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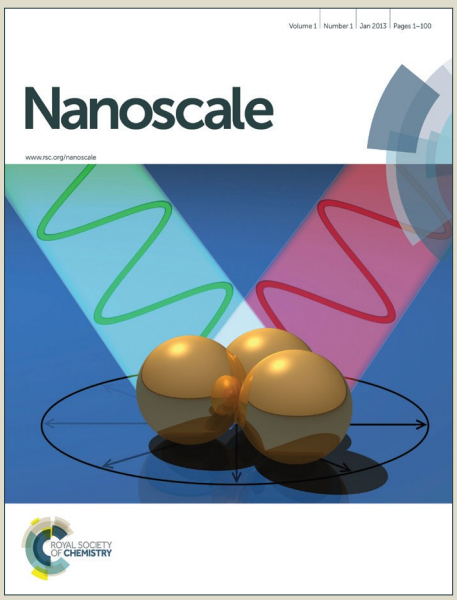

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MPA-capped CdTe quantum dots exposure causes neurotoxic effects through affecting the transporters and receptors of glutamate, serotonin and dopamine at the genetic level and increasing ROS or both of them in nematode *Caenorhabditis elegans*

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Abstract: As quantum dots (QDs) are widely used in biomedical, the number of study focusing on their biological matters is increasing. While several studies have attempted to evaluate the toxicity of QDs on neural cells, the *in vivo* toxic effects on the nervous system and the molecular mechanisms are unclear. The aim of the present study was to investigate the neurotoxic effects and the underlying mechanisms of water-soluble cadmium telluride (CdTe) QDs capped with 3-mercaptopropionic acid (MPA) in *Caenorhabditis elegans* (*C. elegans*). Our results showed that exposure to MPA-capped CdTe QDs induced behavioral defects, including alterations to body bending, head trashing, pharyngeal pumping and defecation interval, as well as impaired learning and memory behavior plasticity based on chemotaxis or thermotaxis in a dose-, time- and size-dependent manner. Further investigations suggested that MPA-capped CdTe QDs exposure within 24 h inhibited the transporters and receptors of glutamate, serotonin and dopamine at the genetic level in *C. elegans*, while showed opposite results at 72 h. Additionally, excessive ROS generations were observed in the CdTe QD-treated worms, which confirmed the common nanotoxicity mechanism of oxidative stress damage, and might overcome the improved gene expression of transporters and receptors of neurotransmitters induced by long-term QD-exposure in the *C. elegans*, resulting in severer behavioral impairments.

Key words: MPA-capped CdTe quantum dot, *Caenorhabditis elegans*, neurotransmission, nanotoxicology, neurotoxicity

1. Introduction

Quantum dots (QDs), considered as alternatives of conventional fluorescent dyes, are an increasingly used class of nanoparticles in biomedical imagery and target drug delivery [1-3]. Owning to their unique photophysical properties, they become an ideal noninvasive probes to visualize biological processes of live organisms at molecular and cellular levels in both real time and three dimensions [4]. However, lagging behind the rapid rate of progress and application of QDs has been the consideration of the potential risks associated with human exposure to these particles. Concern about the potential adverse effects of QDs on biological systems, especially the nervous system, has been increasing in recent years, resulting in calls for much more thorough neurotoxicity assessment.

Studies on the toxicity of QDs to various neural cells and little animals have reported that cadmium-containing QDs exposure was associated with cell death and apoptosis, oxidative stress damage, and altered neurobehaviors, such as impaired spatial learning and reference memory [5-8]. Our previous work have found that 3-mercaptopropionic acid (MPA)-capped CdTe QDs exposure resulted in rat primary cultured hippocampal neurons apoptosis and death by inducing intracellular oxidative stress and elevation of cytoplasmic calcium level (Wu et al., 2015 in revision). Although *in vitro* studies are fast and suitable for evaluating QD's toxicity, they are limited in capturing subtle organism impacts. Systematic in-depth studies of the neurotoxicity induced by QDs in whole organisms are imperative for their future biological applications. Given the vital but limited *in vivo* research on nervous system damage in association with QD exposure and the potential value of QDs' application in the field of neuroscience, here, we presented an *in vivo* study of the model animal *C. elegans* to

address these issues.

C. elegans has been considered an excellent test organism to evaluate toxic responses to several chemicals due to its multitude of advantages, such as inexpensive maintenance, its size, short life cycle, and large brood size. Additionally, *C. elegans* has 302 neurons that signal through 890 electrical junctions, 1410 neuromuscular junctions and 6393 chemical synapses, where its neuronal lineage is fully described. And it uses the same neurotransmitter systems, such as glutamatergic, dopaminergic (DAergic), serotonergic, cholinergic and gamma-amino butyric acid (GABA) ergic, that are expressed in vertebrates, which allows findings from *C. elegans* to be extrapolated and further confirmed in vertebrate systems. Therefore, *C. elegans* has been the reliable subject of a number of neurotoxicological studies focused on various chemicals [9]. As the genome of *C. elegans* has been sequenced completely, it also offers an attractive experimental platform to design and implement studies to evaluate molecular gene mechanisms of tested chemicals.

This study investigated the potential neurotoxic effects of a water-soluble CdTe QDs capped with MPA on *C. elegans*. Quantitative methodologies were applied to assess multiple end points relevant to their nervous system health after short- and long-term exposure to MPA-capped CdTe QDs. The results suggested that changes in the locomotion behavior and plasticity of learning and memory behaviors indicated the adversely neurological effects of MPA-capped CdTe QDs with both two sizes, which might be related with QD-induced changes on transporters and receptors of glutamate, serotonin and dopamine at the genetic level and excessive ROS generation or both of them in *C. elegans*.

2. Materials and methods

2.1. QDs preparation and characterization

The water-soluble MPA-capped CdTe QDs used in this study were prepared as described previously [10-12]. Before the study, we evaluated the physicochemical properties of these QDs. High-resolution transmission electron microscopy (HR-TEM) revealed that the average size of CdTe QDs were 2.2±0.25 nm and 3.5±0.49 nm, respectively. Otherwise, dynamic light scattering (DLS) confirmed that comparable dimensions were 7.39±0.74 nm and 9.82±1.14 nm for 2.2 nm and 3.5 nm CdTe QDs in water solution, respectively. The MPA coating contributed substantially to the size of QDs in water. The surface charges through ξ-potential measurement were -31.84±3.06 mV and -26.46±4.75 mV for 2.2 nm and 3.5 nm CdTe QDs in water solution, respectively. Three concentrations of MPA-capped CdTe QDs suspensions (400, 800 and 1600 μg/mL) were prepared by diluting the stock solution (2200 μg/mL) of QDs, which was sonicated for 20 min before, with K medium (32 mM KCl, 5 mM NaCl) just prior to exposure. The used concentrations for QDs here were selected based on our preliminary lethal experiments, which showed the LD_{50} of exposure to MPA-capped CdTe QDs for 72 h is approximately 8,000 μg/mL (data not shown), and a series of previous studies [10] (Wu et al., 2015 in revision).

2.2. Strains and culture conditions

Nematodes used in this study were wild-type N2 *C. elegans*. The *C. elegans* strain and the *Escherichia coli* OP50 strain were obtained from the Caenorhabditis Genetics Center

(CGC) (University of Minnesota, MN, USA). *C. elegans* culture and manipulation were performed using standard methods [13], which is that the wild-type N2 *C. elegans* was cultured at 20 ^{o}C on nematode growth media (NGM) agar plates seeded with *Escherichia coli* OP50. Synchronization of nematode cultures was achieved using a bleaching buffer (5% 5 M NaOH, 12% NaClO) treatment of gravid hermaphrodites.

2.3. Preparation of plates and exposure conditions

Experiments were carried out on 24-well plates with 0.5mL of NGM-agar added into each well, which were seeded with fresh overnight-culture of OP50. To the surface of each well, 30 μL various concentration MPA-capped CdTe QDs dissolved in K-medium were added to the corresponding coagulated NGM and covered the bacterial lawn. After about 30 min at 20 ^{o}C, synchronized L4-stage nematodes were placed into each well cultivated in a 20 ^{o}C incubator for 24 and 72 h.

2.4. Bioimaging

The uptake and distribution of MPA-capped CdTe QDs by *C. elegans* were observed by a fluorescence microscope (Olympus, FSX100). Worms exposed to 1600 μg/mL CdTe QDs for 24 h and 72 h were washed five times and transferred to an agar pad to which was added sodium azide previously in order to immobilized the worms. 2.2 nm CdTe QDs showed green fluorescence while 3.5 nm CdTe QDs showed red. All images were acquired and processed using the FSX-BSW PC software.

2.4. Behavior assay

2.4.1. Locomotion behavior assay

The body bend frequency and the head thrash frequency were determined to monitor the locomotion behavior. A body bend was counted as a change in the direction of the part of the nematodes corresponding to the posterior bulb of the pharynx along the Y axis, assuming the nematode travelling along the X axis [14, 15]. A head trash was defined as a change in the direction of bending at the mid body [9, 16-18]. The nematodes were transferred to a second plate without food and scored for the number of body bends in an interval of 20 s, and the head thrashes were scored for 1 min. A minimum of 30 nematodes was examined per treatment using a stereo microscope (Olympus, SZ61) and the tests were performed at least three times.

2.4.2. Pharyngeal pumping assay

The pharyngeal pumping rate represents the ability of food intake of *C. elegans*. 30 nematodes of the different exposure groups were randomly selected and their pharynx pumping frequency was determined three times over a 1 min timespan at room temperature [19]. The test was performed a minimum of three times.

2.4.3. Defecation assay

The whole defecation process includes four steps, but we measured the interval between the initiations of two successive posterior body-wall muscle contraction steps as a cycle period of defecation [20, 21]. 30 nematodes were used for each defecation cycle length assay and three replicates were performed.

2.4.4. Chemotaxis assay

The nematodes *C. elegans* are usually attracted to NaCl when they are cultured on normal NGM, but their chemotaxis towards NaCl will fall dramatically if nematodes are

148 staved on plates that contain NaCl [22, 23]. Thus, the plasticity of learning behavior could be
149 indicated by pairing presentation of a chemoattractant and starvation in *C. elegans*. The
150 specific test method was according to Ju et al. with some modifications [15, 18]. An agar plug
151 with additional of 100 mM NaCl was placed on one side (N) of the assay plate, which was
152 then left overnight (Fig. 1A). Just before the test, the NaCl plug was removed and one drop of
153 0.5 M sodium azide was spotted on the same position to anaesthetize the nematodes. The
154 sodium azide was also spotted onto the position (C) 3 cm away from the NaCl spot. Exposed
155 nematodes were cultured in the presence of NaCl and the absence of food at 20 ^{o}C for 4 h.
156 About 100 worms of each group were placed on the starting point (S), which was 3 cm away
157 from the N and C. And then nematodes were left to move freely on the assay plate for 60 min
158 at 20 ^{o}C, where the number of nematodes within 1.5 cm of each spot was counted (N_n and N_c).
159 The chemotaxis index (CI) was equal to $(N_n-N_c)/(N_n+N_c)$. Assays were performed in triplicate,
160 and the CI value was averaged.
161 2.4.5. Thermotaxis assay
162 The nematode *C. elegans* memorizes temperatures, and this stored information modifies
163 its subsequent migration along a temperature gradient (Temperature Sensing by an Olfactory
164 Neuron in a Circuit Controlling Behavior of *C. elegans*) [24, 25]. A radical temperature gradient
165 was created by placing a vial containing frozen glacial acetic acid on the bottom of the assay
166 plate for 30 min at 25 ^{o}C (Fig. 1B). Exposed nematodes were transferred to NGM agar with a
167 plenty of food at 20 ^{o}C overnight, and then gotten starved for 0, 0.5, 1, 3, 7, 12 and 18 h. At
168 least 50 worms of every treatment were placed on the assay plate and allowed to move freely

for 45 min at 25 ^{o}C. The percentage of animals performing isothermal tracking (IT) at 20 ^{o}C was determined by the tracks left on the agar surface after removal of the worms from the plate. A trace is considered as IT if more than half of the trace length left on the agar surface by a single worm is circular, or presents an arc of a circle near the isotherm of the cultivation temperature [25].

2.5. Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) analysis

Total RNA from worms of each group was isolated using TRIzol, and the synthesis of cDNA was performed by a reverse transcriptase reaction using Mastercycleasr gradient PCR system (Eppendorf, USA). The qRT-PCR analysis was carried out on selected genes relevant to three transmissions of glutamate, serotonin and dopamine on a StepOnePlus^{TM} real-time PCR systems (Version 2.2.2, Applied Biosystems, Carlsbad, CA, USA) using the SYBR Green qRT-PCR master mix (TOYOBO, Japan). The qRT-PCR primers were designed based on sequences retrieved from the *C. elegans* database (www.wormbase.org) and National Center for Biotechnology Information (NCBI), and detailed base sequences were on Table 1. Three replicates were conducted for each qRT-PCR analysis. The relative quantities of mRNA were determined using comparative cycle threshold methods, and were normalized against the mRNA of *act-1*, which encodes actin isoforms. The fold change of the mRNA level was normalized to that observed in non-exposed control samples.

2.6. Determination of ROS generation

We used the oxidation-sensitive fluorescence probe 2',7'-dichlorofluorescin diacetate (DCFH-DA) (Sigma, MO, USA) to measure the levels of reactive oxygen species (ROS) in nematode *C. elegans*. After treatment with QDs for 24 h and 72 h, the worms were washed twice with M9 buffer and then loaded with 10 μM DCFH-DA and incubated at 20 ^{o}C for 30 min in dark. After washed three times with M9 buffer, random ten worms of each group were mounted on an agar pad for the examination with a fluorescence microscope (Olympus, FSX100) at 488 nm of excitation wavelength and 525 nm of emission filter. All images were analyzed by ImageJ Software to measure the florescence intensity of the highly fluorescent dichlorofluorescein (DCF) oxidized. The semiquantified ROS was expressed as relative fluorescent units (RFU). In *C. elegans*, the DCFH-DA labeled fluorescent signals were much stronger than the autofluorescence signals of MPA-capped CdTe QDs.

2.7. Data analysis

All data were displayed as the mean ± standard deviation (SD). Statistical analysis was performed using SPSS Statistics 19.0 Software. One-way analysis of variance (ANOVA) was used to determine the statistical significance between control and exposed groups, followed by the *Dunnett's t*-test to determine the significance of differences between the groups. Probability levels of <0.05 and <0.01 were considered statistically significant.

3. Results

3.1. *In vivo* imaging of distribution of MPA-capped CdTe QDs in *C. elegans*

Before any MPA-capped CdTe QD toxicity could be interpreted, it was necessary to

evaluate the uptake and distribution of QDs in *C. elegans*. The transparent body of *C. elegans* and the bright fluorescence from MPA-stabilized CdTe QDs enabled us to directly visualize them in a live and intact worm using florescent microscopy. As the QDs were added to the surface of an agar plate supporting the worms, we anticipated that the main route of exposure would be through the digestive tract. We found green (2.2 nm CdTe QDs) and red (3.5 nm CdTe QDs) fluorescence in the whole body, mainly digestive tissue of the nematodes (Fig. 2B1, 2C1, 2D1 and 2E1). Additionally, in the amplified images (Fig. 2B2, 2C2, 2D2 and 2E2), MPA-capped CdTe QDs were noticeably translocated into head areas, which might be trough the biological barrier along the intestine. Compared to 3.5 nm CdTe QDs, 2.2 nm CdTe QDs showed brighter and more dispersed in head region. Longer exposure time also resulted in brighter fluorescence. Moreover, the QDs remained bright and consistent over the duration of the study, which indicated that the MPA-capped CdTe QDs did not appreciably change while ingested.

3.2. Toxicity responses to MPA-capped CdTe QDs on behavior in *C. elegans*

As locomotion behavior is a relatively sensitive endpoint for neurotoxicity evaluation in nematodes, firstly, we investigated the effects of MPA-capped CdTe QDs exposure on locomotion behavior as indicators of body bends and head thrashes. Apart from the low-dose 3.5 nm CdTe QDs group, exposure to 400~1600 μg/mL CdTe QDs with two sizes for 24 or 72 h decreased the body bends and head thrashes of nematodes significantly compared with the control (Fig. 3A and 3B). And both the body bends and head thrashes of nematodes exposed to 2.2 nm CdTe QDs were more severely reduced than those exposed to 3.5 nm CdTe QDs.

The time course analysis further indicated that the more significant reductions of body bends and head thrashes were observed after nematodes were exposed to MPA-capped CdTe QDs at the L4-larval stage for 72 h.

As pharyngeal pumping and defecation in *C. elegans* were controlled by specific neurons and MPA-capped CdTe QDs were mainly accumulated in the alimentary system, we chose pharyngeal pumping frequency and defecation interval as endpoints to reflect neurotoxic effects of QDs. After exposed to CdTe QDs for 24 h, significant decrease in the number of pharyngeal pumping in *C. elegans* over a 1min timespan was observed in the middle- and high-dose treatments compared to control (Fig. 4A). And for 72 h exposure, every treatment groups showed a significant decrease in pharyngeal pumping frequency. For the defecation activity in *C. elegans*, MPA-capped CdTe QDs exposure significantly increased the interval between two posterior body-wall muscle contractions in a dose- and time-dependent manner, compared with the control (Fig. 4B).

Considering the important value of plasticity in learning and memory, we next examined the possible adverse effects of MPA-capped CdTe QDs exposure on capacity of learning and memory in *C. elegans* based on their chemotaxis and thermotaxis. Our results showed that the CIs increased along with the crescent concentrations of CdTe QDs, indicating the number of nematodes that have learned pairing presentation of a chemoattractant and starvation was decreasing (Fig. 5). Compared with the control, CIs of nematodes exposed to middle- and high-dose CdTe QDs for 24 or 72 h increased significantly. Moreover, the 2.2 nm CdTe QDs and the longer exposure time exerted severer learning deficits in *C. elegans*.

In the control group, the percentage of animals performing IT at 20 ^{o}C was decreasing during starvation, from 85.42±1.43% and 86.11±3.46% at the beginning to 45.36±9.36% and 44.63±6.93% at the end of the experiment for 24 h and 72 h, respectively. As shown in Figure 6A and 6B, the data of each treatment group followed the similar tendency of the controls, and exposure to high-dose of MPA-capped CdTe QDs caused significant decreases in the percentage of IT behaviors in *C. elegans* at 20 ^{o}C at each time point, compared with the controls, while only some time points suggested significantly different data between the middle-dose groups and the controls. However, the thermostatic behavior was unaffected by low-dose MPA-capped CdTe QDs. Moreover, when the exposure dose was same, 2.2 nm CdTe QDs always leaded to severer toxic effects.

3.3 The expression of transporters and receptors of glutamate, serotonin and dopamine in regulating the formation of adverse effects in MPA-capped CdTe QD-exposed *C. elegans*

C. elegans contain many classic neurotransmissions found in vertebrates. To identify which neurotransmission(s) mediates the adverse effects of MPA-capped CdTe QDs, we further examined the expression of genes that are required for the transmissions of glutamate, serotonin and dopamine, which might be affected by MPA-capped CdTe QD exposure. As shown in Table 1, selected genes can be separated into encoding transporter and receptor. The mRNA levels were determined using quantitative real-time RT-PCR and normalized to the expression of *act-1*. The fold change was normalized to that observed in untreated control group.

The results showed that the mRNA levels of *glr-1, glr-2, glt-7, eat-4, mgl-1, ser-1, mod-1,*

dat-1 and *dop-3* were significantly decreased, but the mRNA level of *mod-5* was significantly increased, compared to those in the control, when worms were exposed to three doses of MPA-capped CdTe QDs with two size for 24 h (Fig. 7A). Among them, *glr-1, glr-2, glt-7, eat-4* and *dat-1* showed dose-effect relationship to 2.2 nm CdTe QDs, while *glr-2* and *mod-5* showed dose-effect relationship to 3.5 nm CdTe QDs. Otherwise, 2.2 nm CdTe QDs exposure significantly decreased the mRNA levels of *nmr-1* and *dop-1*, while 3.5 nm CdTe QDs exposure significantly influenced the mRNA levels of *glr-6, glt-3* and *glt-6*, where low-dose caused increased expressions but middle-dose caused reduced expressions.

In contrast, when nematodes were exposed to MPA-capped CdTe QDs for 72 h, the mRNA levels of most genes, including *glr-1, glt-3, glt-6, eat-4, mgl-1, ser-1, mod-1, mod-5, dat-1* and *dop-1*, were significantly increased compared with the control, of which *glt-3, glt-6, eat-4, mgl-1, ser-1, mod-1, mod-5* and *dat-1* showed dose-effect relationship to 2.2 nm CdTe QDs, while *glt-3, glt-6, eat-4, mgl-1, dat-1* and *dop-1* showed dose-effect relationship to 3.5 nm CdTe QDs (Fig. 7B). However, except that the mRNA level of *dop-1* decreased dose-dependently, tendencies of others were opposite. Otherwise, only was the expression of gene *glr-2* significantly decreased after CdTe QDs exposure when compared to the control. Significantly increased mRNA levels of *glr-6* and *dop-3* were merely observed in 2.2 nm CdTe QDs treatment groups. The results suggested that MPA-capped CdTe QDs exposure influenced the expression of many genes that are required for the transmissions of glutamate, serotonin and dopamine in *C. elegans*.

3.4. Generated ROS production

The connection between nanotoxicity and excessive oxidative stress has been widely accepted and the levels of ROS serve as reliable indicators of oxidative stress [26-28]. DCFH-DA is a common detection reagent to measure ROS generation. Exposure to 1600 μg/mL CdTe QDs for 24 h significantly enhanced ROS production in nematodes (P<0.001, Fig. 9). Prolonged exposure to over 400 μg/mL of CdTe QDs also significantly increased the levels of ROS production (P<0.001, Fig. 9), and the fluorescent intensities of nematodes in 72 h control looked like stronger than those in 24 h control (Fig. 8).

4. Discussion

Neurobehavioral tests are traditionally used in mammals, but it costs a plenty of time, resources and money. *C. elegans* was originally developed as an experimental model to study nervous system development in 1974 [29]. Furthermore, its nervous system has been systematically investigated, and differentiation and migration patterns have been described, as well as the main neurotransmission systems are phylogenetically conserved which permits the analysis of changes in neurotransmitter expression in response to various neurotoxins. Moreover, researchers have found that *C. elegans* was equally affected by positive neurotoxins, but responded much faster than vertebrates, which indicated the validity and robustness of this applied model animal [15, 18, 19].

So far, *C. elegans* has been used widely to investigate neurotoxicity of assorted nanoparticles with locomotion behavior, pharyngeal pumping, defecation and chemotaxis as the endpoints [14, 16, 30-32]. Although there is limited but growing evidence linking QDs exposure

315 to neurobehavioral outcomes [7, 14], the effects and mechanisms of QDs toxicity on neuronal
316 functions require further explanation. Knowledge having gained on the *C. elegans* behavior
317 will facilitate the use of these worms in understanding the behavior effects of interventions.
318 Therefore, the extensive advantage of using a *C. elegans* model provides the capability of
319 examining various endpoints of nervous system function, including the behaviors and gene
320 transcription.

321 Firstly, benefit from the bright fluorescence of water-soluble MPA-capped CdTe QDs, we
322 used fluorescent microscopy to observe the uptake and distribution of a high level of
323 MPA-capped CdTe QDs and found that QDs were mainly accumulated within the digestive
324 lumen, and some may enter into the adjacent cells in *C. elegans*. The results implied that
325 MPA-capped CdTe QDs were capable of crossing the intestinal barrier and translocated into
326 nerve nets in head area, which was similar to some previous studies: Contreras et al.
327 suggested that *C. elegans* might have the capacity to acclimate low levels of QDs [33]; Zhao et
328 al showed that MPA-capped CdTe QDs could be translocated through intestinal barrier and
329 deposited in RMEs motor neurons in nematodes, resulting in abnormal foraging behavior and
330 deficits in development of RMEs motor neurons [14]; and in the investigation of Qu et al., the
331 transfer of QDs from alimentary system to the reproductive system also verified the capacity
332 of QD's distribution from the intestine to the adjacent cell and causing adverse effects [34].
333 Along with exposure time, the accumulation and biodistribution effects of QDs were severer.
334 And 2.2 nm CdTe QDs seemed like tend to cause more obvious effects than 3.5 nm CdTe
335 QDs. Therefore, MPA-capped CdTe QDs could accumulate in the body of *C. elegans*,

336 especially the head region containing the nerve nets, which was more likely to cause several
337 neurotoxic effects.

338 Biodistribution of QDs is only the beginning on the long road to unravel their biological
339 effects. After confirming the accumulation of QDs within the organ systems, including the
340 nervous system in *C elegans*, we can further investigate the corresponding toxicological
341 impact on its physiological behavior. In this study, we chose endpoints of body bending and
342 head thrashing to evaluate the locomotion behavioral defects caused by MPA-capped CdTe
343 QDs in *C. elegans*, because locomotion behavior is a relatively sensitive endpoint for
344 neurotoxicity evaluation in nematodes. The results showed that MPA-capped CdTe QDs
345 exposure could restrain the body bending and head thrashing in a dose- and time-dependent
346 manner. Additionally, several well-characterized behaviors of *C. elegans* controlled by
347 specific neurons, including pharyngeal pumping and defecation, were easily assessed [35, 36].
348 We found that the pharyngeal pumping behavior and defecation system of nematodes in CdTe
349 QDs treatment groups were be damaged in different degrees, where the longer exposure time
350 and the smaller size of QDs gave worms more damage.

351 *C. elegans* has developed chemical induction system, which is used to sense many
352 environmental stimuli. According to different chemical signals in the environment, *C. elegans*
353 integrates various sensory signals within the nervous system, showing different forms of
354 learning behavior plasticity [37]. In the chemotaxis asssy, the conditioning required both the
355 presence of NaCl and the absence of a bacterial food source, indicating it was a form of
356 associative learning not merely adaptation or habituation. This assay system provided an

opportunity to study the changes of learning behavioral plasticity in *C. elegans* exposed to MPA-capped CdTe QDs, which reflected neurological functions.

The results from the control group showed that starvation in the presence of NaCl for 4 h resulted in a strong avoidance of high NaCl concerntration. This response was very similar to that reported by Saeki et al., suggesting that starvation sensitized the ability to associate NaCl with negative cues [22]. However, the proportion of worms that failed to learning the association with NaCl and starvation was increasing with the raising expose dose and time. The changes in the learning behavior plasticity of *C. elegans* exposure to MPA-capped CdTe QDs were identified as neurotoxic compounds by tests.

The thermotaxis assay is used to investigate nematodes' memory capacity based on their thermotaxis behavior, which is the response to temperature on spatial thermal gradients in an experience-modulated manner. The results suggested that the percentage of nematodes performing IT at 20 ^{o}C in MPA-capped CdTe QDs treatment groups decreased more obviously over time, compared to the control, which indicated that exposure to MPA-capped CdTe QDs caused severe deficits in thermotaxis memory in nematodes. The decrease in thermotaxis-dependent memory capacity occurred quickly over 3~4h, which was confirmed by previous studies [25, 38]. Otherwise, nanoparticles are often expected that the smaller size exert stronger toxicity. In this study, smaller sized CdTe QDs (2.2 nm) really seemed to be more toxic than larger sized ones on the observed neurotoxic effects in *C. elegans*.

As any individual reduction of the locomotive activity, pumping or defecation activity, or impairment of the behavior plasticity must be taken as an individual indication of

neurotoxicity, the neurotoxic effects of MPA-capped CdTe QDs have been identified. However, we still know little about the genetic and molecular mechanisms explaining the formation of neurotoxicity after MPA-capped CdTe QDs exposure. The effects and mechanisms of MPA-capped CdTe QDs toxicity on neuronal functions require further explanation.

Recently, gene transcription has been widely used in toxicological studies, because it is able to provide high sensitivity and mechanistic values to investigate adverse effects of chemicals. Attributed to *C. elegans*'s genome and metabolic pathways highly conserved with mammals, it is suitable for studying genetic mechanisms of MPA-capped CdTe QDs neurotoxicity [39]. Here, in order to assess whether the defects of locomotion, feeding, defecation, capacities of learning and memory were caused by neuron physical blockage, we used qRT-PCR to analysis the alteration of gene transcription related to the transporters and receptors of three common neurotransmitters: glutamate, serotonin and dopamine based on the functions of them in neuronal migration.

Glutamate acts as both an excitatory and inhibitory neurotransmitter in *C. elegans*, and glutamate transporters and receptors are responsible for the glutamate-mediated postsynaptic excitation of neural cell, which is important for neural communication, memory formation, learning and regulation. MPA-capped CdTe QDs exposure for 24 h caused significant reduction of the mRNA levels of genes *glt-3, glt-6, glt-7* and *eat-4,* encoding glutamate transporters; genes *glr-1* and *glr-2*, encoding non-NMDA-like ionotropic glutamate receptors; and gene *mgl-1*, encoding metabotropic glutamate receptors; but no NMDA glutamate

receptors gene (*nmr-1*).

Serotonin (5-hydroxytryptamine, or 5-HT) is another common neurotransmitter in vertebrates and invertebrates. In *C. elegans*, serotonin stimulates pharyngeal pumping while inhibits locomotion and defecation [40]. Our results showed that MPA-capped CdTe QDs exposure for 24 h elicited significant decrease of the mRNAs levels of genes *mod-5*, *ser-1* and *mod-1*. The gene *mod-5,* which encodes the only serotonin reuptake transporter in *C. elegans*, modulates locomotion defective [41]. The findings from Li et al. demonstrated that mutations of *mod-5* gene resulted in deficits in thermotaxis memory behavior in *C. elegans*, indicating that deficits in uptake of serotonin would noticeably influence the thermotaxis memory behavior [38]. Several studies have found that serotonin-gated chloride channel MOD-1 modulated locomotion behavior [42], and G protein coupled serotonin receptor SER-1 might be responsible for the effects of serotonin on pharyngeal pumping, and learning and memory [43, 44].

Dopamine (3,4-dihydroxyphenylethylamine) signaling has established roles in the modulation of locomotion behavior and in learning [45]. Exposed to MPA-capped CdTe QDs for 24 h significantly decreased the mRNAs levels of genes *dat-1*, *dop-1* and *dop-3*. The *dat-1* encoding presynaptic dopamine transporter proteins (DATs) is a dispensable part of chemical signaling by small molecule neurotransmitters [46]. Chase et al have found that two classes of dopamine receptor: D1-like receptor DOP-1 and D2-like receptor DOP-3 antagonized each other to control locomotion behavior in *C. elegans* by acting in the same motor neurons of the ventral nerve cord, which were not postsynaptic to dopaminergic neurons [47].

Thus it was obvious that short-term exposure of MPA-capped CdTe QDs decreased the expression of most genes, suggesting the inhibitory effect of QDs on neurotransmissions in the mRNA levels. However, after treatment of MPA-capped CdTe QDs for 72 h, the expression of most genes was completely opposite. We speculated that it might be due to the recovery response to MPA-capped CdTe QDs and aging- and QD-induced oxidative stress in *C. elegans.* The recovery response formation has been found in nanoparticle exposed *C. elegans*, which was regulated by both defecation behavior and intestinal barrier [14, 48]. Zhao et al. also found that animals undergoing prolonged exposure to nanoparticles would be difficult to exhibit the recovery response because the deficit in intestinal structure might be irreversible, which could explain the outcomes seemed to be contradicting.

Otherwise, oxidative stress damage has been considered as one of major contributors to QD-induced toxicity [49]. The activation of glutamate or dopamine receptors located and expressed in neurons has been reported capable of reducing oxidative stress and supporting neurons viability [50-52]. Therefore, long-term exposure of MPA-capped CdTe QDs could increase the expression of genes encoding transporters and receptors to fix QD-induced neurotransmission inhibition and oxidative stress, even though the outcomes were not obvious. However, as extensive oxidative stress occur in the aging process, and aging is accompanied with decline in behavioral and cognitive functions [53-55], the effects of overexpression of neurotransmitter-relevant genes might not overwhelm the aging-related ROS generation and behavioral deterioration in the nematodes of 72 h treatment groups which were experiencing aging.

All in all, these data suggested an obvious link or correlation between various neurotoxicity endpoints, and gene transcription relevant to transporters and receptors of three neurotransmitters and ROS generation in *C. elegans* exposed to MPA-capped CdTe QDs. Therefore, physiological disturbances in the nervous system may be considered as a progression of toxicity in the complex consequence of abnormal gene transcriptions relevant to neurotransmitters and ROS generation induced by MPA-capped CdTe QDs. However, owning to the complex of neurotransmissions, some specific genes and definite mechanisms of MPA-capped CdTe QDs disturbing neurotransmissions should be further investigated, perhaps by corresponding RNAi knockdown strains.

5. Conclusion

Our results suggested that MPA-capped CdTe QDs exposure tended to inhibit locomotion behaviors, pharyngeal pumping and defecation, as well as impair learning and memory behavior plasticity based on chemotaxis or thermotaxis in *C. elegans*, by which reflected the neurotoxic effects of MPA-capped CdTe QDs in a dose-, time- and size-dependent manner. The potential mechanisms of causing these adverse effects seemed complicated. We observed that short-term MPA-capped CdTe QDs exposure was capable of inhibiting the expression of several genes encoding transporters and receptors of three important neurotransmitters: glutamate, serotonin and dopamine without requiring the ROS increment. However, the neurological deficits of *C. elegans* in the long-term QD-treatment groups could not be merely explained by the expression of transporters and receptors of neurotransmitters, which was

most probably attributed to the excessive ROS generation. Some other factors, such as recovery response to QDs and aging effects, were possibly involved in the long-term mechanisms of MPA-capped CdTe QDs inducing neurotoxicity, which was worthy of investigating. As we know, understanding the neurotoxcity of MPA-capped CdTe QDs is the key requirement to use them in the living organisms, further studies into the underlying complex mechanisms of QDs in this inexpensive but valuable nematodes should be considered.

Acknowledgements

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Declaration of interest

Authors of this article declare that there are no conflicts of competing financial interest.

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Table 1: Functions and designed qRT-PCR primers of genes required for glutamate, serotonin, and dopamine neurotransmissions in *C. elegans*

Neurotrans-mitter	Gene	Functions	Designed qRT-PCR primers	
			Forward	Backward
Glutamate	*eat-4*	Glutamate transporter	5'-TCTTATTAGCCAGTCTTATTCAC-3'	5'-GACCATTCTTCCTCCTCTT-3'
	glt-3	Glutamate transporter	5'-CGTTGCCAGTAACATTCC-3'	5'-TTCCGTCCATTGTAATTGTG-3'
	glt-6	Glutamate transporter	5'-TTGCTGCTTCAATGGTTC-3'	5'-GTGAATGATGGTCTTCTACTG-3'
	glt-7	Glutamate transporter	5'-CGAATTGAAGTTAGAGGAAGG-3'	5'-CGATGGAATGTGATGGAAG-3'
	glr-1	Non-NMDA glutamate receptor	5'-CCTACGACCAAGAGATGTT-3'	5'-CTCCACCTCCTAATGAAGAT-3'
	glr-2	Non-NMDA glutamate receptor	5'-TCTCTTCATACACGGCTAAT-3'	5'-CCTTCATTGACACCATACAG-3'
	glr-6	Non-NMDA glutamate receptor	5'-ACCAATCACGAAGGAGTT-3'	5'-TGCCAACACGAGTAAGAT-3'
	mgl-1	Metabotropic glutamate receptor	5'-ACTGTAGTTCCCGATTATGT-3'	5'-AGATGTCTTGCGTGATGA-3'
	nmr-1	NMDA glutamate receptor	5'-GGAGATAATCGTCTGGAATTG-3'	5'-AGTGTATATGCTGATGATGTAAC-3'
Serotonin	*mod-5*	Serotonin transporter	5'-ACTACCTCCATCATTAACTGT-3'	5'-ACTACCTCCATCATTAACTGT-3'
	mod-1	Ionotropic serotonin transporter	5'-ATTATTCAAGCCTATGTTCCAA-3'	5'-GAGATGAGATTCCGACAGT-3'
	ser-1	Metabotropic serotonin receptor	5'-TTCTCACCTCATCAACACT-3'	5'-TTCTCACCTCATCAACACT-3'
Dopamine	*dat-1*	Dopamine transporter	5'-ATGGTAATTGGAATTGCTATGT-3'	5'-CATTGCTTCACAGAACACT-3'
	dop-1	D1-like receptor	5'-GACGAGCATTCAAGAAGATT-3'	5'-ATTGTCAGGAGCAGCATA-3'
	dop-3	D2-like receptor	5'-CCTGAATGTGGAAGTATGC-3'	5'-TAGTGAGAATTGCCGAAGA-3'

Figure 1: Methological details. (A) The 9 cm plates with a piece of NaCl plug for chemotaxis assay. (B) The 9 cm radial thermal-gradient plates was performed using a vital containing frozen acetic acid (the middle glass vial) for thermotaxis assay, in which adult animals were allowed to move freely for 45 min on the assay plate. The stable radial temperature-gradient ranging from ~17 ^{o}C to 25 ^{o}C was established for at least 50 min on the agar surface. Worm tracks were categorized into six groups after thermotaxis assay. Typical tracks of each category are shown in (a)~(f). They were classified as "17 ^{o}C", "17/20 ^{o}C", "20 ^{o}C", "20/25 ^{o}C", "25 ^{o}C" and "17/25 ^{o}C" depending on the area to which worms moved.

Figure 2: Uptake and distribution of 2.2 nm and 3.5 nm CdTe QDs in *C. elegans*. (A) The image taken by a fluorescence microscope (Olympus, FSX100) of the entire nematode representing the anatomy: head, body and tail. (B1, B2) Images of an entire worm and the amplified head region after 1600 μg/mL 2.2 nm CdTe QDs treatment for 24 h, respectively. (C1, C2) Images of an entire worm and the amplified head region after 1600 μg/mL 3.5 nm CdTe QDs treatment for 24 h, respectively. (D1, D2) Images of an entire worm and the amplified head region after 1600 μg/mL 2.2 nm CdTe QDs treatment for 72 h, respectively. (E1, E2) Images of an entire worm and the amplified head region after 1600 μg/mL 3.5 nm CdTe QDs treatment for 72 h, respectively.

Figure 3: Effects of 2.2 nm and 3.5 nm CdTe QDs exposure for 24 h (left) and 72 h

(right) on locomotion behaviors in *C. elegans*. (A) The number of body bends in 20 s. (B) The number of head thrashes in 1 min. The results were performed as the mean ± SD of randomly selected thirty worms from each treatment. One-way analysis of variance followed by *Dunnett's post hoc* test was used for statistical analysis. *p<0.05 or **p<0.01 indicates a statistically significant difference when compared to the control.

Figure 4: Effects of 2.2 nm and 3.5 nm CdTe QDs exposure for 24 h (left) and 72 h (right) on pharyngeal pumping and defecation system in *C. elegans*. (A) The frequency of pharyngeal pumping in 1 min. (B) The length of defecation interval. The results were performed as the mean ± SD of randomly selected thirty worms from each treatment. One-way analysis of variance followed by *Dunnett's post hoc* test was used for statistical analysis. *p<0.05 or **p<0.01 indicates a statistically significant difference when compared to the control.

Figure 5: Effects of 2.2 nm and 3.5 nm CdTe QDs exposure for 24 h (left) and 72 h (right) on chemotaxis in *C. elegans*. The results were performed as the mean ± SD of three independent experiments. One-way analysis of variance followed by *Dunnett's post hoc* test was used for statistical analysis. *p<0.05 or **p<0.01 indicates a statistically significant difference when compared to the control.

Figure 6: Time course of changes on thermotaxis in C. elegans after exposed to 2.2

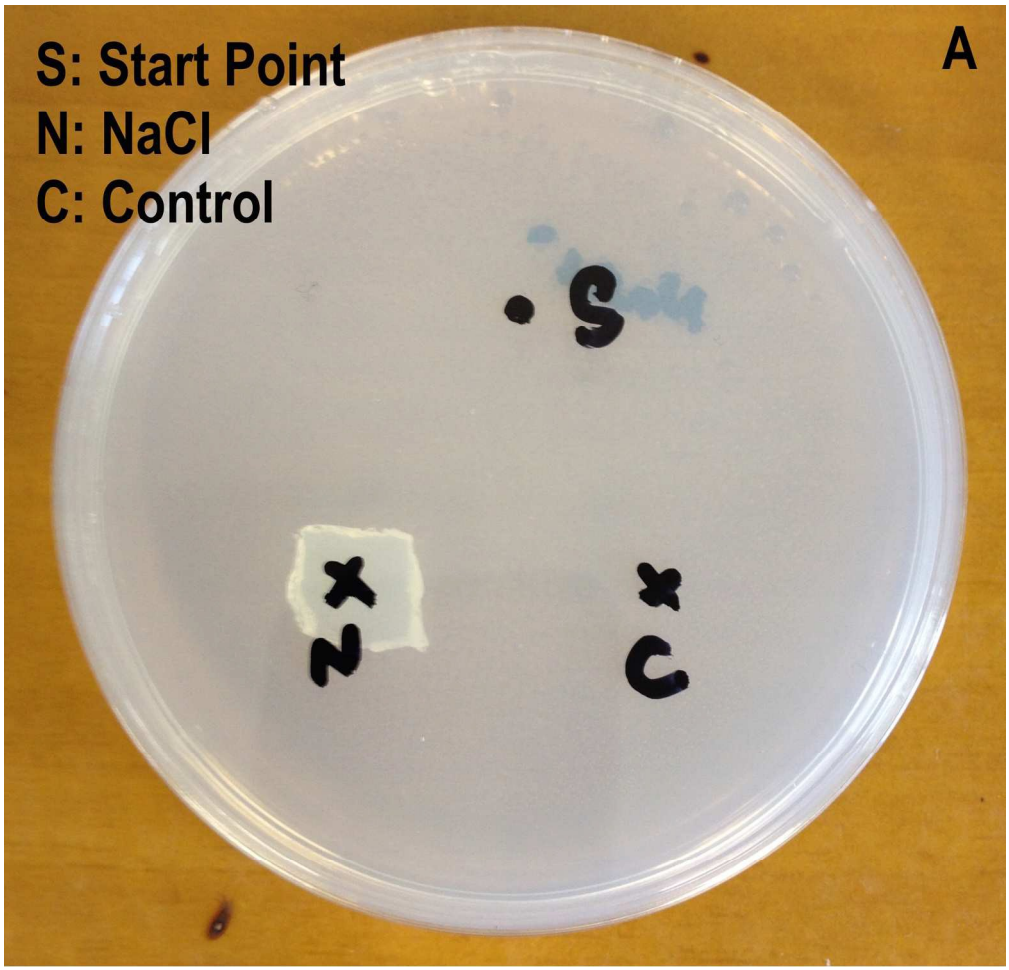
nm and 3.5 nm CdTe QDs exposure for 24 h (A) and 72 h (B). The traces show mean ± SD of animals performing IT behavior at 20 ^{o}C of three independent experiments. One-way analysis of variance followed by *Dunnett's post hoc* test was used for statistical analysis at each testing time point. *p<0.05 or **p<0.01 indicates a statistically significant difference when compared to the control.

Figure 7: Effects of 2.2 nm and 3.5 nm CdTe QDs exposure for 24 h (A) and 72 h (B) on the gene expression of neurotransmitters including glutamate, serotonin and dopamine in *C. elegans*. The results were performed as the mean ± SD of three independent experiments. One-way analysis of variance followed by *Dunnett's post hoc* test was used for statistical analysis at each testing time point. *p<0.05 or **p<0.01 indicates a statistically significant difference when compared to the control (control=1).

Figure 8: Representative fluorescent images taken by a fluorescence microscope (Olympus, FSX100) showing changes of the DCF inflorescence intensity in *C. elegans* treated with CdTe QDs for 24 h and 72 h. (A1 and A2) Images of worm in controls for 24h and 72 h, respectively. (B1~D1) Images of worms after 400, 800 and 1600μg/mL 2.2 nm CdTe QDs treatment for 24 h. (B2~D2) Images of worms after 400, 800 and 1600μg/mL 2.2 nm CdTe QDs treatment for 72 h. (E1~G1) Images of worms after 400, 800 and 1600μg/mL 3.5 nm CdTe QDs treatment for 24 h. (E2~G2) Images of worms after 400, 800 and 1600μg/mL 3.5 nm CdTe QDs treatment for 72

h.

Figure 9: Effects of 2.2 nm and 3.5 nm CdTe QDs exposure for 24 h (left) and 72 h (right) on ROS generation in *C. elegans*. The results were performed as the mean ± SD of randomly selected thirty worms from each treatment. One-way analysis of variance followed by *Dunnett's post hoc* test was used for statistical analysis. *$p<0.05$ or **$p<0.01$ indicates a statistically significant difference when compared to the control.



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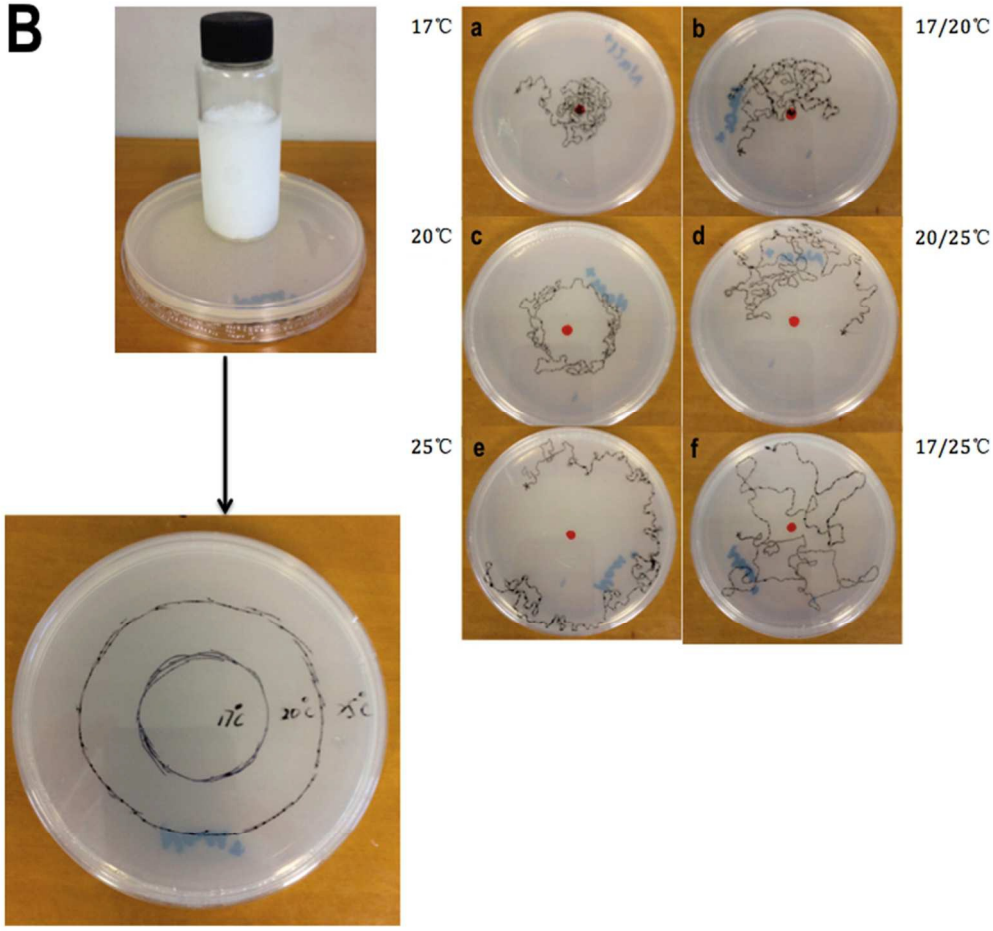

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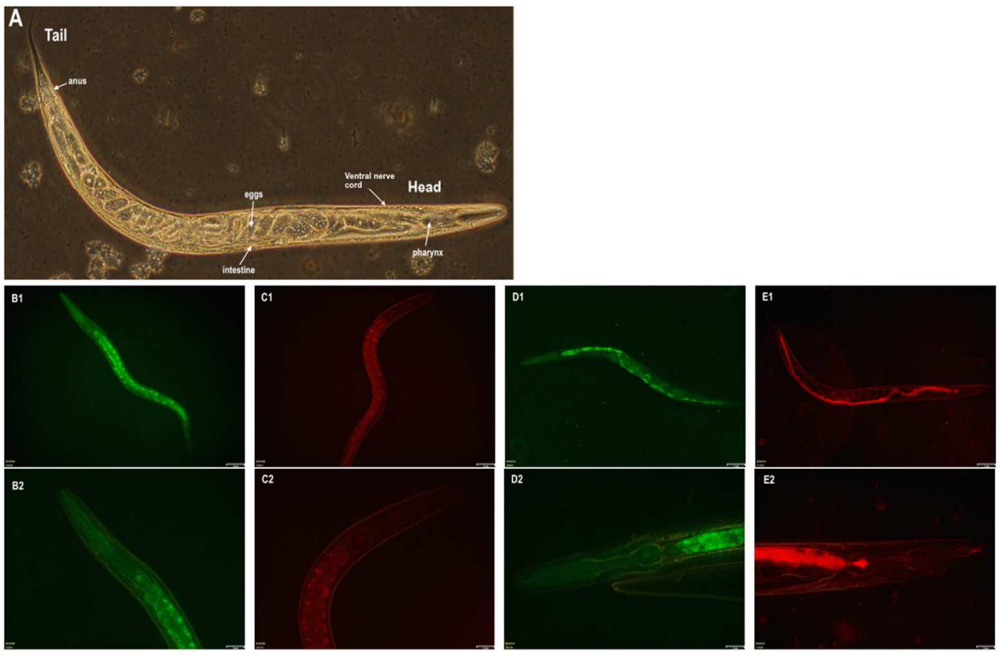
266x251mm (72 x 72 DPI)



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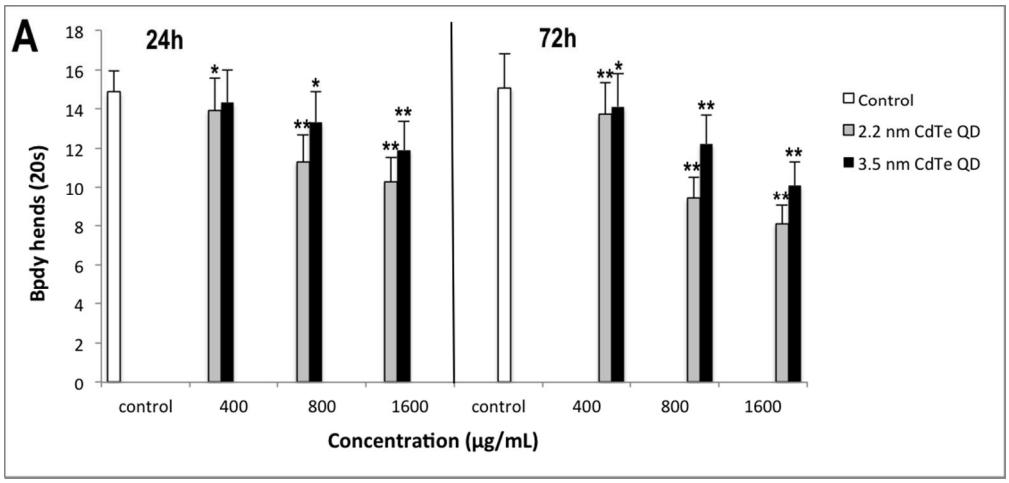
398x259mm (72 x 72 DPI)



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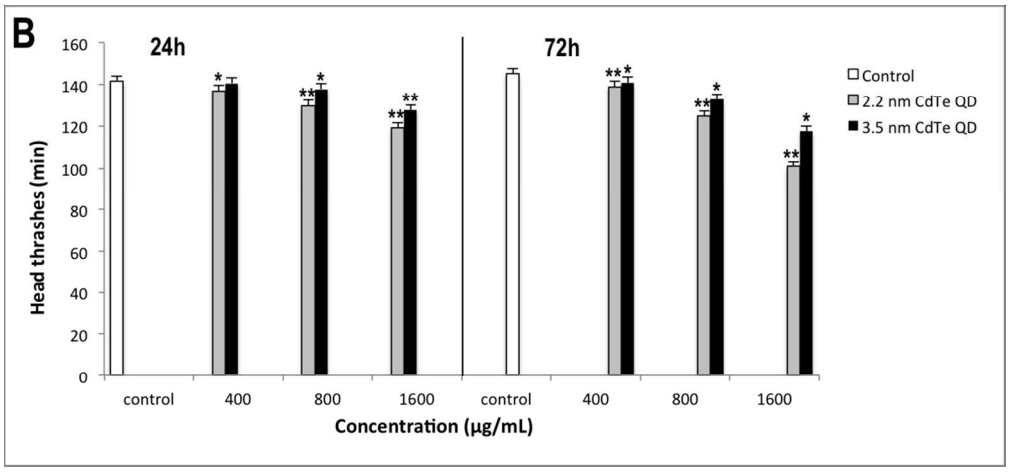

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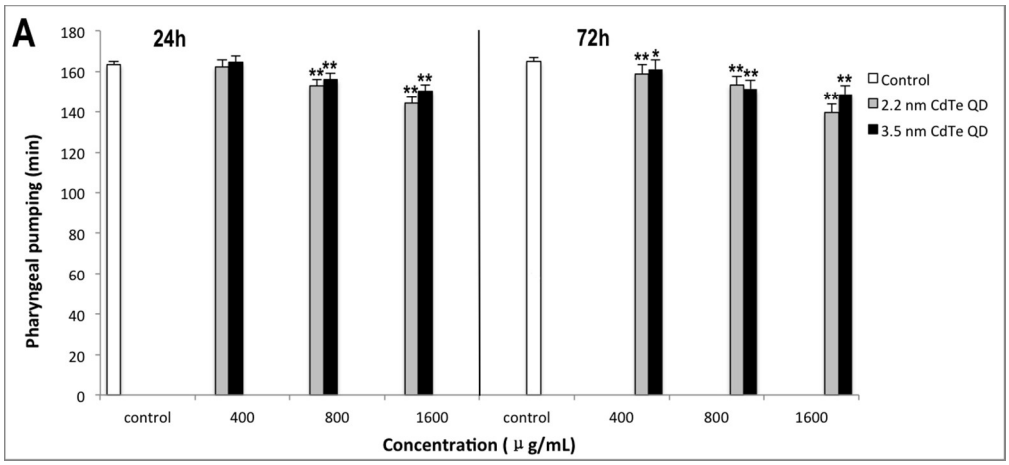

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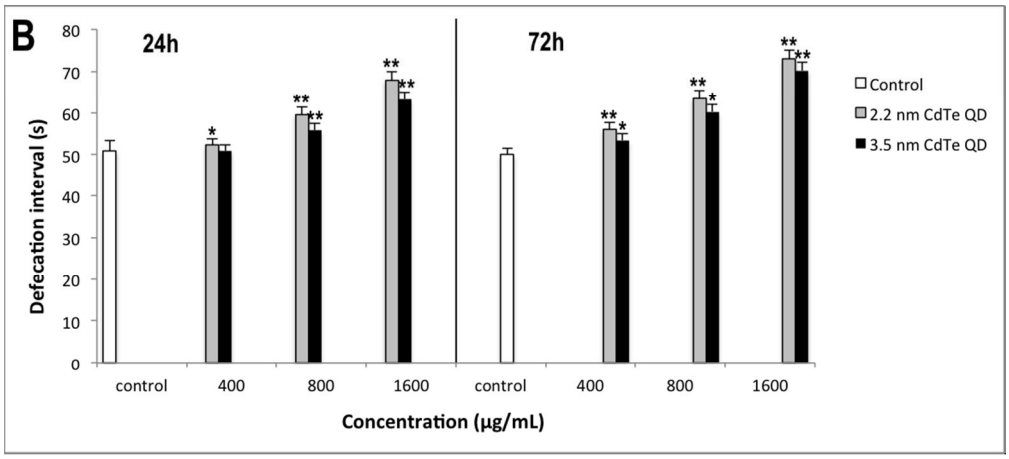

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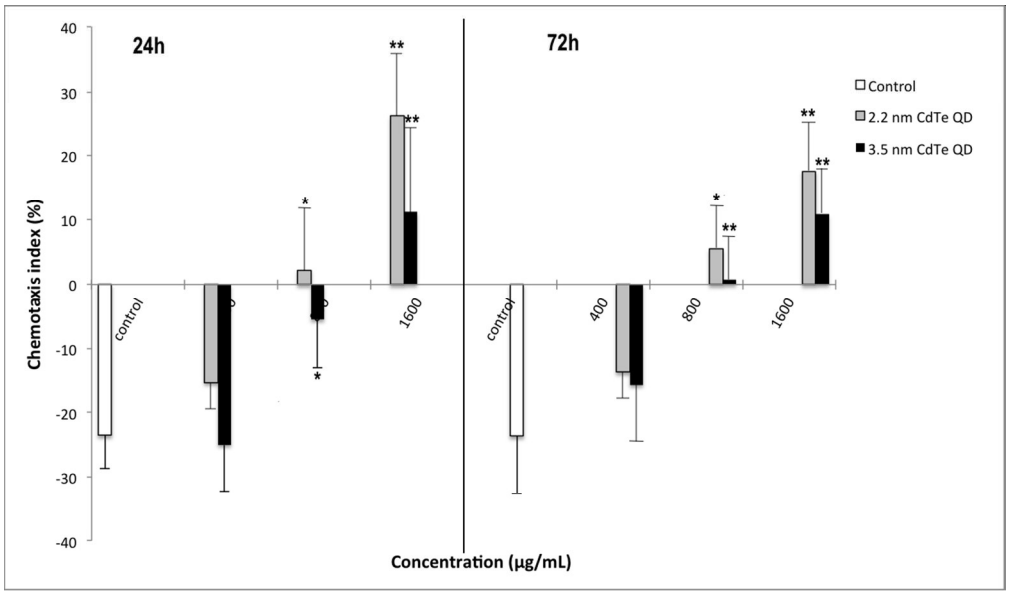

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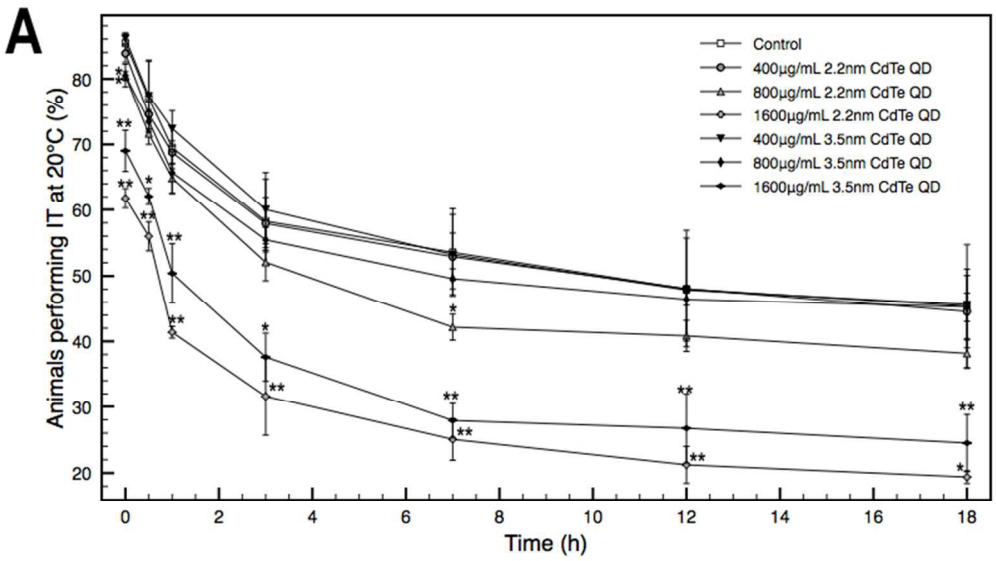

Figure 6: Time course of changes on thermotaxis in C. elegans after exposed to 2.2 nm and 3.5 nm CdTe QDs exposure for 24 h (A). The traces show mean ± SD of animals performing IT behavior at 20 oC of three independent experiments. One-way analysis of variance followed by Dunnett's post hoc test was used for statistical analysis at each testing time point. *p<0.05 or **p<0.01 indicates a statistically significant difference when compared to the control.
282x168mm (72 x 72 DPI)

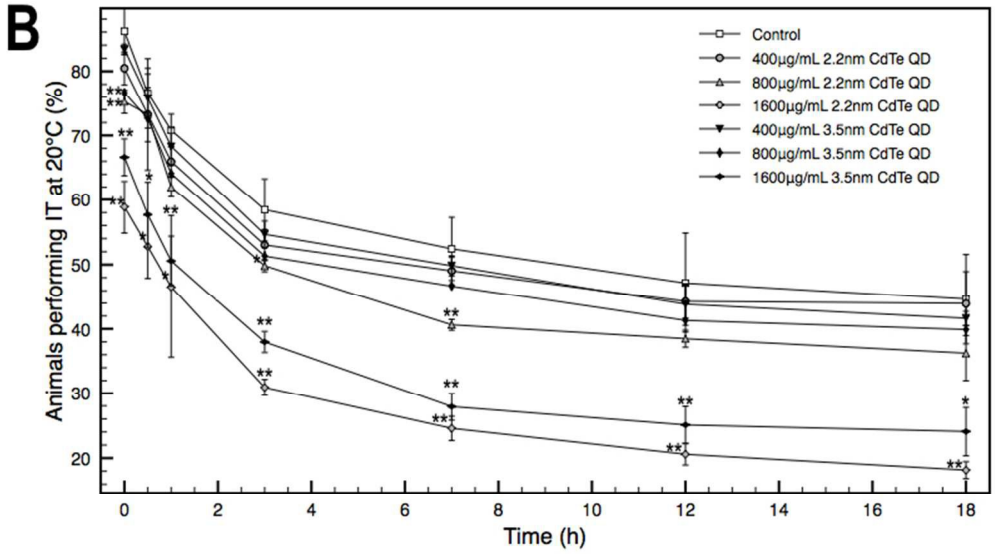

Figure 6: Time course of changes on thermotaxis in C. elegans after exposed to 2.2 nm and 3.5 nm CdTe QDs exposure for 72 h (B). The traces show mean ± SD of animals performing IT behavior at 20 oC of three independent experiments. One-way analysis of variance followed by Dunnett's post hoc test was used for statistical analysis at each testing time point. *p<0.05 or **p<0.01 indicates a statistically significant difference when compared to the control.
282x168mm (72 x 72 DPI)

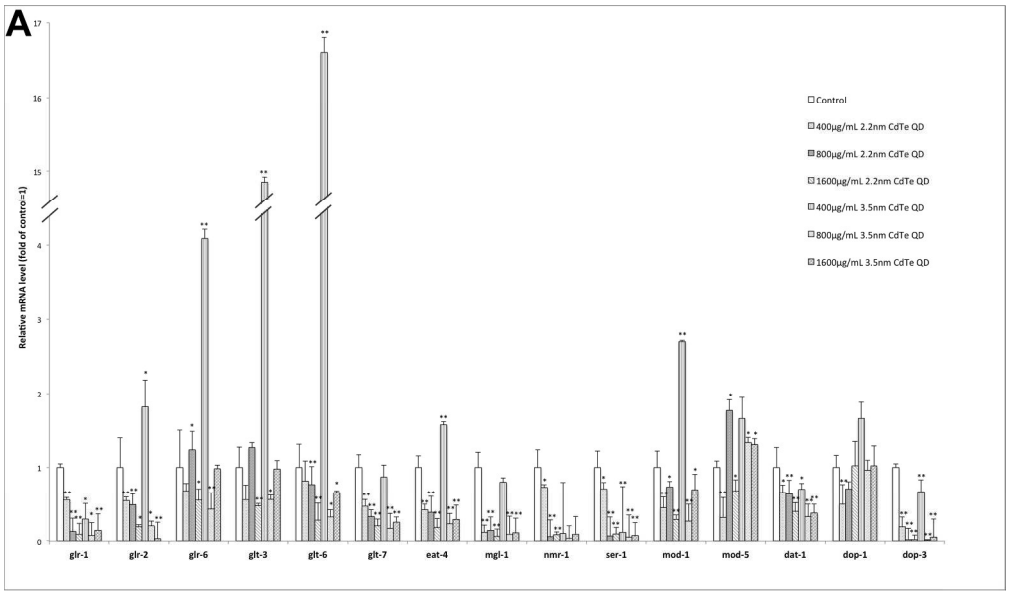

Figure 7: Effects of 2.2 nm and 3.5 nm CdTe QDs exposure for 24 h (A) on the gene expression of neurotransmitters including glutamate, serotonin and dopamine in C. elegans. The results were performed as the mean ± SD of three independent experiments. One-way analysis of variance followed by Dunnett's post hoc test was used for statistical analysis at each testing time point. *p<0.05 or **p<0.01 indicates a statistically significant difference when compared to the control (control=1).
878x513mm (72 x 72 DPI)

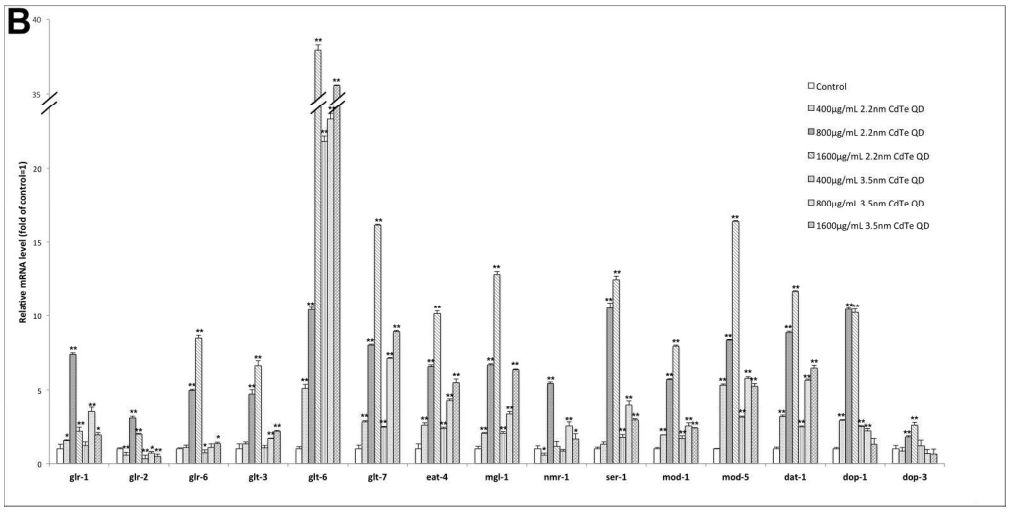

Figure 7: Effects of 2.2 nm and 3.5 nm CdTe QDs exposure for 72 h (B) on the gene expression of neurotransmitters including glutamate, serotonin and dopamine in C. elegans. The results were performed as the mean ± SD of three independent experiments. One-way analysis of variance followed by Dunnett's post hoc test was used for statistical analysis at each testing time point. *p<0.05 or **p<0.01 indicates a statistically significant difference when compared to the control (control=1).
878x439mm (72 x 72 DPI)

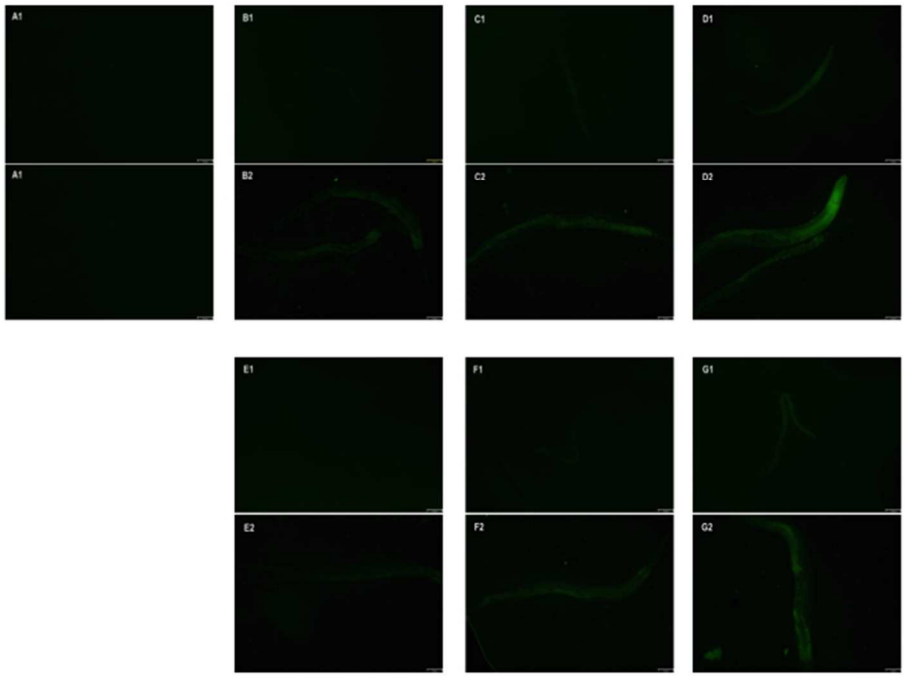

Figure 8: Representative fluorescent images taken by a fluorescence microscope (Olympus, FSX100) showing changes of the DCF inflorescence intensity in C. elegans treated with CdTe QDs for 24 h and 72 h. (A1 and A2) Images of worm in controls for 24h and 72 h, respectively. (B1~D1) Images of worms after 400, 800 and 1600μg/mL 2.2 nm CdTe QDs treatment for 24 h. (B2~D2) Images of worms after 400, 800 and 1600μg/mL 2.2 nm CdTe QDs treatment for 72 h. (E1~G1) Images of worms after 400, 800 and 1600μg/mL 3.5 nm CdTe QDs treatment for 24 h. (E2~G2) Images of worms after 400, 800 and 1600μg/mL 3.5 nm CdTe QDs treatment for 72 h.

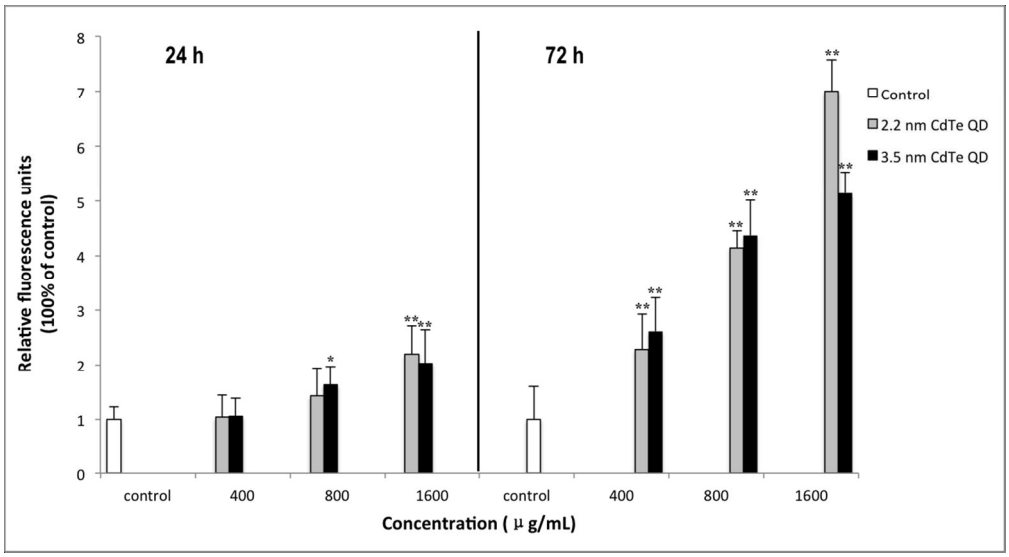

Figure 9: Effects of 2.2 nm and 3.5 nm CdTe QDs exposure for 24 h (left) and 72 h (right) on ROS generation in C. elegans. The results were performed as the mean ± SD of randomly selected thirty worms from each treatment. One-way analysis of variance followed by Dunnett's post hoc test was used for statistical analysis. *$p<0.05$ or **$p<0.01$ indicates a statistically significant difference when compared to the control.