrifuged at 12,298 g for 20 minutes to collect erythrocytes. A 48 h in-vitro incubation (at 37°C) of 0.4% hemotocrit were done in ringer solution containing (in mM) MgSO4 1, NaCl 125, KCl 5, Glucose 5, CaCl2 1, N-2-hydroxy-piperacine-N-2-ethanesulphonic acid (HEPES) 32. For the treatment of erythrocytes under in-vitro conditions, different concentrations (0.5-1.5 μM) of bifenthrin were prepared by using water as solvent. In the current research work, physiological concentrations of bifenthrin were used as the highest concentration (1.5 µM) was lower than its reported plasma concentration.

**Oxidative Stress Measurement**

Antioxidant enzyme assays were performed for the confirmation of oxidative stress in bifenthrin exposed erythrocytes.

1. **Superoxide dismutase**

   The superoxide dismutase activity was assessed in compliance with the protocol of Shabir et al. The concentrations of individual components in the reaction mixture were; 0.15 g NBT in 17.5 mL H2O2, 0.22 g Methionine in 15 mL H2O, 0.0375 mL Triton-X 100 in 17.5 mL H2O, and 0.2 M phosphate buffer.

2. **Glutathione peroxidase**

   A 0.5 mL sample and reaction mixture were loaded on 96 well plate and absorbance was monitored at 560 nm by spectrophotometer.

3. **Catalase**

   Catalase activity was estimated by following the methodology proposed by Ullah et al. A 0.1 mL of sample and 0.1 mL of reaction mixture were loaded onto 96 well plate and absorbance was measured at 240 nm wavelength. Reaction mixture comprised of 50 mM phosphate buffer (pH 5), 19.5 μL Guaiacol in 10 mL water, and 350 μL H2O2 in 10 mL water.

**Mean Cell Volume Determination**

Mean cell volume (MCV) of the control and bifenthrin treated erythrocytes was determined to confirm the membrane blebbing. Hematology analyzer was used for the measurement of MCV.

**Confirmation of Ca2+2 Role**

Erythrocytes were treated with 10 μM amlodipine (Ca2+ channel blocker) for the confirmation of the calcium role in the bifenthrin
induced eryptosis. Mean cell volume was estimated by using hematology analyzer to confirm the inhibition of eryptosis.\textsuperscript{22}

**Hemolysis Measurement**

The blood samples were centrifuged (20 g for 3 min, at 25 °C temperature) and the supernatant in test tube was separated in order to measure the percentage of hemolysis. To calculate the hemolysis percentage in bifenthrin treated erythrocytes, the hemoglobin concentration in the collected supernatant was measured at wavelength 405 nm.\textsuperscript{23} For comparison, the absorption of hemoglobin that was present in d.H\textsubscript{2}O (hemolysed cells) was considered as 100% hemolysis.\textsuperscript{24}

**Statistical Analysis**

The data is arithmetically expressed as ±SEM. For statistical analysis, ANOVA was used with Tukey’s test as post-test.\textsuperscript{17} Software GraphPad InStat was used for statistical analysis of the data.

**Results**

Results of the investigations are presented in figures, prepared with mean ± SEM values with proper indication of statistical significance. Oxidative stress is among the stimulatory mechanisms of eryptosis. Bifenthrin triggered oxidative stress was checked by measuring the antioxidant enzymes activities in treated and control cells. Figure 1 is indicating a decrease in superoxide dismutase activities in treated cells after 48 hour treatment with bifenthrin (.5-1-1.5 μM). A significant decrease in SOD activity is evident in the figure at 1.5 μM bifenthrin in comparison to the control cells. Figure 2 is illustrating that the 48 hour treatment of erythrocytes with above mentioned concentrations of bifenthrin resulted in

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**Figure 1.** Determination of superoxide dismutase activity (U/gHb) in bifenthrin-treated erythrocytes. Arithmetic means ± SEM (n = 20) of the means in erythrocytes exposed to ringer solution without (white bar) or with (black bar) .5-1.5 μM bifenthrin for 48 hours. *(p<0.05)* indicates significant difference in the absence of bifenthrin (ANOVA).

**Figure 2.** Determination of glutathione peroxidase activity (U/gHb) in bifenthrin-treated erythrocytes. Arithmetic means ± SEM (n = 20) of the means in erythrocytes exposed to ringer solution without (white bar) or with (black bar) .5-1.5 μM bifenthrin for 48 hours. ***(p<0.001)* denotes a statistically significant difference from the control values (ANOVA).**

**Figure 3.** Determination of catalase activity (U/gHb) in bifenthrin-treated erythrocytes. Arithmetic means ± SEM (n = 20) of the means in erythrocytes treated with ringer solution without (white bar) or with (black bar) .5-1.5 μM bifenthrin for 48 hours. ***(p<0.001)* denotes a statistically significant difference from the control values (ANOVA).**

**Figure 4.** Measurement of erythrocytes mean cell volume (fL) in bifenthrin-exposed erythrocytes. Arithmetic means ± SEM (n = 10) of the means in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with 1.5 μM bifenthrin (black bar). ***(p<0.001)* indicates a highly significant difference between untreated and treated samples (t-test).
highly significant decrease in glutathione peroxidase activities at 1 and 1.5 μM concentrations in comparison to the control cells. Likewise, Figure 3 is demonstrating that the exposure of erythrocytes with same concentrations of bifenthrin for 48 hour, leads to moderate and highly significant decrease in catalase activity at 1 μM and 1.5 μM concentrations, respectively.

Figure 4 is indicating that the 48 hour exposure of erythrocytes with bifenthrin leads to a highly significant increase in the mean cell volume of erythrocytes at 1.5 μM, may be due to the membrane blebbing. In the next experiment, amlodipine, a calcium channel blocker, was used to confirm the role of calcium in the induction of membrane blebbing. Figure 5 is depicting a significant decrease in the mean cell volume of erythrocytes in the presence of amlodipine compared to non-amlodipine treated cells that may be due to the blockage of calcium entry in the cells. The hemolytic effect of bifenthrin on erythrocytes was determined by hemolysis % measurement. Figure 6 is indicating a significant increase in hemolysis % after 48 hours exposure of erythrocytes to bifenthrin (1.5 μM).

Discussion

The main objective of this study was to unravel the oxidative and eryptotic effects of bifenthrin on erythrocytes. In order to meet this objective, antioxidant enzyme’s activities, cell volume of erythrocytes and the role of calcium in triggering eryptosis were determined. The concentrations (.5-1-1.5 μM) of bifenthrin used in the current study were quite lower than the plasma concentration reported by Gammon et al.18 Oxidative stress is among the main causes of eryptosis25 and increased oxidative stress leads to decrease in antioxidant enzyme activity.7 The decrease in enzyme activity may be due to the production of superoxide radicals as superoxide dismutase catalyzes high efficiency dissemination of O2, leading to the formation of H2O2. Eraslan et al,26 also found a decrease in superoxide dismutase enzyme levels in rats on exposure to pyrethroid compound. Similar effect was also observed by Dar et al,27 in different organs of rat due to exposure of bifenthrin. Glutathioneperoxidase is an important H2O2 scavenger and important player in minimizing lipid peroxidation. The reduction in glutathione peroxidase activity indicates the inability of cells to remove reactive oxygen species.26 It is reported in an in-vivo study that bifenthrin induces oxidative stress by decrease in glutathione peroxidase levels.7 It is reported in previous research studies, pesticides can produce free radicals that lead to oxidative stress and changes in the activity of antioxidant enzymes.29 Catalase enzyme catalyzes the degradation of hydrogen peroxide into water and oxygen molecules.30 A decrease in catalase activity indicates an increased production of hydrogen peroxide.2 Yousef et al,31 reported an oxidation activity due to significant decrease in catalase levels in pyrethroid deltamethrin exposed rats. Similarly, Dar et al,27 noticed low catalase levels due to the induced oxidative stress in rat models after bifenthrin administration.

Erythrocyte membrane blebbing is an indicator of eryptosis.11 It is an apparent swelling or protrusion of the cells.32 Oxidative stress activates non-selective cation channels that led to the intake of Ca2+ in the cells.33 High intracellular calcium levels results in the activation of calcium-dependent endopeptidase calpain, enzymes which degrades the erythrocyte cytoskeleton that subsequently results in blebbing of the membrane.34 Rana et al,35 in their study investigated that oxidative stress breaks down the cytoskeleton asymmetry leading to the formation of membrane bleb. Similarly, Illyas et al. observed that xenobiotics induced oxidative stress results in membrane blebbing of erythrocytes.36 Inhibition of the membrane blebbing confirms the role of calcium in the oxidative stress induced eryptosis. Naveed et al,37 shared the
similar information that calcium channel blockage results in a decrease of the mean cell volume of erythrocytes indicating a stoppage of oxidative stress stimulated membrane blebbing.

The release of defective erythrocytes from the system prior to hemolysis is a significant physiological function of eryptosis. 38 Released hemoglobin by the ruptured erythrocytes is either filtered by the kidney or it gets precipitated in the acidic lumen of renal tubule. 12 The release of hemoglobin from erythrocytes lead to a decrease in NO bioavailability during hemolysis, which can promote vasomotor instability, systemic vasoconstriction, and endothelial dysfunction and contribute to serious clinical conditions such as high blood pressure, pulmonary complications, cardiovascular disorders, renal dysfunction, and inflammation. 39 Observed hemolytic activity was may be due to oxidative stress as high ROS generation was noticed during the episodes of hemolysis. 40

**Conclusion**

The findings of our research concluded that the indicated concentrations of bifenthrin may increase the rate of erythrocyte removal from circulation by eryptosis and hemolysis by inducing oxidative stress. Future prospects related to this work include the unmasking of further mechanisms involved in the induction of eryptosis by physiological doses of bifenthrin.

**Acknowledgments**

Authors are thankful to the Department of Biochemistry, University of Agriculture, Faisalabad for support in conducting this research study.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Funding**

The author(s) received no financial support for the research, authorship, and/or publication of this article.

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