

# Protective Effect of Melatonin Against Malathion Induced Alterations in Antioxidant Defense System and Morphology of Erythrocytes in Wistar Rats

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**Abstract:** Malathion intoxication has been shown to produce oxidative stress due to the generation of free radicals and alter the antioxidant defense system in erythrocytes. Previous studies have shown the ameliorative role melatonin against oxidative stress induced by generation of free radicals. Present study was designed to investigate the protective effect of melatonin against malathion induced oxidative stress. For this purpose, male Wistar rats were randomly divided into four groups: Control; Melatonin (10mg/Kg body weight) group; Malathion (250mg/Kg body weight) treated group; Malathion + Melatonin treated group. Animals were administered an acute dose of malathion orally. The result of this study shows that *in vivo* administration of malathion caused inhibition in AChE activity in erythrocytes. Malathion intoxication also significantly increased the oxidative damage as evidenced by increased level of LPO and GSH content. The inhibition of GSH level and increased lipid peroxidation in erythrocytes was relieved in malathion + melatonin group. Enhanced activities of SOD, CAT, GR and GPx were observed in erythrocytes of malathion treated rats as compared to control group. Moreover, melatonin supplementation in malathion treated rats maintain normal level of antioxidant enzymes as compared to malathion treated rats which indicates that melatonin provide protection against malathion-induced oxidative stress in erythrocytes. No significant change in the membrane bound enzymes such as Na<sup>+</sup>/K<sup>+</sup>-ATPase, Mg<sup>2+</sup>-ATPase and Ca<sup>2+</sup>-ATPase was observed in malathion intoxicated rats. Findings of scanning electron micrographs of erythrocytes revealed that both the malathion treated and malathion+melatonin treated groups exhibited morphological changes in erythrocytes. However, concomitant melatonin supplementation normalized the morphological alterations in erythrocytes induced by malathion toxicity. In conclusion, melatonin supplementation may ameliorate malathion-induced oxidative imbalance by enhancing the glutathione level, reducing lipid peroxidation and normalizing antioxidant enzyme activities in erythrocytes.

**Keywords:** Malathion, Melatonin, Erythrocytes, Lipid peroxidation.

## INTRODUCTION

Pesticides are widely used in agriculture and industry to enhance the production of food by eradicating unwanted insects and controlling disease. Because of their widespread use and easy accessibility, pesticide poisoning has become an important global health problem especially in the developing countries [1, 2]. Every year, millions of severe poisoning and deaths occur due to excessive use or misuse of pesticides in the developing world [3, 4].

Malathion (O,O-dimethyl S-1,2 bis-ethoxy carbonylethyl phosphorodithioate), is one of the most extensively used Organophosphate (OP) insecticides in agricultural, veterinary and public health practices. Various studies have demonstrated that OPs exposure inhibits acetylcholine esterase activity and induces oxidative stress in rats [5, 6] and humans [7, 8] due to excessive generation of free radicals and alterations in cellular antioxidant defense system. Production of ROS

is part of normal oxidative metabolism, but when produced in excess, they cause tissue injury including lipid peroxidation, alteration in the activities of various antioxidant enzymes [9]. Malathion is known to inhibit acetyl cholinesterase activity in various tissues [10, 11]. Previous studies reported that malathion intoxication leads to lipid peroxidation in the erythrocytes, liver, and brain of rats [12, 13]. Other studies indicate that malathion could induce cytotoxicity and genotoxicity *in vivo* and *in vitro* [14, 15].

Melatonin (*N*-acetyl-5-methoxytryptamine) is a naturally occurring compound, synthesized in various organs such as the pineal gland, retina, intestine, bone marrow cells and skin [16]. Because of its lipophilic nature, melatonin can easily cross cell membranes and the blood-brain barrier [17]. Melatonin has strong antioxidant and prophylactic properties against oxidative stress [18-25]. Several experimental studies have shown the ameliorate action of vitamins C and E against Malathion induced toxicity [26-29] but very few studies have been carried out to study the role of melatonin against pesticide toxicity in rats. The present study was undertaken to elucidate the antioxidant action of melatonin against the Malathion induced oxidative stress in rats.

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## MATERIALS AND METHODS

All the chemicals used in this study were of analytical grade and purchased from Sigma, St. Louis, USA and Sisco Research Laboratory (SRL), Mumbai, India.

### Experimental Animals and Study Design

Male Wistar rats, weighing 150-200 grams were procured from Central Animal House of Panjab University, Chandigarh. Animals were housed in plastic cages, fed a standard laboratory diet and water *ad libitum*, exposed to a 12 h light/dark cycle. Animals were allowed to acclimatize in the laboratory environment for 10 days before start of treatments. The protocols were approved by Animal Ethics Committee of Panjab University, Chandigarh. The animals were randomly divided into 4 groups, each comprising of 4 animals and orally administered the respective doses of Malathion and Melatonin.

Group 1 (Control): Animals were given 1.0 ml of corn oil.

Group 2 (Melatonin treated): Animals were orally supplemented with melatonin (10 mg/kg body weight).

Group 3 (Malathion treated): Animals were treated with Malathion (250 mg/kg body weight).

Group 4 (Malathion+ Melatonin): Animals were given Malathion (250 mg/kg body weight) along with Melatonin (10 mg/kg body weight).

After 6 hours of acute treatment, animals were given weak ether anaesthesia and blood was withdrawn from the rat. The blood was then centrifuged and erythrocytes were washed twice with 0.1 M phosphate buffered saline (pH 7.4). Erythrocyte lysate was prepared and used for the assay of antioxidant enzymes.

### Estimation of Proteins

The protein content in erythrocyte membrane were quantified by the method of Lowry *et al.* [30], using bovine serum albumin (BSA) as a standard.

### Measurement of Lipid Peroxidation

The lipid peroxidation in erythrocytes was determined by the method of Wills [31]. This method is based on the formation of a pink chromophore, at 532 nm, following the reaction of thiobarbituric acid (TBA) with degradation products of peroxidized lipids,

releasing malondialdehyde (MDA). TBA-MDA chromophore has been taken as an index of lipid peroxidation. Lipid peroxidation was calculated using molar extinction coefficient of MDA at 532 nm. The results were expressed as n moles of malondialdehyde/mg protein.

### Estimation of Reduced Glutathione (GSH) Content

GSH content was quantified in erythrocytes using 5, 5'-dithiobis-2-nitrobenzoic acid (DNTB) as the complexing agent [32]. This method is based on reduction of DNTB by free -SH groups to form a yellow colored complex, 2-nitro-5-mercaptobenzoic acid. The glutathione contents were expressed as nmoles GSH/mg protein using the molar extinction coefficient of GSH ( $13.6 \times 10^6 \text{ M}^{-1} \text{ cm}^{-1}$ ) at 412nm.

### Assay of Acetylcholine Esterase (AChE) Activity

AChE activity in erythrocytes was measured spectrophotometrically using acetylthiocholine iodide as a substrate [33].

### Catalase (CAT) Activity

CAT activity was spectrophotometrically measured by the rate of decomposition of  $\text{H}_2\text{O}_2$  by catalase [34]. CAT activity was expressed as  $\mu\text{moles of H}_2\text{O}_2$  decomposed /min/mg protein.

### Superoxide Dismutase (SOD) Activity

SOD activity was assayed by a method based on generation of superoxide anions by the oxidation of hydroxylamine hydrochloride [35]. The reduction of nitro blue tetrazolium (NBT) to blue formazon by superoxide anions was measured at 560 nm under aerobic conditions. SOD activity was expressed as U/mg protein.

### Glutathione Peroxidase (GPx) Activity

GPx activity in erythrocytes was measured by the method by the oxidation of glutathione by GPx [36]. In the presence of glutathione reductase and NADPH, the oxidized glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to  $\text{NADP}^+$ . The decrease in absorbance of NADPH was measured at 340 nm wavelength. GPx activity was expressed as nmol NADPH oxidized/min/mg protein.

### Glutathione Reductase (GR) Activity

GR activity in erythrocytes was determined by following the oxidation of NADPH to NADP during the

reduction of oxidized glutathione [37]. GR activity was expressed as nmol of NADPH oxidized/min/mg protein.

### Total ATPase Activity

$\text{Na}^+/\text{K}^+$  ATPase and  $\text{Mg}^{2+}$  ATPase enzymes activities were assayed in the erythrocyte membranes [38].  $\text{Na}^+/\text{K}^+$ -ATPase activity was measured under two conditions; in the presence of  $\text{Mg}^{2+}$ ,  $\text{Na}^+/\text{K}^+$  ATPases (total ATPase) and secondly in the presence of  $\text{Mg}^{2+}$ ,  $\text{Na}^+/\text{K}^+$  ATPases and ouabain.

### Scanning Electron Microscopy (SEM) of Erythrocytes

A drop of blood was fixed immediately in 2.5% glutaraldehyde prepared in 0.2M phosphate buffer (pH 7.2). After 2 hours of fixation, the cells were centrifuged at 1000-1500 rpm. The fixative was discarded and the pellet was resuspended in the phosphate buffer. This process was repeated 2 times, every time the supernatant was discarded. The pellet was then suspended in the triple distilled water and again centrifuged and reconstituted for 1-2 times in triple distilled water. Finally, the pellet was suspended in a minimum amount of triple distilled water and a drop of sample was smeared on the metallic SEM stubs, which was loaded with a conductive silver tape on its top. These stubs were then coated with gold to a thickness

of 100Å using sputter ion coater, with gold source for 4-5 minutes. These specimens were finally observed under electron microscope (JSM-6100, Jeol, Japan) at Regional Sophisticated Instrumentation Centre (RSIC), Panjab University, Chandigarh, India.

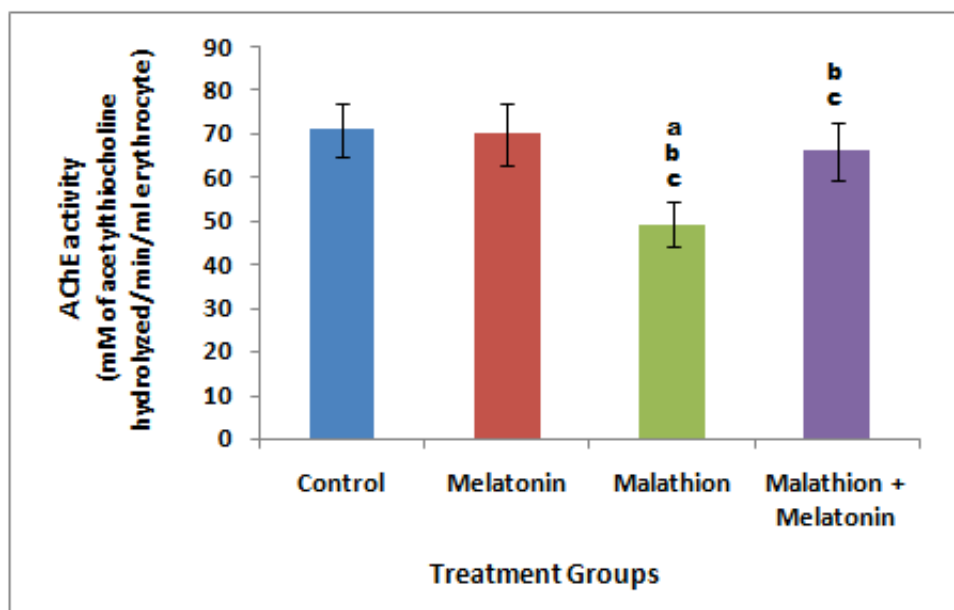
### Statistical Analysis

The data are expressed as Mean±Standard Deviation (SD). Differences among experimental groups were calculated by using one-way analysis of variance (ANOVA). In all experiments,  $P < 0.05$  was considered statistically significant. The data were analyzed by using SPSS 16.0 for Windows.

## RESULTS

### Effect of *In Vivo* Administration of Malathion on AChE Activity

Figure 1 shows the effect of acute administration of malathion on AChE activity in rat erythrocytes. Present study results shows that Malathion treatment significantly reduced the AChE activity in the erythrocytes when compared with the control group. No significant change in AChE activity was observed in the Melatonin supplemented rats and control group. However, melatonin supplementation along with malathion resulted in a small recovery in AChE activity.



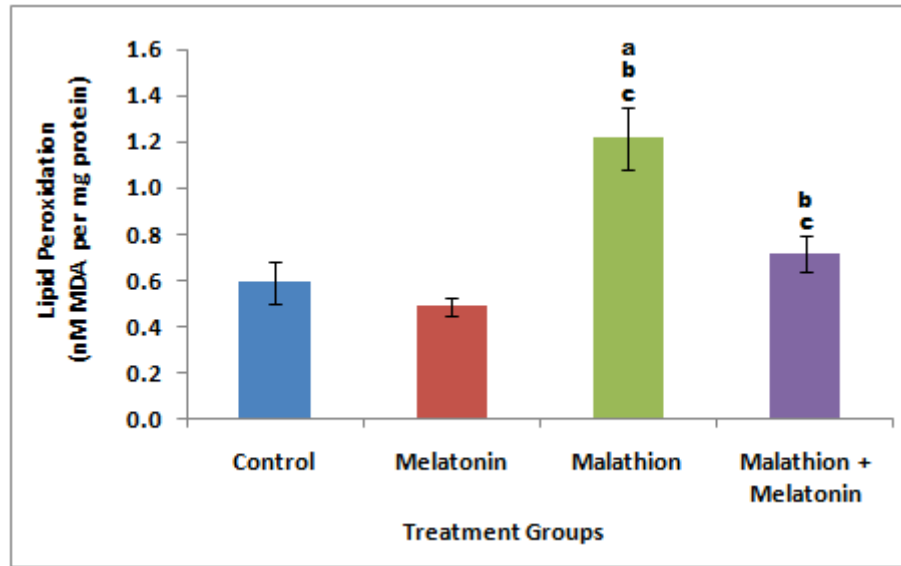
**Figure 1: Effect of *in vivo* administration of Malathion on AChE activity in rat erythrocytes.**

Values are mean ± SD of six rats in each group. Significance at  $P < 0.05$ .

<sup>a</sup>Comparison of control and other groups.

<sup>b</sup>Comparison of Melatonin treated group with Malathion group/Melatonin + Malathion group.

<sup>c</sup>Comparison of Malathion treated group with Melatonin + Malathion group.



**Figure 2: Effect of *in vivo* administration of Malathion on lipid peroxidation in rat erythrocytes.**

Values are mean  $\pm$  SD of six rats in each group. Significance at  $P < 0.05$ .

<sup>a</sup>Comparison of control and other groups.

<sup>b</sup>Comparison of Melatonin treated group with Malathion group/Melatonin + Malathion group.

<sup>c</sup>Comparison of Malathion treated group with Melatonin + Malathion group.

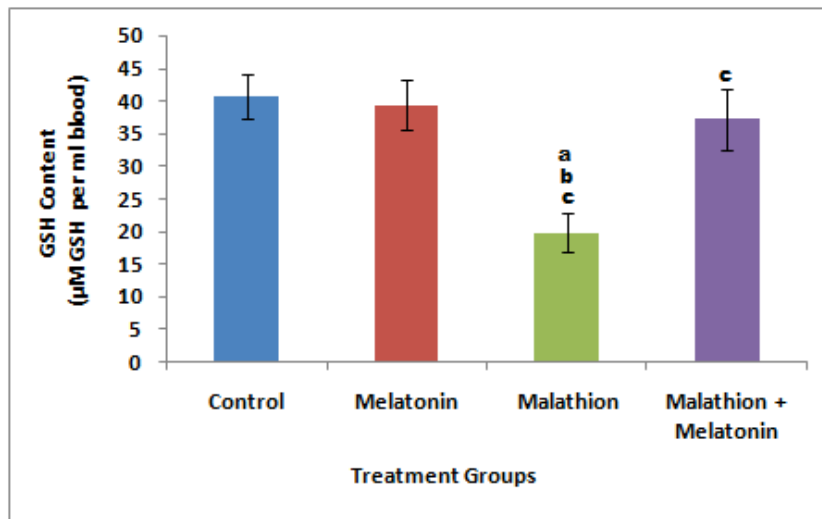
### Effect of *In Vivo* Administration of Malathion on Lipid Peroxidation

The effect of *in vivo* administration of malathion on lipid peroxidation in erythrocytes is shown in Figure 2. Exposure of animals with malathion resulted in a significant increase in MDA level compared to control animals. A significant decrease in the LPO was observed when animals were supplemented with

melatonin along with Malathion, compared to Malathion treated rats. However, treatment with melatonin was more effective in terms of reducing lipid peroxidation.

### Effect of *In Vivo* Administration of Malathion on GSH Content

The GSH content in erythrocytes of experimental groups are given in Figure 3. Comparison of GSH



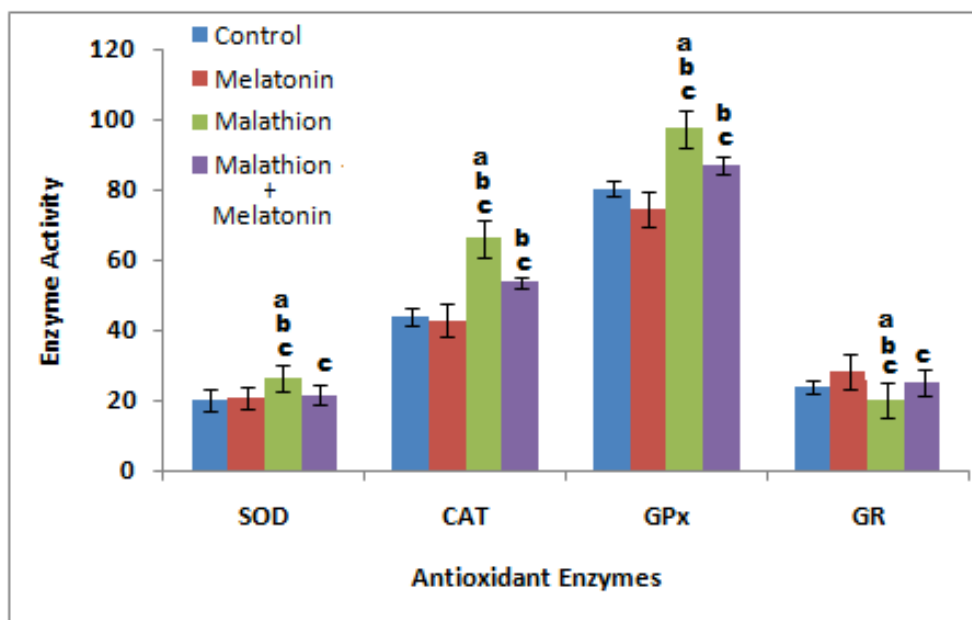
**Figure 3: Effect of *in vivo* administration of Malathion on GSH Content in rat erythrocytes.**

Values are mean  $\pm$  SD of six rats in each group. Significance at  $P < 0.05$ .

<sup>a</sup>Comparison of control and other groups.

<sup>b</sup>Comparison of Melatonin treated group with Malathion group/Melatonin + Malathion group.

<sup>c</sup>Comparison of Malathion treated group with Melatonin + Malathion group.



**Figure 4: Effect of *in vivo* administration of Malathion on activities of antioxidant enzymes.** SOD activity was expressed as U/mg protein. CAT activity was expressed as  $\mu$ moles of  $H_2O_2$  decomposed /min/mg protein. GPx activity was expressed as nmol NADPH oxidized/min/mg protein. GR activity was expressed as nmol of NADPH oxidized/min/mg protein.

Values are mean  $\pm$  SD of six rats in each group. Significance at  $P < 0.05$ .

<sup>a</sup>Comparison of control and other groups.

<sup>b</sup>Comparison of Melatonin treated group with Malathion group/Melatonin + Malathion group.

<sup>c</sup>Comparison of Malathion treated group with Melatonin + Malathion group.

contents in the experimental groups showed a significant decrease in Malathion treated rats than in the control group. There was a significant increase in GSH levels in Malathion + melatonin administered rats when compared to those in the malathion treated rats. These results indicate that Malathion intoxication decreased the GSH content in erythrocytes and administration of melatonin along with malathion showed recovery in their GSH content as compared to malathion treated group.

#### Effect of *In Vivo* Administration of Malathion on Antioxidant Enzymes

Significant increase in CAT, SOD and GPx activity was observed after acute administration of malathion when compared to control or melatonin treated rats (Figure 4). However significant recovery in antioxidant enzymes was found in malathion + melatonin treated rats.

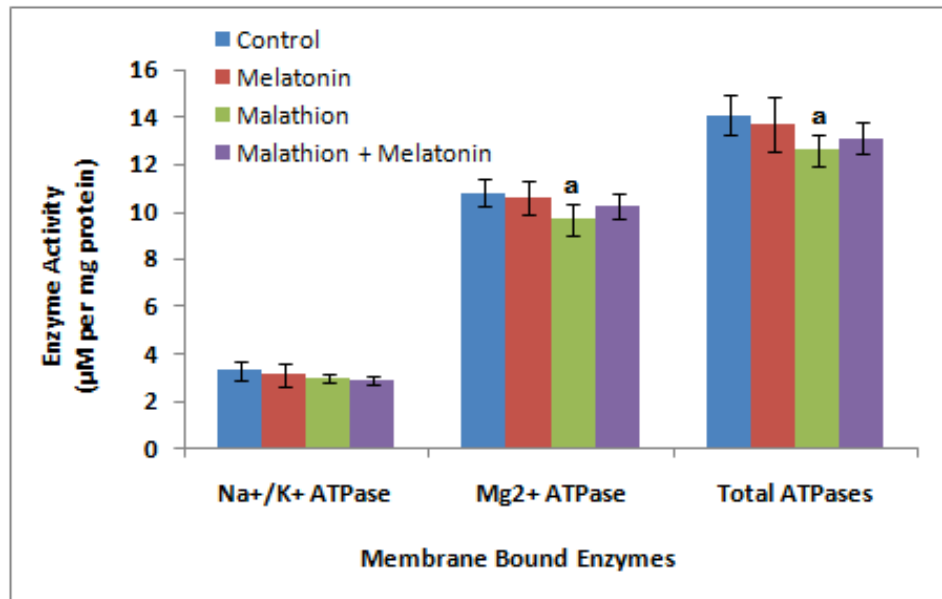
No significant change in GR activity was observed in malathion treated rats as compared to control group. But a slightly increased GR activity was observed in Melatonin supplemented rats when compared with control group.

#### Effect of *In Vivo* Administration of Malathion on ATPases

Figure 5 shows the effect of *in vivo* administration of malathion on ATPase activities in rat erythrocytes. No significant change in total ATPases ( $Na^+/K^+$  ATPase and  $Mg^{2+}$  ATPase) activity was observed in melatonin supplemented rats as compared to control group. But a slight decrease in total ATPases was observed in Melathion treated rats when compared with control or melatonin supplemented rats.

#### SEM of Erythrocytes

Scanning electron micrographs of erythrocytes revealed that administration of malathion resulted in prominent morphological changes in rat erythrocytes (Figure 6). It is evident from the electron micrographs that the erythrocytes of the control group were perfect discocytes, that is, typical biconcave disks. Distortions of normal discocytes to different pathological forms were observed after acute administration of malathion. Most of the erythrocytes became spherocytes. Erythrocytes of melatonin+malathion treated group shows some improvement in erythrocytes topography as compared to malathion treated group.



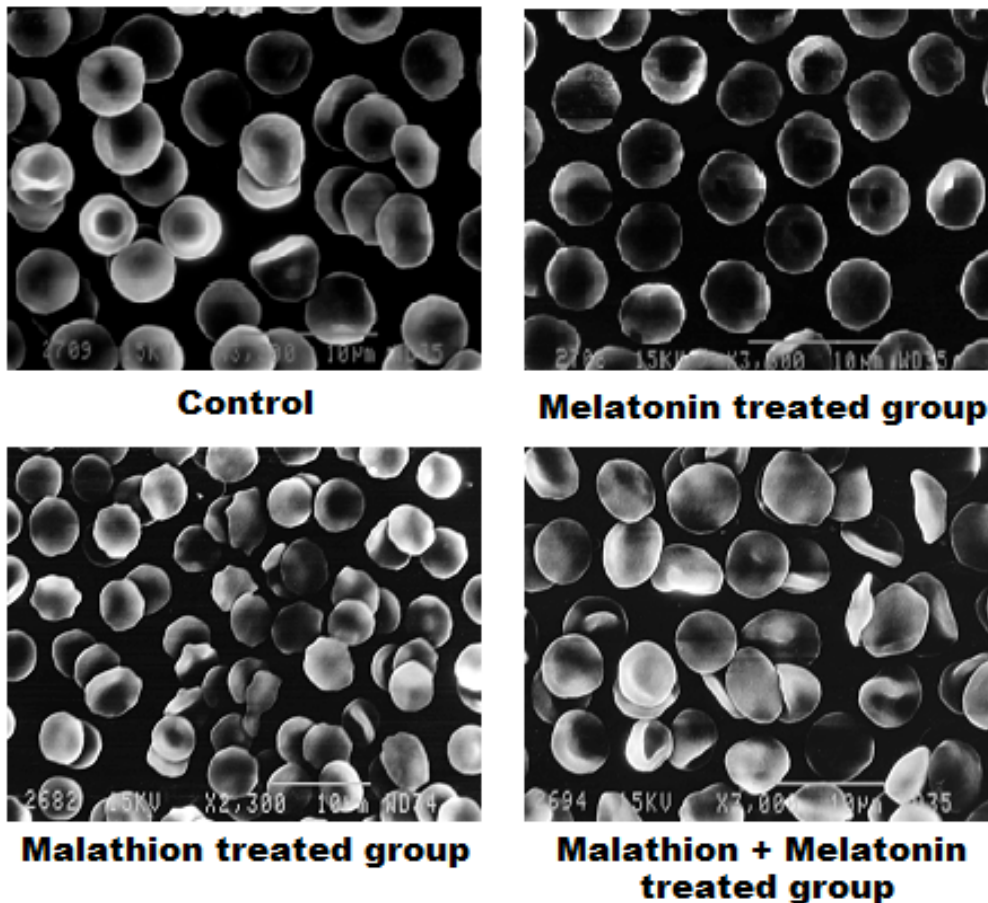
**Figure 5: Effect of *in vivo* administration of Malathion on membrane bound enzymes.**

Values are mean ± SD of six rats in each group. Significance at P < 0.05.

<sup>a</sup>Comparison of control and other groups.

<sup>b</sup>Comparison of Melatonin treated group with Malathion group/Melatonin + Malathion group.

<sup>c</sup>Comparison of Malathion treated group with Melatonin + Malathion group.



**Figure 6: Scanning Electron Micrographs of erythrocytes.**

## DISCUSSION

The frequent and widespread use of OP insecticides in agriculture has resulted in their distribution in environment and exerts deleterious effects on biological systems. Erythrocytes are highly susceptible to oxidative damage due to the presence of heme iron, PUFA and oxygen radicals which may initiate the reactions that induce oxidative changes in the red blood cells [5, 9]. AChE activity in erythrocytes was inhibited by acute administration of malathion, however, this activity was recovered to some extent by melatonin supplementation. These results show that melatonin may reduce the OP-induced oxidative stress in erythrocytes upto some extent but it may not be directly involved in relieving the inhibition of AChE activity.

Lipid peroxidation (LPO) is one of the main manifestations of oxidative damage and has been found to play an important role in the deleterious effects of many xenobiotics. Elevation of LPO in erythrocytes as evidenced by the increased production of MDA in the present study, suggests participation of free radical induced oxidative cell injury in mediating the toxicity of Malathion. Peroxidation of lipids seems to be an unavoidable process in tissue injury and may impair antioxidant defense system which leads to oxidative stress [39]. Malathion might induce oxidative stress by directly enhancing the production of reactive oxygen species (ROS) or decreasing the antioxidant ability of the cell. Previous studies reported that exposure to malathion enhanced the lipid peroxidation and alter physiological and biochemical characteristics of erythrocytes [12, 13, 40]. A significant decrease in LPO in erythrocytes in melatonin supplemented rats shows the strong antioxidant properties of melatonin against malathion induced ROS generation. A recent study described the ameliorative action of melatonin against atrazine induced oxidative stress in erythrocytes [20].

GSH plays an important role in protecting cells from xenobiotic induced tissue injury [41]. Significant decrease in GSH content after malathion exposure indicated pro-oxidant condition in erythrocytes, which may result in oxidative damage. The reduced levels of GSH in malathion treated rats may be due to augmented consumption of GSH for conjugation and/or participation of GSH as an antioxidant in terminating free radical production. However, the recovery in GSH levels in melatonin supplemented rats administered malathion may be due to the stimulation of GSH

synthesis by melatonin as reported by a previous study [25]. Various studies reported ameliorative role of vitamins as well as other antioxidants against pesticide toxicity [20, 29, 40, 42].

Antioxidant enzymes provide a major line of defense against free radical damage either by metabolizing them to less reactive species or to non-toxic byproducts. Alterations in antioxidant defense system are one of the possible alternative targets of OP toxicity. The increased activities of SOD, CAT and GPx in malathion treated rats was probably due to enhanced production of ROS which caused oxidative stress in erythrocytes. The significant recovery in antioxidant enzymes in malathion + melatonin treated rats indicated that melatonin limits the accumulation of peroxides in tissues and contributes to antioxidant protection against oxidative damage induced by malathion intoxication. Melatonin is known to scavenge various free radicals such as hydroxyl radical, peroxynitrite, singlet oxygen and peroxy radical [43-46], which are generated during the oxidation of unsaturated fatty acids and lead to lipid peroxidation in erythrocytes. Various studies have reported that melatonin may reduce oxidative stress also by stimulating antioxidant enzymes such as SOD, GR and GPx [47-49]. GPx reduces free radical damage by metabolizing  $H_2O_2$  to  $H_2O$ . GSH is oxidized to its disulfide, GSSG which is then quickly reduced back to GSH by GR, an enzyme which has also been shown to be stimulated by melatonin. The recycling of GSH may well be a major action of melatonin in curtailing oxidative stress. The ability of melatonin to regulate the GSH/GSSG balance by modulating enzyme activities appears to involve an action of melatonin [50]. The high efficacy of melatonin in preventing oxidative damage of essential biomolecules suggests that melatonin can play a vital role in curtailing the oxidative stress induced by pesticide toxicity.

The observed morphological changes in erythrocytes of Malathion intoxicated rats might be due to the altered lipid composition of cell membrane. The decrease in the cholesterol content reduces the membrane fluidity and in turn also affects the membrane permeability which further leads to the alteration in the cell shape with adverse hematological consequences [42]. Supplementation of melatonin recovers the cell damage induced by malathion exposure. Numerous *in vitro* and *in vivo* studies have documented the ability of melatonin to protect against free radical destruction. Previous studies described that



melatonin play a vital role in stabilizing the cell membrane because of interaction with unsaturated fatty acids.

In conclusion, Malathion intoxication inhibited the acetylcholine esterase activity and induces oxidative imbalance as evidenced by enhanced lipid peroxidation, reduced GSH content and altered antioxidant defense system in erythrocytes. However, supplementation of melatonin ameliorates the deleterious effects of Malathion in rat erythrocytes.

## ACKNOWLEDGEMENT

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