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Original Article

Effect of Cypermethrin Induced Antioxidant Enzymes Systems in Response to Melatonin Administration in *Drosophila Melanogaster*

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ABSTRACT

Introduction: Melatonin, the main hormonal product of the pineal gland, has pleiotropic bioactivity that encompasses numerous endocrinological and behavioral processes. Melatonin and several of its metabolites function as free radical scavengers and broad-spectrum antioxidants. Due to their small size and amphiphilic nature they can easily reach all cellular compartments. The aim of the present study was to investigate the effect of melatonin on cypermethrin induced free radical damage in *Drosophila melanogaster*. **Methods:** The pro-or antioxidant enzyme activities of superoxide dismutase (SOD), glutathione–S-transferase (GST), lipid peroxidation (LPO), peroxidase (POX), catalase (CAT) and cytochrome P450 were assayed. **Results:** SOD and cytochrome P450 enzymes show significant increase in the melatonin+synthetic pyrethroid treated group, while GST shows significantly decreased activity in melatonin+synthetic pyrethroid group with respect to control. Lipid peroxidation activity was significantly increased in the synthetic pyrethroid group and also in melatonin+synthetic pyrethroid treated groups. **Conclusion:** In conclusion, melatonin administered the resulted in activation/increased expression of Cyt P450, GST and SOD titers, showing that melatonin in addition to being an antioxidant molecules, it also participates in eliciting other antioxidant systems in insects.

Key words: Melatonin, Antioxidant enzymes, Fruit fly, Free radical scavenging, Chronobiology, Detoxification Enzymes.

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INTRODUCTION

Synthetic pyrethroids constitute a unique group of insecticides having pyrethrum like structures with better performance characteristics and account for over 30% of insecticides used globally.[1] Based on the symptoms produced in animals, pyrethroids fall into two distinct classes: types I and II. While type I pyrethroids affect sodium channels in nerve membranes, producing repetitive neuronal discharge and prolonged negative after-potential, type II pyrethroids produce even longer delay in sodium channel inactivation leading to a persistent depolarization of the nerve membrane without repetitive discharge[2] and their target site is biological membrane. In addition, type II syndrome implicates the central nervous system, while type I involve the peripheral nerves.[3] In general, insecticide sprayers and industrial worker are rarely exposed to single insecticides in their day-to-day life. Mostly, they are exposed to multiple classes of pesticides altogether. Several epidemiological and experimental studies have been performed to assess the health risks associated with cypermethrin exposure and measured cypermethrin level in the blood and urine of the pesticides sprayers and exposed individuals. Cypermethrin has been identified as one of the important constituent pesticides associated with human health risks.[4]

Cypermethrin is a class II synthetic pyrethroid which crosses the blood brain barrier and exerts neurotoxicity in the central nervous system and also induces motor deficits. Cypermethrin acts by hyper polarization of neurons by prolonged opening of sodium channels[5] and by modulating GABA levels.[6] Furthermore, cypermethrin-mediated neurotoxicity is contributed by its ability to induce free radical generation.[7]

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent, resulting in production of free radicals. In turn, these radicals can start chain reactions. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions.[8,9] Plants and animals maintain complex systems of multiple types of antioxidants such as glutathione, melatonin, Vitamin C, Vitamin A and Vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxides. In inhibition of certain body antioxidants and free radical generation leads to cellular damage and dysfunction of organs in synthetic pyrethroids toxicity. Hence, antioxidant would be beneficial to reduce the toxic effect of synthetic pyrethroids.

Melatonin plays a role in the regulation of biological rhythms and ovarian functions. In this role it is secreted in a rhythmic pattern, with peak levels occurring at night. The peak secretion level serves as a biochemical signal for "darkness", after which melatonin levels gradually decline until the daytime hours, or, alternatively, secretion can be abruptly suppressed if there is a sudden exposure to environmental light. Melatonin is known to be synthesized in many vertebrate tissues, in bacteria, protozoa, plants, fungi and invertebrates.[10] Melatonin (N-acetyl-5-methoxytryptamine) is a potent hydroxyl radical scavenger.[11] Melatonin cross can cell membrane to enter the cytosol and it also has access to every subcellular compartment due to its high solubility in lipids and its hydrophilicity.[12] Removal has pineal gland in rats significantly reduces the free radical scavenging ability, while exogenous administration increased the antioxidant status.

Melatonin as a natural antioxidant, can serve as a suitable indicator of the dark period.[13] The production of melatonin decreases with age and it is suggested that maintaining melatonin at a high level could slow age related alterations. In addition to scavenging reactive oxygen species, melatonin stimulates the activity of antioxidant enzymes superoxide dismutase, glutathione, peroxidase and catalase.[14]

Melatonin is known hormone to play a role in regulating circadian and seasonal rhythms in rats.[15] Several circadian rhythms, such as the rest/activity cycle, core body temperature, neuronal electrical activity and locomotor activity, are driven by melatonin.[16] Melatonin has been shown to reduce the symptoms of improve jet lag and other sleep disturbances.

Melatonin is a versatile molecule whose functions have been shown to include prevention of aging, analgesic and anti-inflammatory activity and neuroprotection against excitotoxicity. Numerous reports have documented protective actions of melatonin. Previous studies revealed that melatonin stimulates a host of antioxidative enzymes and protect the cells against oxidative damage induced by pesticide toxicity in *Spodoptera litura*.[17] Further numerous studies have verified the influence of melatonin in the antioxidant defense system, and, more specifically, its regulatory actions on certain antioxidant enzymes like superoxide dismutase and glutathione peroxidase (GPx), catalase (CAT), Mn-superoxide dismutase (Mn-SOD), and Cu/Zn-superoxide dismutase (Cu/Zn-SOD).[18] Melatonin also acts directly as a scavenger of reactive oxygen species (ROS) such as the hydroxyl radical, the peroxyl radical,[19] hydrogen peroxide and singlet oxygen.[20] To date, numerous investigations have demonstrated melatonin's protective action against oxidative stress induced by free radicals and other reactive oxygen species.

Consistent with the fact that variation in the aerobic metabolism do occur and also daily variations in the antioxidant system in insects. The crab *Neohelice granulata* displays a biphasic profile in the daily variations of oxygen consumption both in the gills and the hepatopancreas, with one peak at the photophase and another at the scotophase, as well as daily variations in some components of the antioxidant defense system and lipid peroxidation.[21]

Drosophila melanogaster is an excellent model organism for studying mechanisms like oxidative stress, aging, neurodegenerative diseases, cancer etc. Melatonin and the enzyme arylalkylamine N-acetyltransferase, which catalyzes the rate limiting step in melatonin synthesis, are present in *D. melanogaster*. On the other hand, the antioxidative effect of this hormone in *D. melanogaster* has been demonstrated that melatonin prevent the increase in protein carbonyl when oxidative stress from endogenous sources was enhanced by the administration of the catalase inhibitor 3-amino-1, 2, 4-triazole.[22]

The present study was investigated that ameliorating the impact of oxidative stress induced by melatonin and synthetic pyrethroid against *Drosophila melanogaster*. It was presumed that given the antioxidant and free radical scavenger properties of this hormone, its administration would prevent oxidative damage to the fly tissues especially to the central nervous system, which is highly susceptible to damage by oxygen-base radicals.

MATERIALS AND METHODS

Drosophila melanogaster stock and culture

D. melanogaster, wild adult male flies (8-10 days old) were obtained from the *Drosophila* stock culture facility at University of Mysore, Karnataka, India. The flies were maintained at 25 ± 1°C and 70–80% relative humidity, and fed on a standard wheat flour-agar diet with yeast granules as the protein source. Diet was prepared according to a standard protocol. One liter semisolid diet contained 100 g wheat flour, 100 g jaggery (carbohydrate source), 10 g agar agar (solidifying agent) and 7.5 ml propionic acid (antifungal agent) few granules of yeast were added to ensure availability of protein source.[23] After 24 h, flies were transferred to stock bottles to avoid sticking of flies to media.

Chemicals

Cypermethrin (10% EC) was obtained from insecticides at SIDCO. Melatonin was purchased from Himedia, Mumbai.

Experimental design

Melatonin was incorporated into an artificial diet [24] after dissolving in acetone and diluting; the final concentration of acetone was 1% and the concentrations of melatonin were 4.3×10^{-5} M. Control larvae were fed melatonin-free diets (without melatonin and Cypermethrin), or a diet containing 1% acetone. The *D. melanogaster* larvae were fed the regulated diets from the first instar stage to adult stage (9 days) under constant photoperiods (12L: 12D) at 25°C with refreshment of diet in every 3-4 days. Cypermethrin was administered into artificial diet at (5 g a.i/hectare) field recommended dosages.

Enzyme preparation

Treated fruit fly larvae were homogenized on ice in homogenization buffer (0.1 M phosphate buffer, pH 7.2) containing 1 mM EDTA, 1 mM DTT, 1 mM PTU, 1 mM PMSF and 20% glycerol. Larvae were chilled on ice before homogenization. Tissues were homogenized in 2 ml buffer, the homogenate was centrifuged at 4°C, 10, 000 rpm for 15 min, and the solid debris and cellular material were discarded. The supernatant was decanted into a clean eppendorf tube, placed on ice and used immediately for Glutathione–S-transferase (GST), Catalase (CAT), Peroxidase (POX), Cytochrome P450, Lipid Peroxidation and Superoxide dismutase (SOD).

Biochemical assay

Total Protein

Total protein content of the tissue homogenate was determined by the method[25] using bovine serum albumin as the standard.

Glutathione S-transferase Assay (GST)

GST activity was estimated using the method[26] with minor modifications. 50 µl of 50 mM 1-chloro-2, 4-dinitrobenzene (CDNB) and 150 µl of 50 mM reduced glutathione (GSH) were added to 2.78 ml of sodium phosphate buffer (100 mM, pH 6.5). Twenty µl of enzyme stock was then added. The reaction was carried out in triplicate. The contents were shaken gently, incubated 2-3 min at 20°C and then transferred to a cuvette in the sample cuvette slot of a UV-Visual Spectrophotometer (Systronics). Reaction mixture (3 ml) without enzyme was placed in the reference slot for setting zero. Absorbance at 340 nm was recorded for 10-12min employing kinetics (time scan) menu. The GST activity was calculated using the formula: CDNB-GSH conjugate (µM mg protein^{-1} min^{-1}).

Superoxide dismutase assay (SOD)

SOD activity was assayed using the method.[27] Reaction mixtures were prepared in 3-ml glass spectrophotometer cuvettes by adding 2.8 ml of Tris-EDTA (50 mM Tris and 10 mM EDTA, pH 8.2) buffer and 50µl of enzyme supernatant. The content was mixed and the final volume was adjusted to 2.9 ml with Tris-EDTA buffer. Reaction in the cuvette was started with the addition of 100 µl of Pyrogallol (15 mM). The rates of autoxidation were followed at 440 nm in the UV-Visible spectrophotometer (Systronics), and absorbance was measured for 3 min. One unit total SOD activity was calculated as the amount of protein per milligram causing 50% inhibition of pyrogallol autoxidation. The total SOD activity was expressed as units per milligram of protein.

Catalase assay

CAT activity was spectrophotometrically measured by the rate of decomposition of H_2O_2 by catalase.[28] CAT activity was expressed as µmoles of H_2O_2 decomposed /min/mg protein.

Peroxidase assay

POX activity was determined[29] using UV–Vis spectrophotometer at 430 nm by catalyzing the oxidation in the presence of H_2O_2 of a substrate. One unit of POX activity was defined as the amount that catalyses 1 mg substrate per minute per mg protein. POX activity was expressed as U mg^{-1} protein.

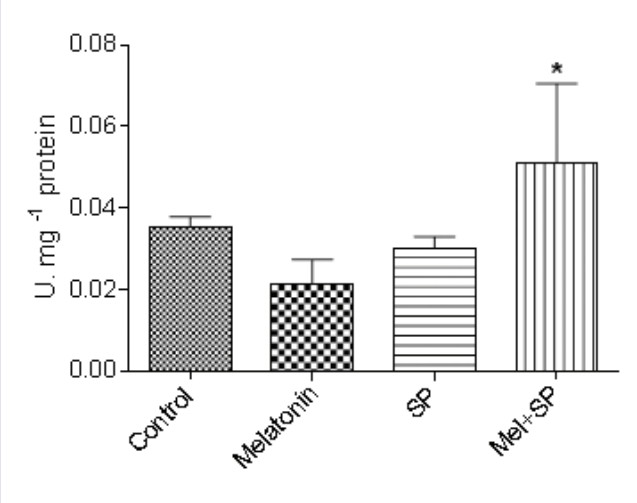

Figure 1: Effect of melatonin on SOD enzyme levels in the larvae of Drosophlia melanogaster treated with synthetic pyrethroid. Asterisk (*) indicates significant difference among treatments with respect to control (p<0.05). Control; Melatonin; SP-Synthetic Pyrethroid.

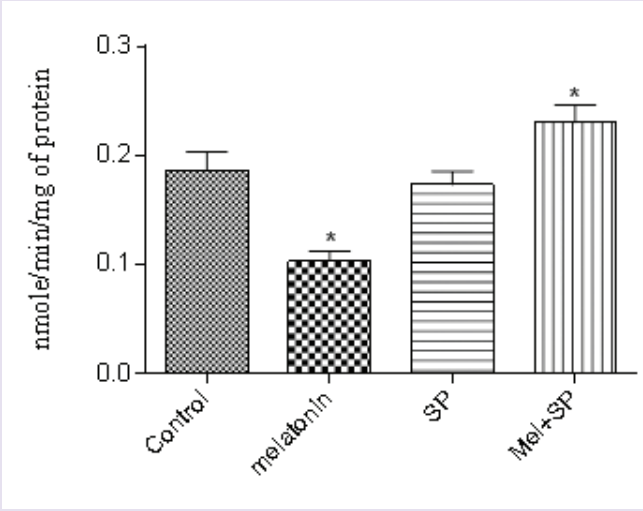

Figure 2: Effect of melatonin on Cyt P450 enzyme levels in the larvae of *Drosophila melangastor* treated with synthetic pyrethroid. Asterisk (*) indicates significant difference among treatments with respect to control (p<0.05). Control; Melatonin; SP-Synthetic Pyrethroid.

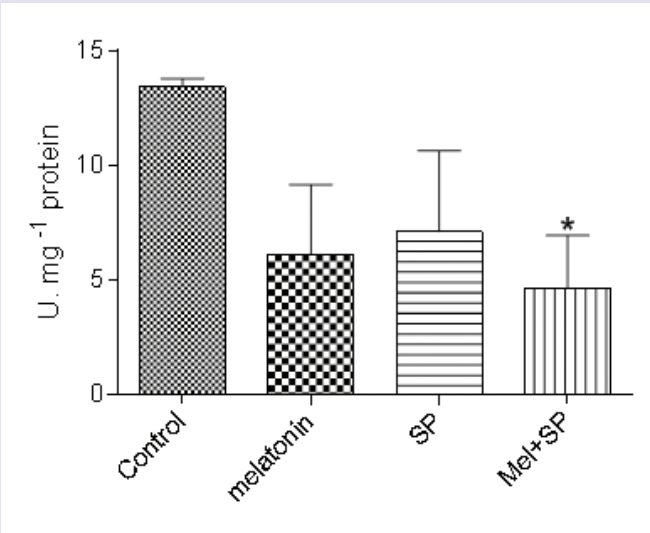

Figure 3: Effect of melatonin on GST enzyme levels in the larvae of *Drosophila melangastor* treated with synthetic pyrethroid. Asterisk (*) indicates significant difference among treatments with respect to control (p<0.05). Control; Melatonin; SP-Synthetic Pyrethroid.

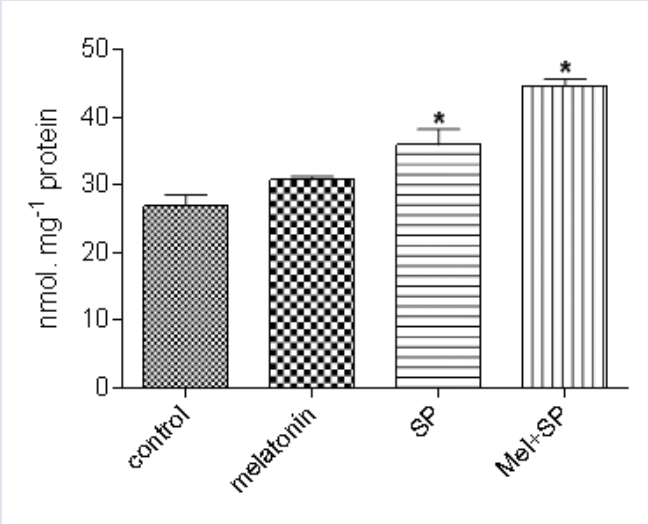

Figure 4: Effect of melatonin on MDA enzyme levels in the larvae of *Drosophila melangastor* treated with synthetic pyrethroid. Asterisk (*) indicates significant difference among treatments with respect to control (p<0.05). Control; Melatonin; SP-Synthetic Pyrethroid.

Cytochrome P450 assay

Cytochrome P450 activity was done by using method.[30] 20 µl of supernatant was taken in the test tube containing 200 µl of 6.3 mM TMBZ solution (10 mg TMBZ dissolved in 5 ml absolute methanol mixed with 15 ml of 0.25 M sodium acetate buffer ($NaC_2H_3O_2$), pH 5.0, prepared fresh daily). Then to each of test tubes 80 µl of 0.0625 M potassium phosphate buffer ($KHPO_4$) pH 7.2 and 25 µl of 3% hydrogen peroxide (H_2O_2) were added. Two controls were prepared per sample, with 20 µl of homogenizing buffer with all the ingredients excluding the enzyme source. Absorbance was recorded at 630 nm after 30 min at room temperature. Recorded absorbance was converted to end product formation from a standard curve of Cytochorme C. Total cytochrome P450 activity was expressed as nmol equivalent cytochrome P450 mg protein^{-1} min^{-1}.

Lipid peroxidation assay

Lipid peroxidation activity was determined.[31] The process of lipid peroxidation results in the formation of malondialdehyde (MDA). Thiobarbituric acid reactive substances are formed as a byproduct of lipid peroxidation, which can be detected by the TBARS assay using

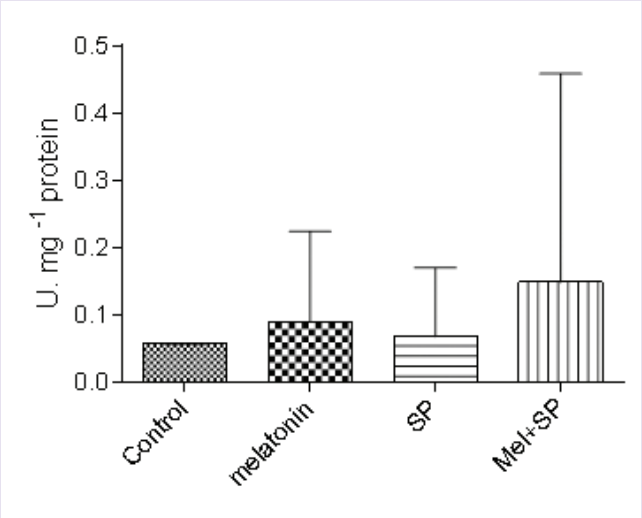

Figure 5: Effect of melatonin on POX enzyme levels in the larvae of *Drosophila melangastor* treated with synthetic pyrethroid. Asterisk (*) indicates significant difference among treatments with respect to control (p<0.05). Control; Melatonin; SP-Synthetic Pyrethroid.

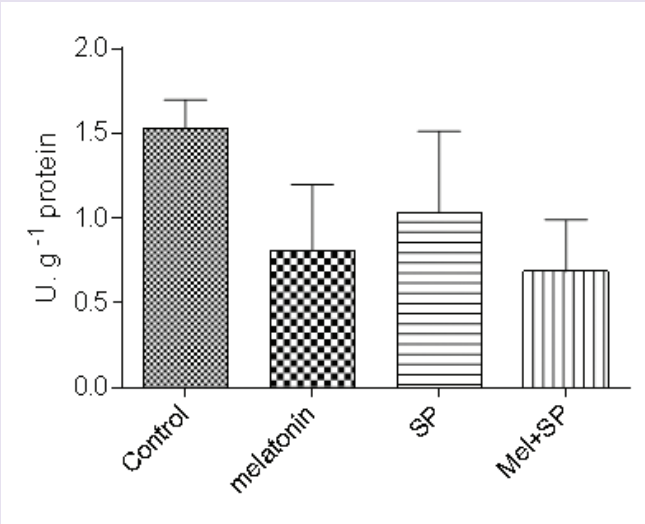

Figure 6: Effect of melatonin on Catalase enzyme levels in the larvae of *Drosophila melangastor* treated with synthetic pyrethroid. Asterisk (*) indicates significant difference among treatments with respect to control (p<0.05). Control; Melatonin; SP-Synthetic Pyrethroid

thiobarbituric acid as a reagent. 0.1 ml of the enzyme solution was taken.1.9 ml of 0.1 M sodium phosphate buffer at the pH was added to the enzyme solution. Then the mixture was incubated at 37°C for one hour. This mixture was precipitated with 10% TCA and centrifuged at 5000 rpm for 15 minutes and supernatant was collected. 1 ml of 1% TBA was added to the supernatant collected after centrifugation. The sample was boiled in water bath for 15 minutes. After boiling the supernatant was cooled and absorbance was taken at 532 nm.The MDA level was expressed as nmol/h/mg of protein.

Statistical Analysis

The data are summarized as means ± their respective standard deviations (SD). Differences among experimental groups were calculated by using a one-way analysis of variance (ANOVA) with dunnett multiple comparison tests. In all experiments, the value $P<0.05$ was used as the standard of statistical significance for hypothesis testing. The data were analyzed using the SPSS 16.0 statistical package for Windows.

RESULTS

Antioxidant enzymes

A significant ($p<0.05$) increase in SOD activity in Melatonin+cypermethrin treated samples as compared with respect to control. However there are no changes in Melatonin and cypermethrin treated samples (Figure 1). Cytochrome P450 activity was significantly ($p<0.05$) increases in melatonin and melatonin+cypermethrin treated groups as compared with control (Figure 2). GST activity was decline in the cypermethrin, melatonin and melatonin+cypermethrin treated groups as comparison with control, and a significant ($p<0.05$) decrease activity was found in melatonin+cypermethrin treated groups (Figure 3). Lipid peroxidation activity was significantly ($p<0.05$) increases in cypermethrin and melatonin+cypermethrin treated groups as compared with control (Figure 4). POX activity was no changes between melatonin and cypermethrin as compared with control (Figure 5). In CAT enzyme activity was decline in cypermethrin, melatonin and melatonin+cypermethrin treated groups as comparison with control (Figure 6).

DISCUSSION

Oxidative stress plays an important role in governing the life span of *D. melanogaster*.[32] A modest amount of research has been carried out on the relationship between life span and antioxidant defense in *Drosophila*. Massie *et al.*[33] have noted that the activity of the enzyme superoxide dismutase (SOD), which catalyzes dismutation of the superoxide radical,[34] is not significantly different between wild strains of *Drosophila* whose life span may differ by as much as similar. Melatonin's protective effects against oxidative damage to lipids and DNA are well known.[35] It also prevents free radical damage to proteins.[36] Additionally, the antioxidant protection provided by melatonin could also be carried out through the stimulation of antioxidative enzymes like glutathione peroxidase,[37] superoxide dismutase, glutathione reductase and catalase and the increase in the efficiency of the electron transport chain thereby reducing electron leakage and the generation of free radicals.[38] Melatonin also stimulates c-glutamyl-cysteine synthetase, the rate-limiting enzyme in the synthesis of glutathione.

Melatonin and the enzyme arylalkylamine N-acetyl transferase, which catalyzes the rate-limiting step in melatonin synthesis, are present in *D. melanogaster*.[39] The antioxidative effect of melatonin in Drosophila was demonstrated by a preferential suppression of oxidative damage to proteins, as compared to lipids, when the catalase inhibitor 3-amino-1,2, 4-triazole was used to enhance oxidative stress from endogenous source. Earlier several studies have shown the involvement of mitochondrial oxidative stress in PQ toxicity in a Drosophila model. Flies over expressing catalase in mitochondria were reported to be resistant to PQ, while enhancement of cytosolic catalase was not protective.[40] RNA interference silencing of Mn-SOD in flies caused hypersensitivity to PQ.[41] Since PQ is used in experimental models of PD to generate mitochondrial oxidative damage,[42] it is important to assess interaction of PQ with mitochondria among flies. Similarly the protective effect of melatonin against paraquat toxicity to *D. melanogaster* has been previously demonstrated.[43]

The frequent and widespread use of Synthetic Pyrethroid insecticides in agriculture has resulted in their broad distribution in the environment

and resulting deleterious effects on biological systems. GST has the ability to detoxify several chemical classes of insecticides such as organophosphates, pyrethroids, carbamates and chlorinated hydrocarbons. GST in insects can be considered a primary antioxidant enzyme, in as much as it is effective in metabolizing lipid peroxides.[44] However significantly decreased GST activity occurred in cypermethrin+melatonin treated samples as compared with control. Our studies suggest that there was an decreased level in synthetic pyrethroid against melatonin in lepidopteran insects. Various studies have reported that vitamins as well as other antioxidants have an ameliorative effect against pesticide toxicity.[45] Antioxidant enzymes provide a major line of defense against free radical damage either by metabolizing them to less reactive species or to nontoxic byproducts. Alterations in antioxidant defense system are one of the possible alternative targets of cypermethrin toxicity. The increased activities of SOD, cyt P450 and lipid peroxidation in melatonin treated insects were probably due to enhanced production of ROS which caused oxidative stress in insects. Melatonin is known to scavenge various free radicals such as hydroxyl radical, peroxynitrite, singlet oxygen and peroxyl radical, 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.[46] 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 regulation of GSH/GSSG balance through the modulation of enzyme activities appears to involve melatoninergic action.[47] Previous study reported that protective effect of melatonin against cypermethrin induced antioxidant enzymes in *Spodoptera litura*.[17] Our results show that POX and CAT activity was decreased in melatonin and melatonin+cypermethrin treated samples as compared with control. Our study suggests that melatonin and synthetic pyrethroid can play a vital role in curtailing the degradation of antioxidative enzymes inhibited by pesticide toxicity.

It is interesting note that the antioxidant protective effect of melatonin on biological systems could also be carried out through the regulation of antioxidant defense system. This indoleamine increases the activity of glutathione peroxidase, an antioxidative enzyme, which metabolizes potentially damaging molecules to non-toxic products.[48] Furthermore, melatonin stimulates other antioxidant enzymes including superoxide dismutase, glutathione reductase and catalase. Melatonin also enhances intracellular glutathione levels by stimulating the rate-limiting enzymes in its synthesis γ-glutamylcysteine synthetase.[49] The antioxidant activity of phenolic compounds is principally due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers. In addition, they have a metal chelation potential.[50] Finally melatonin inhibits the prooxidative enzymes nitric oxide synthetase and lipoxygenase and increases the efficiency of the electron transport chain thereby reducing electron leakage and the generation of free radicals.

CONCLUSION

In addition to melatonin's ability to interact with directly and indirectly neutralize free radicals, as well as non-radical reactants. Melatonin has several additional features that may make it of interest in antioxidant enzyme system. When exogenously administered melatonin is readily taken into the blood and is distributed throughout the organisms. In conclusion, the present study demonstrated that the experimental evidence of dietary intake of melatonin and synthetic pyrethroid activated free radical scavenging and antioxidant system in *Drosophila melanogaster*.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

ABBREVIATION USED

SOD:Superoxide dismutase; **CAT:** Catalase; **GST:** Glutathione-S-Transferase; **LPO:** Lipid Peroxidase; **POX:** Peroxidase.

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SUMMARY

- Melatonin acts as an antioxidant molecules in invertebrates and vertebrates.
- Antioxidant system of insects in SOD, CAT, GST, POX and LPO were assessed.
- Synthetic pyrethroid cypermethrin insecticide mostly used in toxicity and target biological site in insects.

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