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Dichlorvos induced toxicity and oxidative stress in the nematode *Caenorhabditis Elegans*

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Abstract
Oxidative stress and other effects induced by sublethal concentration of dichlorvos (0. 5, 50, 100, 150 and 200μM) on non-target, invertebrate, model nematode *Caenorhabditis elegans* exposure for 4h, resulted in increased levels of ROS (12.5 − 50%), alterations in antioxidant system, increased activity of superoxide dismutase (6 − 55.56%), glutathione-*S*-transferase (9 − 33%), decreased levels of catalase (34 − 50%), variations in glutathione peroxidase, glutathione reductase (26.58 − 57%) and glutathione was reduced with varying degree, a non –enzymatic anti-oxidant. Similarly the target enzyme of organophosphorous insecticide (OPI) acetylecholinesterase inhibtition observed (45 − 85%) and study was extended to understand the detoxification of dichlorvos by carboxylesterase as a measure of inhibition (17 − 58%). Further the physiological parameters examined as decrease in brood size and life span observed was dose dependent (15 − 46.5%) and the decreased life span as a measure of worm survival on 21st day. These results clearly demonstrated that the dichlorvos induces oxidative stress in the model organism and there by alterations in antioxidant enzyme system and effect on physiological parameters were observed on sub lethal exposure.

Keywords: dichlorvos, *Caenorhabditis elegans*, oxidative stress, acetyelecholinesterase

Introduction
Organophosphorus insecticides (OPI) are indiscriminately used due to their high toxicity to insects and low environmental persistence. Humans get exposed to these pesticides as non-target organisms either during agricultural practices or as a result of residues in foods. OPI have potential to produce several different forms of neurotoxicity [1]. The acute intoxication resulting from AChE inhibition is well understood, which when severe can have longer lasting consequences [2]. Dichlorvos and lindane administration caused Oxidative stress due to abnormal production of reactive oxygen species (ROS) in wistar rats [3].

Oxidative stress caused by excessive reactive oxygen species production has been shown to lead to cellular dysfunction culminating in cell death. Cell death in neuronal tissues has been implicated in numerous neurodegenerative diseases [4]. ROS are part of normal oxidative metabolism, but when produced in excess, they cause tissue injury including lipid peroxidation, DNA damage, and enzyme inactivation. In addition, oxidative stress is also a process related to xenobiotic exposure and different levels of environmental contamination [5]. And leading to oxidative damage by changing the balance between oxidants and antioxidants [6]. Involvement of oxidative stress following acute exposure to OPI has been reported recently [7]. And it has been demonstrated unequivocally that lipid peroxidation is one of the molecular mechanisms involved in OPI-induced cytotoxicity [8].

The nematode *C. elegans* has become an important model organism for the study of development, aging, host-pathogen relationship, and certain diseases [9]. This organism possesses simple nervous system, consisting of 302 neurons and considerable toxicity testing has been performed employing *C. elegans* as a bioassay system and various endpoints have been assessed in both aquatic and soil environments on exposure to toxicants. Recent studies have reported the toxicity of dichlorvos in *C. elegans* with special reference its lethality, comparing LC-50, EC-50 with that of rat LD50 [10]. Numerous physiological parameters such as reproduction, development, growth rate and inhibition of feeding, fecundity and motility [11]. Alterations in global gene expression following OP exposure was readily observed in c elegans [12].

In our earlier study [13], We showed that sublethal concentrations of dichlorvos induce heat shock proteins, inhibit feeding and AChE activity in transgenic *C. elegans*. and comparative Kinetics of ACHE in *C. elegans* and rat brain system invitro [14], Similarly we have also demonstrated the pattern of AChE inhibition and accumulation of ACh on sublethal exposure to dichlorvos and possible correlation between AChE inhibition, ACh accumulation and the

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incidence of neurobehavioral responses.

Dichlorvos an OPI, widely used for pest control has been shown to induce oxidative stress in fish [15], and rats [16]. There is a lack of data pertaining to the effect of dichlorvos on non-target organism and effect on aquatic and invertebrate organism on exposure. Hence the study was designed to understand the potential of dichlorvos to induce oxidative stress, effect on antioxidant enzyme system and there by effect on brood size and life span in C. *elegans.*

Materials and Methods
Chemicals
Dichlorvos (Technical grade, 97.6% pure) was a gift from Hyderabad Chemical Supplies Ltd., Hyderabad, India. Acetylthiocholine iodide, BSA (fraction V), 2, 7-Dichlorofluorescin diacetate (DCFH-DA), 5, 5-dithio-bis-2-nitrobenzoic acid (DTNB), 5-fluoro-2'-deoxyuridine (FudR), β-mercaptoethanol, *p*-nitrophenyl acetate (PNPA) and Quercetin were procured from Sigma Chemical Co., (St. Louis, MO, USA). 1-chloro-2,4-dinitrobenzene (CDNB), Ethylenediaminetetracetic acid (EDTA), Folin's reagent, Hydrogen peroxide (H_2O_2), Nicotine amide adenine dinucleotide phosphate reduced (NADPH), Reduced glutathione (GSH), Trichloroacetic acid (TCA) and Oxidised glutathione (GSSG) were procured from Sisco Research Lab, (Mumbai, India). All other chemicals used were of analytical grade.

Worm culture
The N2 (Wild-type) strain of *Caenorhabditis elegans* was procured from Caenorhabditis Genetics Center (NIH funded National Center for Research Resources, Minneapolis, MN, USA). The worms were transferred to nematode growth medium (NGM) plate pre-seeded with a lawn of *Escherichia coli* (strain OP50) and cultured for 3 days at 20ºC until they reached adulthood. The worms were washed off from the plate with cold K-medium (53 mM NaCl,32 mM KCl),pelleted by centrifugation (3000 *g*,5 min), washed again twice with cold K-medium, and finally suspended in K-medium to obtain 30 to 50 nematodes per 10 µl [17].

Exposure conditions
Synchronized adult worms (L4 stage) were exposed to the Dichlorvos (0, 5, 50, 100 and 200µM) in 12-well tissue culture plates for 4 h at 20º C. Each well contained 1.0 ml of K-medium with Dichlorvos. Three replicate wells were maintained for each assay as described below.

Brood size
After exposure, the worms were washed with K-medium and a single worm was picked and transferred to a 12-well tissue culture plate containing 1.0 ml K-medium, and OP50 at a dilution of 10.0 OD at 550 nm. The plate was incubated at 20° C for 72 h. After incubation, the worms were washed, pelleted and the progeny were counted under the dissecting microscope. 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.

Lifespan
After exposure period (4 h at 20º C) [18], the 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 bottom 24-well plates, each well containing 500 µ l of K-medium (OD at 550 nm adjusted to ≈ 1.0 with OP50) and FudR at a final concentration of 50 µ M. These selected worms were raised and maintained at 20°C and 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 [19].

Study on oxidative stress parameters
Synchronized worms at gravid stage(pre-incubated with DCFH-DA (1mM) for 1h 30min) were exposed to dichlorvos (5, 50,100,150 and 200µM), in 1 ml K-medium in 12-well tissue culture plate incubated for 4 h at 20º C. After exposure period, the worms were washed three-times with K-medium and the worm pellet homogenized for fixed time in 50 mM Tris–HCl buffer (pH 7.4). The worm homogenate centrifuged at 10000 rpm for 10 min, and the supernatant used for the following assays.

The levels of ROS generated in the worms were determined by DCFH oxidation method [20]. The amount of DCF (resulting from the ROS mediated oxidation of DCFH, which is produced by hydrolytic cleavage of DCFH-DA by cellular esterases) was determined using the DCF standard graph. Results were expressed as nmol of DCF/mg protein

Reduced glutathione (GSH) level in *C elegans* homogenate was quantified by the method [21]. And the results were expressed as µ g GSH/mg protein. The activities of various antioxidant enzymes such as SOD [22], catalase [23], glutathione peroxidase (GPx) [24], glutathione reductase (GR) [25] glutathione s-transferase (GST), Acetylcholinesterase and Carboxylesterase were also measured. Total protein in the homogenate was measured according to Lowry [26].

Statistical analyses
Results were expressed as mean ± standard error of the means with 'n' denoting the number of experiments performed. Significant difference was tested using ANOVA, followed by Post-hoc Duncan (at P< 0.05)

Table 1: Oxidative stress markers in *C. elegans* exposed to various treatments [Values are mean ± SEM (n = 9)]

Dichlorvos (µM)	0	5	50	100	150	200
ROS (nmol DCF/ mg protein)	1.04 [a] ± 0.12	1.17 [a] ± 0.10	1.25 [a] ± 0.18	1.37 [a] ± 0.13	1.46 [a] ± 0.35	1.56 [a] ± 0.25
GSH (µg / mg protein)	178.2 [a] ± 7.50	135.9 [d] ± 8.00	140.4 [cd] ± 14.5	153.6 [b] ± 8.50	144.9 [c] ± 9.50	137.1 [d] ± 14.5
AChE (nmol substrate hydrolysed / min/ mg protein)	21.71 [a] ± 0.89	11.89 [b] ± 0.89	8.95 [c] ± 1.58	5.18 [d] ± 1.03	4.60 [de] ± 1.03	3.33 [e] ± 0.90
CaE (nmol substrate hydrolysed/ min/ mg protein)	4.20 [a] ± 0.93	3.51 [a] ± 0.39	2.50 [a] ± 0.36	2.25 [a] ± 0.38	1.95 [a] ± 0.37	1.75 [a] ± 0.33
SOD (units/mg protein)	22.20[a]±1.48	23.57[a]±1.20	24.20[a] ±0.9	30.70[a]±2.58	32.40[a]±3.58	34.57[a]±2.69
CAT (µ mol H2O2 consumed/min/mg protein)	12.30±1.13	8.13±0.68	7.72±0.60	6.45±1.12	6.24±0.81	6.12±0.70
GPx (nmol NADPH oxidized/min/mg protein)	9.73±0.44	7.23±0.41	7.84±0.49	5.30±0.25	6.43±0.30	6.12±0.19
GR (nmol NADPH oxidized/min/mg protein)	26.33±3.43	19.33±3.32	15.33±1.95	13.33±2.32	12.33±2.14	11.33±1.57
GST (nmol adduct/min/mg protein)	11.27±1.73	11.93±1.30	12.58±0.51	12.65±0.80	13.61±1.78	13.94±1.59
AChE (nmol/min/mg protein)	21.71±1.85	11.89±0.89	8.94±1.69	5.17±1.59	4.60±1.02	3.33±0.65
CaE (nmol/min/mg protein)	4.19±0.93	3.51±0.39	2.50±0.36	2.25±0.38	1.95±0.37	1.75±0.33

Groups in each row with different letters are significantly different (ANOVA, *post-hoc*, Duncan SPSS 10.05, P< 0.05).

Results and Discussion

Reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anion and hydroxyl radical are produced in a number of cellular reactions and by enzymes such as lipoxygenases, peroxidases and dehydrogenases. ROS are part of normal oxidative metabolism, but when produced in excess, lead to oxidative stress (OS) and they cause tissue injury including lipid peroxidation, DNA damage, and enzyme inactivation [27]. More recently, it has been postulated that OPI induce oxidative stress in different tissues through the formation of reactive oxygen species (ROS) [28]. In such cases, peroxidation of membrane lipids seems to be an unavoidable process in tissue injury, and may impair antioxidant defenses, leading to oxidative damage by changing the balance between oxidants and antioxidants [29].

Oxidative stress induced by a test compound is evident by a significant elevation in ROS and alterations in antioxidant enzyme activities and content of GSH. A significant increase in ROS generation is an indicator of the prooxidant action of the compound in question. In the present study, we found increase in ROS (12.5 - 50%) was observed in worms exposed to dichlorvos. However, a negative correlation between ROS generation vs. GSH content is indicative of utilization of GSH as a non-enzymatic antioxidant in scavenging free radicals. Depletion of GSH, the most abundant cellular non-protein thiol, is associated with oxidative stress and cytotoxicity of pro-oxidant xenobiotic. Glutathione is presumed to be an important endogenous defense against the peroxidative destruction of cellular membranes. GSH can act either to detoxify activated oxygen species such as H_2O_2 or to reduce lipid peroxides themselves [30]. Tissue GSH concentration reflects the potential for detoxification and the levels may decrease due to an increased use of glutathione to detoxify. Indeed, a depletion in GSH induces an increase in the sensitivity of the organisms to xenobiotics or overall, with generating processes of radicals [31]. Interestingly, we observed a marginal decrease in GSH content in worms exposed to dichlorvos. This observation strengthens the fact dichlorvos possesses the potential to induce OS in *C. elegans.*

The worms exposed continuously at 2 or 8 h resulted in time-dependent increase in the expression of genes involved in stress responses and at later times, an immune-like response and cellular repair mechanisms dominated the expression pattern [32]. Dichlorvos induced LPO in erythrocyte and alterations in antioxidant enzyme activities, suggesting that reactive oxygen species (ROS) may be involved in the toxic effects of DDVP [33]. Under normal physiological conditions, a delicate balance exists between the rate of formation of H_2O_2 via dismutation of $O_2 \bullet^-$ by SOD activity and the rate of removal of H_2O_2 by CAT and GPx. Therefore, any impairment in this pathway will affect the activities of other enzymes in the cascade [34].

In *C. elegans*, antioxidant protection involves anti oxidant enzymes such as SOD, Catalase, Glutathione peroxidase and non-enzymatic anti-oxidant glutathione [35]. SOD and to a lesser extent, catalase have been shown to be crucial for defense against oxygen toxicity in *C. elegans*. These two enzymes detoxify the reactive compounds superoxide and hydrogen peroxide. SOD converts superoxide into water and hydrogen peroxide, and catalase produces water and oxygen from hydrogen peroxide.

GSTs are also important as antioxidants in many organisms including nematodes. The enzyme in mammals and insects is inducible by exposure to potential substrates. *C. elegans* Gst -

1 mutant has been shown by differential display to be induced by paraquat, which induces oxidative stress in adults and larvae [36]. In the present study, an increase in GST activity was observed. This can be understood in view of the fact that OPI consumes GSH through GST catalyzed reaction as a major way of detoxification and these chemicals are expected to induce the activity of GST as a potent protection mechanism of the organism.

Glutathione peroxidases are enzymes that catalyze glutathione dependent reduction of both hydrogen peroxide and fatty acid peroxides. In the present study, we observed decrease in the activities of catalase, glutathione peroxidase and glutathione reductase in worms exposed to dichlorvos. Similar decrease in activities of GPx and catalase has also been recently reported in rat erythrocytes after exposure to sublethal concentration of methidathion [37].

Exposure of worms to sublethal concentrations of dichlorvos also resulted in significant inhibition in the activities of both AChE (45 - 85%) and carboxylesterase (17 - 58%). This confirms the exposure of the worms to effective concentrations of dichlorvos since AChE and CaEs are considered to be important targets for organophosphorus compounds and inhibition of CaE by OPI has also been well documented in several tissues / organisms [38].

In the present study, exposure to dichlorvos significantly decreased the brood size by 16 – 46% at the tested concentrations. Nematodes can change their reproductive speed, life cycle and other properties while exposed to stress surroundings [39]. Hence, fecundity and reproduction rates are suggested as good end points to evaluate the generational effects of steroids and synthetic hormones on *C. elegans* [40]. Reproduction and various life-cycle parameters have been used routinely as toxicity endpoints for *C. elegans* in several studies [41]. Decreased brood size can be the result of lowered fecundity, increased generation time and an increased generation time may be the consequence of retarded post embryonic development, delayed gonad maturation, abnormalities in gamete, or inhibition of embryogenesis. Hence the results obtained in the present study are indicative of the impact of dichlorvos on the above processes in *C. elegans*.

Under optimal laboratory conditions, the average life span of *C. elegans* is 2-3 wk. Studies have shown that environmental factors influence aging and lifespan of *C. elegans* [42]. Both acrylamide and cadmium have been reported to shorten life span of *C. elegans* [43]. In the presence of copper or cadmium or SDS, the life span of *C. elegans* was found to be significantly decreased in concentration dependent manner [44]. In the present study, a concentration-related decrease in survivability was evident in the worms exposed to dichlorvos after 5 d and decreased life span was observed on 21st day (15-46.5% decrease).

Several studies have been revealed closed relationship between stress tolerance and longevity in *C. elegans* [45]. Oxidative stress appears to be a major factor limiting lifespan in both *C. elegans* and humans [46].

These findings show that studies of aging in *C. elegans* provide useful insights for identifying genes and compounds that can prolong or decrease lifespan in humans. A mutant of *C. elegans* with half the normal SOD activity was hypersensitive towards oxygen and paraquat and had a shortened life span [47]. A long-lived strain of *C. elegans* contained elevated SOD and catalase [48]. Hence the decrease in life span in worms exposed to dichlorvos could be linked to the OS induced.
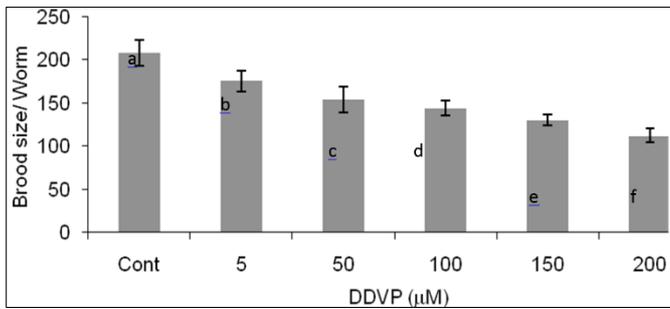
Data analyzed by post-hoc test (Duncan); Means in the same column with different superscript differ significantly (p< 0.05)

Fig 1: Effect of dichlorvos (DDVP) on brood size in *C. elegans*: outcome of 4h of exposure of L4 stage worms
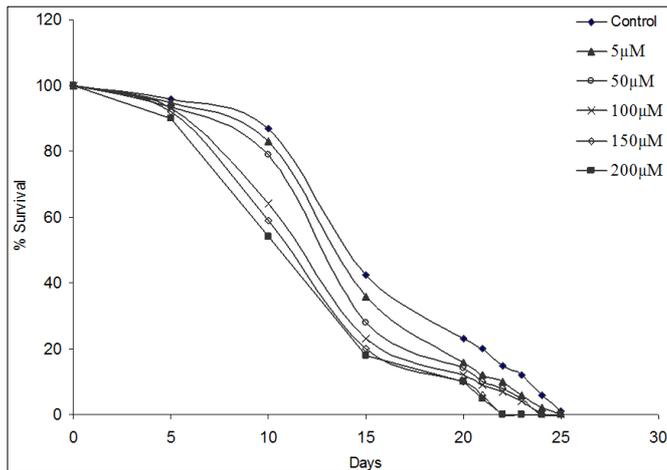
Values are mean ± S.E of 3 observations with 3 replicates each.

Fig 2: Impact of dichlorvos exposure for 4h on life span in *C. elegans*

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