

Cellular Localization and Toxicity Assessment of CdTe-COOH Quantum Dots in HeLa Cells

Anahí Rodríguez-López¹, V Agarwal², Jorge Reyes-Esparza¹ and Lourdes Rodríguez-Fragoso^{1*}

¹Facultad de Farmacia, Universidad Autónoma del Estado de Morelos, Av Universidad 1001, Col Chamilpa, Morelos 62209, Mexico

²Centro de Investigación en Ingeniería y Ciencias Aplicadas, Universidad Autónoma del Estado de Morelos, Av Universidad 1001, Col Chamilpa, Morelos 62209, Mexico

*Corresponding author: Dra. Lourdes Rodríguez Fragoso, Facultad de Farmacia, Universidad Autónoma del Estado de Morelos, Col. Chamilpa, Cuernavaca, Morelos CP 62209, Mexico, Tel: (777) 3297000 ext 2321; E-mail: mrodriguezf@uaem.mx

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Abstract

Purpose: In order to determine material toxicity, we treated cells at low concentrations for both short and long time periods, and examined cell death and genotoxic effects.

Materials and Methods: CdTe QDs functionalized with carboxyl were evaluated on HeLa cells. To determine cytotoxicity, we treated cells at 0.1 to 1000 ng/mL for 1 and 5 days, and examined cell death and genotoxic effects. Cellular and sub cellular localization of QDs was also studied.

Results: Present results demonstrate that CdTe-COOH QDs at high concentrations (1000ng/mL) are cytotoxic in HeLa cells. CdTe-COOH QDs induced apoptosis and necrosis (>100 ng/mL) at 5 days. The *in vitro* results showed that cells incorporated QDs efficiently. QDs were found in cytoplasm and nucleus and the intensity of fluorescence and cell distribution was in a dose-dependent manner. Present results demonstrated that CdTe-COOH QDs were genotoxic at concentrations higher to 100 ng/mL and when cells were treated for long time periods.

Conclusion: The CdTe-COOH QDs used in this study could be used for imaging applications in bio medicine, but only at low concentrations and over short time periods. But additional studies are needed to confirm whether long-term exposure in other experimental model can lead to increased toxicity.

Keywords: Quantum dots; CdTe; Cytotoxicity; Genotoxicity; Apoptosis

Abbreviations

AU: Arbitrary units; CdTe: Cadmium telluride; CdTe-COOH: Carboxylated cadmium telluride; DMSO: Dimethyl sulfoxide; DNA: Deoxyribonucleic acid; EDTA: Ethylenediaminetetraacetic acid; HCl: Hydrochloride acid; H₂O₂: Hydrogen peroxide; MTT: Methyl tetrazolium; NaOH: Sodium hydroxide; PBS: phosphate buffered saline; PDT: Proliferation duplication time; QDs: Quantum Dots; ROS - Reactive Oxygen species

Introduction

Nanotechnology has been highly influential across different fields, prompting substantial progression a relatively short time. In biomedicine, nonmaterial's can be potentially used as tools for immunohistochemical detection and bioimaging, as biosensors and new modes of drug delivery [1-3]. Many research laboratories currently work with nonmaterial's, which results in greater occupational exposure and, certainly, greater environmental pollution [4]. However, knowledge of their toxic potential is limited and there is no appropriate regulatory measures regarding their use [5].

Quantum dots (QDs) are semiconductor nanomaterials with particular optical and physicochemical properties. They are synthesized with different sizes and coating, so current research has focused on how these properties affect their fate and how they interact with their cellular environment [6-8]. CdTe QDs has shown evidence of cytotoxicity *in vitro* [9-12]. Nevertheless, there is little information regarding their potential effect on cellular systems and how this is affected by concentration, exposure time and functionalization. Short- and long-term dose-response pharmacological studies of new molecules are necessary in order to know whether there is accumulation or toxicity, and if these molecules can be potentially used in humans.

Cadmium selenide or cadmium telluride particles are considered the most suitable emitting 'core' materials because of their bright emission

in the visible range and near the infrared region of the electromagnetic spectrum [13-15]. However, there are problems regarding suitable capping agents, retention of particles over a certain size, biological magnification, and the breakdown and decomposition products of these inorganic materials. Protecting the core can, to some degree, control the toxicity related to cadmium and selenium leakage. However, the change in the physicochemical and structural properties of engineered quantum dots could be responsible for a number of material interactions that could also have toxicological effects.

This study employed CdTe quantum dots (QDs) with a carboxyl-group for the surface coating (CdTe-COOH QDs) and evaluated their cytotoxicity on HeLa cells. In order to determine cytotoxicity, we treated cells at 0.1 to 1000 ng/mL for short and long time periods, and examined cell death and genotoxic effects. Cellular and sub cellular uptake were also studied.

Materials and Methods

Quantum dots (QDs)

CdTe QDs functionalized with carboxyl (QD-COOH, 777935 Sigma Chemical Co., USA) group were purchased from Sigma-Aldrich. The quantum dot wavelength was λ_{em} 520nm. The concentration of QD stock solution was 100 μ g/mL. Before dilution, QD stock solutions were mixed for 20 min, followed by a short centrifugation at low rpm to remove particles from the tube lids.

Cell culture

HeLa cells (cervix adenocarcinoma) (ATCC[®] No. CCL-2[™]) were cultured in RPMI (GIBCO, USA), with 10% FBS (GIBCO, USA) and 100 U/ml penicillin/100 µg/ml streptomycin (GIBCO, USA), in a humidified 5% CO₂ atmosphere at 37°C.

Cell viability and cell proliferation assays

Cell viability and cell proliferation were determined using a MTT (methyl tetrazolium, Sigma Aldrich, USA) assay [16]. For cell viability, HeLa cells were seeded into a 96-well plate (10,000/well) and incubated for 24 h at 37°C and 5% CO₂. The culture medium was substituted by a new one supplemented with different concentrations of CdTe-COOH QDs (0.1, 1, 10, 100 and 1000 ng/mL) and incubated for 24 h. After treatment, the medium was gently removed and replaced with 20 µL MTT (5 mg/mL) and 150 µL of non-phenol-red medium, and incubated for 4 h. Medium from each well was discarded, followed by the addition of 200 µL DMSO and 25 µL Sorensen's glycine buffer (glycine 0.1 M, NaCl 0.1 M, pH 10.5) to each well. When the formazan crystals were dissolved, the optical density was determined on a micro plate reader (Bio-Rad) at a wavelength of 590 nm. Untreated cells served as non-treated cell viability control. The results were graphed as percentage of the relative viability of cells.

For cell proliferation, HeLa cells were seeded into a 96-well plate (1,000/well) and incubated for 24 h and then treated as described above everyday for 5 days. In order to know the proliferation duplication time (PDT) we used the following formula:

$$PDT = \frac{\ln(n/N_0)}{t}$$

Where:

N₀ = Initial number of cells

N = Final number of cells

T = Interval of time between N₀ and n

Assessment of cell death by fluorescence microscopy

Cell death analysis was undertaken using acridine orange and ethidium bromide-staining assay, as previously described [17]. Briefly, HeLa cells were prepared into a 6-well plate (250,000 cells/well) and incubated for 24 h at 5% CO₂ and 37°C. The culture medium was changed for a fresh medium containing CdTe-COOH QDs at 0.1, 1, 10, 100 and 1000 ng/mL, and the cells were incubated for another 24 h. Cells were washed with DPBS, and 250 µL of a mixture of acridine orange/ethidium bromide (100 µg/mL each one) (Sigma Aldrich, USA) were added to each well. The cells were maintained at room temperature for 10 seconds and examined under a fluorescence microscope. Images of fluorescent cells were photographed with an Olympus digital camera. The data represents the average number of live, apoptotic or necrotic cells observed across at least 15 images for each treatment. Cells incubated in culture medium without QDs were used as control. 1 µL/mL of 30% H₂O₂ served as apoptosis control and smashed cells were used as necrosis control. Cells were classified as normal (green cells), apoptotic cells (yellow-orange cells), or necrotic cells (orange-red cells).

Analysis of CdTe-COOH QDs absorption in HeLa cell

HeLa cells were used to verify the absorption of CdTe-COOH QDs into cells. The cells (10⁵) were plated onto 20mm sterile cover slips in a 6-well plate. The cells were incubated for 24 h, washed with phosphate buffered saline (PBS), and then treated with CdTe-COOH QDs (0.01, 1, 10, 100 and 1000 ng/mL) for 24 h. After washing with PBS, the cells were fixed with 200 µL of 4% for 20 min. After a time, paraformaldehyde was removed and cells were washed again. The cover slip with fixed cells was covered with a glass slide with a drop of 10 µL of 50% glycerol/PBS

(v/v) and observed with a confocal microscope (Nikon AI, Nikon, Japan). CdTe-COOH QDs were excited with 488 nm laser, and the emission of fluorescence was at 515 nm. In order to analyze the cellular absorption of CdTe-COOH QDs, the cover slip with fixed cells was examined under fluorescence microscopy and using Image-Pro Insight 9 software (Media Cybernetics Inc.).

Genotoxicity study

We used the comet assay to evaluate whether CdTe-COOH QDs induce DNA damage in HeLa cells [18]. Cells were cultured in RPMI-1640, supplemented with nonessential amino acids, 10% FCS, L-glutamine (2 mol/L), and antibiotics (100 U/mL penicillin and 100 mg/ml streptomycin). 10⁵ viable cells were seeded in a six well plate and maintained at 37°C under an atmosphere of 5% CO₂ and 95% air. Cells were treated with CdTe-COOH QDs at 0.1, 1, 10, 100 and 1000 ng/mL for 24 h. Other cells were treated with hydrogen peroxide 300 µM and used as positive control. Cells were harvested using 5 min centrifugation at 1000 rpm, washed with PBS for 20 min on ice, and then washed twice with PBS. Slices were incubated overnight in a lysis solution (30 mM NaOH, 1,2 M NaCl, 1% (w/v) laurylsarcosine, 0.05% triton × 100, 1% DMSO, pH 12.4). The slices were washed for 20 min each in electrophoresis buffer (30 mM NaOH, 10 mM EDTA, pH 12.4) to remove lysis solution and allow the unfolding of the nuclear DNA. The samples were subjected to agarose gel electrophoresis for 20 min at 25 V and 300 mA. After electrophoresis, the gels were neutralized in 400 mM Tris-HCl, pH 7.5 for 15 min. The slices were stained with ethidium bromide (10 µg/mL) and 100 randomly selected nuclear images of each slice were observed with an epifluorescent microscope (Olympus I×81) using a 515-560 nm excitation filter at 100 × magnification. For DNA damage analysis, 100 cells were analysed per slice. Different damage degrees were assigned based on the intensity of the comet tail (Class 0 = no damage; Class 1 = mild damage; Class 2 = moderate damage; Class 3 = high damage; and Class 4 = severe damage). The percentage of cells with damage was calculated and an arbitrary unit (AU) was used to express the extent of DNA damage.

Statistical analysis

The data are presented as the mean ± SD of 3 independent experiments conducted with eight replicates. The data was statistically analyzed using the SPSS 10.0 software (SPSS Inc., Chicago, Ill., USA), the *t*-test and ANOVA. Differences were considered significant if the p < 0.05.

Results

As we can see in Figure 1, CdTe-COOH QDs did not produce significant changes in viability when measured by MTT assay. Concentrations of 1000 ng/mL were accompanied by a tendency toward decreased cell viability, but this was not statistically significant. However, due to the observed cytotoxic effect with the higher concentration at 24 h, we decided to do another other assay in order to characterize the lethal effects produced by CdTe-COOH QDs in HeLa cells. Acridin orange/ethidium bromide staining was employed to differentiate between healthy cells and damaged cells. The microscopic analysis revealed the absence of cell death in HeLa cells treated with 0.01 to 100 µg/mL CdTe-COOH QDs, but those treated with 1000 ng/mL at 24 h showed numerous apoptotic cells. On the other hand, cells treated with CdTe-COOH QDs for 5 days showed a scattering of apoptotic and necrotic cells when they were treated with 100 ng/mL; concentrations of 1000 ng/mL led to numerous necrotic cells (Figure 2).

CdTe-COOH QD absorption was analyzed using a fluorescence microscope in cells incubated with 0.1 to 1000 ng/mL of CdTe-COOH QDs for 24 h and then washed to remove any QDs not incorporated into the cells. The morphology of the untreated epithelial HeLa cells was like the usual epithelial cells and showed a light fluorescence. Interestingly, almost all the treated cells revealed a uniform fluorescence pattern under

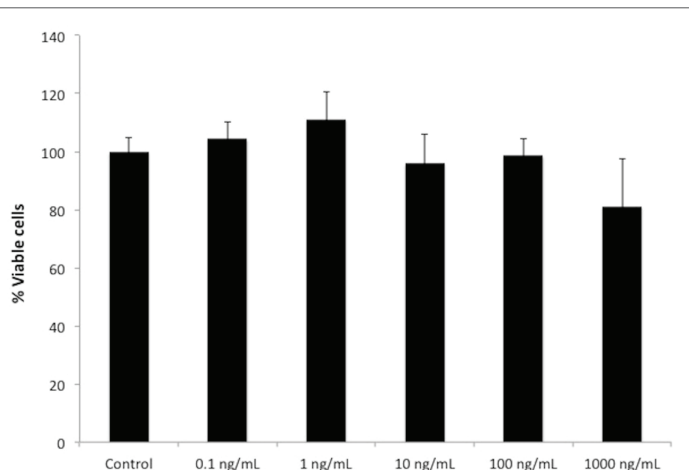


Figure 1: Effects of CdTe-COOH on cell viability of HeLa cells. Cells were exposed in culture medium using different concentrations of CdTe-COOH QDs for 24 h. Results are expressed as the percentage of cell viability as compared to the control group. Data are presented as the mean \pm SD of at least three independent experiments (n=8). *p < 0.05 as compared with control group.

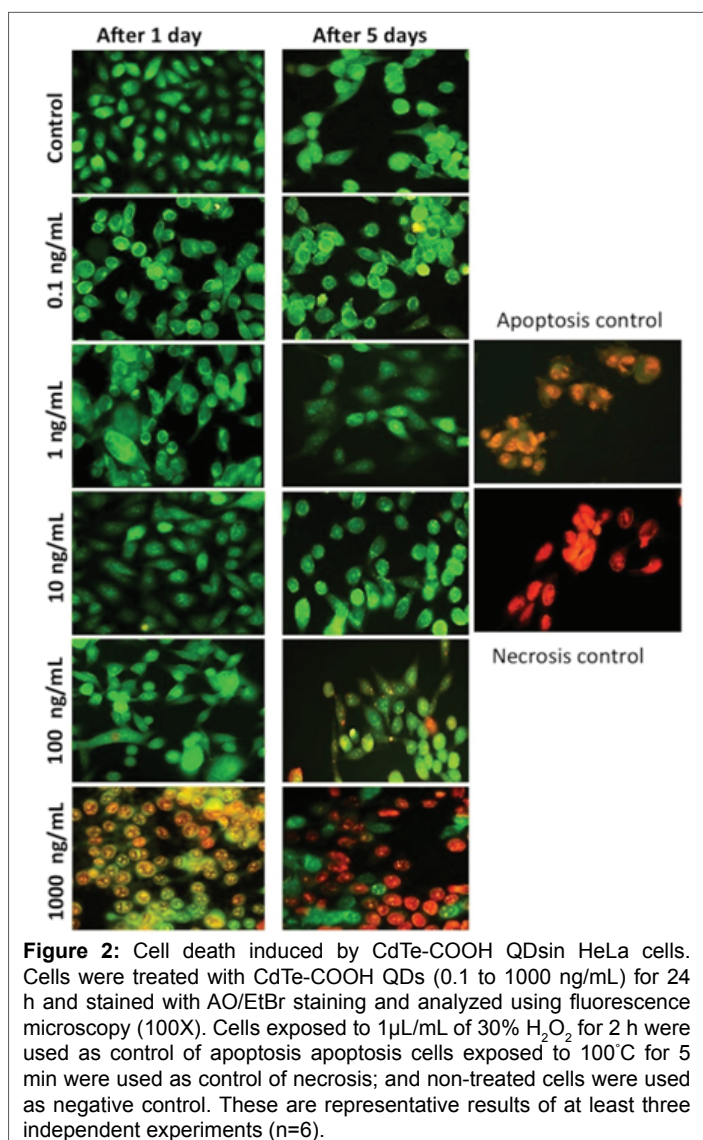


Figure 2: Cell death induced by CdTe-COOH QDs in HeLa cells. Cells were treated with CdTe-COOH QDs (0.1 to 1000 ng/mL) for 24 h and stained with AO/EtBr staining and analyzed using fluorescence microscopy (100X). Cells exposed to 1 μ L/mL of 30% H₂O₂ for 2 h were used as control of apoptosis; cells exposed to 100°C for 5 min were used as control of necrosis; and non-treated cells were used as negative control. These are representative results of at least three independent experiments (n=6).

confocal fluorescence microscope (Figure 3). Further analysis of this fluorescence showed it was dose dependent (Figure 3). In cells treated with 0.1 ng/mL, fluorescence was mainly observed in the cytoplasm; but in cells treated with 1, 10 and 100 ng/mL, fluorescence was observed in both the cytoplasm and nucleus. However, in cells treated with 1000 ng/mL, QDs were absorbed more thoroughly and fluorescence was more intense in the nucleus of some cells.

The analysis of cell proliferation in HeLa cells showed different effects depending on the concentration of CdTe-COOH QDs; lower concentrations (0.1 and 1 ng/mL) increased (5-20%) cell proliferation at 5 days (p<0.05). Cells incubated with 10 and 100 ng/mL produced a significant reduction of cell proliferation on the third and fourth day; however, there was increased cell proliferation (7-24%) (p<0.05) on the fifth day. Higher concentrations (1000 ng/mL of CdTe-COOH QDs) led to non-cell proliferation for all 5 days of treatment (Figure 4), p<0.05.

To evaluate whether CdTe-COOH QDs can induce DNA damage, we employed the comet assay. In the control group (untreated cells), damaged remained within the 0 and 1 categories, meaning no damage or mild damage. The positive control group (H₂O₂) showed ordinary DNA injury

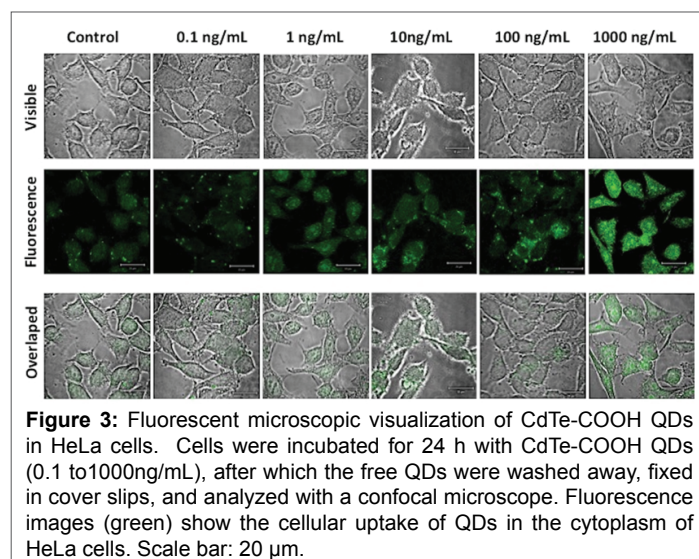


Figure 3: Fluorescent microscopic visualization of CdTe-COOH QDs in HeLa cells. Cells were incubated for 24 h with CdTe-COOH QDs (0.1 to 1000 ng/mL), after which the free QDs were washed away, fixed in cover slips, and analyzed with a confocal microscope. Fluorescence images (green) show the cellular uptake of QDs in the cytoplasm of HeLa cells. Scale bar: 20 μ m.

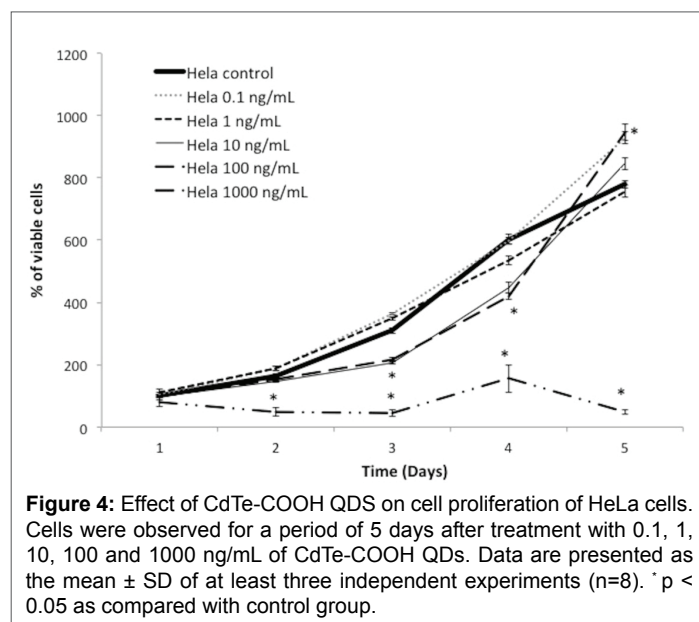


Figure 4: Effect of CdTe-COOH QDs on cell proliferation of HeLa cells. Cells were observed for a period of 5 days after treatment with 0.1, 1, 10, 100 and 1000 ng/mL of CdTe-COOH QDs. Data are presented as the mean \pm SD of at least three independent experiments (n=8). *p < 0.05 as compared with control group.

of 46% and included all categories of DNA damage, proving the sensitivity of the method. HeLa cells ($AU 2.25 \pm 0.7$ and 4.25 ± 2) treated with CdTe-COOH QDs at 0.1 and 1 ng/mL showed no damage. At 10 ng/mL, the damage registered in the 1 and 2 categories ($AU 11.5 \pm 3.8$) (Figure 5, Table 1) ($p < 0.05$). However, in cells incubated with 100 and 1000 ng/mL, the recorded damage was mainly 1 and, to a lesser extent, 2 and 3 when the cells were treated for 24 h ($AU 18.7 \pm 2$ and 24.5 ± 2.8) ($p < 0.05$). On the other hand, cells incubated with CdTe-COOH QDs for 5 days showed a larger number of cells in the 4 category (Table 1).

Discussion

As happens with other toxic agents, QD cytotoxicity depends on the physicochemical properties of each nanomaterial [19]. This diversity in physicochemical properties reflects in our current inability to reproduce observed results across different experimental models. Consequently, we require more toxicological research to demonstrate their safety. Most extant CdTeQD toxicity research includes reports of a wide variety of new QDs recently synthesized with different surface coatings, and these have

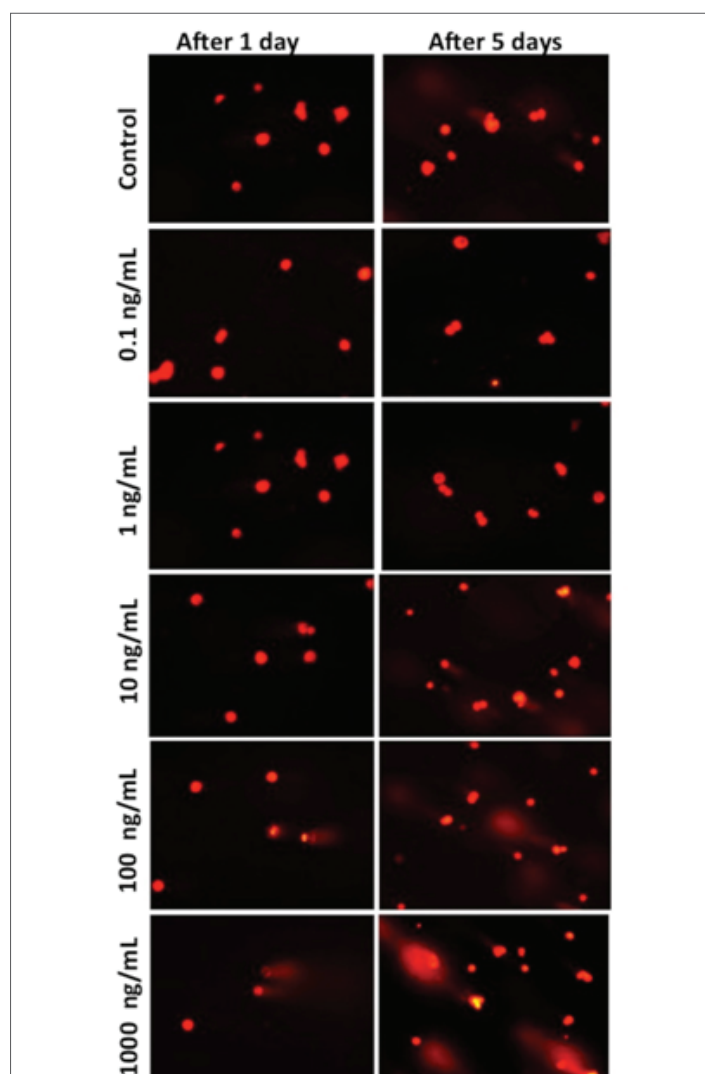


Figure 5: Analysis of β -HCP genotoxicity. HeLa cells were treated with CdTe-COOH at 0.1, 1, 10, 100 and 1000 ng/mL for 1 or 5 days. H_2O_2 300 μ M was used as positive control (control +). Results are expressed as average percentage of tail DNA \pm d.s. Data are presented as the mean \pm SD of at least three independent experiments ($n = 6$). * $p < 0.05$ as compared with control group.

GROUP	DNA damage at 1 day				AU
	0	1	2	3	
Negative	96	4	0	0	0.5 ± 1.41
Positive	0	0	0	32	$46 \pm 2.82^*$
0.1 ng/mL	82	18	0	0	$2.25 \pm 0.70^{**}$
1 ng/mL	80	12	4	2	$4.25 \pm 2.12^{**}$
10 ng/mL	56	16	16	4	$11.5 \pm 8.48^{**}$
100 ng/mL	38	26	6	8	$18.75 \pm 2.12^{**}$
1000 ng/mL	28	28	2	4	$24.5 \pm 2.82^{**}$
GROUP	DNA damage at 5 days				AU
Negative	98	2	0	0	
Positive	0	0	0	84	$48 \pm 1.41^*$
0.1 ng/mL	82	14	4	0	$2.75 \pm 0.70^{**}$
1 ng/mL	78	10	6	2	$5.25 \pm 3.53^{**}$
10 ng/mL	50	14	10	14	$15.75 \pm 0.70^{**}$
100 ng/mL	34	24	4	30	$22.25 \pm 0.70^{**}$
1000 ng/mL	20	16	6	50	$31.5 \pm 1.41^{**}$

Table 1: Analysis of CdTe-COOH genotoxicity

* $p < 0.05$ as compared with control group

** $p < 0.05$ as compared with positive control

been studied in different cell lines under a wide range of experimental conditions [20-23]. Surface modifications may not provide sufficient protection from cytotoxicity, as present results demonstrate. There are currently no reports regarding the cytotoxicity of CdTe QDs functionalized with carboxyl surface coating. Our studies agree with others on the fact that cells with different tissue origin have varying thresholds for CdTeQD-induced toxicity. It is evident that surface coating is related to the degree of toxicity observed in cells. This study shows evidence of the cellular distribution and the cytotoxic effect produced by CdTe-COOH QDs on HeLa cells.

It has been suggested that most of QDs, including CdTe QDs, induce oxidative stress. For example, studies using endothelial and fibroblast cells have shown that CdTe QDs-mediated mitochondrial-dependent apoptosis is accompanied by the presence of reactive oxidative species (ROS) [24,25]. Lai et al. have suggested that degradation of CdTe in lysosomes and lysosomal destabilization induce cell necrosis [26]. Recent *in vivo* studies using CdTe QDs have also shown that QD-induced damage is time-dependent and reversible. Those effects have been associated with ROS generation *in vivo* [27].

The fluorescent properties of CdTeQDs allow us to directly observe their diffusion and distribution into the cells. The role of the surface coating on intracellular distribution has been reported before [28]. QD-induced cellular disorders might trigger several pathophysiological processes depending on concentration and exposure duration [29]. The toxic consequences of CdTe QDs on cells are not only the result of free cadmium ions but also the molecular events generated by the intracellular presence of QDs in cells. It has been reported that CdTeQDs reduce the expression of important proteins that control the cell cycle, affecting cell division [29]. CdTe QDs functionalized with gambogic acid have displayed the ability to internalize cancer cells and inhibit cell proliferation [30].

The genotoxicity of cadmium-based quantum dots has been previously reported [31-33]. Cadmium ions are known to induce DNA damage and, for this reason, it is important to research the effect of cadmium-based QDs [34]. Several reports have shown that QDs with different coating induce oxidative stress, and it has been suggested that ROS generation is a consequence of the photo-activation of QDs, which would have catastrophic effects on DNA [35]. CdTe QDs and cadmium ions can activate heat shock protein $_{70}B'$ promoter and induce the expression of metal-responsive genes [36], as well as the formation of molecular

intermediates for DNA double-strand breaks [37]. Although it has been reported that functionalized CdTe QDs generated much less DNA damage when compared with CdTe [38], the present study showed that CdTe-COOH were genotoxic to HeLa cells, giving evidence of the genotoxicity of CdTe-COOH QDs.

Conclusions

Our data indicate that CdTe-COOH QDs have cytotoxic and genotoxic effects and those they may affect cell proliferation in HeLa cells. Present results indicate that CdTe-COOH QDs induce dose-dependent effects. Like other nanomaterials, CdTe-COOH QDs only induce cytotoxic and genotoxic at high concentrations (<100 ng/mL). Therefore, the CdTe-COOH QDs used in this study can be potentially employed in biomaging. That said, additional studies using animal models are needed in order to find out if long-term exposure can lead to increased toxicity *in vivo*.

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