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Involvement of acetylcholinesterase inhibition in paralyzing effects of monocrotophos in *Caenorhabditis elegans*

Apurva Kumar Ramesh Joshi[1,2*], Raju Nagaraju[1,3] and Padmanabhan Sharda Rajini[1]

Abstract

Background: Organophosphorus insecticides (OPI) are among the most commonly used class of pesticides in agricultural and domestic scenarios. Clinical presentations of acute OPI toxicity are attributable to acetylcholinesterase inhibition and ensuing cholinergic stress. Consequently, acetylcholinesterase reactivators and acetylcholine receptor antagonists are used to treat clinical cases of OPI toxicity. The nematode worm *Caenorhabditis elegans* is emerging as an attractive alternative model for toxicity evaluations. This study was carried out to understand the involvement of acetylcholinesterase inhibition in paralyzing effects of monocrotophos (an OPI) in *Caenorhabditis elegans*.

Results: Exposure of *C. elegans* to monocrotophos was associated with strong toxicity in an acute toxicity test as revealed by LC_{50} of 35.5 ± 1.2 mM. Sub-lethal concentrations of monocrotophos were found to elicit severe acetylcholinesterase inhibition and paralysis. Co-exposure of worms to pralidoxime was found to rescue worms from the paralyzing effects of monocrotophos along with partial reactivation of acetylcholinesterase activity.

Conclusion: Our work demonstrates that acetylcholinesterase inhibition is responsible for the paralyzing effects of monocrotophos in *Caenorhabditis elegans*, and paralysis can be used as an experimental marker of cholinergic acute toxicity of OPI in *C. elegans*.

Keywords: Acetylcholinesterase inhibition, *Caenorhabditis elegans*, Monocrotophos, Paralysis, Pralidoxime

Background

Many non-mammalian alternative models have been explored as options for reducing the use of higher organisms needed for experimental studies and to achieve rapidity in time of assessment. *Caenorhabditis elegans* (nematode worm) has emerged as a highly preferred alternative model in various areas of research. There are certain aspects of *C. elegans* that make it an attractive model. In addition to low cost and ease of maintaining worm cultures in the laboratory, the nematode has a short life cycle (3 days) with the ability to produce a large number of offspring. This allows rapid assessments that may also include multi-generation studies. Since the body size is very small (1 mm), *in vivo* assays can be performed using 96-well formats, which makes the nematode suitable for medium- and high-throughput methodologies. The transparent body allows clear observation of cells in immature and developing animals. Furthermore, intensive knowledge of the genome and established genomic, transgenic, and RNA interference methodologies provide a variety of options that could be of immense use in toxicological research (Leung et al., 2008). With the above mentioned advantages, *C. elegans* will most definitely serve well as an alternative toxicological model as it exhibits comparable responses to that of higher mammalian models. Indeed, *C. elegans* has been found to predict the mammalian toxicity of substances like metals and organophosphorus insecticides (Cole, Anderson, & Williams, 2004; Rajini et al., 2008). With an extensively studied nervous system, the nematode is most suitable for studying toxic effects of neurotoxic insecticides, particularly OPI. *Caenorhabditis elegans* has four different genes yielding four different AChE enzymes (Combes, Fedon, Grauso, Toutant, &

* Correspondence: apurvakmr@gmail.com
[1]Food Protectants and Infestation Control Department, CSIR-Central Food Technological Research Institute, Mysore, Karnataka, India
[2]Food Chemistry Division, National Institute of Nutrition, Jamai-Osmania, Hyderabad, India
Full list of author information is available at the end of the article

Arpagaus, 2000). Acetylcholine-mediated processes are involved in locomotory, egg-laying, andpredict the mammalian toxicity of substances like metals pharyngeal pumping (McKay, Raizen, Gottschalk, Schafer, & Avery, 2004; Rand, 2007) behaviors in *C. elegans*.

Organophosphorus insecticides (OPI) are one of the largely used classes of compounds for pest control in various scenarios. The use of OPI has increased owing to high insecticidal potency and low persistence in the mammalian system. OPIs act via inhibition of acetylcholinesterase (AChE; EC 3.1.1.7), an enzyme involved in regulation of neurotransmission by hydrolysis of the neurotransmitter, acetylcholine (ACh). Subsequent accumulation of ACh and consequential over stimulation of ACh receptors have been deemed the chief mechanism of their acute toxicity (Costa, 2006). Since acute toxicity resulting from acetylcholinesterase inhibition is a hallmark effect of OPIs, our lab has been trying to understand the far-reaching consequences of AChE inhibition in various experimental models. We have demonstrated that OPIs have the propensity to elicit hyperglycemia in rats after a single dose (Joshi, Nagaraju, & Rajini, 2012; Joshi & Rajini, 2009). Recently, we provided evidence for mechanistic involvement of cholinergic stress in hyperglycemic outcomes in rats treated with a single dose of monocrotophos (Joshi & Rajini, 2012). Studies conducted in our lab reveal that paralysis manifests in worms after acute exposure to dichlorvos and monocrotophos (Jadhav & Rajini, 2009b; Leelaja & Rajini, 2013). Previous studies from our laboratory have reported toxicity of OPI to *C. elegans*, characterized by AChE inhibition, stress response, and neurophysiological impairments (Jadhav & Rajini, 2009a, 2009b; Leelaja & Rajini, 2013). Cholinergic stress is a critical determinant of acute toxicity of OPI, and hence, a behavioral parameter mechanistically underlined by cholinergic stress presents a valuable tool for the better utility of *C. elegans* as a model for studying OPI toxicity. Further, the paralysis model in *C. elegans* could be exploited for the development of medium- and high-throughput assay systems for rapid screening of AChE reactivators, with the possibility of obtaining both biochemical and physiological perspectives on the efficacy of AChE reactivators. In view of this, the present study was conducted to understand whether acetylcholinesterase inhibition in *C. elegans* shares a causal relationship with paralysis in worms exposed to monocrotophos, an organophosphorus insecticide.

Methods

Worms and *Escherichia coli*

The wild-type (N2) strain of *C. elegans* and uracil auxotroph of *E. coli*, OP25 were received from the Caenorhabditis Genetics Centre (CGC, Minneapolis, MN, USA).

Chemicals

5, 5-Dithio-bis-2-nitrobenzoic acid (DTNB), acetylthiocholine iodide (ATCI), and bovine serum albumin (BSA), were procured from Sisco Research Laboratories (Mumbai, India). Pralidoxime iodide (25 mg/mL) was procured from a local pharmacy. Technical grade (76%) sample monocrotophos (dimethyl (*E*)-1-methyl-2-(methylcarbomoyl) vinyl phosphate) was a gift from Hyderabad Chemical Supplies Ltd. (Hyderabad, India). All other chemicals used in the study were of analytical grade.

Worm culture

Under laboratory conditions, the worms were fed *Escherichia coli* (*E. coli*) strain-OP50, which is auxotrophic for uracil. All developmental stages of the worm are maintained on the Nematode Growth Medium (0.032 M KCl, 0.051 M NaCl, 2.5% bactopeptone, 0.17% bactoagar in distilled water, sterilized by autoclaving) seeded with *E. coli* (Brenner, 1974). Gravid worms (that have laid eggs) were washed off the NGM plate using K-medium (50 mM NaCl and 30 mM KCl). The adherent eggs were salvaged into K-medium by gentle rubbing and were poured into centrifuge tubes. The eggs were facilitated to settle to the bottom by gentle centrifugation. The excess K-medium was discarded, and 5.0 ml fresh hypochlorite solution (20% *v*/*v* hypochlorite solution containing 1% NaOH) was added and mixed at intervals for 4 min. The suspension was centrifuged at 3500 rpm for 6 min. The egg pellet was washed with K-medium thrice and transferred to NGM plates seeded with OP50 (Fabian & Johnson, 1994). The plates were then incubated at 20 °C to facilitate hatching and growth of worms to the L4 stage. Worms were then transferred to fresh NGM plates seeded with OP50 to maintain appropriate nutritional status.

Determination of toxicity of monocrotophos to *C. elegans*: mortality profile

All exposures were carried out in K-medium, which is a reliable medium for carrying out the exposure of worms in liquid medium (Williams & Dusenbery, 1990). Acute toxicity of monocrotophos to *C. elegans* was determined in K-medium using a 4-h exposure period. Briefly, 10 ± 1 L4 (age-synchronized) stage worms were exposed to varying concentrations monocrotophos (10–60 mM, dissolved in K-medium) in 24-well plates in 0.5 ml of test solution for 4 h at 20 °C. The number of survivors were counted at the end of the exposure period. The worms were counted as dead if they were non-responsive to touch. Median lethal concentration (LC_{50}) was calculated by Probit analysis of the data.

Impact of sub-lethal concentrations monocrotophos on acetylcholinesterase activity in *C. elegans*

Approximately 5000 worms/well were exposed to sub-lethal concentrations (1/80, 1/40, 1/20, and 1/10th LC_{50}) of monocrotophos in 12-well plates in a test volume of 1.0 ml for 4 h at 20 °C. At the end of the exposure period, the worms were washed into microcentrifuge tubes and facilitated to settle to the bottom by brief centrifugation. The worm pellet was washed thrice with K-medium. The worms were then homogenized in Tris-HCl (50 mM, pH 7.4), and 10000 rpm supernatants were used for the acetylcholinesterase (EC. 3.1.1.7) activity assay. Briefly, acetylthiocholine iodide was added to a mixture containing suitable amounts of worm homogenate (as a source of enzyme) and DTNB. Change in absorbance was monitored over 3 min in a microplate reader at 405 nm. The amount of enzyme causing a change of 0.001 units of absorbance per minute was considered as one unit of enzyme, and the results were expressed as units per milligram protein (Galgani & Bocquene, 1991). The protein content of worm homogenates was determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951).

Induction of paralysis in *C. elegans* by monocrotophos

25 ± 5 gravid (age-synchronized) worms were exposed to sub-lethal concentrations (1/40, 1/20 and 1/10 LC_{50}) of monocrotophos in a test volume of 0.5 ml for 4 h at 20 °C. At the end of the exposure period, the number of paralyzed worms was counted under the microscope, and the results were expressed as percent worms paralyzed. Worms incapable of achieving whole body wave propagation despite showing a small twitch in the anterior (head) region of the body upon being prodded with a soft plastic bristle were scored as paralyzed. Worms capable of whole body wave propagation without external aid were scored as non-paralyzed.

Effect of pralidoxime iodide on paralysis and acetylcholinesterase inhibition in monocrotophos-treated worms

For studying the effect of pralidoxime on paralysis in monocrotophos treated worms, 25 ± 5 gravid (age-synchronized) worms were exposed to sub-lethal concentrations (1/20 and 1/10 LC_{50}) of monocrotophos in a test volume of 0.5 ml for 4 h at 20 °C in the presence and absence of pralidoxime (20mM). At the end of the exposure period, the number of paralyzed worms was counted under the microscope, and the results were expressed as percent worms paralyzed. The AChE reactivation potential of pralidoxime (PAM) was studied under co-exposure conditions. Five thousand worms/well were exposed to 1/20th LC_{50} monocrotophos in the presence or absence of 20 mM PAM for 4 h at 20 °C. The concentration of PAM was based on our preliminary studies (data not shown). Pralidoxime was not found to induce mortality or paralysis at the tested concentrations. At the end of the exposure period, the worms were washed as described above, and AChE activity was determined.

Lysis of acetylthiocholine by pralidoxime

The ability of pralidoxime to cause lysis of acetylthiocholine was measured in a microplate at 405 nm in an enzyme-free system. To ATCI and DTNB taken from Tris-HCl (50 mM, pH 7.4), PAM was added at 0.25 and 1.0 mM concentration. The optical density was measured immediately after adding PAM and 1 min later, and the results were expressed as absorbance at 405 nm.

Statistical analysis

Mean and SE was calculated for all data, and data has been depicted as mean ± SE. The degree of significance between different groups was calculated by ANOVA and Tukey test.

Results

Toxicity of monocrotophos to *C. elegans*

The acute toxicity of monocrotophos to *C. elegans* measured after 4 h of exposure is depicted in Fig. 1. No mortality was observed in control worms. In worms exposed to monocrotophos, mortality began to manifest at a concentration of 20 mM and 96% mortality was observed at 60 mM. Probit analyses of the mortality data revealed the median lethal concentration (LC_{50}) of monocrotophos as 35.5 ± 1.2 mM (95% confidence 33.2–37.8 mM).

AChE inhibition and paralysis in *C. elegans* exposed to monocrotophos

Impact of sub-lethal concentrations (1/80, 1/40, 1/20, and 1/10th LC_{50}) of monocrotophos on AChE activity in *C. elegans* following 4 h in vivo exposure is depicted in Fig. 2. As evident from the illustration, monocrotophos

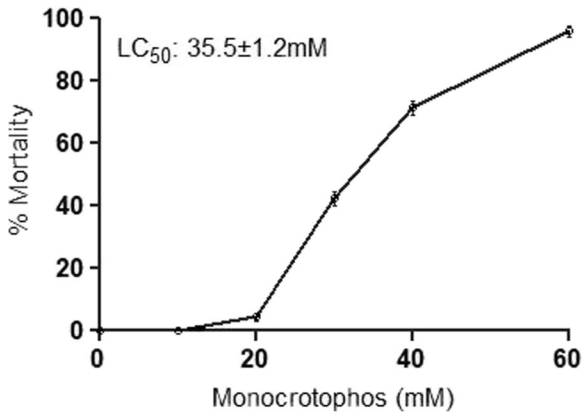

Fig. 1 Toxicity of monocrotophos to *Caenorhabditis elegans* after 4 h of exposure. Values are mean ± SE ($n = 9$)

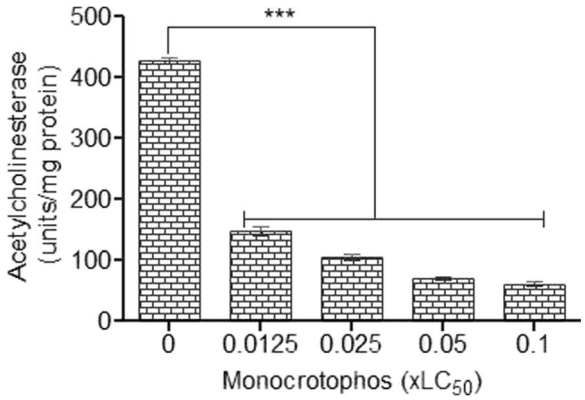

Fig. 2 Acetylcholinesterase inhibition in *Caenorhabditis elegans* by monocrotophos after 4 h of exposure. Values are mean ± SE ($n = 9$). ***$p < 0.001$

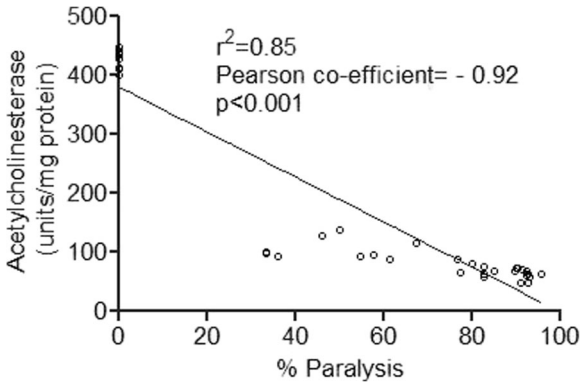

Fig. 4 Correlation between acetylcholinesterase activity and extent of paralysis in *Caenorhabditis elegans*. Data from control and monocrotophos-treated worms was used for obtaining Pearson correlation

caused marked inhibition (65%) even at 1/80th of LC_{50}, while 1/10th LC_{50} was associated with severe AChE inhibition (86%). Data on the paralyzing effects of monocrotophos in *C. elegans* is depicted in Fig. 3. No paralysis was observed among unexposed worms, while exposure of worms to sub-lethal concentrations of monocrotophos (1/40, 1/20, and 1/10th LC_{50}) for 4 h elicited significant paralysis (49–92%), characterized by loss of wriggling movements. We observed a strong negative correlation between AChE activity and extent of paralysis with a Pearson coefficient of – 0.92 (Fig. 4).

Effect of pralidoxime iodide on AChE activity and paralysis in worms exposed to monocrotophos

Figure 5 depicts the effect of co-exposure of worms to PAM on the paralyzing effects of monocrotophos in *C. elegans*. Co-exposure of worms to PAM (20 mM) significantly rescued worms from paralyzing effects of monocrotophos. Monocrotophos at 1/20 and 1/10th LC_{50} concentrations elicited 82 and 92% paralysis respectively, while worms exposed to both monocrotophos and PAM (20 mM) exhibited minimal paralyses (10 and 15% paralysis at 1/20 and 1/10th LC_{50} monocrotophos in the presence of PAM). Effect of co-exposure of worms to PAM and monocrotophos for 4 h on AChE activity is depicted in Fig. 6. Co-exposure of worms with PAM (20 mM) was associated with partial but significant reactivation of monocrotophos-induced inhibited AChE. Monocrotophos (1/20th LC_{50}) per se caused 81% inhibition of AChE, while co-exposure with PAM was associated with the lower degree of inhibition (46%).

Lysis of acetylthiocholine by pralidoxime

Data on lysis of ATCI by PAM is depicted in Fig. 7. The interpretation of this experiment is based on the liberation of thiocholine from ATCI. Thiocholine thus produced

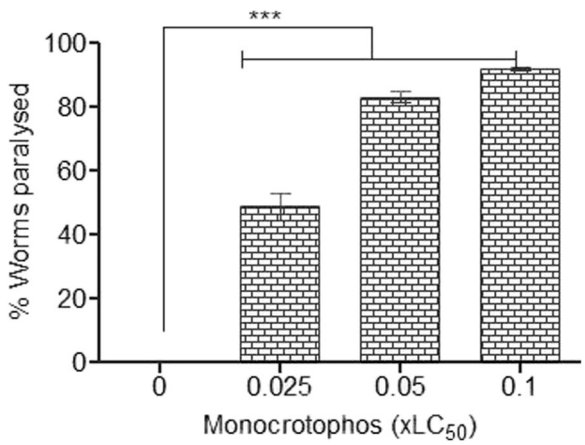

Fig. 3 Paralysis in *Caenorhabditis elegans* exposed to monocrotophos for 4 h. Values are mean ± SE ($n = 9$). ***$p < 0.001$

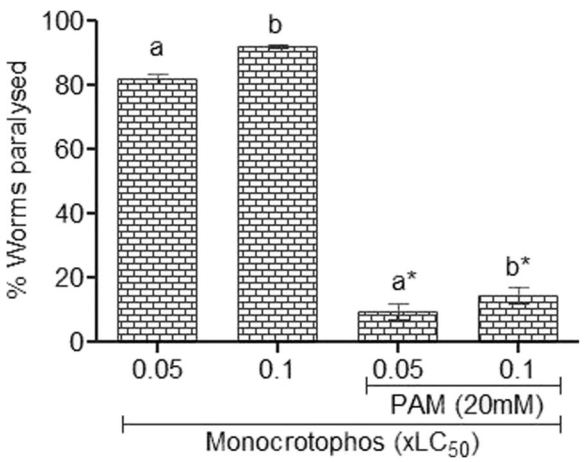

Fig. 5 Rescue of *Caenorhabditis elegans* from the paralyzing effects of monocrotophos by pralidoxime (PAM). Values are mean ± SE ($n = 9$). $p < 0.001$ for a v/s a* and b v/s b*

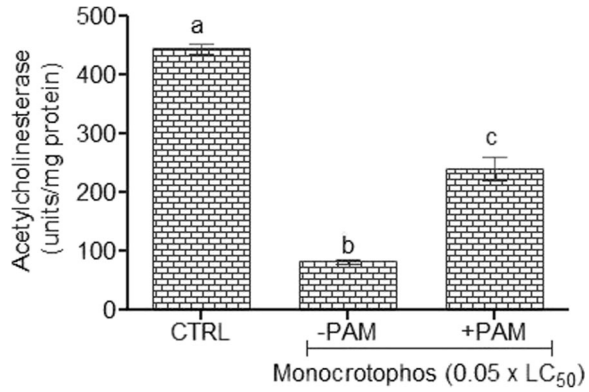

Fig. 6 Effect of pralidoxime (PAM, 20 mM) on AChE inhibition by monocrotophos in *Caenorhabditis elegans*. Bars with different letter are significantly different from each other ($p < 0.001$)

reacts with DTNB to produce 5-mercapto-2-nitrobenzoic acid, a yellow-colored chromophore. PAM caused rapid lysis of ATCI as evidenced by an increase in absorbance at 405 nm (1.08 and 3.39 absorbance units). The cleavage for acetylthiocholine affected by PAM is equivalent to 1080 and 3390 (0.25 and 1.0 mM respectively) units of the esterase-like activity.

Discussion

Our study reveals the toxic effects of monocrotophos (OPI) to *C. elegans*, characterized by mortality, and AChE inhibition and paralysis at sub-lethal concentrations after 4 h exposure. We observed a strong inverse correlation between AChE activity and extent of paralysis in worms. Organophosphorus insecticides are derivatives of phosphoric, phosphorous, or phosphinic acid (Abou-Donia, 2003). The toxicity of active OPI is attributed to their ability to inhibit acetylcholinesterase (AChE, choline hydrolase, EC 3.1.1.7), an enzyme that catalyzes the hydrolysis of the neurotransmitter acetylcholine (ACh). AChE inhibition leads to

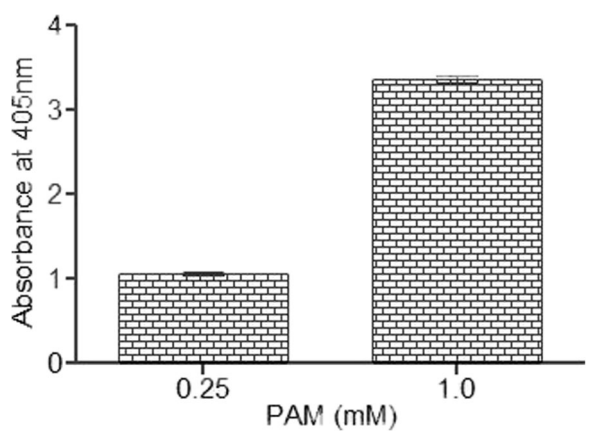

Fig. 7 Esterase-like activity of pralidoxime (PAM) towards acetylthiocholine. Absorbance measured at 405 nm ($n = 5$)

cholinergic stress as a result of stimulation of muscarinic and nicotinic ACh receptors (Abou-Donia, 2003; Fukuto, 1990; Sogorb & Vilanova, 2002). The ubiquitous presence of AChE in invertebrates and vertebrates and its crucial role in nervous system predisposes not only insects but also animal and human health to toxic action of OPIs.

With respect to toxicology studies, an attractive feature of the worm as a model is its ability to predict mammalian toxicity. The order of acute toxicities of some metallic salts to *C. elegans* has been demonstrated to be comparable to the order of their acute toxicities to rats and mice (Williams & Dusenbery, 1988). Cole et al. (2004) determined the acute behavioral toxicities of 13 organophosphorus insecticides to *C. elegans* and compared the values to acute toxicity values of the same insecticides for rats and mice. They reported that the order of toxicity in *C. elegans* significantly correlated with that of rats and mice. A similar correlation between the toxicities in *C. elegans* and rats has been reported for ten OPIs (Rajini, Melstrom, & Williams, 2008). Our data on acute toxicity of monocrotophos to *C. elegans* (LC_{50} of 35.5 mM) is in close agreement with other studies that evaluated acute toxicity of monocrotophos in *C. elegans* (Leelaja & Rajini, 2013; Rajini et al., 2008).

The extent of AChE inhibition and paralysis was studied as indices of the toxic effect of sub-lethal concentrations of monocrotophos in *C. elegans*. Assessment of AChE activity in whole body homogenate revealed that monocrotophos possessed the propensity to cause AChE inhibition even at low doses. The sensitivity of AChE activity in *C. elegans* to exposure to OPI *in vivo* clearly establishes that the worm is a reliable model for studying the toxicity of OPIs. AChE-inhibiting OPI and carbamate insecticides have been known to induce paralysis in infective juveniles of the entomogenous nematode *Neoaplectana carpocapsae* (Hara & Kaya, 1983). Further, previous reports from our laboratory have demonstrated paralyzing effects of OPIs in *C. elegans* (Jadhav & Rajini, 2009b; Leelaja & Rajini, 2013). This suggests that cholinergic overstimulation may result in paralysis in *C. elegans*. Further, levamisol (acetylcholine receptor agonist) is reported to induce paralysis in *C. elegans* (Lewis, Wu, Levine, & Berg, 1980; Nonet, Saifee, Zhao, Rand, & Wei, 1998). Aldicarb, an AChE inhibitor, has also been demonstrated to induce paralysis in *C. elegans* (Nonet et al., 1998). Together, these studies lend strong support to the view that AChE inhibition and consequent cholinergic stress may be the chief mechanism underlying the paralyzing effects of monocrotophos in *C. elegans*. Our study reveals a negative correlation between AChE activity and extent of paralysis caused by monocrotophos in *C. elegans*. The work of Melstrom and Williams clearly demonstrated the propensity of pharmacological AChE inhibition to alter the movement behavior in *C. elegans*

as evidenced by the correlation between the extent of AChE inhibition and a decrease in movement in worms exposed to each tested carbamate (Melstrom & Williams, 2007). These observations are supportive of the fact that paralysis manifests in *C. elegans* as a result of AChE inhibition and ensuing cholinergic stress after exposure to OPI.

A combination of anticholinergic drugs, AChE reactivators (oximes), and anticonvulsants (symptomatic drugs) is considered the most promising treatment of organophosphate poisonings (Marrs, Rice, & Vale, 2006). Pralidoxime (PAM), trimedoxime (TMB4 or TMC4), obidoxime (LüH-6), methoxime (MMB4 or MMC4), and HI-6 (HI-6 2Cl or HI-6 DMS) are examples of AChE reactivators available for treating OPI intoxication (Antonijevic & Stojiljkovic, 2007). However, there is no single broad-spectrum AChE reactivator with potency to activate AChE inhibited by many OP compounds, and hence, new reactivators are being still being synthesized and evaluated (Chambers et al., 2016; Chambers, Meek, & Chambers, 2016; Kliachyna et al., 2014; McHardy, Wang, McCowen, & Valdez, 2017). In order to understand the involvement of AChE inhibition in monocrotophos-induced paralysis in *C. elegans*, we co-incubated the worms with both monocrotophos and pralidoxime iodide (PAM), an AChE reactivator. PAM is one of the many oxime-based substances clinically used to reactivate AChE inhibited by OPI. The mechanism by which PAM activates the enzyme is based on nucleophilic attack followed by displacement of the phosphoryl group from the enzyme as the oxime-inhibitor complex (Kuca, RC, Musilek, Jun, & Pohanka, 2009). We observed that PAM rescued worms from the paralyzing effects of monocrotophos. PAM activated, albeit partially, AChE activity inhibited by monocrotophos. This further suggests that AChE inhibition is the underlying mechanism of monocrotophos-induced paralysis. More importantly, the OPI model of paralysis described in the present study utilizing paralysis as a visualizable physiological end point with AChE assay can be further developed as a good model for screening AChE reactivators.

Further work is needed to explain how pralidoxime rescues worms from paralysis while offering partial recovery of the inhibited enzyme. It is well known that PAM is capable of cleaving ATCI, a factor that could contribute to esterase-like activity leading to the erroneous recording of AChE activity. While we took sufficient precaution to remove excess PAM by repeated washing of worm pellet prior to homogenization, the possibility of residual PAM trapped inside the cuticle contributing to esterase activity must be considered. Despite well documented reports on cleavage of ATCI by PAM (Nadarajah, 1992; Sakurada, Ikegaya, Ohta, Akutsu, & Takatori, 2006; Zhang, Miyata, Wu, Wu, & Xie, 2007), it is surprising that there is no data on whether PAM is capable of cleaving ACh in vivo. It is known that PAM causes lysis of ATCI in a process where PAM becomes acetylated, while thiocholine is set free (Sakurada et al., 2006). If such a lysis by PAM is also true for ACh, the physiological efficacy may also be explained by the esterase-like activity of PAM. Data from two studies substantiate the view that PAM may not possess the propensity to cause lysis of ACh in vitro (Sakurada et al., 2006; Zhang et al., 2007). One of these studies suggesting the view of the inability of PAM to cause oximolysis of ACh was based on the pH-based assay for the release of acetic acid from ACh. However, it is known that PAM is acetylated as it causes oximolysis of acetylthiocholine (Sakurada et al., 2006). It remains to be seen whether pH measurement is sensitive to the appearance of acetylated PAM as opposed to acetic acid. However, HPLC analysis of an assay system in which ACh was incubated with PAM revealed that acetylated PAM does not appear in the system, indicating that PAM may not cause oximolysis of ACh in vitro. Based on these observations, the rescue of worms from the paralyzing effects of monocrotophos (in the presence of PAM) observed by us may be attributed to partial reactivation of the enzyme. Nonetheless, we opine that more studies are needed in the direction of addressing whether PAM is capable of causing lysis of *ACh* in vivo.

Conclusions

Our study shows the propensity of monocrotophos to elicit strong inhibition of acetylcholinesterase in *C. elegans* at sub-lethal concentrations in an acute toxicity test. A strong negative correlation was discernible between AChE activity and the extent of paralysis. Our study clearly shows that co-exposure to pralidoxime (AChE reactivator) rescues worms from the paralyzing effects of monocrotophos. This is suggestive of the fact that paralysis observed in monocrotophos-treated worms is a classical outcome of acute toxicity of OPI caused by AChE inhibition. Our study also suggests a need for revisiting the question whether the esterase-like activity of PAM operates as a mechanism in vivo responsible for its therapeutic effects. The paralysis model described in this study provides the proof-of-concept for the development of a robust and rapid in vivo model for studying the efficacy of cholinesterase activators. The *Caenorhabditis elegans* model will be certainly of great advantage over traditional in vitro models as it gives both physiological and biochemical perspectives on AChE reactivators.

Abbreviations

Ach: Acetylcholine; AChE: Acetylcholine esterase; ATCI: Acetylthiocholine iodide; DTNB: 5, 5-Dithio-bis-2-nitrobenzoic acid; OPI: Organophosphorus insecticide; PAM: Pralidoxime

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Authors' contributions
AKRJ and PSR conceived and designed the study. Experiments and data acquisition were performed by AKRJ and NR. AKRJ and PSR wrote the manuscript. All authors have read and approved the manuscript.

Ethics approval
This study did not require any institutional ethical approval.

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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Author details
[1]Food Protectants and Infestation Control Department, CSIR-Central Food Technological Research Institute, Mysore, Karnataka, India. [2]Food Chemistry Division, National Institute of Nutrition, Jamai-Osmania, Hyderabad, India. [3]Work Physiology and Sports Nutrition, National Institute of Nutrition, Jamai-Osmania, Hyderabad, Telangana 500007, India.

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