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Cypermethrin-induced alterations in vital physiological parameters and oxidative balance in *Caenorhabditis elegans*

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ABSTRACT

Cypermethrin belongs to the class of synthetic pyrethroids, which are being widely used as an insecticides in agricultural practices. The toxicity of cypermethrin is well studied in *Drosophila melanogaster*, fish, rats, mice, and is reported to cause neurotoxicity and oxidative stress during its metabolism. In this study, we evaluated the biological consequences of 4 h exposure to cypermethrin at sublethal concentrations (1, 5 and 15 mM) in *Caenorhabditis elegans* on physiological parameters such as egg laying, brood size, feeding and lifespan and oxidative stress parameters such as ROS, hydrogen peroxide levels, protein carbonyl, enzymatic antioxidants and glutathione levels. There was a significant and dose-dependent decrease in brood size (18–53%), egg laying (54–67%), feeding (29–58%) and marked decrease in lifespan (20%) at 15 mM of cypermethrin. Increase in levels of oxidative markers such as ROS (21–56%), intracellular hydrogen peroxide (17–62%), protein carbonyl (8–29%) and alteration in the activity of enzymatic antioxidants as well as depletion of glutathione (13–38%) were also observed. Our study offers evidence to show that cypermethrin induces significant oxidative stress in *C. elegans* and alters several physiological parameters in these worms, which can lead to impaired functioning, and survival of these worms.

1. Introduction

Pyrethroids are widely used as pest control agents in a wide array of indoor and outdoor applications, including medicinal, veterinary and agricultural scenario [1] and are the synthetic derivative of natural pyrethrins with greater potency and environmental stability [2]. They are subdivided into two groups according to their chemical structures – type-I pyrethroids are devoid of a 'cyano' moiety at the α-position while type-II pyrethroids have an α-cyano moiety [3]. Action of these two classes is primarily on the sodium channels of nerve. While type-I produces repetitive neuronal discharge and prolonged negative after potential, type-II pyrethroids produce even longer delay in sodium channel inactivation leading to persistent depolarization of the nerve membrane [4].

Cypermethrin (CYP), the α-cyano-3-phenoxybenzyl ester of 2,2-dimethyl-3-(2,2-dichlorovinyl)-2-2-dimethyl cyclopropane carboxylate, is the most commonly used type-II pyrethroid insecticide. It is a photostable, potent synthetic pyrethroid exhibiting broad-spectrum insecticidal action with low mammalian toxicity [5] and is employed to control many pests including pests of fruits and vegetable crops. CYP exerts its neurotoxic effects through voltage-dependent sodium channel [3] and integral protein ATPase in the neuronal membrane [6]. Recent *in vitro* and *in vivo* studies have shown that CYP produces reactive oxygen species (ROS) and thus induces oxidative stress [7–11]. Induction of oxidative stress during its metabolism is also based on the evidence that excitatory events may stimulate ROS production during the cleavage of CYP [10]. Thus although oxidative stress may not be the fundamental cause of toxicity of CYP, a variety of specific mechanism may lead to oxidative damage on exposure to CYP. CYP is also reported to possess the potential to cause endocrine disruption [12]. CYP was well studied in *Drosophila melanogaster* [13], fish [14] and higher mammalian systems viz., rat [8] and mice [15] but there are no reports on its toxicity in the invertebrate model, the nematode *Caenorhabditis elegans*.

Over the past decade, issues of animal use and care in toxicology research and testing have become one of the fundamental concern for both science and ethics, and emphasis has been placed on the use of alternatives to mammals in testing, research and education. *C. elegans* is one among the widely used invertebrate model organism because of its well-elucidated genetics and developmental biology. The use of *C. elegans* has been recommended by the European Center for the Validation of Alternative Methods (ECVAM), whose goal is to promote the scientific and regulatory acceptance of alternative methods, which reduce, refine or replace the use of laboratory animals [16]. *C. elegans* is a saprophytic nematode species, possessing many traits of favorable usage as a biological model. *C. elegans* has a transparent body approximately 1 mm in length, and serves as a reliable model organism because it possesses (1) short life cycle (3 days at 20 °C), (2) short life span and (3) ease rearing in the laboratory with large number progeny

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of 300–350 per worm [17]. In addition, it is a simple multicellular eukaryote whose developmental process and behavior can be easily monitored. *C. elegans* has been frequently used in ecotoxicolgical studies [18,19]. Toxicity studies with *C. elegans* have been focused from the beginning on the mortality but more other sensitive physiological parameters such as reproduction (more sensitive experiment then LC_{50} determination) [20], movement [21,22], feeding and growth [19,21], lifespan [23] and expression of small heat shock proteins [24].

Recently, *C. elegans* has been employed to study the oxidative stress inducing potential of several xenobiotics such as juglone and paraquat [25,26]. *In vivo* exposure to these toxicants induced oxidative stress by increasing reactive oxygen species and altering antioxidant enzymes and antioxidant molecules. In *C. elegans* the defense mechanism involves enzymatic antioxidants viz., superoxide dismutase, catalase, glutathione peroxidase glutathione reductase, glutathione-*S*-transferase and reduced glutathione are well studied [27].

In the present study, we demonstrated the toxicity of cypermethrin in *C. elegans* on sublethal exposure based on physiological parameters viz., egg laying, brood size, life span and feeding and also on the extent of oxidative stress induction by measuring the levels of reactive oxygen species (ROS), intracellular hydrogen peroxide levels, protein carbonyl content and activity of antioxidant enzymes and non-enzymatic antioxidant levels.

2. Materials and methods

2.1. Materials

Cypermethrin (Technical grade, 92% pure) was a gift from Hyderabad Chemicals Ltd., Hyderabad, India. 2,7-Dichlorofluorescein diacetate (DCFH-DA), 2,7-dichlorofluorescein (DCF), 5-fluro-2-deoxyuridine (FudR), quercetin, *N*,*N*,*N*,*N*-tetramethyethylenediamine (TMEDA), ethylenediamine tetraacetic acid (EDTA), hydrogen peroxide (H_2O_2), glutathione reductase (GR) and amino trizole (ATZ) were procured from Sigma Chemical Co. (St. Louis, MO, USA). Xylenol, sorbitol, 2,4-dinitro-phenyl hydrazine (DNPH), sodium dodecyl sulfate (SDS), nicotinamide adenine dinucleotide phosphate (NADPH), trichloroacetic acid (TCA), reduced glutathione (GSH), oxidized glutathione (GSSG). 1-Chloro-2,4-dinitrobenzene (CDNB), *t*-butylhydroperoxide (*t*-BHP), Tris–HCl and dimethylsulfoxide (DMSO) were procured from Sisco Research Laboratories (Mumbai, India). All other chemicals used were of analytical grade.

Caenorhabditis elegans, wild-type strain (N2), was obtained from the Caenorhabditis Genetics Center (CGC, Minneapolis, MN, USA), which is funded by the NIH National Center for Research Resources (NCRR).

2.2. Worm culture

The worms were cultivated on NGM plates (3 g l^{-1} NaCl, 2.5 g l^{-1} proteose peptone, 5 mg l^{-1} cholesterol, 1 mmol l^{-1} $CaCl_2$, 1 mmol l^{-1} $MgSO_4$, 25 mmol l^{-1} potassium phosphate, pH 6.0, 17 g l^{-1} agar) on an established lawn of *Escherichia coli* strain OP50 [28] and maintained at 20 °C. To obtain synchronized culture, gravid hermaphrodites were lysed in an alkaline hypochlorite solution [29] and the eggs were seeded in fresh NGM plates. The culture was grown for 3 days at 20 °C until the newly hatched worms reached adulthood. The L4 stage worms/gravid worms were washed with K-medium (53 mM NaCl, 32 mM KCl) [30], pelleted by centrifugation (3000*g*, 5 min), washed again thrice with cold K-medium and finally suspended in K-medium to obtain 30–50 nematodes per 10 μl.

2.3. Egg laying assay

Egg laying rates were determined by placing age-synchronized adult worms in 24-well tissues culture plates with NGM agar media (10 worms per well) with CYP incorporated at varying concentrations (0, 1, 5 and 15 mM). For each test condition tested, the assays were repeated 12 times, thus using a total of 120 worms. The number of eggs laid during 1 h of exposure to CYP was counted [31].

2.4. Brood size

Age synchronized adult worms (L4 stage) were exposed to different concentrations of cypermethrin (0, 1, 5 and 15 mM) for 4 h at 20 °C. After exposure, the worms were washed with K-medium and a single worm was picked and transferred to 12-well tissue culture plates containing 1 ml K-medium, which contained *E. coli* at a dilution of 1 OD at 550 nm. The plates were incubated at 20 °C for 72 h. After 72 h, the worms were washed, pelleted and the progeny was counted under the dissecting microscope [32]. For each test concentration and control the average number of mean progeny from three wells was obtained for each test replicate and the testing was repeated three times.

2.5. Feeding assay

Assays were performed in 12-well plates. Aliquots of K-medium with *E. coli* suspended at a concentration of 1.0 OD (at 550 nm) were dispensed into the wells. CYP was added to the wells at different concentrations (0, 1, 5 and 15 mM). The worms suspended in K-medium were added to the wells such that each well had 5000 worms. The total volume in each well was 1 ml. The plate was incubated at 25 °C with constant shaking. One milliliter sample was drawn at 1 h intervals, spun gently for 20 s at 800 rpm and the absorbance of the supernatant was determined at 550 nm. The samples were returned to the culture plate for further incubation [33].

2.6. Lifespan

Age synchronized adult worms (L4 stage) were exposed to different concentrations of cypermethrin (0, 1, 5 and 15 mM) for 4 h at 20 °C. After the exposure period, worms were washed thrice with K-medium and 20 ±1 L4 worms (designated as Day 0 of life span estimate) were placed in a well of 15 mm flat bottomed 24-well plates, each well containing 500 μl of K-medium (OD at 550 nm adjusted to 1.0 with *E. coli* cells) and FudR at a final concentration of 50 μM [34]. Worms were raised and maintained at 20 °C. The survivability was scored every day by gentle touching with platinum wire. The worms, which failed to move in response to touch, were considered as dead.

2.7. Measurement of oxidative stress markers

Age synchronized worms (gravid stage) were exposed to different concentrations of CYP (0, 1, 5 and 15 mM)) in 1 ml K-medium in 12-well tissues culture plate and incubated for 4 h at 20 °C. After the exposure period, the worms were washed three times with K-medium, and the worm pellet was homogenized for fixed time in 50 mM Tris–HCl buffer (pH 7.4). The worm homogenate was centrifuged at 10,000 rpm for 10 min and the supernatant was used for the various assays as described below.

2.7.1. Assay of reactive oxygen species (ROS)

The levels of reactive oxygen species generated in the worms were determined by DCFH oxidation method by Keston and Brandt

[35]. *C. elegans* homogenate was centrifuged at 1000 rpm for 5 min, the supernatant incubated with 5 μM DCFH-DA in final volume of 2 ml Tris–HCl for 45 min at room temperature. The intensity of florescence was measured at 530 nm following excitation at 485 nm. The amount of DCF (resulting from the ROS mediated oxidation of DCFH, which is produced by hydrolytic cleavage of DCFH-DA by cellular esterases) is determined using the DCF standard graph. Results were expressed as nmol of DCF/mg protein.

2.7.2. Measurement of H_2O_2

Hydrogen peroxide (H_2O_2) levels were measured by the method of Wolff [36]. The worm homogenate was centrifuged at 2500 rpm for 10 min. Briefly, 0.1 ml of the worm supernatant was added to 900 μl of FOX1 reagent (100 μM xylenol orange, 100 mM sorbitol, 250 μM ammonium ferrous sulfate and 25 mM H_2SO_4), vortexed and incubated at room temperature for 30 min. The sample was then centrifuged at low speed for 3 min and the absorbance of the supernatant was read at 560 nm against reagent blank. The enzyme activity was calculated based on molar extinction coefficient at $2.24 \times 10^{-5}\ M^{-1}\ CM^{-1}$. Results were expressed as nmol H_2O_2 generation/mg protein.

2.7.3. Reduced glutathione (GSH)

Glutathione levels in *C. elegans* homogenate were quantified by the method of Benke et al. [37]. Worms after exposure were washed thrice with K-medium and then homogenized in 5% TCA containing 1 mM EDTA solution in micro-centrifuge tubes using a micro-pestle followed by centrifugation at 3000 rpm. Two hundred microliters of the supernatant was added to 4.75 ml of sodium phosphate buffer (0.1 M, pH 8.0) with 1 mM EDTA to which 50 μl of DTNB (in phosphate buffer, 0.1 M, pH 7.0) was added and mixed. The absorbance of the solution was read at 412 nm. Results were expressed as μg GSH/mg protein.

2.7.4. Protein carbonyl levels

The protein carbonyl levels were measured by the method of Levine et al. [38]. After incubation, the assay mixture was centrifuged at 10,000 rpm for 10 min at 4 °C and 0.2 ml of the supernatant was precipitated with an equal volume of 20% TCA and then centrifuged. The pellet was re-suspended in 1 ml of 2,4-dinitrophenylhydrazine (10 mM, in 2 M HCl) and allowed to stand at room temperature for 60 min with occasional vortexing. Proteins were then precipitated by adding 0.5 ml of 20% TCA. The resulting mixture was centrifuged and the pellet obtained was washed three times with 1 ml acetone. One milliliter of 2% SDS (in 20 mM Tris–HCl, 0.14 M NaCl, pH 7.4) was added to the pellet and incubated overnight at 37 °C. The absorbance of the solution was read at 360 nm and the carbonyl content was calculated using a molar extinction coefficient of 22,000 $M^{-1}\ cm^{-1}$. Results were expressed as nmol carbonyl/mg protein.

2.7.5. Superoxide dismutase (SOD)

SOD activity was measured by a method on inhibition of auto-oxidation of quercetin by the enzyme at pH 10 [39]. Auto-oxidation of quercetin was followed by measuring decrease in absorbance at 406 nm. The reaction mixture contained: 0.016 M phosphate buffer, 0.8 mM *N,N,N′,N′*-tetramethylethylenediamine and 0.08 mM EDTA in a total volume of 3.0 ml. The reaction was started by addition of 0.1 ml of quercetin solution (1.5 mg/10 ml). For the assay of SOD, suitable aliquot of supernatant was incorporated in the assay mixture in a final volume of 3.0 ml, and inhibition of quercetin auto-oxidation was monitored. The results are expressed as U/mg protein. One unit of enzyme is defined as the amount of enzyme that inhibits quercetin auto-oxidation by 50%.

2.7.6. Catalase (CAT)

Catalase activity was measured by the method of Beers and Sizer [40]. Briefly, supernatant derived from worms after exposure equivalent to 250 μg protein along with 25 μl of H_2O_2 (3%) was added to 3 ml phosphate buffer (50 mM, pH 7.4) and the decrease in absorbance due to H_2O_2 degradation was monitored at 240 nm for 5 min. The enzyme activity was calculated based on molar extinction coefficient at 43.6 $M^{-1}\ cm^{-1}$ and the results were expressed as μmol of H_2O_2 consumed/min/mg protein.

2.7.7. Glutathione peroxidase (GPx)

Glutathione peroxidase was measured according to the method of Flohe and Gunzler [41]. The supernatant derived from worms after exposure equivalent to 100 μg protein was incubated with 0.575 ml phosphate buffer (0.1 M, pH 7.0, containing 0.5 mM EDTA). One hundred microliters of glutathione reductase (2.5 units/ml), 100 μl of GSH (10 mM) and 100 μl of NADPH (1% in sodium bicarbonate solution) at 37 °C for 10 min. Finally, 100 μl of 12 mM *t*-butylhydroperoxide was added to the above mixture and the oxidation of NADPH over 5 min was recorded at 340 nm. Results were expressed as nmol of NADPH oxidized/min/mg protein.

2.7.8. Glutathione reductase (GR)

The reaction was started by adding 100 μl homogenate to the reaction mixture (final volume 1 ml) containing 0.2 M sodium phosphate buffer (pH 7.0) of 2 mM EDTA, 1 mM oxidized glutathione (GSSG) and 0.2 mM NADPH. The enzyme activity was measured indirectly by monitoring the oxidation of NADPH following decrease in OD/min for a minimum of 3 min at 340 nm. One unit enzyme activity was defined as nmol of NADPH consumed/min/mg protein based on molar extinction coefficient [42].

2.7.9. Glutathione-S-transferase (GST)

Potassium phosphate buffer (0.5 ml, 0.2 M, pH 7.2) were added 0.1 ml of GSH (10 mM), 0.01 ml of 1-chloro-2,4-dinitro benzene (CDNB, 0.1 M) and 390 μl of distilled water and mixed. Supernatant derived from worms after exposure equivalent to 100 μg protein was then added to the above mixture and the formation of the adduct of CDNB (*S*-2,4-dinitrophenyl glutathione) was monitored by measuring net increase in absorbance at 340 nm. The enzyme activity was calculated based on absorption coefficient of 9 $mM^{-1}\ cm^{-1}$. Results were expressed as μmol of adduct formed/min/mg protein [43].

2.8. Protein estimation

Ten microliters of homogenate was added to 490 μl of distilled water and incubated with 2.5 ml alkaline copper sulfate solution for 10 min at room temperature. Two hundred and fifty microliters of Folin's reagent (1:1 diluted with water) was added to the mixture, mixed well and the color was read after 30 min at 670 nm. BSA was used as the standard [44].

2.9. Statistical analysis

Data were expressed as means ± SE. Statistical significance was assessed either by Student's *t*-test ($P < 0.001$) or by ANOVA and post hoc Duncan's Multiple Range Test (DMRT) ($P < 0.05$) using SPSS 10.05 FOR Windows (SPSS, Chicago, IL, USA).

3. Results

3.1. Effect of CYP on egg laying in C. elegans

Worms exposed to CYP exhibited significant reduction in the egg laying, in a concentration-dependent manner (Table 1). The average number of eggs laid per worm in control group was 3.0 ± 0.04/h while that in worms exposed to the highest concentration of CYP (15 mM) was 1.0/h. The percent decrease in egg laying was 55%, 64% and 67% at 1, 5 and 15 mM cypermethrin, respectively.

3.2. Brood size

Exposing *C. elegans* to graded doses of cypermethrin caused significant decrease in mean progeny (brood size) per worm (286 ± 4 in control vs 233 ± 3, 172 ± 3 and 133 ± 5 at 1, 5 and 15 mM), respectively. Percent decrease in brood size over control was – 18%, 40% and 53% at the above mentioned concentrations of CYP, respectively (Fig. 1).

3.3. Feeding inhibition

There was a gradual, time-related decrease in bacterial density in control worm suspension indicating normal feeding by these worms. CYP at all the tested concentrations induced significant decrease in feeding in *C. elegans* after 4 h of exposure. The extent of feeding inhibition after 4 h in worms exposed to CYP was – 1 mM, 29%; 5 mM, 41%; 58%, 15 mM (Fig. 2). However, even after 4 h of exposure, the worms showed no cessation in feeding.

3.4. Lifespan

Cypermethrin decreased the life span in *C. elegans* in a dose-dependent manner compared to control (Fig. 3). The untreated worms survived till for 25 days while the CYP treated worms survived for 23, 22, 20 days at 1, 5 and 15 mM, respectively.

3.5. Effect of cypermethrin on oxidative balance

Fig. 4 shows the effect of CYP on reactive oxygen species generation in worms after 4 h exposure period. The worms revealed an increase in ROS in a dose-dependent manner (21%, 32% and 56% increase at 1, 5 and 15 mM, respectively). The effects of CYP on intracellular hydrogen peroxide levels in the worms under various concentrations are depicted in Fig. 5. There was a significant and a dose-dependent increase of 17%, 50% and 62% in hydrogen peroxide levels at 1, 5 and 15 mM of CYP, respectively. CYP induced oxidative stress resulted in a dose-dependent decrease in the levels of reduced glutathione in the CYP exposed worms (Fig. 6). Concomitantly, as shown in Fig. 7, CYP also increased protein carbonyl levels a dose-dependent manner (8%, 17% and 29% at 1, 5 and 15 mM, respectively) (Fig. 7).

Table 1
Inhibition of egg laying in *C. elegans* on exposure to cypermethrin.

Cypermethrin (mM)	Mean eggs/worm*
0	3.0 ± 0.041[a]
1	1.4 ± 0.036[b]
5	1.1 ± 0.028[b,c]
15	1.0 ± 0.026[b,c]

Data analyzed by post-hoc test (Duncan); Means in the same column with different superscript differ significantly ($P < 0.05$).
* Eggs laid by a single worm after 1 h of exposure to cypermethrin ($n = 120$).

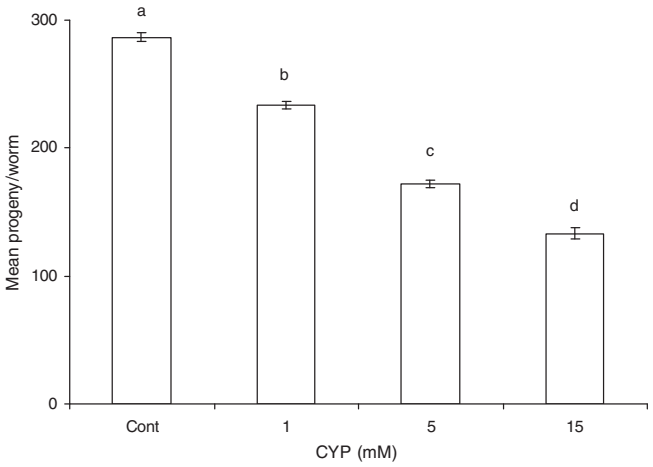

Fig. 1. Effect of cypermethrin (CYP) treatment on brood size in *C. elegans*. Each data point is means ± SEM ($n = 9$). Groups with different letters are significantly different (ANOVA, post hoc, Duncan SPSS 10.05, $P < 0.05$).

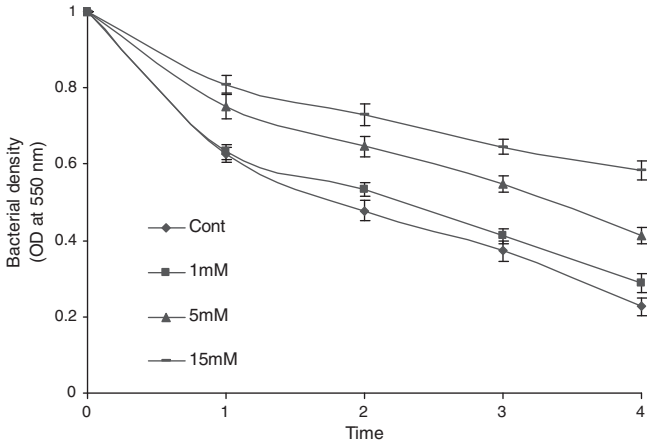

Fig. 2. Effect of cypermethrin (CYP) on feeding in *Caenorhabditis elegans*. Worms were suspended in K-medium with bacterial density adjusted to 1.0 OD (at 550 nm) initially. Feeding was monitored by decrease in OD at different time intervals. Each data point is means ± SEM of three replicates from three independent experiments.

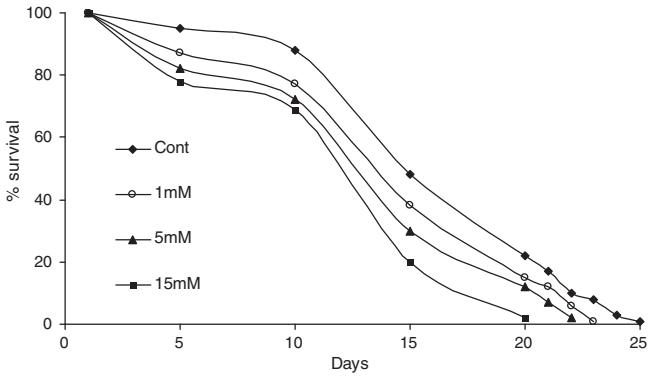

Fig. 3. Shortened lifespan of *C. elegans* resulting from exposure to cypermethrin (CYP). Lifespan of worms was determined by counting the live worms in the absence (control) or presence of varying concentrations of CYP on different days.

The effect of CYP on antioxidants enzymes in *C. elegans* after 4 h exposure is presented in Figs. 8–11. SOD activity was significantly decreased (21%) at the highest concentration of CYP (15 mM) (Fig. 8) while an increase in the activity of CAT at 15 mM (45%)

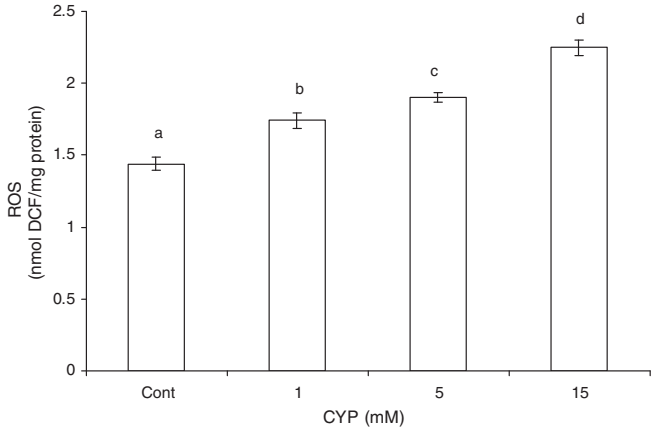

Fig. 4. Reactive oxygen species levels in *C. elegans* exposed to cypermethrin (CYP). All values are means ± SEM ($n = 9$). Groups with different letters are significantly different (ANOVA, post hoc, Duncan SPSS 10.05, $P < 0.05$).

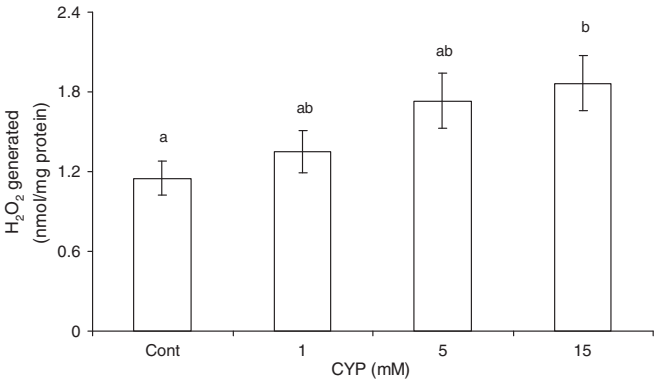

Fig. 5. Hydrogen peroxide levels in *C. elegans* exposed to cypermethrin (CYP). All values are means ± SEM ($n = 9$). Groups with different letters are significantly different (ANOVA, post hoc, Duncan SPSS 10.05, $P < 0.05$).

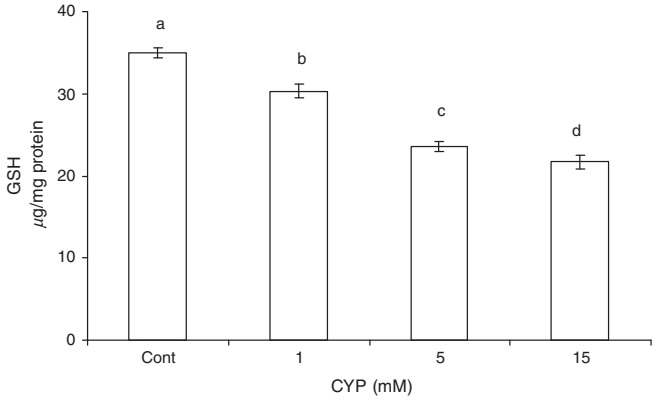

Fig. 6. Glutathione levels in *C. elegans* exposed to cypermethrin (CYP). All values are means ± SEM ($n = 9$). Groups with different letters are significantly different (ANOVA, post hoc, Duncan SPSS 10.05, $P < 0.05$).

was evident (Fig. 9). In *C. elegans*, the activity of GPx is reported to be less and our study showed a significant decrease in GPx activity on exposure to CYP (1 mM, 10%; 5 mM, 20%; 15 mM, 27%) and an increase in the GR activity in *C. elegans* exposed to CYP was evident which was marked only at 15 mM (25%) (Fig. 10). The activity of glutathione-*S*-transferase showed a significant and dose-dependent increase at all tested concentrations (1 mM, 14%; 5 mM, 32%; 15 mM, 38%) (Fig. 11).

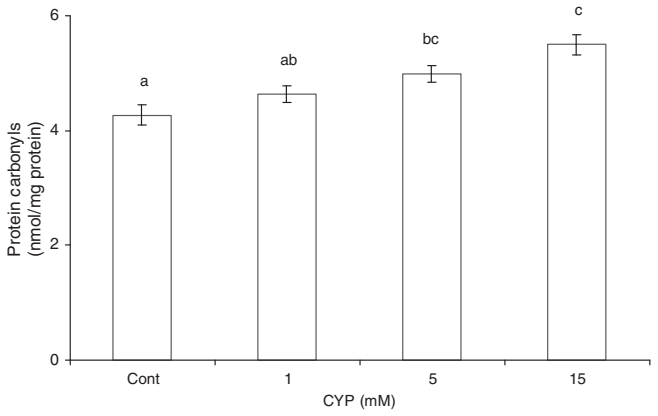

Fig. 7. The effect of cypermethrin (CYP) treatment on protein carbonyls levels in *C. elegans*. All values are means ± SEM ($n = 9$). Groups with different letters are significantly different (ANOVA, post hoc, Duncan SPSS 10.05, $P < 0.05$).

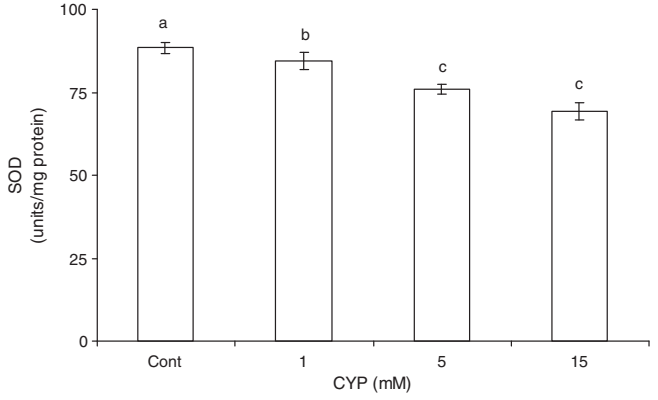

Fig. 8. Superoxide dismutase activities in *C. elegans* exposed to cypermethrin (CYP). All values are means ± SEM ($n = 9$). Groups with different letters are significantly different (ANOVA, post hoc, Duncan SPSS 10.05, $P < 0.05$).

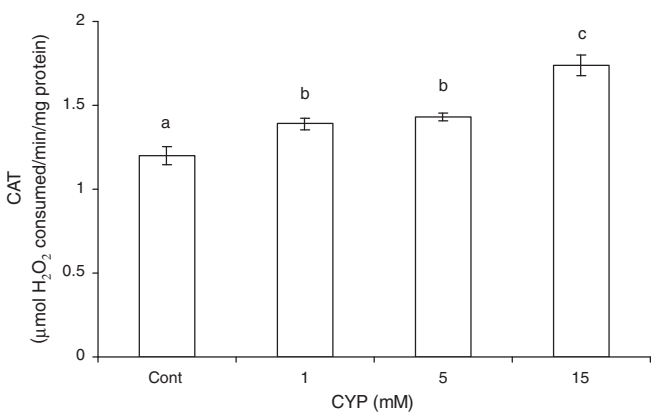

Fig. 9. Catalase activities in *C. elegans* exposed to cypermethrin (CYP). All values are means ± SEM ($n = 9$). Groups with different letters are significantly different (ANOVA, post hoc, Duncan SPSS 10.05, $P < 0.05$).

4. Discussion

The results of the present study provides evidences to show that although CYP at the tested concentrations did not induce any symptoms of toxicity or mortality in *C. elegans*, it elicited toxicity by affecting physiological parameters viz., egg laying, brood size, feeding rate, life span and also induced oxidative stress in worms

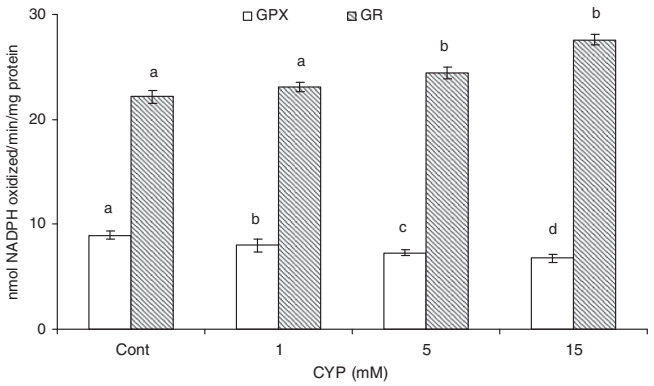

Fig. 10. Glutathione peroxidase and glutathione reductase activities in *C. elegans* exposed to cypermethrin (CYP). All values are means ± SEM ($n = 9$). Groups with different letters are significantly different (ANOVA, post hoc, Duncan SPSS 10.05, $P < 0.05$).

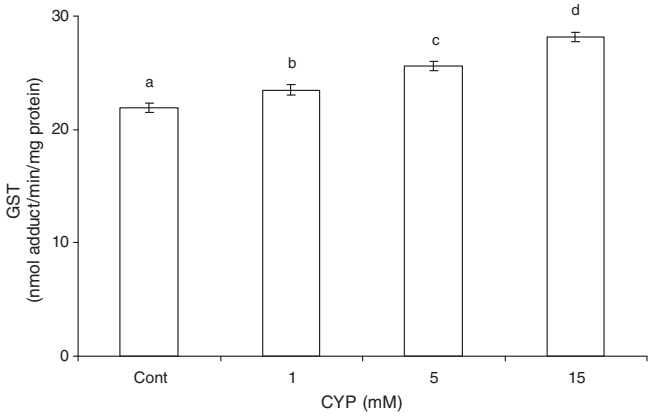

Fig. 11. Glutathione-*S*-transferase activity in *C. elegans* exposed to cypermethrin (CYP). All values are means ± SEM ($n = 9$). Groups with different letters are significantly different (ANOVA, post hoc, Duncan SPSS 10.05, $P < 0.05$).

by increasing free radicals, decreasing reduced glutathione levels, increasing protein carbonyl levels and altering the activities of antioxidant enzymes at all tested concentrations. To the best of our knowledge, this is the second report on the toxicity of CYP in *C. elegans*. Earlier we had reported CYP toxicity and its amelioration by α-tocopherol supplementation in *C. elegans* [45].

The primary action of CYP is on the nervous system of vertebrates and invertebrates by induction of repetitive activity and production of nerve impulses as a result of alteration in permeability of nerve membrane [4]. Further, CYP being a lipophilic compound can penetrate into the lipid bilayer of tissues, disturbing the phospholipid orientation thus causing changes in the fluidity of the membrane [46]. Cleavage of CYP releases cyanohydrins, which are unstable under physiological conditions. These cyanohydrins decompose to cyanides and aldehydes, which produce free radicals and cause oxidative stress [10]. The overall results of the present study showed that exposure of *C. elegans* to CYP resulted in significant oxidative stress in the worms. Increased levels of ROS/H_2O_2 and protein carbonyl content associated with reduced level of GSH and alteration in antioxidant enzymes clearly demonstrated the oxidative insult.

GSH is also an important intracellular molecule that protects cells against endogenous and exogenous OS [47]. Depletion of GSH by oxidants may alter the redox status of the cell and present a stressful and toxic situation since GSH plays a critical role in maintaining cellular redox homeostasis. Oxidative stress (OS) is also known to damage proteins by the introduction of carbonyl groups. Hence the significant depletion of GSH and marked elevation in protein carbonyl content in the worms exposed to CYP clearly suggest oxidative stress.

Various organisms can respond to oxidative stress by increasing their antioxidant enzyme activities. In the present study, exposure to CYP resulted in decrease in the activities of SOD and GPx and increase in the activities of CAT, GR and GST. For *C. elegans*, there is some information on transcription factors involved in up-regulating antioxidant enzymes (SOD and GST) in response to oxidants [48,49] however, the mechanism of catalase regulation is not known. Catalase detoxifies the ROS/H_2O_2 and therefore it acts as an antioxidant enzyme [50] and it is inducible by OS in *C. elegans* as well as in mammals [51,52].

GPx is involved in protecting cytosol and plasma membrane from lipid peroxidation (LPO) [53]. In fact, this enzyme transforms lipid hydroperoxides produced at the membrane level into less reactive species. The presence of GPx has not been earlier demonstrated in *C. elegans* [27]. However, we could conveniently assay the activity of GPx in the worms, although the basal activity was comparatively low. Our data are in accordance with reports in rats wherein reduced activity of GPx was observed in CYP exposed rats [54]. The lower enzymatic activity of GPx in our study could indicate facilitation of increased LPO due to the lack of the protective effect of this antioxidant enzyme. GST is an enzyme that participates in the detoxification process facilitating the conjugation reactions between GSH and xenobiotics [55]. Hence GST plays a critical role in protection against electrophiles and products of oxidative stress [56]. In the present study, concomitant with the decrease in GSH levels due to CYP exposure, the activity of GST increased further corroborating the oxidative insult of CYP.

There is ample correlative evidence that indirectly supports the oxidative damage theory and aging such as observed increased with increasing age of molecular damage and ROS production and correlations among animal species between life span and ROS production rates [57]. It has also been shown that treatments that increase oxidative stress promote molecular damage and reduce life span [58,59]. In the present study, we have shown that treatment of *C. elegans* with CYP decreased the life span marginally which correlated well with the extent of oxidative stress induced by CYP in these worms.

In the present study, feeding rate was significantly decreased following exposure to CYP. Feeding effect could be observed by microscopy as a shut down of the pumping action of pharynx. The rate of nematode feeding could be quantified by measuring the rate of reduction of the optical density (at 550 nm) of the bacterial suspension. On addition of a toxicant, the rate of reduction of the optical density was markedly reduced, as a consequence of feeding inhibition [33]. A physiological mechanism that inhibits feeding may be essential for survival when animals are exposed to severe chemical stress. Such a mechanism would serve both to limit the intake of toxins/toxicant. Thus feeding inhibition also has implications for toxicological studies in *C. elegans* since it imposes a limit on the concentration of toxicant that can be ingested.

The fitness of an organism is determined by its success in reproduction [60]. In the present study worms exposed to CYP showed decreased egg laying and decreased brood size compared to the controls. In contrast to the lack of lethality of CYP treatment in adult worms, non-lethal concentrations of CYP reduced the brood size of *C. elegans* and reduced the fertility suggesting the potential genotoxic effects of CYP to the germ cells of the worms. The genotoxicity of CYP has been well demonstrated in *D. melanogaster* [13] and mouse [15].

Egg laying in *C. elegans* serves as an excellent behavioral parameter, since it has been extensively characterized. It can be effectively quantitated and more importantly, it has a simple

anatomical basis. Eggs are laid when the two hermaphrodite-specific neurons (HSNs) stimulate the contraction of 16 egg-laying muscle cells to push eggs through the uterus and out the vulva [61]. In the present study, we have not investigated in detail the specific effect of cypermethrin on HSNs. We attribute this effect of CYP to its ability to alter nerve function. Cypermethrin has been earlier shown to stimulate reproductive physiology in the freshwater snail *Lymnaea acuminata* resulting in increased egg masses by altering the hormonal levels [57]. However, although CYP significantly increased fecundity in that study, it equally reduced survivability of hatchlings in the snails.

Reductions in egg laying concomitant with decreases in food consumption has been reported in birds exposed to dietary methamidophos, an organophosphorus insecticide [62]. To the best of our knowledge, similar effects have not been reported in animals exposed to CYP. In the present study, we observed reduced egg laying/brood size concomitant with reduced feeding in worms exposed to CYP. However, whether these parameters are interdependent in *C. elegans* is not clear.

5. Conclusions

In conclusion, we have shown that CYP induces oxidative stress in *C. elegans*. We have also demonstrated that at non-lethal concentrations, cypermethrin also induces subtle physiological alterations, which may or may not be the consequence of oxidative stress induced therein, which can largely impair the normal functioning and survival of the worms. However, further studies are warranted to correlate the observed biochemical and physiological responses elicited by cypermethrin.

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