

Attenuation of Dopaminergic Neuronal Dysfunction in *Caenorhabditis elegans* by Hydrophilic Form of Curcumin

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Abstract

Curcumin (CUR), the principal curcuminoid and the “yellow coloring matter” of turmeric, has been demonstrated to exhibit protective activity against various neurologic diseases. Owing to its hydrophobic nature, CUR is insoluble in water, and its poor solubility in aqueous solution remains a major barrier in its bioavailability and clinical efficacy. In view of this, in the present study, we rendered the CUR hydrophilic by making an ‘inclusion compound’ with gamma cyclodextrin (γ CD) and evaluated its neuroprotective efficacy by employing the model system *Caenorhabditis elegans*. Both wild-type and transgenic strain of *C. elegans* [expressing green fluorescent protein (GFP) specifically in the dopaminergic neurons [BZ555 (Pdat-1: GFP)] were treated with either the well-known neurotoxin, 6-hydroxydopamine (6-OHDA) or Monocrotophos (MCP), a neurotoxic insecticide. We examined the ameliorative effect of CUR on phenotype (in terms of locomotion), reproductive parameters (brood size, egg-laying) and longevity. Further, neurotoxicity was assessed by monitoring neurodegeneration of dopaminergic neurons and acetylcholinesterase activity. We found that CUR treatment marginally increased egg laying, brood size and the survival (by 3d). Interestingly, it also significantly decreased the dopaminergic neurodegeneration with a marginal restoration of acetylcholinesterase activity among worms exposed to either OHDA or MCP. Our findings provide evidence for the potential of CUR to ameliorate neuronal dysfunction in the worm model and suggest its use as a therapeutic molecule against PD.

Keywords: Curcumin; Monocrotophos; *C. elegans*; Neurodegeneration; γ cyclodextrin

Introduction

Dopaminergic neuronal dysfunction is the characteristic feature of neurodegenerative disorders such as Parkinson’s disease (PD). PD is typically characterized by the loss of nigrostriatal dopamine neurons, and the formation of intraneuronal inclusions termed ‘Lewy bodies’ [1]. Although the exact etiology of dopaminergic neurodegeneration in PD is unknown, genetic susceptibility and environmental factors [2] that mediate mitochondrial dysfunction, inflammation, abrogation of the autosomal-lysosomal autophagy system [3] and endoplasmic reticulum stress [4] are implicated to play a role in the disease development. Currently, there is no effective cure for PD and the drugs used for the treatment are dopamine antagonists and monoamine oxidase-B (MAO-B) inhibitors, which provide only symptomatic relief. A growing body of evidence suggests that nutritional components such as green tea, blueberries, resveratrol, and *Ginkgo biloba* extract offer protection against neurodegenerative disorders [5,6]. Several herbal medicines and plant extracts have been evaluated in animal models for their potential to ameliorate biochemical and physiological abnormalities under experimentally-induced dopaminergic neurodegeneration [7,8].

Curcumin (CUR) is a yellow coloring component present in the spice turmeric (*Curcuma longa*) that belongs to the family, Zingiberaceae. In the recent past, CUR has acquired tremendous importance in modern medicine due to its anti-inflammatory and chemoprotective property [9]. CUR has been used as traditional medicine for several decades in India and China. CUR has been demonstrated to exhibit marked protective activity against various neurologic diseases, including Alzheimer’s disease, multiple sclerosis, epilepsy, cerebral injury, age-associated neurodegeneration, schizophrenia, spongiform encephalopathies, neuropathic pain and depression [10-17]. Low concentrations of CUR were shown to inhibit dopamine toxicity *in vivo*

[18] and inhibit MPP+ toxicity in PC12 neuronal cell line [19]. CUR has been demonstrated to exhibit neuroprotection in the 6-OHDA rat model of PD [20]. Treatment of dopaminergic neuronal cells and mice with CUR was shown to restore depletion of GSH levels, protect against protein oxidation, and preserve mitochondrial complex I activity [21].

Being hydrophobic in nature, CUR is insoluble in water but soluble in ethanol, dimethylsulfoxide, and acetone [22]. Poor solubility in aqueous solution remains a major limitation for the bioavailability and clinical efficacy of CUR. Various strategies such as encapsulation in liposomes, biodegradable microspheres, cyclodextrin, and hydrogels have been explored in order to increase its solubility and bioavailability [22]. Solubility and phase-distribution studies have shown that curcuminoids with side groups on the phenyl moiety have a higher affinity for the γ CD [23]. The uniqueness of the present study lies in the fact that we rendered the CUR hydrophilic by making an ‘inclusion compound’ with gamma cyclodextrin (γ CD) [23]. Several laboratories have employed *C. elegans* as a model organism to inquire the molecular and genetic mechanisms contributing to PD [24-26]. The translucent worm is an excellent model for monitoring the physiology and pathophysiology of dopaminergic neurons *in vivo*, by selective expression of green fluorescent protein driven by dopamine transporter (*dat-1*) promoter [27-29]. Accordingly the

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primary aim of the study was to examine the therapeutic potential of CUR under neurotoxin exposure employing *C. elegans* as the model system. The neuroprotective efficacy of CUR was investigated against the standard neurotoxin- OHDA which induces dopaminergic neuronal dysfunction, as well as a neurotoxic insecticide, monocrotophos (MCP) which has been shown to affect the dopaminergic neuronal functions [30] to compare the extent of protection offered under two different neurotoxic insults. We employed the wild-type (N2) worms to determine the locomotor phenotype, reproductive parameters, life span and used transgenic strain BZ555 (Pdat-1: GFP; bright GFP observable in dopamine neuronal soma and processes) to determine the extent of protection offered by CUR against neurodegeneration of dopaminergic neurons.

Materials and Methods

Chemicals

Curcumin (Analytical Grade, 96% pure) was gifted by Dr. Pura Naik (CSIR - CFTRI, Mysore, India). Both Monocrotophos (MCP, 97%) and 6-OHDA and 2-Nonanone were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Worm and *Escherichia coli* strains

Wild-type *C. elegans* (N2), transgenic strain (BZ555) and *E. coli* (OP50, a uracil auxotroph) were provided by *Caenorhabditis* Genetics Center (CGC, Minneapolis, MN, USA) funded by the National Center for Research Resources (NCRR).

Preparation of γ Cyclodextrin (γ CD)+Curcumin (CUR) inclusion compound

Inclusion compounds of γ CD and CUR were prepared as described by Kashima et al. [31] with minor modifications. A sterile γ CD solution was prepared by filtering a nearly saturated γ CD (200 mg/ml) solution, and this was mixed with curcumin (50 μ M, dissolved in Ethanol) in a shaker at 120 rpm overnight to obtain the inclusion compound. The inclusion compound was mixed with M9 buffer and OP50 for 12 h. The final solution was spread on a peptone-free NGM plate with known amount of test compounds (Monocrotophos, MCP; 0.75 mM in distilled water and 6-OHDA; 25 mM in distilled water). Age synchronised L4 worms were introduced into the plates and incubated at 20°C. The worms were recovered after 24 h for evaluating physiological endpoints. For biochemical parameters, inclusion compound was mixed with K-medium and OP50. L4 worms were exposed to the test compounds for 24 h in 12 well microtitre plate containing 1 ml K-medium in each of the well with test compounds as above.

Worm maintenance and treatment

All developmental stages of *C. elegans* were maintained in 70 mm Petri plates on Nematode Growth Medium (NGM) (0.032 M KCl, 0.051 M NaCl, 0.1 M CaCl₂, 0.1 M MgSO₄, 2.5% Bacto-peptone, 0.17% Bacto-agar and 0.01% cholesterol) seeded with OP50 (*Escherichia coli* - uracil auxotroph) as the food source [32]. The gravid worms were washed out of the NGM plates using K-medium (50 mM NaCl, 30 mM KCl in distilled water) and the eggs synchronised by alkali-bleaching method [33]. Age synchronised L4 worms were exposed to varying concentrations of MCP (50, 100 and 200 μ M in distilled water) for 24 h in 12 well microtitre plate containing 1 ml K-medium in each of the wells.

Nonanone repulsion assay

Nonanone repulsion assay [34] was employed for indirect measurement of the DA contents since any deviation from the

normal DA levels affects response time of the worms to the volatile repellent nonanone. This assay was carried out to select the efficient concentration of CUR for further studies. For the assay, we dipped an eyelash hair glued on a toothpick in nonanone and placed it in front of a forward-moving animal on a NGM plate without food. The time taken by the worm to reverse the path was noted. The assay was carried out for ten worms in each treatment group, three times in triplicates and the response time was calculated in seconds.

Treatment of worms with 6-hydroxydopamine (6-OHDA), Monocrotophos (MCP) and Curcumin

To induce neurodegeneration, worms (L4 stage) were exposed to 6-OHDA (25 mM) (for dopaminergic neurodegeneration) and MCP (0.75 mM) (for neurotoxicity) mixed with OP50, plated in NGM. The MCP concentrations used in the present study were selected based on our earlier study wherein these levels were found to affect the motility and acetylcholinesterase activity in the worms [30]. For co-exposure paradigm, CUR solution (50 μ M) was added along with OP50 before plating. The concentration of CUR was selected based on the nonanone repulsion study. The exposure time for all the experiments was 24 h.

Phenotype measurement (analysis of locomotion)

To quantify the development of phenotype, we analysed the locomotor behaviour of worms of various treatment groups. After treatment, the worms were transferred to agar plates with bacterial food. Locomotion rate was quantified by counting the number of body bends produced by a worm in 20 sec under a stereomicroscope. Ten worms were studied in each exposure group [35].

Egg laying activity

Ten treated worms were randomly picked from each exposure group including control and were transferred to NGM plates. The number of eggs laid by each worm during 1 h after exposure period was counted [36].

Determination of brood size

Following exposure, the worms were washed with K-medium and a single worm was picked and transferred to a 12-well tissue culture plates containing 1 ml K-medium, which contained *E. coli* at a dilution of 1 O.D 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 [37]. The average numbers of offspring from three wells were obtained for each test replicate, and the testing was repeated three times.

Effect on life span

Following exposure, the worms were washed thrice with K-medium. 20 \pm 1 L4 worms were re-exposed in a 12 well plate containing K-medium with OP50 and 5-fluoro-2-deoxyuridine (FudR, 50 μ M). Worms were maintained at 20°C. The survival was scored every day. Worms that failed to respond to touch were considered as dead. The survival was checked until all the worms were dead [38].

Determination of acetylcholinesterase (AChE) activity

After the exposure period, the worms were washed thrice with K-medium, pelleted and then homogenised in buffer (100 mM Tris-HCl, pH 8) followed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant was used for assaying acetylcholinesterase activity employing the method of Ellman et al. [39] rendered suitable for microplate reader analysis [40].

Quantification of dopamine (DA) levels

After exposure, the worms were washed thrice with K-medium and homogenised in buffer A (50 mM Tris-HCl buffer pH 7.4 ± 0.1% Tween 20) followed by precipitation with 10% TCA and centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant was filtered through 0.25 µm nylon filters. Dopamine was quantified in the supernatant by high-performance liquid chromatography (Shimadzu HPLC) with an electrochemical detector (ECD) consisting of a high-pressure isocratic pump, a 20 µl sample injector valve, C18 reverse phase column, and electrochemical detector. Retention time (RT) for dopamine peak was between 11.9 and 13.3 min of the run time. DA levels were calculated from an external standard curve and expressed as pg/mg protein [41].

Analysis of degeneration of dopaminergic (DA) neurons

L4 stage transgenic worms BZ555 (P_{dat-1} : GFP; bright GFP observable in dopamine neuronal soma and processes) were exposed to different concentrations MCP and OHDA mentioned above for 24 hours. After the exposure period, the worms were washed thrice in K-medium to get rid of any adhering bacteria. The worms were pelleted by centrifugation at 3000 rpm for 3 min. The pellets were suspended in a minimum volume of K-medium. Microscopy of living young adults was performed by placing the worms on glass slides with small aliquots of sodium azide (55 mM) used as an anesthetic, and visualising GFP with a CX31 (Olympus) fluorescence microscope fitted with a camera (C-7070 Wide Zoom, Olympus). All images were acquired at 40X objective and aperture size 4.8 and processed with Image J software for enumerating the green pixel number [26]. The integrity of DA neurons was evaluated by visual scoring of GFP intensity separately in two pairs of cephalic neurons (CEPs) and one pair of anterior deirid neurons (ADEs). Three independent experiments were conducted for each exposure with at least ten worms per exposure group.

Statistical analysis

All the experiments were conducted at least three times with three replicates each. Mean and standard error (SE) were determined for all the parameters, and the results were expressed as mean ± SE. The data were analysed employing analysis of variance (ANOVA) followed by the Tukey's multiple comparison tests. The mortality data were subjected to Kaplan-Meier survival analysis to prepare survival curves and the data were compared with untreated worms. All the calculations were carried out by Graph Pad Prism version 5 (computer software program).

Results

In the present study, initially we used three concentrations of curcumin (25, 50 and 100 µM) to screen the protective effect. CUR alone (25 and 50 µM) did not show any significant difference from control worms in terms of repulsion time (3.0 ± 0.01 s). However, worms exposed to both the neurotoxins exhibited marked increase in repulsion time (6-OHDA: 10.0 ± 0.03 s; MCP: 7.3 ± 0.17 s). CUR treatment significantly reduced the repulsion time in worms exposed to both 6-OHDA and MCP compared to the time taken by the neurotoxin treated worms (Figure 1). CUR did not seem to exert a concentration-dependent protective effect. Since CUR at 50 µM offered optimum protection, this concentration was selected for all the further studies.

Assessment of phenotype (Locomotion rate)

CUR (50 µM) treatment did not induce any significant alterations in the phenotype as the locomotion rate was highly comparable to those of untreated control worms. However, worms treated with the neurotoxin, 6-OHDA exhibited marked decrease in the locomotion

rate (64%), while worms exposed to MCP showed a 52% reduction in locomotion rate. Interestingly, in the co-exposure paradigm, CUR treatment significantly restored the locomotion rate among worms exposed to both 6-OHDA and MCP (Figure 2).

Egg-laying activity

The egg laying activity was comparable between control and CUR treated worms. In contrast, a significant decrease (56%) in the egg laying function was evident among worms exposed to either 6-OHDA or MCP. However, CUR treatment significantly enhanced (22%) the egg laying function in the worms exposed to either 6-OHDA or MCP (Figure 3A).

Brood size

A marked reduction in brood size was evident among worms exposed to neurotoxins (6-OHDA-25%; MCP-31%). However, in the co-exposure paradigm, CUR treatment marginally increased the brood size among worms exposed to either 6-OHDA or MCP (Figure 3B).

Life span

In our laboratory, N2 worms grown under standard laboratory conditions (22°C) have a mean lifespan of 28 days. However, worms maintained on NGM containing CUR (50 µM) showed a statistically

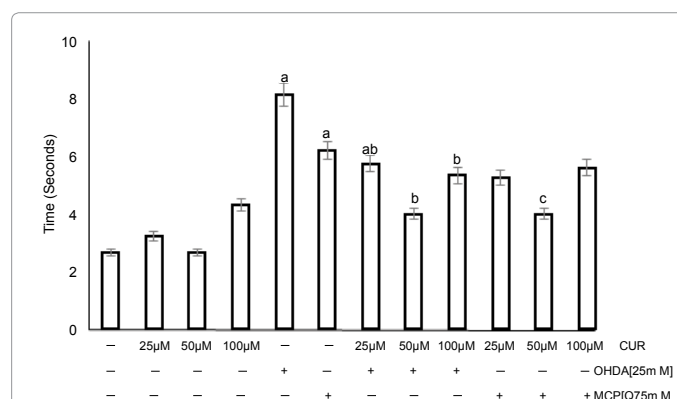


Figure 1: Effect of Curcumin (CUR) treatment on dopamine content as estimated by nonanol repulsion assay in *C. elegans*. Values represent mean ± S.E (n=9). ^aControl vs OHDA and MCP ($P \leq 0.05$). ^bOHDA vs OHDA+CUR ($P \leq 0.05$). ^cMCP vs MCP+CUR ($P \leq 0.05$).

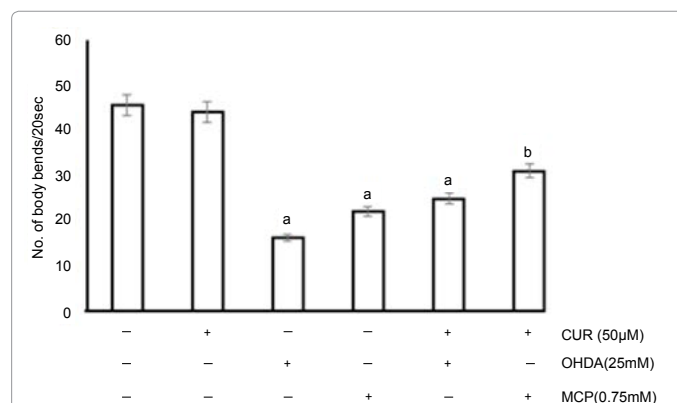
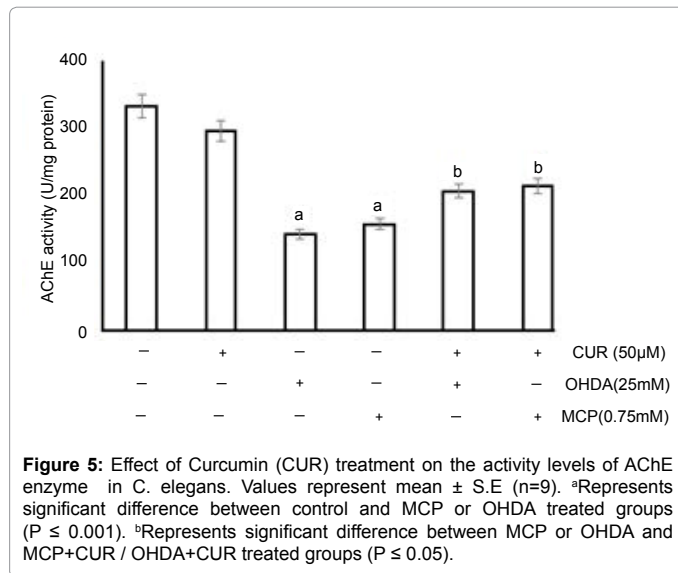
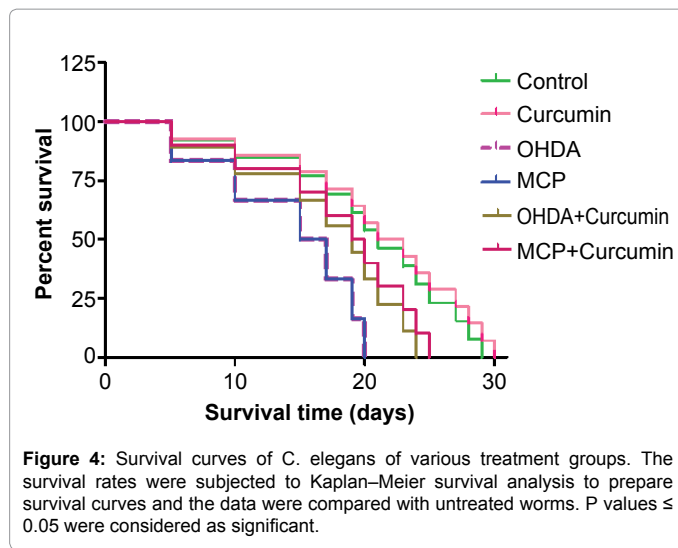
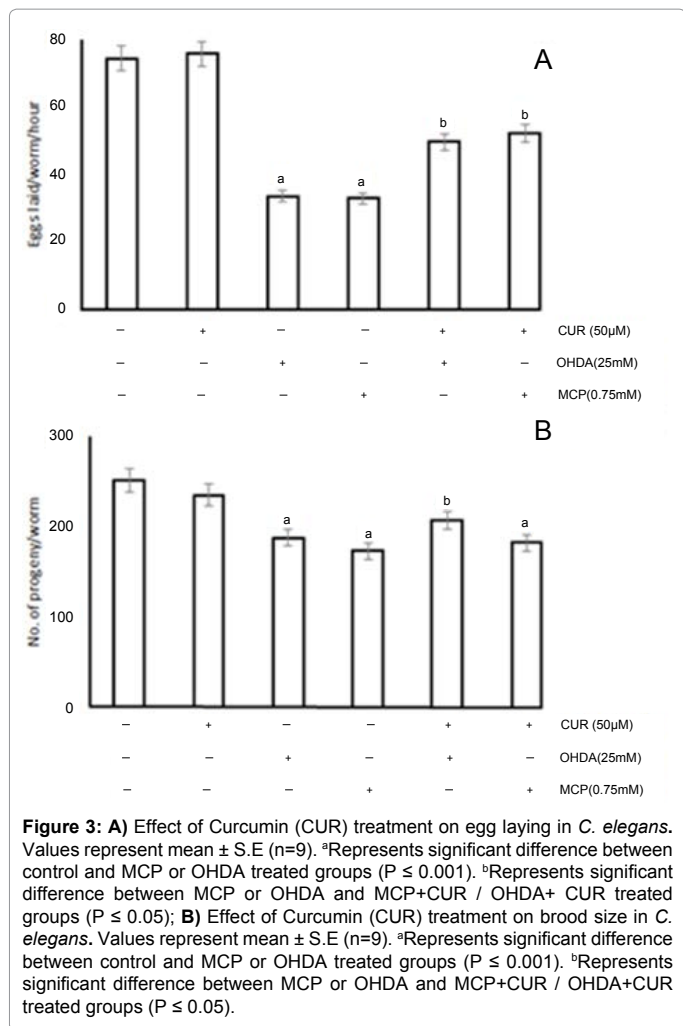


Figure 2: Effect of Curcumin (CUR) treatment on the locomotory response in *C. elegans*. Values represent mean ± S.E (n=9). ^aRepresents significant difference between control and MCP or OHDA treated groups ($P \leq 0.001$).; ^bRepresents significant difference between MCP and MCP+CUR treated groups ($P \leq 0.05$).



significant increase in lifespan compared to controls, with a lifespan of 31 days. In contrast, 6-OHDA and MCP exposure caused a significant reduction in the lifespan to 18 days. Interestingly, in the co-exposure paradigm, CUR treatment enhanced mean lifespan of worms exposed to 6-OHDA (22 days) and MCP (24 days) (Figure 4) suggesting its potential to improve survival.

Acetylcholinesterase (AChE) activity

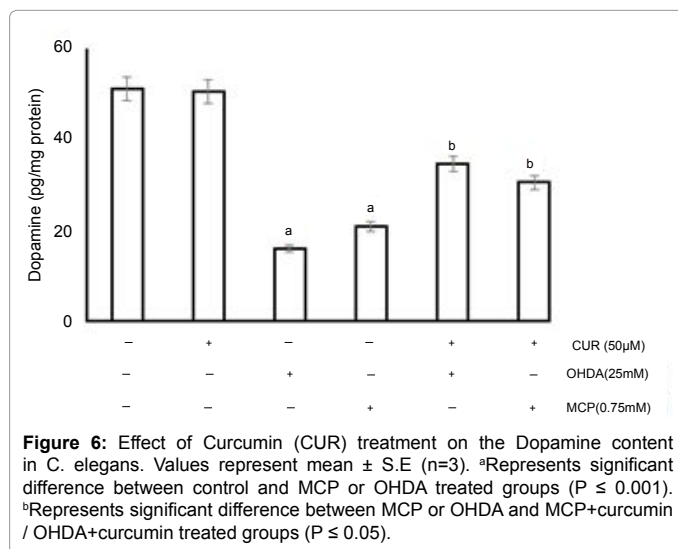
While CUR alone treatment had no significant effect on the activity levels of AChE, exposure of worms to neurotoxins significantly (6-OHDA-57%; MCP-52%) reduced the AChE activity. In the co-exposure paradigm, CUR treatment marginally restored the activity among worms exposed to either 6-OHDA or MCP (Figure 5).

Dopamine (DA) content

CUR alone treatment did not show any significant effect on the DA levels. However, worms exposed to the neurotoxins exhibited markedly reduced DA levels (6-OHDA-64%; MCP-60%). In the co-exposure paradigm, CUR treatment significantly restored the DA levels among worms exposed to either 6-OHDA or MCP (Figure 6).

Analysis of degeneration of dopaminergic (DA) Neurons

Worms treated with 6-OHDA and MCP showed significant loss within processes of CEP and ADE neurons compared to the untreated



worms (Figure 7A-7E). Further, a visibly marked reduction in GFP expression was also evident with neurotoxin exposed (72-77% respectively) was also evident in these worms (Figure 7G). However, worms co-exposed to CUR with either 6-OHDA or MCP showed a marginal increase in fluorescence intensity compared to those treated with either 6-OHDA or MCP alone (Figure 7D and 7F).

Discussion

The primary objective of the present study was to assess the protective potential of hydrophilic CUR against experimentally induced neurodegeneration in *C. elegans*. CUR is fat soluble, and several previous studies have either used DMSO or added it to culture media.

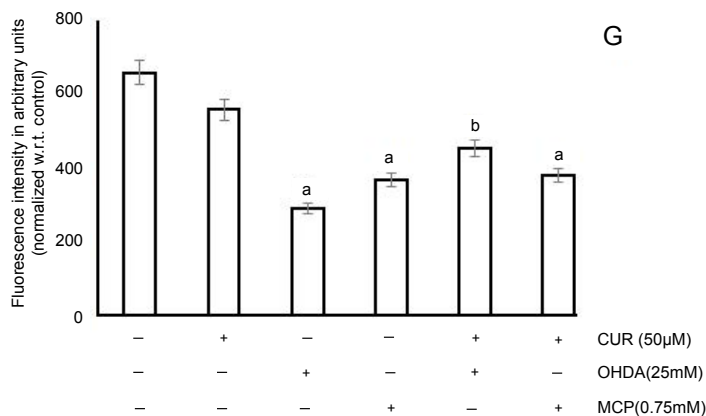
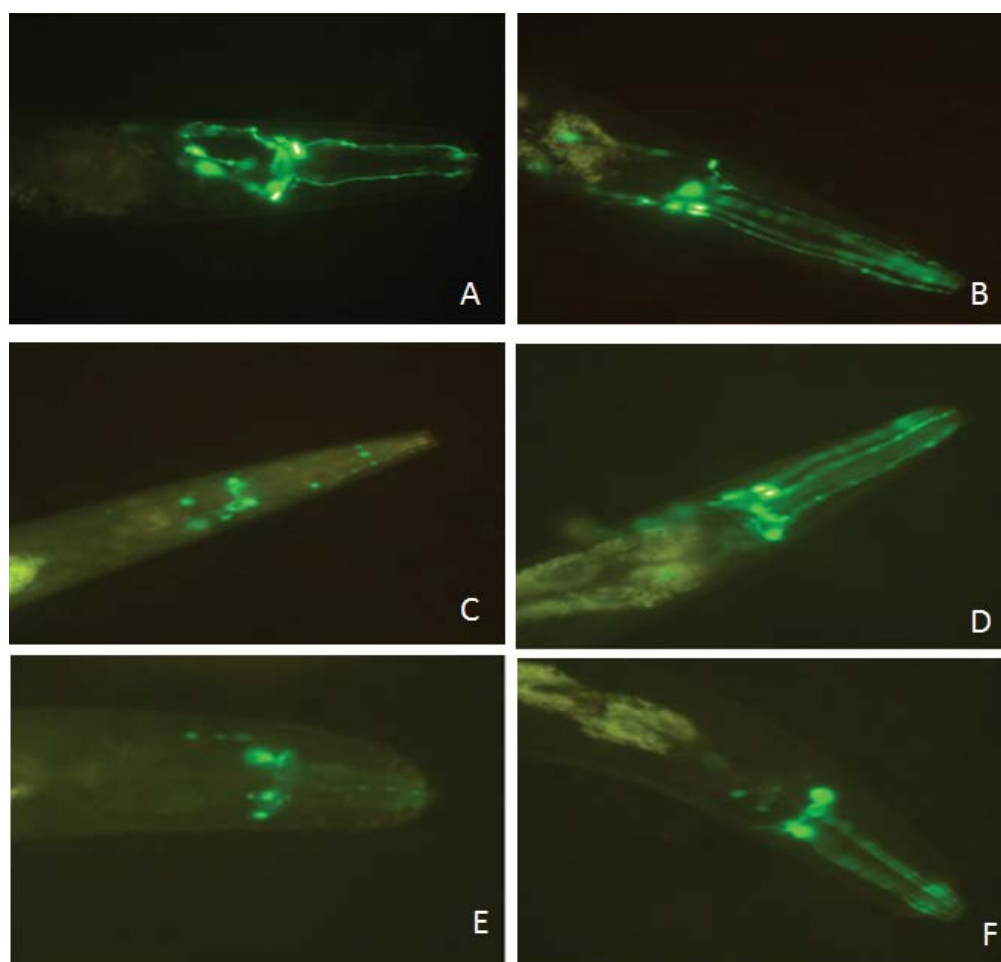


Figure 7: Modulatory effect of Curcumin (CUR) treatment on GFP expression in dopaminergic neurons of transgenic *C. elegans* (BZ555). A- Control, B- Curcumin (50 μM), C- MCP (0.75 mM), D- MCP (0.75 mM)+Curcumin (50 μM), E- OHDA (25 mM), F- OHDA (25 mM)+Curcumin (50 μM). (G) Graphical representation of fluorescence intensity of GFP in dopaminergic neurons of transgenic *C. elegans* as quantified using Image J software; Values represent mean ± S.E (n=9). ^aRepresents significant difference between control and MCP or OHDA treated groups ($P \leq 0.001$). ^bRepresents significant difference between OHDA and OHDA+curcumin treated groups ($P \leq 0.05$).

In the present study, we employed γ -cyclodextrin as a delivery system for administration of CUR to *C. elegans*. γ CD has been demonstrated to be an excellent vehicle for quantitative oral administration of hydrophobic chemicals to *C. elegans* [23,31]. Using this method, oral supplementation with hydrophobic antioxidants such as tocotrienol, astaxanthin, or α -tocopherol was found to prolong the lifespan of worms more efficiently than conventional delivery methods. Further, this method limits the use of an organic solvent for the treatment of worms.

Initially, we employed the 'nonanone repulsion assay' which gives an indirect measure of the dopamine content in the worms. The response time of the worm to the volatile repellent nonanone is an indicator of the DA levels. Worms with higher DA content responds early by moving back, and the worm with decreased DA content takes longer time to respond towards nonanone [34,42]. It has been clearly demonstrated that dopamine signaling is required for the enhancement of 2-nonanone avoidance [43]. We employed this parameter to select one effective concentration of CUR for studying the protective efficacy. Our repulsion assay showed that CUR offered nearly equal protection at the three tested concentrations. We selected 50 μ M CUR for all the further assays.

Previous studies have reported that both 6-OHDA and MCP cause a significant decrease in the mobility of worms [44]. The model neurotoxin, 6-OHDA has been reported to cause shrinkage of DAergic cells, nuclear condensation and DNA fragmentation (apoptotic characteristics) *in vitro* [45]. In the present study, CUR alone treatment did not cause any appreciable effect on the locomotory behavior in worms. However, we observed a significant decrease in the movement (in terms of body bends) among worms exposed to neurotoxins (6-OHDA: 64% and MCP: 52%). A variety of behaviors has been associated with dopamine in *C. elegans* including inhibition of locomotion, modulation of crawling speed with response to food [46] and controlling of turning frequency [47]. Numerous studies have reported the beneficial effects of CUR the active principle of turmeric, in several neurological disorders [48]. CUR possesses a variety of biological and pharmacological activities which range from antioxidant property, anti-inflammatory, antimicrobial and anticarcinogenic [49,50]. Its potential against neurodegenerative disease is well established [21]. The increased mobility induced by CUR treatment among worms co-exposed to both neurotoxins correlates with amelioration of degeneration in dopaminergic neurons.

In the present model, we observed that exposure of worms (L4) to sublethal concentrations of 6-OHDA and MCP for 24 h resulted in decreased reproductive capability as revealed by reduced (25-31%) brood size. A similar reduction in brood size was demonstrable in *C. elegans* exposed to DDVP [37] and in *Daphnia* exposed to other toxicants such as diazinon and fenitrothion [51]. Decrease in brood size is related to neurodegeneration. CUR treatment provided a significant increase in the brood size, among neurotoxin exposed worms. However, the precise mechanisms underlying this protective effects merit further investigation. Further, we also found an increase in mean life span among worms treated with CUR alone and also among co-exposed to neurotoxicants (MCP-4d; 6-OHDA- 6d) clearly suggesting its potential to enhance survival. A recent study showed that exposure of *Drosophila* to CUR resulted in a significant reduction in the oxidative stress and apoptosis, and increase the lifespan of PD model flies [52]. Our results corroborate with these findings on the potential of CUR to promote survival. CUR is powerful antioxidant and has the power to mitigate age-associated cellular damage induced by production of reactive oxygen species [53]. Evidence from several animal models has shown that CUR improves health span by preventing or delaying the onset of various neurodegenerative diseases [54]. The transcription factor SKN-1 in *C. elegans* which is involved in the

extension of longevity is the orthologue to mammalian Nrf proteins which induce phase2 detoxification responses that defend against oxidative stress [55,56]. We speculate that similar mechanisms may be responsible for the observed extension of longevity under conditions of neurotoxin exposure.

AChE is an enzyme that modulates the amount of ACh, the neurotransmitter at neuronal junctions. AChE acts to hydrolyze acetylcholine released in synaptic clefts, thus ensuring that signalling is rapidly terminated [57]. Acetylcholine (ACh) is an excitatory transmitter at the neuromuscular junctions of nematodes [58], and more than one-third of the cells in the *C. elegans* nervous system are known to release acetylcholine. Further, cholinergic transmission is suggested to be involved directly or indirectly, in many *C. elegans* behaviors, including locomotion, egg laying, feeding and mating [59,60]. The cellular and behavioral consequences of AChE inhibition have been evaluated in terms of egg-laying in *C. elegans* [61]. MCP, being a neurotoxic OPI is known to inhibit AChE activity. In the present study, as expected, MCP markedly inhibited AChE activity in the worms. There are, however, very limited reports on the effect of OHDA on AChE activity in mammals. Following a 6-hydroxydopamine lesion of the rat nigrostriatal pathway, the spontaneous release of AChE was found to be reduced, in both the caudate nucleus and substantia nigra. In both structures, a marked reduction in the release of acetylcholinesterase and nonspecific cholinesterase was reported [62]. It has been reported that there was a tight correlation between cognitive impairment in PD and cholinergic deficit [63]. However, there are no reports to the best of our knowledge on the effect of OHDA on AChE activity in *C. elegans*. Therefore, we also determined the activity of AChE, an indirect indicator to evaluate the cholinergic system. We observed a marked reduction in AChE activity in worms exposed to OHDA. In the present model CUR, alone treatment did not appreciably inhibit acetylcholinesterase (AChE) in the worms. However, it significantly restored the AChE activity levels in worms exposed to both the neurotoxins. This could result in the limiting persistence of acetylcholine in cholinergic synapses leading to repetitive stimulation of muscarinic and nicotinic receptors in target tissues. Although the precise mechanism/s by which CUR treatment partially restored the AChE activity in the co-exposure paradigm is not clear from the study, this is consistent with the observed improvement in the locomotion rate among the neurotoxin exposed worms.

In the present model, CUR treatment did not affect the DA levels in worms. However, it significantly restored the DA content in the worms exposed to the neurotoxins suggesting its specific effect on DA metabolism and its potential to rescue dopaminergic neurodegeneration induced by 6-OHDA and MCP. Our findings in the transgenic worms apparently support this property of CUR. It would be interesting to conduct further mechanistic studies (dose and time -response) so as to confirm the neuroprotective efficacy of the hydrophilic form of CUR in completely alleviating the neurodegeneration in this model.

Acknowledgements

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