Toxicology Research

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/toxicology

Partial protection of N-acetylcysteine against MPA-capped CdTe quantum dot-induced neurotoxicity via oxidative stress and intracellular calcium levels in rat primary cultured hippocampal neurons

Tianshu Wu[a,b,#], Keyu He[a,b,#], Qinglin Zhan[a,b], Shengjun Ang[a,b], Jiali Ying[a,b], Shihan Zhang[a,b], Ting Zhang[a,b], Yuying Xue[a,b] Yilu Chen[c] and Meng Tang[a,b*]

Abstract: As one of the most frequently used quantum dots (QDs), the toxicity of cadmium telluride (CdTe) QDs related to several body systems have been investigated, but the studies on the nervous system are rather limited. It is extremely important to assess QDs' cytotoxicity to neurons by through systematic and thorough quantitative analysis before they are applied in scientific or clinical settings. This study observed that CdTe QDs caused cell death and apoptosis in rat primary cultured hippocampal neurons in a dose-, time- and size-dependent manner. QD-exposed neurons showed an increase in reactive oxygen species (ROS) and intracellular calcium levels that lead to neuron apoptosis and even death, which may be completely or partially protected by a common antioxidant N-acetylcysteine (NAC), respectively. For future research, it is necessary to study the underlying mechanisms by investigating the extrinsic and intrinsic pathways by which CdTe QDs induce neurotoxic effects.

[a] Key Laboratory of Environmental Medicine and Engineering, Ministry of Education; School of Public Health, & Collaborative Innovation Center of Suzhou Nano Science and Technology, Southeast University, Nanjing 210009, China;

[b] Jiangsu key Laboratory for Biomaterials and Devices; Southeast University, Nanjing 210009, China

[c] College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, Nanjing, 211816, China

E-Mails: ninatswu@126.com (T.W.); tm@seu.edu.cn (M.T.)

# The authors contributed equally to the work.

* The author to whom correspondence should be addressed; E-Mail: tm@seu.edu.cn (M.T.); Tel.: +86-025-83272564; Fax: +86-025-83272479

Key words: neurotoxicity; cytotoxicity; CdTe quantum dots; reactive oxygen species; calcium overload; N-acetylcysteine; hippocampal neurons

1. Introduction

Quantum dots (QDs) are essentially nanometer-scale crystalline semiconductors consisting of chemical elements from Groups Ⅲ~Ⅴ or Groups Ⅱ~Ⅵ of the periodic table with sizes range from roughly 1~10 nm [1, 2]. They are well known for their unique optical properties, such as photostability, strong fluorescent intensity, etc. [3-6] and have been widely applied as advanced fluorescent probes in biomedical research or clinical diagnostic and therapeutic settings in recent years [7-10]. So far, cadmium- and tellurium-containing QDs, such as CdTe QDs and CdSe QDs, have been used most frequently as their quantum confinement area crosses the whole spectrum [11].

Neuroscience is one of the important branches of biomedicine that have benefited from QDs as well [12-14]. As the nervous system is a critical organ system, the neurotoxic effects of QDs need to be well understood before they are approved to be used in living organisms. From a limited number of investigations of QDs' neurotoxicity recently, as early as 2005, Lovric et al. observed that CdTe QDs capped with 3-mercaptopropionic acid (MPA) or cysteamine could elicit decreased metabolic activity, chromosome condensation, cell membrane bubbles, and even worse, cell death or apoptosis of rat PC12 cells [15]. Later, Prasad et al. investigated the interaction between thioglycolic acid capped CdTe QDs with differentiated PC12 cells and found

similar deleterious effects [16].

One source of toxicity of CdTe QDs they found is the generation of reactive oxygen species (ROS), which impaired the plasma membrane, mitochondria and nucleus, and affected cellular signal cascades that control different cellular processes leading to severe cell dysfunction and triggering apoptosis. So far, ROS generation has been considered as a major contributor to many types of metal-based QD-induced toxicity [17-21]. However, the exact mechanisms for the production of these reactive species induced by QDs are not well understood.

On the other hand, the increase in intracellular calcium levels is always related to ROS exposure [22, 23]. As a second messenger, Ca^{2+} signaling plays a significant role in physiological as well as pathological functions. An overload of intracellular calcium concentration is considered as a main cause of cytotoxic effects including impairment of mitochondrial function, chromatin damage, and eventually cell death [18, 24, 25].

N-acetylcysteine (NAC) is an antioxidant thiol compound and its neuroprotective effects have been observed in different neuronal and glial *in vitro* models [26-31]. Several studies showed that NAC could easily permeate cell membranes and inhibit cell death via antioxidant, antiangiogenic, anti-inflammatory and anticarcinogenic effects [27, 29, 32, 33]. Another protective mechanism of NAC against QD-induced toxicity is that NAC could adsorb to the surface of cadmium-containing QDs by a mercapto group and stabilize the QDs in culture media [15, 32], which prevented cell death induced by released toxic Cd^{2+} from cadmium-containing QDs.

Although there is an increasing number of studies on the cytotoxicity of CdTe

QDs, such as the toxicity in somatic cells, lungs and immune systems, the potential neurotoxicity is still not clear due to the particularity and complexity of the nervous system. The aim of this study is to investigate the toxic effects of MPA-capped CdTe QDs with two different sizes in rat primary cultured hippocampal neurons in order to gather some preliminary information on the potential mechanisms of neurotoxicity of CdTe QDs. The reason for choosing CdTe QDs is that they are one of most common QDs and we have been studying them for several years [21, 24]. As hippocampus have been reported as one of the target organs of QDs, a rat hippocampal culture model would be suitable for exploring the *in vitro* metabolism and potential neurotoxicity of CdTe QDs initially.

In this study, some fundamental cytotoxicity methods have been used to assess changes in several key biomarkers, which indicate the onset of apoptosis, oxidative stress and a rise in calcium levels. In addition, the protective effects of NAC on neurotoxicity induced by CdTe QDs was evaluated. Overall, our observations in this study led us to conclude that CdTe QDs elicited ROS production and free calcium ion release, resulting in cell apoptosis and death, which could be partially prevented by NAC, in a rat hippocampal culture model in a dose-dependent manner. The results suggested that the part of neurotoxic effects of CdTe QDs might be attributed to the oxidative stress and the elevation of calcium concentration.

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 in detail previously [24, 34]. Before this study, the physicochemical properties of these QDs were evaluated. 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. Otherwise, dynamic light scattering (DLS) confirmed that comparable dimensions were 15.63±1.63 nm and 26.79±1.59 nm for 2.2 nm and 3.5 nm CdTe QDs in cell culture media, respectively. The MPA coating contributed substantially to the size of QDs in water. The surface charges through ξ-potential measurement were -15.73±4.10 mV and -13.86±3.44 mV for 2.2 nm and 3.5nm CdTe QDs in cell culture media, respectively. Just before these experiments, QDs had to be washed with acetone and dissolved in maintain medium for treated groups.

2.2. Cell culture

As the hippocampus is the key learning and memory area of the brain and a previously confirmed target of QDs, a rat primary hippocampal neuron culture model was used in this study. Medium-density cultures of postnatal day 0 Wistar rat hippocampal neurons were prepared according to Fath and D Ke [35] with some modifications. Hippocampi were removed from rat pups and treated with 0.125% trypsin for 12~15 min at 37 °C, followed by the termination of dissociation and washing with Dulbecco's modified Eagle's medium (DMEM)/F-12 (1:1). After gentle trituration with a Pasteur pipette, neurons were planted on poly-$_L$-lysine coated culture flasks or plates (Costar, USA) at densities of 3.5~4×10^5 cells/mL. The plating medium was DMEM/F-12 (1:1) containing L-glutamine supplemented with

10% fetal bovine serum (all from HyClone, USA). Four to six hours after the planting, the culture medium was changed to a maintenance medium containing neurobasal-A medium and 2% B-27 supplement (all from Gibco, USA), which has been confirmed for the optimized survival of hippocampal neurons [36]. Cultures were maintained at 37 °C in a humidified atmosphere of 95% O_2 and 5% CO_2. Experiments were carried out on neurons after incubating 8~10 days *in vitro*, when the vitality of neurons was the best. All the treatments were performed in triplicate in three independent experiments.

2.3. MTT assay

The percentage of cell survival was measured using the (3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide) (MTT) colorimetric assay. After the primary cultured hippocampal neurons were cultured for 7 days, they were then plated into 96-well culture plates with a density of $1\sim2\times10^4$ cells/well in a total volume of 200 μL and allowed to attach and grow for 48 h at 37 °C. The supernatant in each well was then replaced with serum-free maintenance medium containing the following concentrations of QDs: 2.5, 5, 10, 20, 40, 80, 120, 180, 240 and 320 μg/mL, respectively. After 12, 24, and 48 h of incubation, 20 μL of MTT (5 mg/mL; Sigma-Aldrich, Shanghai, China) was added into each well. After additional 4 h incubation at 37 °C, the supernatant was replaced with 150 μL dimethyl sulfoxide (Sigma-Aldrich, Shanghai, China) in each well. Samples were then shaken for 15 min to dissolve the dark blue formazan products. Spectrophotometric data were measured using the Epoch multi-volume spectrophotometer system (BioTek, USA) at a

wavelength of 490 nm. In each experiment, four wells were used and experiments were performed in triplicate. MTT assay was also used to identify the effect of (N-acetylcysteine) NAC on the cell viability of hippocampal neurons.

2.4. QDs exposure conditions

Three concentrations of CdTe QDs suspensions (10, 20 and 40 μg/mL) were prepared by diluting the stock solution (2200μg/mL) of QDs with maintenance medium. For the NAC treatment group, NAC was used at a final concentration of 2 mM and added to neurons for 12h prior to 40 μg/mL QD addition and maintained continuously in the media until the exposure time was over. The used concentrations for QDs here were selected based on MTT assay.

2.5. Morphological changes

Hippocampal neurons were plated in a six-well culture plate at a density of $6\sim7\times10^5$ cells/mL and treated with CdTe QDs for 24 h. After twice washes with phosphate buffered saline (PBS), neurons were double stained with a mixture of Hoechst 33342 and propidium iodide (PI) (All form Sigma-Aldrich, Shanghai, China) at the final concentration of 10 μg /mL and 5 μg /mL, respectively. Neurons were observed using a fluorescence microscope (Olympus, FSX100) after incubation at 37 °C for 15 min. Normal neurons were stained light blue by Hoechst 33342 and the cell morphology was healthy, while apoptotic ones were stained bright blue with light red, showing typical characteristics of apoptosis, and PI stained necrotic neurons bright red. Meanwhile, random six views of every group were selected to observe and take photographs were taken in order to calculate apoptotic rates of neurons with

assistance of ImageJ Ver.1.48 software. At least 1000 cells were counted in each group.

2.6. Determination of intracellular ROS generation

The oxidation-sensitive fluorescence probe 2',7'-dichlorofluorescin diacetate (DCFH-DA) (Sigma, MO, USA) was used to analyze the level of intracellular reactive oxygen species (ROS) in the cultures by measuring the florescence intensity of the highly fluorescent dichlorofluorescein (DCF) oxidized in the presence of ROS, which is formed from the non-fluorescent dichlorodihydroflueorescein (DCFH), a deacetylated product of DCFH-DA by intracellular esterases [37]. After treatment with QDs for 24 h, the neurons were rinsed twice with ice-cold PBS and then loaded with 10 μM DCFH-DA and incubated at 37 °C for 25~30 min in dark. After triple washes with serum-free medium, all samples were resuspended in PBS. The ROS incidence was measured by using flow cytometry (Becton, Dickinson and Company, FACSCanto^{TM}) at excitation and emission wavelengths of 488 and 525 nm for DCF fluorescence.

2.7. Calcium imaging

Changes in cytosolic Ca^{2+} level ($[Ca^{2+}]_i$) were measured with the high-affinity Ca^{2+} fluorescent probe fluo-3/acetoxymethyl ester (fluo-3/AM). Fluorescence intensity after labeling was measured using confocal microscopy. For confocal microscopic analyses, the neurons were planted in 35 mm glass culture dishes at a density of $10\sim15\times10^5$ cells/well and cultured for 10 days. The dishes were washed twice with HBSS containing calcium (HyClone, USA) to remove the culture medium.

Neurons were then loaded with 5 μM fluo-3/AM (Biotium, USA) incubated at 37 ℃ for 40~45 min in the dark in order to make sure that the endogenous esterases converted nonfluorescent fluo-3/AM into fluorescent fluo-3. Neurons were gently rinsed three times with HBSS containing calcium to remove excess fluo-3/AM and incubated at 37 ℃ for 20~25 min before imaging. Micrographs were captured by laser-scanning confocal microscopy (Olympus, FV1000) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

For measuring the change of cytoplasmic calcium levels under acute QD exposure, time-lapse sequences were recorded 20 times with an interval of 20 sec. Neurons were continuously incubated in high glucose DMEM without phenol red, and different doses of QDs were added into individual culture dish after the fifth image was captured. Neuron fluorescence (F) during the first 100 s baseline period was F_0. Fluorescence measurements for each cell were normalized to the average fluorescence intensity. Region of indexes (ROIs) were defined in the first image and the normalized fluorescence changes F/F_0 were measured throughout the image sequence. All settings of the scanning system and the complete data acquisition were controlled and collected by confocal microscopy matching software (Olympus Fluoview Ver.4.1).

2.8. Statistical analysis

A minimum number of three independent experiments were performed for all experimental conditions tested. Experimental data were expressed as mean ± standard deviation (SD). Statistical significance among and between groups was analyzed

using one-way analysis of variance followed by *Dunnett's post hoc* test and Least Significant Difference (LSD) test. A *P*-value of less than 0.05 was considered as significantly different. The statistical analysis was performed using IBM SPSS Statistics (Ver.19.0).

3. Results

3.1. Cell death induced by QDs in a time- and dose-dependent manner

MTT assay was used to examine the viability of primary cultured hippocampal neurons to explore the potential cytotoxicity of CdTe QDs at different concentrations (Fig. 1). The neurons were exposed to 2.2 nm and 3.5 nm CdTe QDs at gradually increasing doses from 2.5 to 320 μg/mL for 12, 24 and 48 h. Both sizes of CdTe QDs influence the cell viability of primary neurons slightly (approximately 80% for 2.2 nm CdTe and 70% for 3.5 nm CdTe, respectively) when the concentration was below 40 μg/mL at the various periods after exposed for 12 and 24 h. However, the viability of neurons exposed to 3.5 nm CdTe QDs decreased markedly when the concentration was higher than 40 μg/mL, while this remarkable decreased cell viability occurred at the concentration of 80 μg/mL in 2.2 nm CdTe QD-exposed groups. When the concentration was above 160 μg/mL, the cell viability decreased to less than 20% for 2.2 nm CdTe QDs and 30% for 3.5 nm ones, and then maintained stable. Both types of CdTe QDs showed cytotoxicity in a time- and dose-dependent manner.

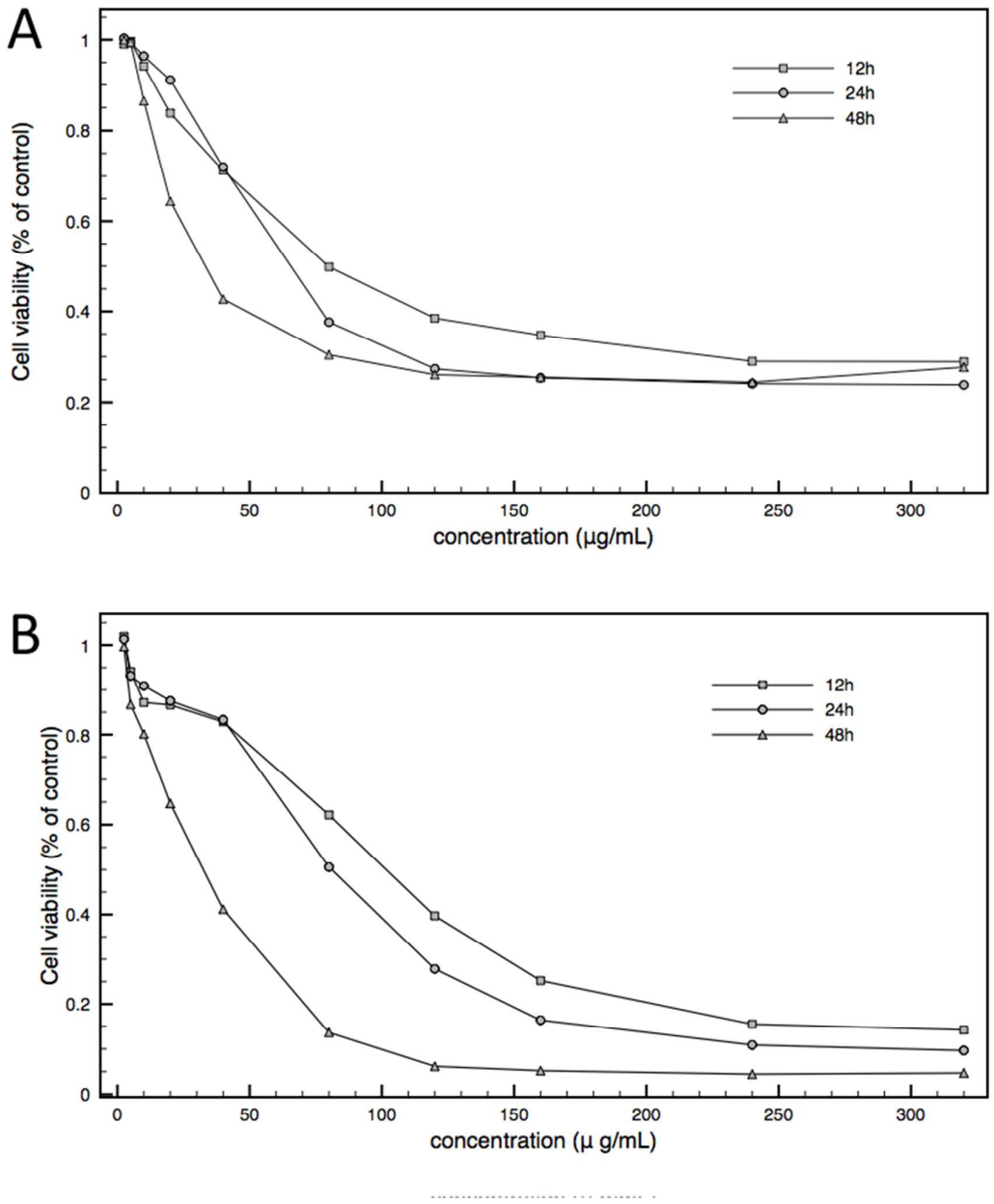


Figure 1. MTT assay of rat hippocampal neuron viability after various treatments. Hippocampal neurons treated with (A) 3.5 nm CdTe QDs and (B) 2.2 nm CdTe QDs at 2.5~320 μg/mL for 12, 24 and 48h. Typical data from one of three independent experiments were shown with similar results compared to the control group.

3.2. Morphological changes against QD toxicity and apoptosis

Light microscopy was used to investigate the influence of different

concentrations of CdTe QDs on morphological changes in rat hippocampal neurons after exposed to 2.2 nm and 3.5 nm CdTe QDs. Pictures of untreated cells, as controls, showed healthy morphology (Fig. 2I). Hippocampal neurons exposed to 3.5 nm CdTe QDs did not show significant changes compared to the control neurons at the concentration of 10 μg/mL. However, the length of neuron processes and the maximum diameter of neuron that exposed to 2.2 nm CdTe QDs decreased at the same dose (Fig. 2A and 2E). When exposed to 20 μg/mL of both CdTe QDs, hippocampal neurons exhibited severe morphological impairment including decreased length of neuron processes and destroyed nervous networks, and showed typical features of early stages of apoptosis including cell shrinkage, membrane fragmentation, irregularity of cell outlines, cell detachment due to loss of adhesion and oval-shaped appearance (Fig. 2B and 2F). Furthermore, hippocampal neurons treated with 40 μg/mL had lost typical morphology of neurons and become floating and agglomerated (Fig. 2C and 2G). However, hippocampal neurons exposed to 40 μg/mL CdTe QDs but pretreated with NAC just showed slight apoptotic morphological changes (Fig. 2D and 2H).

Hoechst 33342/PI double staining was used to obtain more detailed apoptosis information in rat hippocampal neurons after they were exposed to 2.2 nm and 3.5 nm CdTe QDs with different concentrations. Hoechst 33342, a type of blue-fluorescence dye, stains the condensed chromatin in apoptotic cells more brightly than normal chromatin [38], while propidium iodide (PI), a red-fluorescence dye, is only permeating to dead cells [39]. Therefore, the staining pattern resulting from

the simultaneous use of these dyes makes it possible to distinguish normal, apoptotic, and dead cell populations by fluorescence microscopy. We could see that untreated hippocampal neurons were round and light blue in color, while the number of apoptotic neurons showing bright blue and neurotic neurons showing red was increasing along with the increasing dose, but was declining slightly in NAC pretreated neurons.

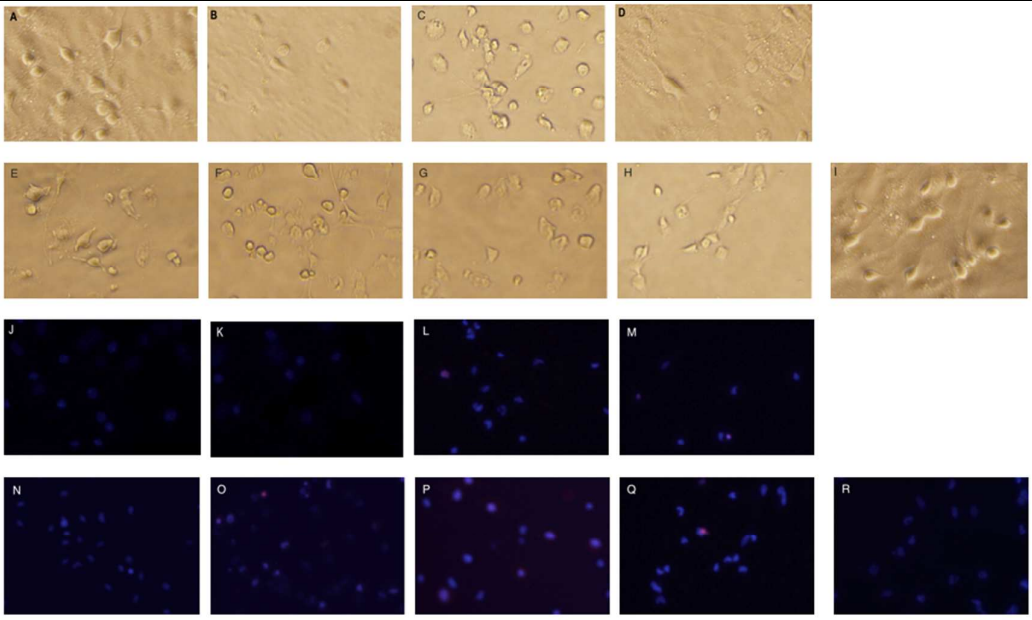


Figure 2. Representative fluorescent images showing morphological changes in rat hippocampal neurons treated with CdTe QDs for 24 h., (A, J)-3.5 nm CdTe QDs 10 μg/mL treatment, (B, K)-3.5 nm CdTe QDs 20 μg/mL treatment, (C, L)-3.5 nm CdTe QDs 40 μg/mL treatment, (D, M)-3.5 nm CdTe QDs 40 μg/mL treatment with NAC preconditioning; (E, N)-2.2 nm CdTe QDs 10 μg/mL treatment, (F, O)-2.2 nm CdTe QDs 20 μg/mL treatment, (G, P)-2.2 nm CdTe QDs 40 μg/mL treatment, (H, Q)-2.2 nm CdTe QDs 40 μg/mL with NAC preconditioning; (I, R)-control group.

Pictures of six random views of each group were taken by fluorescence

microscopy to qualify cell apoptosis by counting total neurons and apoptotic ones. The percentage of apoptotic neurons was 6.18 ± 0.84% in control group, while apoptotic rates were 7.0 ± 1.6%, 11.4 ± 1.6%, 35.4 ± 3.7% and 10.1 ± 2.2% following treatment with 10, 20, 40 μg/mL of 3.5 nm CdTe QDs and 40 μg/mL QDs with NAC pretreatment, and were 11.5 ± 1.8%, 20.9 ± 3.1%, 31.3 ± 3.0%and 20.6 ± 3.5% following treatments with 2.2 nm CdTe QDs (Fig. 3). From these results, exposure to a low concentration of 3.5 nm CdTe QDs had no obvious effect on cell apoptosis and no statistically significant differences were observed when the results were compared to the control group (P=0.987). However, the rest of QD-exposed group showed much higher apoptotic rates in a dose-dependent manner and were statistically significant different from those of the control group (P<0.05). Additionally, 2.2 nm CdTe QDs induced a higher rate of apoptosis in hippocampal neurons than 3.5 nm CdTe QDs at low and medium concentrations, but the results reversed at high concentration. Apoptotic rates of NAC pretreatment groups were statistically significant different from those of the same dose QD-treatment groups [P<0.001(2.2 nm CdTe); P=0.021(3.5 nm CdTe)], implying that NAC pretreatment prevented neurons exposed to 40 μg/mL CdTe QDs from severe apoptosis.

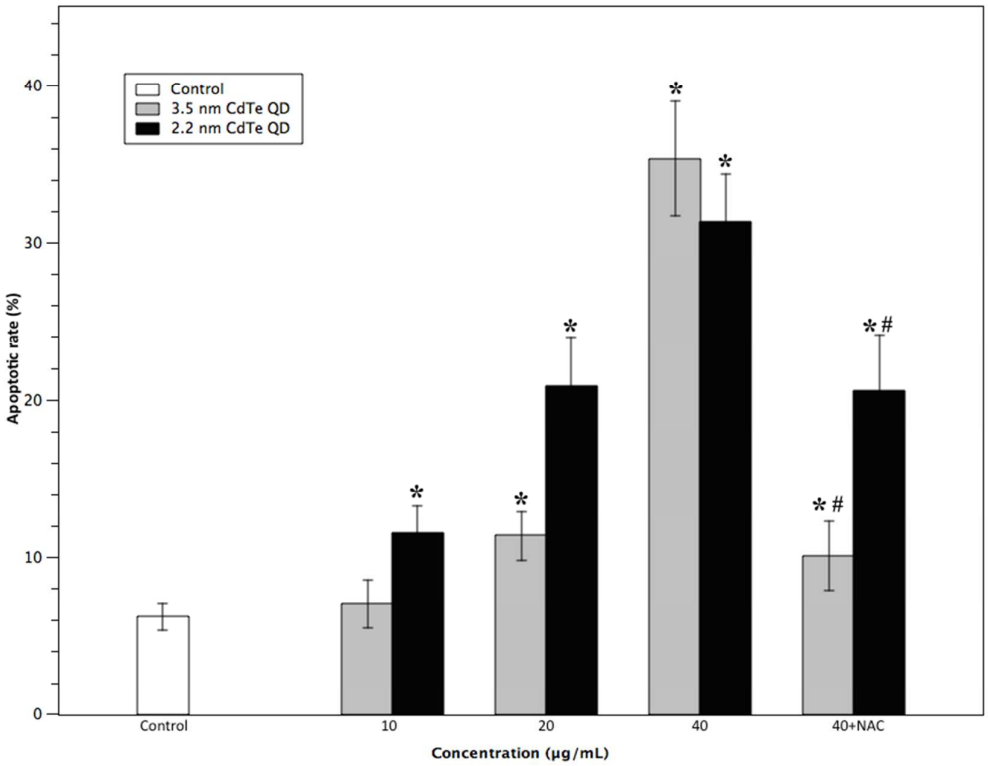


Figure 3. Quantitative analysis of apoptosis in Hoechst 33342/PI double stained hippocampal neurons treated with CdTe QDs for 24 h. Results represented the mean ± SD of three independent experiments. One-way analysis of variance followed by *Dunnett's post hoc* test and LSD test were used for statistical analysis. *p<0.05 indicates a statistically significant difference when compared to the control, and #p<0.05 indicates a statistically significant difference when compared to the same dose treatment group.

3.3. Generated ROS production

Plasma levels of ROS serve as reliable indicators of oxidative stress. Here, we chose the DCFH-DA detection reagent to measure ROS generation in CdTe QD-treated hippocampal neurons. As hydrogen peroxide or low-molecular weight hydroperoxides produced by cells can oxidize DCFH to the highly fluorescent compound DCF, the fluorescence intensity is thus proportional to the amount of

peroxide produced by the cells, therefore we can use flow cytometry to measure the ROS incidence. As shown in Figure 4, the ROS incidences were increasing with the dose and were significantly higher in all QD-treatment groups than in the control (P<0.05). Overall, the intracellular ROS content seemed be higher in 2.2 nm CdTe QD-treated neurons than in 3.5 nm QD-treated neurons. However, in the two NAC-pretreatment groups, the ROS incidence had no significant differences from the control [P=0.375 (2.2 nm CdTe); P=0.998 (3.5 nm CdTe)], but was significantly different from their same dose QD-treatment groups (P<0.001), implying that NAC prevented QD-induced intracellular ROS generation completely.

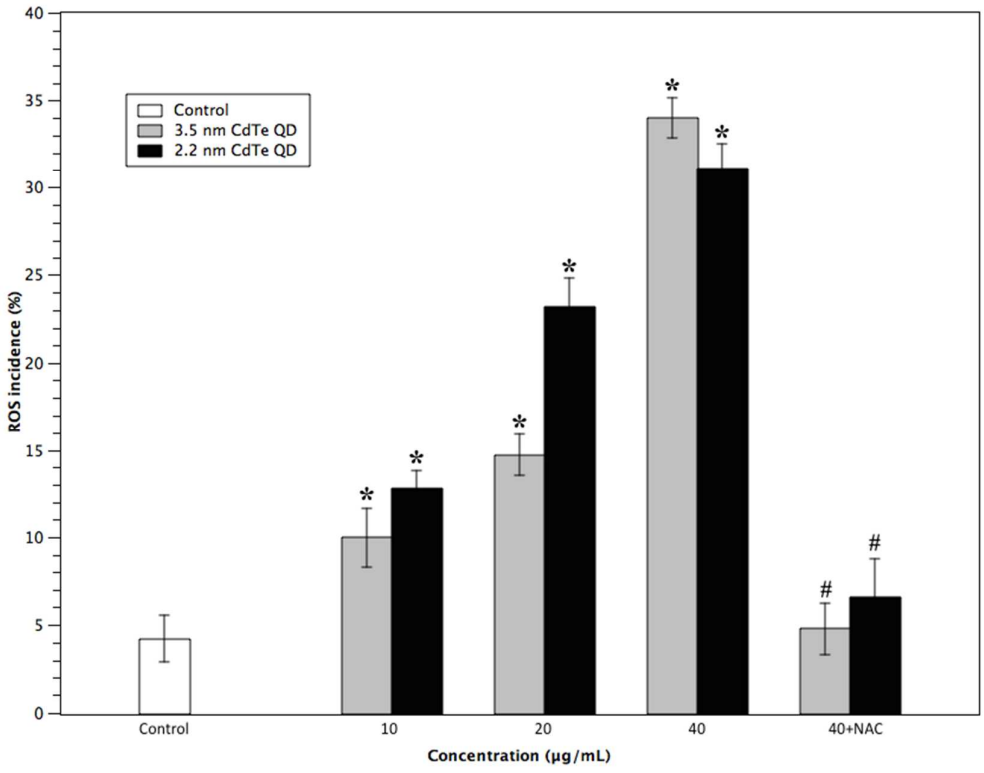


Figure 4. Quantification of fluorescence intensity showing the relative amount of intracellular ROS formation. ROS formation in rat hippocampal neurons exposed to 10, 20 and 40 μg/ml of 2.2 nm and 3.5 nm CdTe QDs for 24 h. The results were quantitatively analyzed for changes in

ROS incidence of each group and expressed as percentages. Results represented the mean ± SD of three independent experiments. One-way analysis of variance followed by *Dunnett's post hoc* test and LSD test were used for statistical analysis. *p<0.05 indicates a statistically significant difference compared with the control, and #p<0.05 indicates statistically significant difference when compared to the same dose treatment group.

3.4. Elevation of cytoplasmic calcium

We examined whether the CdTe QDs could induce sustained elevation of $[Ca^{2+}]_i$ in the cultures of hippocampal neurons, in which both extra- and intracellular Ca^{2+} were involved. The results showed that acute exposure to 40 μg/mL 3.5 nm and 2.2 nm QDs could increase the intracellular calcium levels rapidly (Figure 6). The Fluo-3 fluorescence ratios F/F_0 were up to 1.456±0.003 and 1.497±0.023, respectively, which had a dramatically significant increase compared with control (P<0.001; n=11 and 21, respectively). Otherwise, the rest concentrations of QDs' exposure induced significant rise in $[Ca^{2+}]_i$ level compared to the control (all P<0.05), except for the low exposure group of 3.5 nm CdTe QDs. Although NAC here failed to prevent the rise of intracellular calcium level completely because the NAC-pretreatment groups were significant different from the control, the significant difference from their counter-exposure groups suggested that QD-induced elevation of $[Ca^{2+}]_i$ can be partially reversed by NAC.

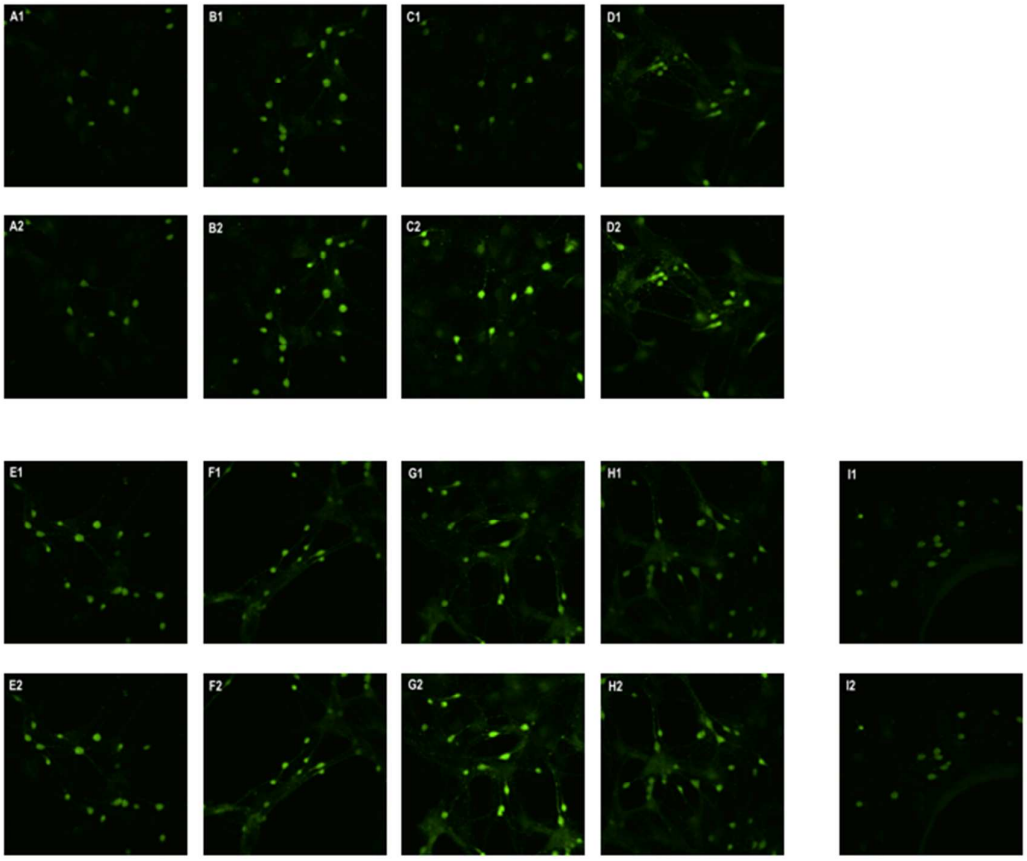


Figure 5. Representative confocal images showing rapid elevation of intracellular calcium concentration in rat hippocampal neurons treated with CdTe QDs. The images were obtained under calcium fluorescence before QD exposure (A1~D1: low, middle, high and QD-NAC treatment groups for 3.5 nm CdTe; E1~H1: low, middle, high and QD-NAC treatment groups for 2.2 nm CdTe) and 200 s after QD exposure (A2~H2); I1 and I2 were the control group).

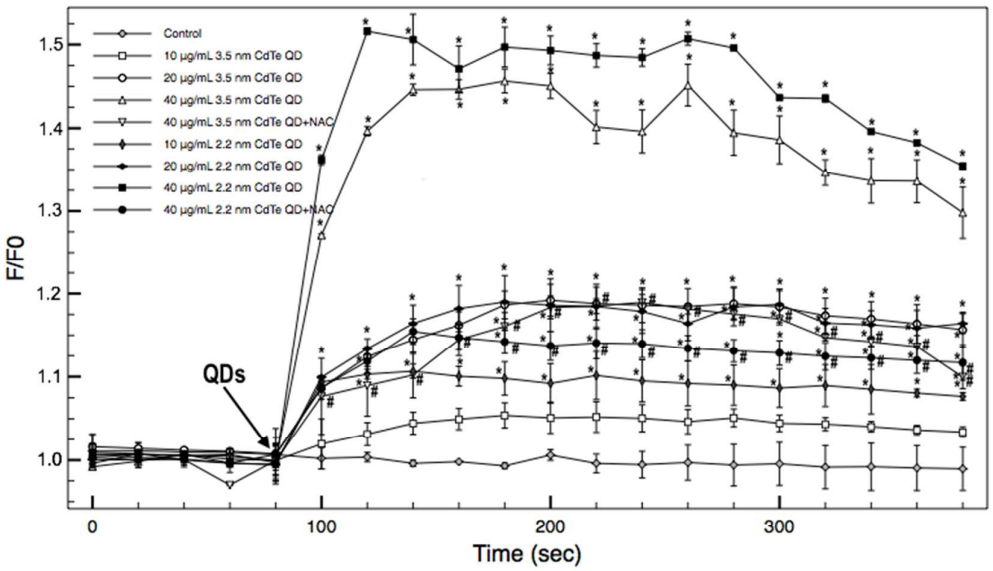


Figure 6. Time courses of changes in the fluo-3influorescence intensity before and after acute CdTe QDs exposure. Traces showed mean ± SD of the normalized fluo-3 fluorescence changes (F/F_0) of three independent experiments. One-way analysis of variance followed by *Dunnett's post hoc* test and LSD test were used for statistical analysis. *p<0.05 indicates a statistically significant difference compared with the control, and #p<0.05 indicates a statistically significant difference when compared to the same dose treatment group.

3.5. Protective effects of antioxidants in hippocampal neurons exposed to CdTe QDs

Previous experiments have suggested that free radical scavenger NAC was able to prevent CdTe QD-induced ROS generation completely, as well as protect partially

against QD-induced elevation of intracellular calcium concentration and apoptosis. Here, we investigated whether QD-induced cell death can be prevented by NAC. Compared to the control, NAC alone had no impact on the cell viability of neurons (P=0.996 and 0.982). However, when NAC was added to hippocampal neurons for 12h prior to QD addition and maintained in the medium, the 3.5 nm CdTe QD-induced reduction in cell viability was significantly prevented compared to the same dose of QD-treatment group (Fig. 7A; P<0.001). For 2.2 nm CdTe QDs, the cell viability of NAC pretreatment group was significantly different from that of control (Fig. 7B; P=0.009), but not significantly different from that of QD-treatment group with the same dose (P=0.064), which meant that partial QD-induced reduction in cell viability was prevented by NAC.

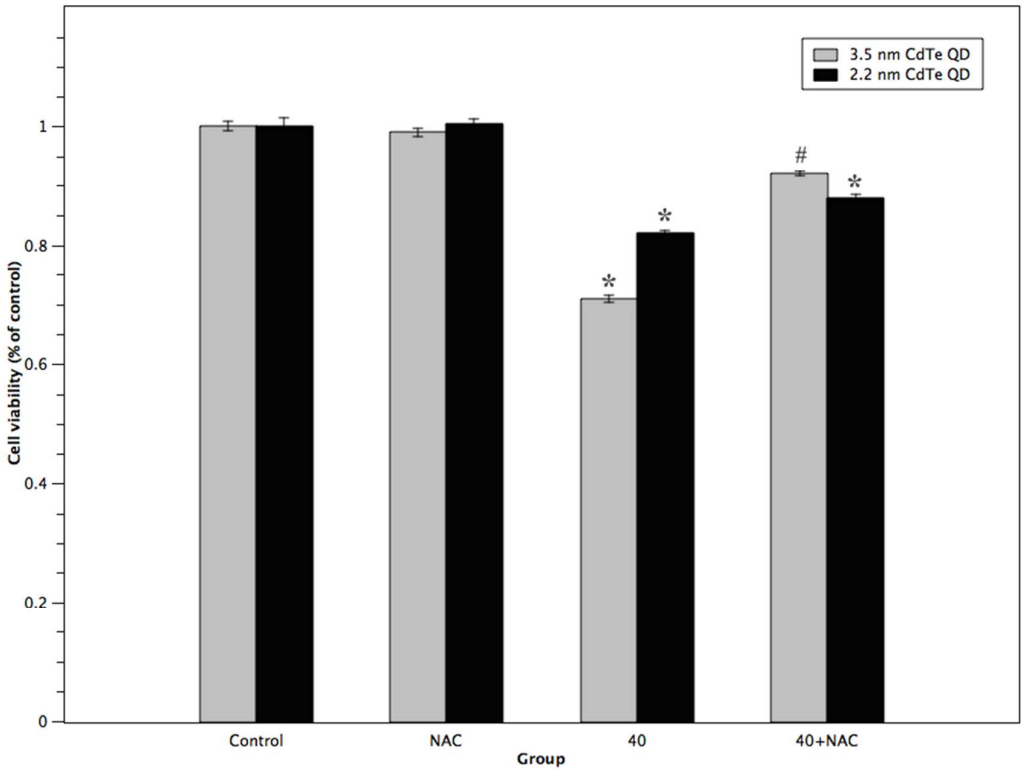


Figure 7. Protective effect of NAC (2 mM) on cell viability after 24 h exposure to (A) 3.5nm CdTe QDs and (B) 2.2 nm CdTe QDs in the continuous presence of the drug. Results represented the mean ± SD of four independent experiments. *p<0.05 indicates a statistically significant difference compared with the control, and #p<0.05 indicates a statistically significant difference when compared to the same dose treatment group.

4. Discussion

As CdTe QDs are capable of crossing the blood brain barrier or moving along neuronal pathways and entering the brain, their biological safety in the nervous system have receiving increasing attention in recent years [40-42]. Additionally, this further prompts us the importance to understand their potential neurotoxicity because QDs are widely applied in neuroscience, such as bioimaging of brain tissues, labeling neuronal cells, and drug delivering crossing the blood-brain barrier (BBB) [40, 43, 44]. Our previous studies have demonstrated that the potential risks are present in subsequent biological and clinical applications of QDs, even if the QDs are well-modified where they are capped with a MPA shell which could prevent the release of toxic components from QDs and reduce their toxicity [21, 24, 45, 46].

In this study, the cytotoxic effects and potential toxicity mechanisms of well-modified MPA-caped CdTe QDs were investigated using the rat hippocampal neurons as a model in order to develop effective and efficient interventions to reduce or even eliminate their adverse responses. Rat primary cultured hippocampal neurons, extensively used for *in vitro* neurotoxicity testing, were chosen for this study because

hippocampus were reported to be a target brain area of QDs and the damage of this brain regain could result in serious health consequences [14, 17, 18, 47]. Just like the previous study to sequentially investigate the effects of CdTe QDs on cytotoxicity and apoptosis [24], our overall findings showed that treatments of 0 to 320 μg/mL CdTe QDs to hippocampal neurons reduced cell viability and increased apoptotic cells in a dose- and time-dependent manner, and QDs with smaller size displayed higher cytotoxicity.

Apart from the quantitative assay, the cell morphology gave a rather clear picture of the trend in cytotoxicity among different doses of two sizes of CdTe QDs. The degeneration of neuritis observable mostly in the case of high concentration of QD-exposures was attributed to hippocampal neuron apoptosis and death as well as directly axonal toxicity. Additionally, other studies have suggested that autophagosomes or lysosomes produced in the cell cytoplasm and neuritis, which destroyed the already impaired mitochondria due to QDs' toxicity, were responsible for the morphological changes as well [48, 49]. Although the CdTe QDs used here are not identical to those used in other studies, the study results are largely consistent with past work using different neuronal or neuronal-like cell lines [15, 16].

To investigate the detailed factors that are possibly responsible for CdTe QD-induced cytotoxicity on neurons, ROS production that has been reported repeatedly to be relevant to nanoparticles' toxicity was measured *in situ* using the fluorescent dye DHE, a specific probe to indicate presence of superoxide anion ($O_2(\bullet-)$). Our results showed that CdTe QDs led to an increase in ROS formation in

hippocampal neurons, which confirmed findings from previous studies that suggested ROS generation results from the CdTe QD-exposure [24, 50]. ROS are highly reactive ions because of the presence of unpaired valence shell electrons and normally play an important role in cell signaling involved in apoptosis induction under both physiological and pathologic conditions [51]. Researchers have found that mitochondria are both sources and targets of ROS, which means that CdTe QDs might increase the ROS level by affecting mitochondria. *In vitro* exposed QDs were capable of entering cells and being distributed and located within the mitochondria, endosome and lysosome after at least 2 h, and then eliciting mitochondrial swelling and network fragmentation as well as disruption of mitochondrial membrane potential ($\Delta\Psi m$) and electron transport chain to impair mitochondrial functions and cause abnormal ROS generation [52-56]. ROS largely mediate cytochrome c release from mitochondria that triggers caspase activation, a certain apoptotic hallmarks, in a direct or indirect way [57]. Excess ROS generation in cells leads to oxidative stress, an important factor for triggering apoptosis [58], which is consistent to our results that the higher ROS incidence, the more apoptotic cells.

Oxidative stress due to excessive ROS production was attributed to the adverse effects of cadmium ions by previous researchers. However, there is evidence showing that capping coats, which prevented leak of cadmium ions from the QD core, reduced the cytotoxicity, while the level of intracellular ROS still elevated [16]. QDs themselves could induce ROS production through localizing to vesicles in the preinucalear region, cytoplasm and mitochondria in order to interact with normal ROS production

mechanisms or decrease the cellular anti-oxidant defense mechanisms [59, 60]. Otherwise, Some researchers thought that QDs' ability of spontaneously inducing ROS production was attributed to their configuration [61]. As photosensitizers, QDs could transfer energy to oxygen molecules resulting in the production of singlet oxygen and superoxide anion under the influence of light [62]. As we know, a number of mechanisms may be explained for generating ROS by CdTe QDs.

In order to verify that the QD-induced toxicity was a consequence of ROS generation, we used a common antioxidant NAC to test its protective effects on cellular damage, because it has been consistently reported that NAC palys a major role in modulating ROS in neuronal cells [26, 31, 63, 64]. In metabolic pathways, NAC acts as a glutathione precursor. It can promote synthesis and the ability of endogenous glutathione to scavenge and neutralize free radicals in order to protect cells against free radicals [31]. Additionally, NAC can modulate cell apoptosis by reverse oxidative DNA damage that is an apoptotic pathway and exert anti-inflammatory effects by inhibition of cyclooxygenase 2 (COX-2) and cytokines secretion [29, 33].

The results showed that the QD-induced reduction in cell viability was partially prevented when millimolar concentrations of NAC was added to hippocampal neurons for 12h prior to QD addition and maintained in the medium, which strongly suggests the involvement of ROS in the toxic processes of QDs. However, as NAC was capable of eliminating ROS completely under the same conditions, it seemed like that NAC merely prevented cell death caused by QD-induced ROS generation. The failure of NAC indicated that ROS were not the exclusive contributors to the

QD-induced cell death, which is similar to what was reposted in a previous study [15].

Recently, researchers found the conflicting effects of NAC that it could act as either antioxidant or prooxidant depending on the milieu. When the rat neurons were cultured under normoxic conditions, the protection by NAC was observed only in cysteine free medium because H_2O_2 was generated to a considerable concentration in cysteine [65]. This finding might explain the incomplete protectiveness of NAC against CdTe QD-induced neuron death, since the maintenance medium used here contains L-cysteine [66].

Ca^{2+} is an important element for cell signaling pathways and some enzymatic activities in cells. The intracellular Ca^{2+} is tightly controlled and the concentration is kept very low in intracellular fluids in healthy cells. Abnormal calcium homeostasis always causes significant physiological dysfunctions. Calcium also plays a critical role in various neuronal functions. For example, calcium participates in synaptic plasticity, a neuronal process presumably associated with cognitive brain functions like learning and memory in hippocampus. Excessive amounts of intracellular calcium levels in hippocampal neurons would cause neuronal damage resulting in severe brain impairment.

Our results showed that elevation of intracellular Ca^{2+} was also observed in CdTe QD-exposed hippocampal neurons with an increase in intracellular ROS levels in a dose-dependent manner, where NAC could partially diminish this rise in the Ca^{2+} concentration provoked by QDs. The findings supported the hypothesis that increasing intracellular ROS induced by CdTe QDs is a direct trigger of the observed

$[Ca^{2+}]_i$ increase attributed to both extracellular Ca^{2+} influx and internal Ca^{2+} release, which have been proposed by many researchers [21, 22, 24, 67].

Otherwise, Naziroglu et al. suggested that neuroprotection against oxidative damages of NAC was associated with the attenuation of a Ca^{2+} influx triggered by oxidative agents. As transient receptor potential vanilloid 1 (TRPV1) channels are involved in the oxidative stress-induced neuronal death, negative modulation of TRPV1 channel activity by NAC pretreatment might account for neuropretective activity against oxidative stress of NAC in neurons of mice [28]. It is likely that influx of extracelllar Ca^{2+} by TRPV channels of the plasma membrane is an oxidative stress dependent mechanism. However, NAC failed to prevent the rise of $[Ca^{2+}]_i$ completely, which also suggests that ROS mechanism was not the only pathway that could affect intracellular calcium steady state.

So far, researchers have proposed four possible mechanisms to explain the toxicity of QDs, including the release of toxic metals from the core of nanoparticles; generation of ROS; nanoparticles aggregation on the cell surface; and cytotoxicity of surface-covering molecules of QDs [50]. Our study confirmed the ROS mechanism and proposed other mechanisms by which CdTe QDs elevated cytoplasmic calcium levels and induced cell apoptosis in hippocampal neurons that remained a matter of conjecture.

5. Conclusion

This study showed that CdTe QDs could induce oxidative stress, which was

supported by the increases in intracellular ROS levels and elevation of cytoplasmatic calcium concentrations. This further results in the reduced cell viability and the increased rates of apoptosis in primary cultured hippocampal neurons of neonatal rats. The death of hippocampal neurons, therefore, severely affected the connection of synapses and signal transduction, leading to learning and memory damages ultimately. However, pretreatment with NAC could protect against CdTe QD-induced neuronal apoptosis and death and inhibit calcium overload partially. The mechanisms for preventing CdTe QD-induced neurotoxicity might involve scavenging ROS and negative modulation of Cd^{2+} relevant channels. Given the particle protective effects of NAC, it was postulated that the toxicity of CdTe QDs arises from multiple sources, including ROS generation, intracellular calcium overload, the intrinsic nanoscale properties of QDs and some other unknown factors. Further investigations of the molecular mechanisms of CdTe QDs neurotoxicity are required. The valuable information from this study provides a good start for understanding the neurotoxicity of CdTe QDs, which is important for broadening their range of applications in the nervous system.

Acknowledgements

This work was supported by National Natural Science Foundation of China (Nos. 30972504, 81172697, 81302461, 81473003), National Important Project on Scientific Research of China (No. 2011CB933404) and Provincial Natural Science Foundation of Jiangsu (No. BK2011606).

Declaration of interest

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

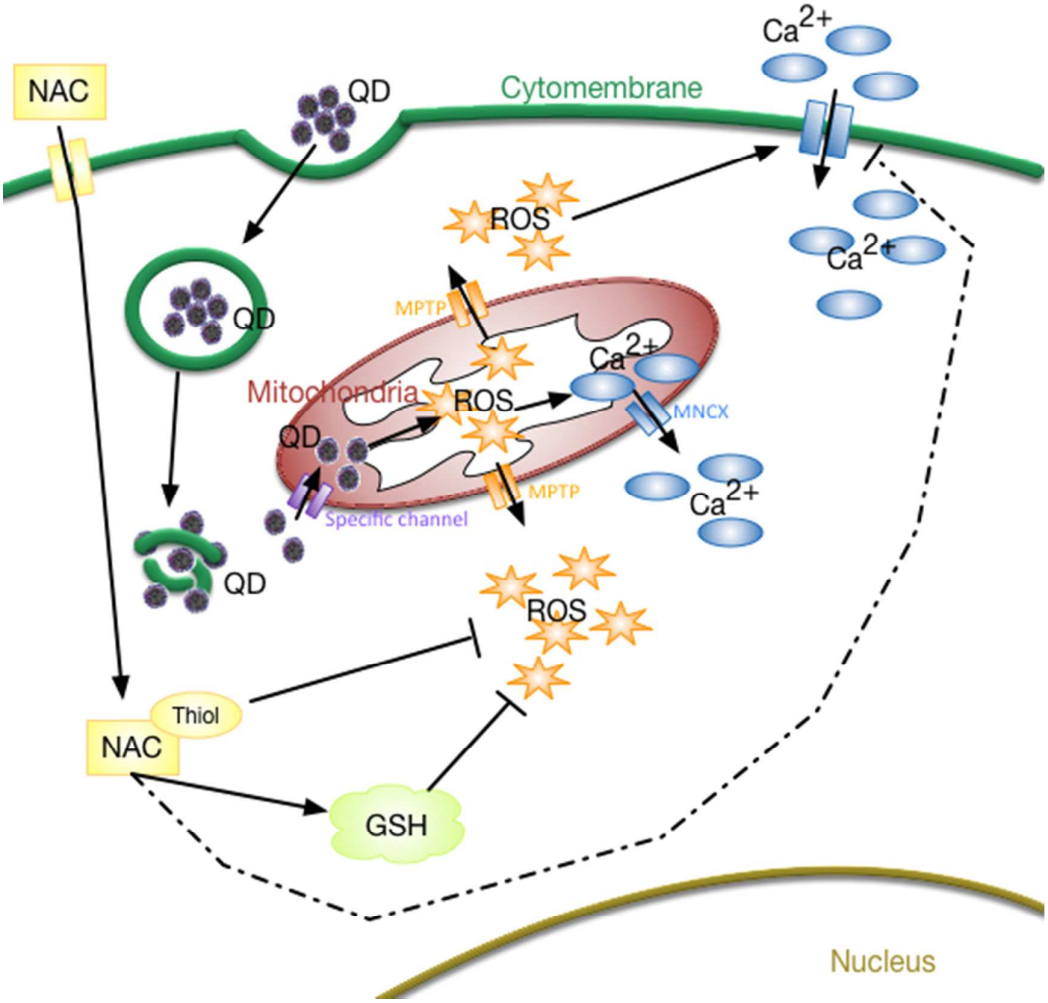
References

1. W. K. Leutwyler, S. L. Bürgi and H. Burgl, *Science*, 1996, **271**, 933.
2. J. L. Pelley, A. S. Daar and M. A. Saner, *Toxicol. Sci.*, 2009, **112**, 276-296.
3. A. Hoshino, S. Hanada and K. Yamamoto, *Arch. Toxicol.*, 2011, **85**, 707-720.
4. B. A. Kairdolf, A. M. Smith, T. D. Stokes, M. D. Wang, A. N. Young and S. Nie, *Annual Review of Analytical Chemistry*, 2013.
5. H. Mattoussi, G. Palui and H. B. Na, *Adv. Drug Del. Rev.*, 2012, **64**, 138-166.
6. L. Shao, Y. Gao and F. Yan, *Sensors*, 2011, **11**, 11736-11751.
7. K. C. Weng, C. O. Noble, B. Papahadjopoulos-Sternberg, F. F. Chen, D. C. Drummond, D. B. Kirpotin, D. Wang, Y. K. Hom, B. Hann and J. W. Park, *Nano Lett.*, 2008, **8**, 2851-2857.
8. I. L. Medintz, H. T. Uyeda, E. R. Goldman and H. Mattoussi, *Nat Mater*, 2005, **4**, 435-446.
9. S. Akhter, I. Ahmad, M. Z. Ahmad, F. Ramazani, A. Singh, Z. Rahman, F. J. Ahmad, G. Storm and R. J. Kok, *Curr. Cancer Drug Targets*, 2013, **13**, 362-378.
10. S. Kato, K. Itoh, T. Yaoi, T. Tozawa, Y. Yoshikawa, H. Yasui, N. Kanamura, A. Hoshino, N. Manabe, K. Yamamoto and S. Fushiki, *Nanotechnology*, 2010, **21**, 335103.
11. K.-T. Yong, W.-C. Law, R. Hu, L. Ye, L. Liu, M. T. Swihart and P. N. Prasad, *Chem. Soc. Rev.*, 2013, **42**, 1236-1250.
12. J. C. Chang, O. Kovtun, R. D. Blakely and S. J. Rosenthal, *Wiley Interdiscip Rev Nanomed Nanobiotechnol*, 2012, **4**, 605-619.
13. G. A. Silva, *Prog. Brain Res.*, 2009, **180**, 19-34.
14. R. Walters, R. P. Kraig, I. Medintz, J. B. Delehanty, M. H. Stewart, K. Susumu, A. L. Huston, P. E. Dawson and G. Dawson, *ASN Neuro*, 2012, **4**, 383-392.
15. J. Lovrić, H. S. Bazzi, Y. Cuie, G. R. Fortin, F. M. Winnik and D. Maysinger, *J. Mol. Med.*, 2005, **83**, 377-385.
16. B. R. Prasad, G. Mullins, N. Nikolskaya, D. Connolly, T. J. Smith, V. A. Gerard, S. J. Byrne, G. L. Davies, Y. K. Gun'ko and Y. Rochev, *Journal of nanobiotechnology*, 2012, **10**, 4.
17. M. Tang, M. Wang, T. Xing, J. Zeng, H. Wang and D. Y. Ruan, *Biomaterials*, 2008, **29**, 4383-4391.
18. M. Tang, T. Xing, J. Zeng, H. Wang, C. Li, S. Yin, D. Yan, H. Deng, J. Liu, M.

Wang, J. Chen and D. Y. Ruan, *Environ. Health Perspect.*, 2008, **116**, 915-922.
19. K. A. Foster, F. Galeffi, F. J. Gerich, D. A. Turner and M. Muller, *Prog. Neurobiol.*, 2006, **79**, 136-171.
20. A. Nel, T. Xia, L. Madler and N. Li, *Science*, 2006, **311**, 622-627.
21. T. Wu and M. Tang, *Inhalation Toxicology*, 2014, **26**, 128-139.
22. H. Wang and J. A. Joseph, *Free Radic. Biol. Med.*, 2000, **28**, 1222-1231.
23. S. Orrenius, M. J. Burkitt, G. E. Kass, J. M. Dypbukt and P. Nicotera, *Ann. Neurol.*, 1992, **32 Suppl**, S33-42.
24. X. Liu, M. Tang, T. Zhang, Y. Hu, S. Zhang, L. Kong and Y. Xue, *PloS one*, 2013, **8**, e59359.
25. N. Macrez and J. Mironneau, *Curr. Mol. Med.*, 2004, **4**, 263-275.
26. M. S. Desole, L. Sciola, M. R. Delogu, S. Sircana, R. Migheli and E. Miele, *Neurochem. Int.*, 1997, **31**, 169-176.
27. B. Gabryel, T. Toborek and A. Malecki, *Neurotoxicology*, 2005, **26**, 373-384.
28. M. Naziroglu, B. Cig and C. Ozgul, *Neuroscience*, 2013, **242**, 151-160.
29. A. P. Stephenson, J. A. Schneider, B. C. Nelson, D. H. Atha, A. Jain, K. F. A. Soliman, M. Aschner, E. Mazzio and R. Renee Reams, *Toxicol. Lett.*, 2013, **218**, 299-307.
30. C. Y. Yan, G. Ferrari and L. A. Greene, *J. Biol. Chem.*, 1995, **270**, 26827-26832.
31. C. Zhang, F. Liu, X. Liu and D. Chen, *Int. J. Dev. Neurosci.*, 2010, **28**, 521-528.
32. I. A. Cotgreave, *Adv. Pharmacol.*, 1997, **38**, 205-227.
33. S. De Flora, A. Izzotti, F. D'Agostini and R. M. Balansky, *Carcinogenesis*, 2001, **22**, 999-1013.
34. J. Liu, Y. Zhang, C. Ge, Y. Jin, S. Hu and N. Gu, *Journal of nanoscience and nanotechnology*, 2010, **10**, 7578-7582.
35. T. Fath, Y. D. Ke, P. Gunning, J. Götz and L. M. Ittner, *Nat. Protoc.*, 2008, **4**, 78-85.
36. G. Brewer, J. Torricelli, E. Evege and P. Price, *J. Neurosci. Res.*, 1993, **35**, 567-576.
37. W. R. Mundy and T. M. Freudenrich, *Neurotoxicology*, 2000, **21**, 1135-1144.
38. I. Parrilla, J. M. Vazquez, C. Cuello, M. A. Gil, J. Roca, D. Di Berardino and E. A. Martinez, *Reproduction*, 2004, **128**, 615-621.
39. T. Suzuki, K. Fujikura, T. Higashiyama and K. Takata, *J. Histochem. Cytochem.*, 1997, **45**, 49-53.
40. G. Xu, S. Mahajan, I. Roy and K. T. Yong, *Front. Pharmacol.*, 2013, **4**, 140.
41. C. J. Wen, L. W. Zhang, S. A. Al-Suwayeh, T. C. Yen and J. Y. Fang, *International journal of nanomedicine*, 2012, **7**, 1599-1611.
42. L. Feng, H. Y. Long, R. K. Liu, D. N. Sun, C. Liu, L. L. Long, Y. Li, S. Chen and B. Xiao, *Cell. Mol. Neurobiol.*, 2013, **33**, 759-765.
43. Q. Zhang, *Methods in molecular biology (Clifton, N.J.)*, 2013, **995**, 179-191.
44. S. S. Minami, B. Sun, K. Popat, T. Kauppinen, M. Pleiss, Y. Zhou, M. E. Ward, P. Floreanig, L. Mucke and T. Desai, *J. Neuroinflammation*, 2012, **9**, 22-22.
45. J. Lee, K. Ji, J. Kim, C. Park, K. H. Lim, T. H. Yoon and K. Choi, *Environ. Toxicol.*, 2010, **25**, 593-600.

46. S. Park, H. Chibli and J. Nadeau, *Journal of visualized experiments : JoVE*, 2012, DOI: 10.3791/3969, e3969.
47. R. D. Rubin, P. D. Watson, M. C. Duff and N. J. Cohen, *Front. Hum. Neurosci.*, 2014, **8**, 742.
48. S. Maday, K. E. Wallace and E. L. Holzbaur, *J. Cell Biol.*, 2012, **196**, 407-417.
49. Y. Yang, K. Xu, T. Koike and X. Zheng, *Autophagy*, 2008, **4**, 243-245.
50. K. G. Li, J. T. Chen, S. S. Bai, X. Wen, S. Y. Song, Q. Yu, J. Li and Y. Q. Wang, *Toxicol. In Vitro*, 2009, **23**, 1007-1013.
51. M. Valko, D. Leibfritz, J. Moncol, M. T. Cronin, M. Mazur and J. Telser, *Int. J. Biochem. Cell Biol.*, 2007, **39**, 44-84.
52. Y.-H. Luo, S.-B. Wu, Y.-H. Wei, Y.-C. Chen, M.-H. Tsai, C.-C. Ho, S.-Y. Lin, C.-S. Yang and P. Lin, *Chem. Res. Toxicol.*, 2013.
53. M. J. Clift, C. Brandenberger, B. Rothen-Rutishauser, D. M. Brown and V. Stone, *Toxicology*, 2011, **286**, 58-68.
54. M. Yan, Y. Zhang, K. Xu, T. Fu, H. Qin and X. Zheng, *Toxicology*, 2011, **282**, 94-103.
55. W. H. Chan, N. H. Shiao and P. Z. Lu, *Toxicol. Lett.*, 2006, **167**, 191-200.
56. K. C. Nguyen, P. Rippstein, A. F. Tayabali and W. G. Willmore, *Toxicol. Sci.*, 2015, **146**, 31-42.
57. H. U. Simon, A. Haj-Yehia and F. Levi-Schaffer, *Apoptosis*, 2000, **5**, 415-418.
58. T. M. Buttke and P. A. Sandstrom, *Immunol. Today*, 1994, **15**, 7-10.
59. S. Hussain, S. Garantziotis, F. Rodrigues-Lima, J. M. Dupret, A. Baeza-Squiban and S. Boland, *Adv. Exp. Med. Biol.*, 2014, **811**, 111-134.
60. I. Pujalte, I. Passagne, R. Daculsi, C. de Portal, C. Ohayon-Courtes and B. L'Azou, *Toxicology Research*, 2015, **4**, 409-422.
61. D. Maysinger and J. Lovric, *Adv. Exp. Med. Biol.*, 2007, **620**, 156-167.
62. B. I. Ipe, M. Lehnig and C. M. Niemeyer, *Small (Weinheim an der Bergstrasse, Germany)*, 2005, **1**, 706-709.
63. A. O. Isaac, I. Kawikova, A. L. Bothwell, C. K. Daniels and J. C. Lai, *Neurochem. Res.*, 2006, **31**, 1305-1316.
64. A. P. Marreilha dos Santos, D. Santos, C. Au, D. Milatovic, M. Aschner and M. C. Batoreu, *Brain Res.*, 2008, **1236**, 200-205.
65. J. Sagara, S. Bannai, N. Shikano and N. Makino, *Neuroreport*, 2010, **21**, 416-421.
66. R. Shrestha, 3537305 Ph.D., Texas A&M University, 2012.
67. E. Y. Chen, M. Garnica, Y. C. Wang, C. S. Chen and W. C. Chin, *PLoS One*, 2011, **6**, e16198.

Graphical abstract:



CdTe QDs exposure caused death and apoptosis of rat primary cultured hippocampal neurons via generating reactive oxygen species and increasing intracellular calcium levels, which could be reversed completely and partially by a common antioxidant N-acetylcysteine (NAC), respectively.