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Article *in* Journal of Nanoscience and Nanotechnology · December 2010

DOI: 10.1166/jnn.2010.2681 · Source: PubMed

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Journal of
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Bioeffects of CdTe Quantum Dots on Human Umbilical Vein Endothelial Cells

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Quantum dots (QDs) hold great potential for applications in nanomedicine, however, their health effects are largely unknown. In the present study, the cytotoxicity and genotoxicity of CdTe QDs were examined in human umbilical vein endothelial cells (HUVECs). The QDs exhibited a dose-dependent inhibitory effect on cell growth. It was shown that after a 12 h treatment QDs at 1, 10, and 50 $\mu g \cdot ml^{-1}$ induced formation of γH2AX foci, indicative of DNA damage, in a dose-dependent manner. Moreover, QD treatment clearly induced the generation of reactive oxygen species (ROS). Pre-treatment with *N*-acetyl-cysteine (NAC), a ROS scavenger, could inhibit the induction of ROS by QDs, as well as the formation of γH2AX foci. Taken together, our data indicate that CdTe QDs have cytotoxic and genotoxic effects on HUVECs, and that ROS generation may be involved in QD induced DNA damage.

Keywords: Quantum Dots, γH2AX, DNA Damage, Reactive Oxygen Species.

1. INTRODUCTION

Quantum dots (QDs) are semiconductor nanocrystals with unique optical and electrical properties. They have applications ranging from biomedical imaging to the electronics industry. Although potentially offering great benefit, QDs may also pose risks to human health and the environment under certain conditions. *In vivo* studies have shown QDs could be systemically distributed and may accumulate in organs and tissues.[1,2] Several *in vitro* and *in vivo* studies have demonstrated a lack of evidence for QD-induced cytotoxicity.[3–7] However, other studies have shown that QDs can affect cell growth and viability.[8–11] For example, CdTe QDs are found in the vicinity of the nucleus after 24 h incubation with MCF-7 cells, causing shrinkage and deformation of the nuclei and chromatin aggregation.[10] Cells treated with QDs also exhibit swelling, rounding of mitochondria and enlarged, fused lysosomes.[10] In addition, CdSe/ZnS QDs can induce DNA nicking,[12] although the mechanism for this remains unknown.[13] With the rapid development of QD-related products, it is important to address their potential risks to health.

Accumulating evidence suggests that exposure to ultrafine particulate air pollution (<100 nm) is linked to increased incidence of cardiovascular disease.[14–16] Even though the mechanisms leading to increased cardiovascular risks have not been fully elucidated, it is believed that endothelial cell dysfunction and inflammation is a key step toward the progression of vascular diseases.[17,18] The possible cardiovascular effects of QDs, in particular on endothelial cells, are of great concern given that one of the most important potential applications for QDs is *in vivo* imaging.

Previously it has been reported that CdSe/ZnS QDs can induce DNA nicking in an aqueous solution, probably through the generation of free radicals.[12] This raises the question of whether QDs can damage DNA in live cells, therefore proving genotoxic to cells. DNA double strand breaks (DSBs) are regarded as one of the most severe types of DNA damage. It has been shown that once DSBs are formed, the histone variant H2AX is rapidly phosphorylated (termed γH2AX) by members of the phosphatidylinositol 3-kinase family (PI-3K) and form "foci" at sites of DSBs.[19] γH2AX is then responsible for the recruitment of many other repair or checkpoint proteins to the damaged sites, such as the Mre11/Rad50/Nbs1 (MRN) complex, BRCA1, and 53BP1 among others.[20] It has been further demonstrated that the number of γH2AX foci detected by immunofluorescence is quantitatively the same as that of DSBs;[21] hence, measuring γH2AX foci formation is a sensitive method for detecting DSBs.[22,23]

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 1533-4880/2010/10/8591/006 doi:10.1166/jnn.2010.2681

In addition, reactive oxygen species (ROS) are known to induce DNA damage, and it has been suggested that ROS play important roles in vascular endothelial injury.[24, 25] Interestingly, QDs have been shown to induce ROS, which are involved in QD-induced cell death.[10, 26, 27] Thus, in this study we used human umbilical vein endothelial cells (HUVECs) as the model system to investigate the bioeffects of CdTe QDs, in particular their genotoxic effects and the roles of ROS on vascular endothelial cells. It is worth mentioning that there is not clear evidence that QD induced damage in endothelial cells *in vitro* correlates with increased incidence of cardiovascular diseases. Therefore, further studies are needed to confirm the cardiovascular toxicity of QDs using *in vivo* assays.

2. EXPERIMENTAL DETAILS

HUVEC cells were obtained from ATCC and routinely subcultured in 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum at 37 °C with 5% humidified CO_2.

2′,7′-dichlorofluorescein diacetate (DCFH-DA), 4,6 diamidino-2-phenylindole (DAPI), *N*-acetylcysteine (NAC), *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), 3-(4,5)-dimethylthiazo (-z-y1) -3, 5 - di-phenytetrazoliumromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). Mouse monoclonal antibody against γH2AX was purchased from Upstate Technology (Lake Placid, NY). FITC-conjugated goat anti-mouse IgG and goat blocking serum were obtained from Beijing Zhongshan Biotechnology Co., China.

CdTe QDs ($\lambda_{em} = 664$) were provided by Institute of Macromolecular Science, Fudan University, with mean diameters of 3.7 nm. For water solubilization, the CdTe QDs were surface coupled with mercaptopropionic acid (MPA) and then suspended in ddH_2O.

The percentage of cell survival was measured using the MTT colorimetric assay. The absorbance was measured at 595 nm using a microplate reader (Infinite M200, Tecan AG, Mannedorf, Switzerland).

To study the effects of QDs on the phosphorylation of H2AX, cells were treated with different concentrations (1, 10, and 50 $\mu g \cdot ml^{-1}$) of QDs for 12 h. Immunofluorescence microscopy was conducted in basically the same manner as described before.[23] In short, 2×10^5 cells were seeded into a six-well culture plate containing a glass cover slip in each well. After treatment, cells were fixed in 4% paraformaldehyde for 15 min, washed with PBS, and permeabilized in 0.2% Triton X-100. After blocking with blocking serum for 1.5 h, samples were incubated with a mouse monoclonal anti-γH2AX antibody (1:1000) for 2 h, followed with FITC-conjugated goat-anti-mouse secondary antibody (1:500) for 1 h. To stain the nuclei, DAPI was added to the cells and incubated for another 15 min. The cover slip was then removed from the plate and mounted on to a glass slide, and observed with an Olympus AX70 fluorescent microscope (Olympus, Tokyo, Japan).

To prevent bias in selection of cells that display foci, all the cells were counted in the field of vision (at least 30 cells). Image Pro Plus (Media Cybernetics, Silver Spring, MD) was used to count the γH2AX foci in each cell. In addition, to exclude relatively weak foci and background spots we used a setting as a standard for quantification in all the cells selected for analysis.[28]

After different treatments, cells (1×10^6) were harvested, fixed in 4% paraformaldehyde for 15 min, washed with PBS, and permeabilized in 0.2% Triton X-100. After blocking with blocking serum for 1.5 h, samples were incubated with a mouse monoclonal anti-γH2AX antibody (1:1000) for 2 h, followed with FITC-conjugated goat-anti-mouse secondary antibody (1:500) for 1 h. Then the fluorescence intensity of FITC was measured using a Beckman Coulter Epics XL-MCL device (Fullerton, CA).

ROS were measured in arbitrary units using DCFH-DA as described previously.[29] 1×10^5 cells were incubated in a 24-well culture plate. After QD treatment, cells were washed, and medium was replaced with serum-free medium. Dihydroethidium was dissolved in methanol (10 $mmol \cdot L^{-1}$) and added to the culture medium at a final concentration of 20 $\mu mol \cdot L^{-1}$, and the cells were incubated for 30 min at 37 °C. Cells were washed with PBS, and fluorescence was then determined at 485 nm excitation and 520 nm emission wavelengths using a microplate reader.

Each experiment was conducted at least three times, data are presented as mean ± S.D. Statistical analysis of quantification of γH2AX foci was performed with χ^2 test and single factor variance analysis. The comparison of ROS levels was done with single factor variance analysis and Student's *t*-test. A probability level of $P < 0.05$ was considered significant.

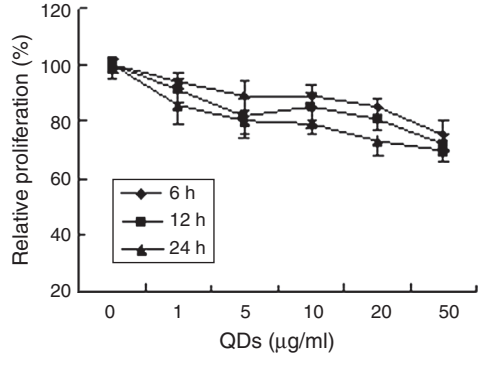
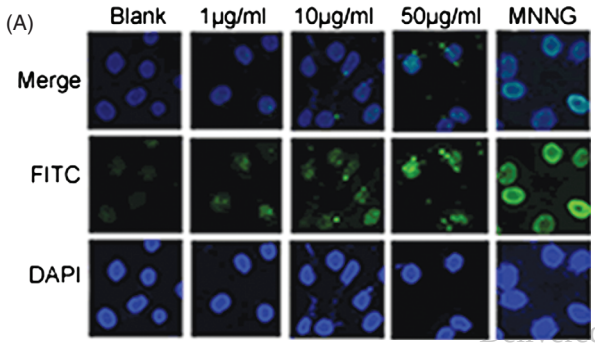


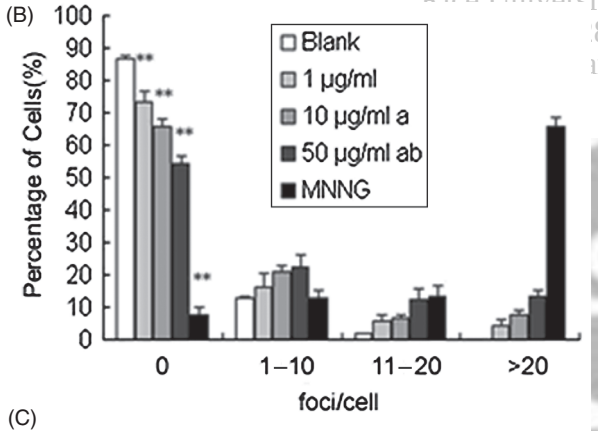
Fig. 1. The cytotoxic effects of QDs on HUVECs. Cells were treated with different concentrations (1, 5, 10, 20, and 50 $\mu g \cdot ml^{-1}$) of QDs, and the MTT assay was conducted at 6 h, 12 h and 24 h to measure cell viability.

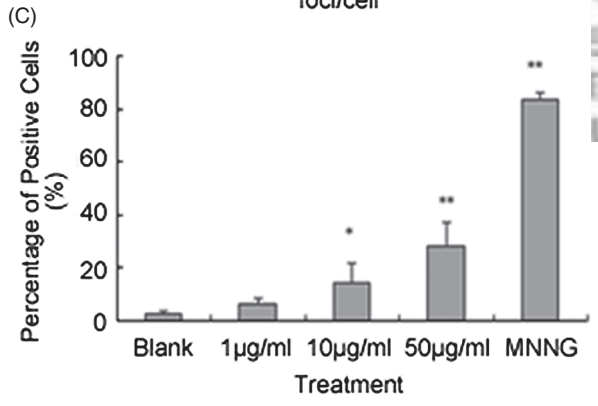
3. RESULTS

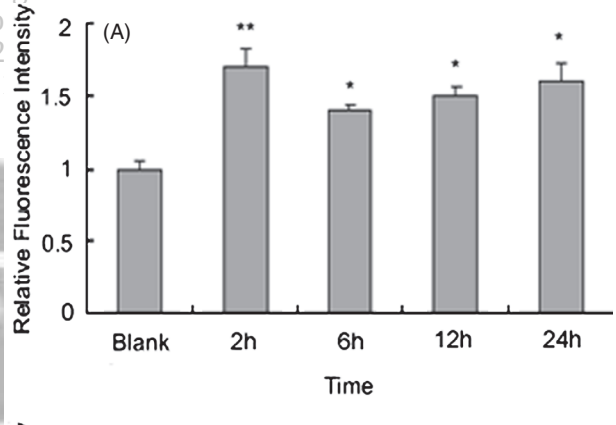
HUVECs were treated with increasing concentrations of QDs (1, 5, 10, 20, and 50 $\mu g \cdot ml^{-1}$) for 6, 12 and 24 h, and then cell viability was measured using an MTT assay. Even though the QDs exhibited a dose-dependent inhibitory effect on cell growth, cell viabilities at the five concentrations were all above 70%. The lowest cell viability at all three time points was observed for the highest treatment concentration (50 $\mu g \cdot ml^{-1}$) (Fig. 1). Based on these results, three concentrations (1, 10, and 50 $\mu g \cdot ml^{-1}$) were chosen for further analyses.

Fluorescent microscopy was employed to observe the formation of γH2AX. MNNG treatment (10 $\mu g \cdot ml^{-1}$) for 1 h is known to induce γH2AX foci formation and was used as positive control.[29] Figure 2(A) presents representative images of γH2AX foci formation following QDs treatment and shows that QDs induce γH2AX foci formation in a dose-dependent manner. Detailed analyses (Fig. 2(B)) revealed that more than 85% of the negative control cells did not contain any foci, and less than 15% of the negative control cells had 1–10 foci. All three concentrations of QDs significantly increased the percentage of cells containing foci as well as the number of foci per cell.









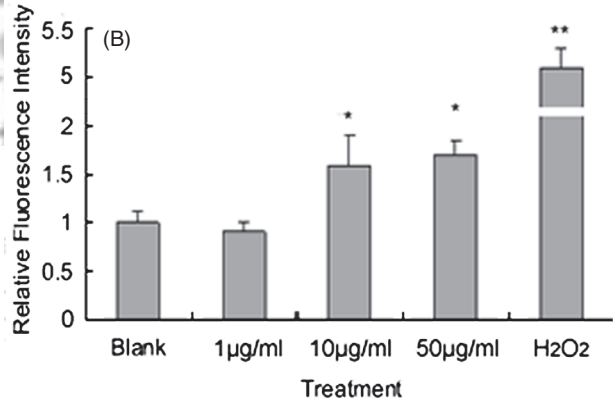


Fig. 2. QDs induce γH2AX foci formation in HUVECs. After various QD treatments, cells were fixed and stained with anti-γH2AX antibody, and subjected to immunofluorescence microscopy. (A) Representative images showing γH2AX foci induced by different concentrations (1, 10, and 50 $\mu g \cdot ml^{-1}$) of QDs at 12 h. (B) Quantitative analyses of γH2AX foci formation induced by different concentrations ($\mu g \cdot ml^{-1}$) of QDs at 12 h. Statistical significance was determined by χ^2 test. $^{**}P < 0.01$, compared with blank; $^{a}P < 0.05$, compared with 1 $\mu g \cdot ml^{-1}$; $^{b}P < 0.05$, compared with 10 $\mu g \cdot ml^{-1}$. (C) Percentage of positive cells after treated with different concentrations ($\mu g \cdot ml^{-1}$) of QDs, measured by flow cytometry. Statistical significance was determined by ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with blank.

Fig. 3. QDs induce ROS generation in HUVECs. After the QD medium was removed, cells were washed with PBS, and then incubated with 20 μM of DCFH-DA for 30 min in a 96-well plate. Dichlorofluorescein fluorescence was determined at an excitation wavelength of 485 nm and emission wavelength of 520 nm. (A) Generation of ROS at different times when treated with 10 $\mu g \cdot ml^{-1}$ QDs. Statistical significance was determined by ANOVA. Differences were considered significant where $^{*}P < 0.05$, $^{**}P < 0.01$, compared with blank. (B) Generation of ROS at 2 hours after treatment with different concentrations ($\mu g \cdot ml^{-1}$) of QDs. Statistical significance was determined by ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with blank. The mean fluorescence intensity of the positive control is 22208.33.

Flow cytometry was applied to quantitatively measure γH2AX foci formation. In order to test whether emission of red light by QDs would interfere with the flow cytometry analysis, we first detected the fluorescence intensity of QDs that had been taken up by HUVECs. Results showed that their emission was negligible (data not shown). After treatments at three concentrations (1, 10, and 50 $\mu g \cdot ml^{-1}$) for 12 h, flow cytometry showed similar results to that obtained by fluorescent microscopy. The percentage of γH2AX-positive cells was 6.5%, 14.1% and 28.0% for 1, 10, and 50 $\mu g \cdot ml^{-1}$ QDs, respectively (Fig. 2(C)).

ROS are known to play important roles in DNA damage and vascular endothelial injury. Therefore, we investigated the intracellular ROS generation of cells using the DCFH-DA method with H_2O_2 (1 $mmol \cdot L^{-1}$) as the positive control. When HUVECs were exposed to QDs (10 $\mu g \cdot ml^{-1}$) for various times (2, 6, 12, and 24 h), ROS generation peaked at 2 h (Fig. 3(A)). Thus 2 h was chosen as the time point to examine ROS generation in HUVECs treated with all three concentrations of QDs (1, 10, and 50 $\mu g \cdot ml^{-1}$). Compared with the control, the ROS level was significantly increased by QD treatments at 10 and 50 $\mu g \cdot ml^{-1}$ but not 1 $\mu g \cdot ml^{-1}$ (Fig. 3(B)). Together, these data suggest that QDs can transiently induce the generation of ROS.

To determine whether ROS were indeed involved in QDs-induced DNA damage, we assessed the effect of a ROS scavenger, NAC, on γH2AX foci formation in QD-treated HUVECs. Cells were pre-treated with 6 $mmol \cdot L^{-1}$ NAC for 30 min, and then treated with different concentrations of QDs for different times. NAC significantly decreased the intracellular ROS level of cells at all concentrations of QDs (Fig. 4(B)). In addition, NAC pre-treatment also decreased ROS levels of cells treated with 50 $\mu g \cdot ml^{-1}$ QDs at all time points (Fig. 4(A)). Furthermore, NAC pre-treatment significantly, though not completely, decreased γH2AX foci formation induced by QD treatment (Fig. 5). This suggests that ROS are at least partially involved in QD-induced γH2AX foci formation.

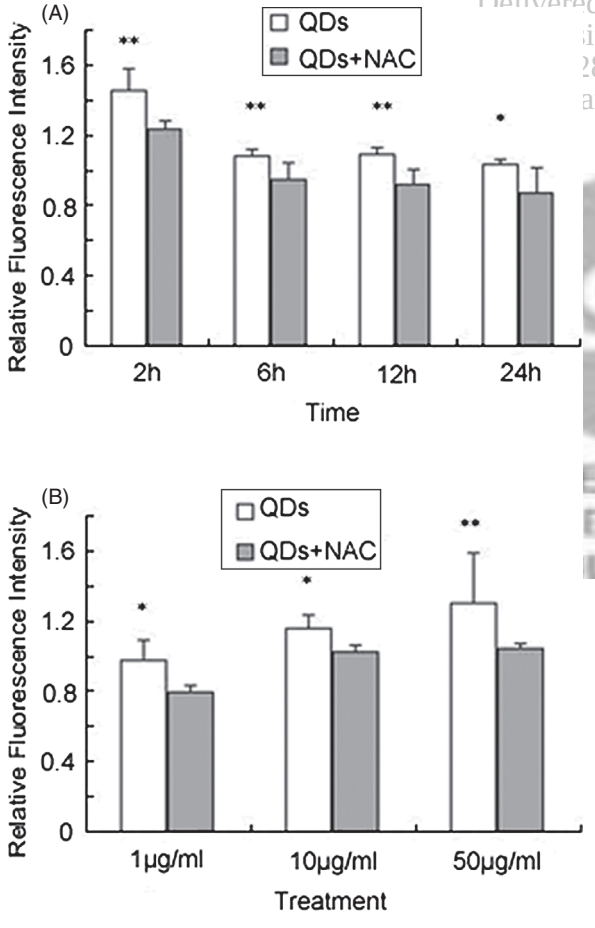


Fig. 4. Effect of NAC on ROS generation induced by QDs. Data was reported as a fold-increase in fluorescence intensity relative to the control. (A) Cells were pre-treated with NAC (6 mM), and then incubated with 50 $\mu g \cdot ml^{-1}$ of QDs at different times. Statistical significance was determined by Student's-*t* test. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the groups treated with QDs only. (B) After pre-treatment of NAC (6 mM), cells then incubated with different concentrations (1, 10, and 50 $\mu g \cdot ml^{-1}$) of QDs for 2 h. Statistical significance was determined by Student's-*t* test. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the groups treated with QDs only.

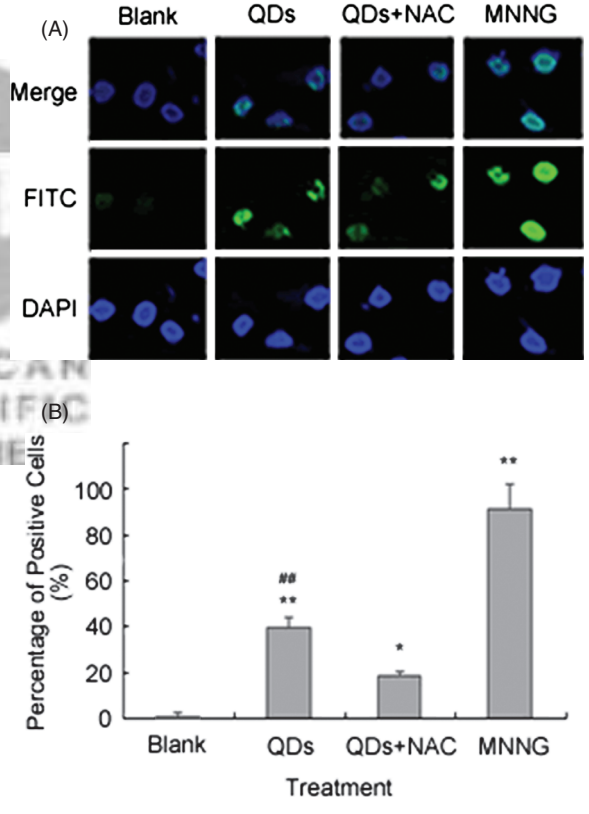


Fig. 5. Effect of NAC on γH2AX foci formation induced by QDs (50 $\mu g \cdot ml^{-1}$) at 6 h. MNNG (10 $\mu g \cdot ml^{-1}$) was used as the positive control. Cells were first treated with 6 mM of NAC, then incubated with 50 $\mu g \cdot ml^{-1}$ of QDs for 6 h. (A) Representative images showing γH2AX foci induced by QDs. (B) Quantitative analyses of γH2AX foci formation induced QDs. Cells with more than five foci/cell were considered to be positive. Statistical significance was determined by χ^2 tests. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with blank; $^{\#\#}P < 0.01$, compared with NAC pre-treatment.

4. DISCUSSION

Previous studies in a variety of cell models have shown that QDs can enter cells and exert cytotoxic effects, such as nuclear condensation, decreased metabolic activity, and cell proliferation inhibition.[8–11, 26] We selected HUVECs to study the toxicity of unmodified CdTe QDs which are stabilized by MPA on vascular endothelial cell. QDs exhibited a dose-dependent cytotoxic effect on HUVECs. Together with previous studies, this suggests that caution is required in the medical applications of QDs, and a more thorough evaluation of QD cytotoxicity using systemic methods is necessary.

Using DCFH-DA to examine ROS generation, our results revealed that QDs increased intracellular ROS content in HUVECs in a time- and dose-dependent manner. It is firmly established that ROS play very important roles in vascular injury. The mechanism of oxidative stress induced endothelial cell damage is complicated, mainly attributed to subcellular organelle damage and lipid peroxidation of cell membranes induced by ROS. In addition, ROS regulate the expression of several classes of genes, including adhesion molecules and chemotactic factors, of which the up-regulation is closely related to vascular injury. Thus, besides HUVECs, QDs can also induce ROS generation in IMR-32 cells,[31] SH-SY5Y cells,[32] and MCF-7 cells,[27] suggesting that oxidative stress may be a common mechanism for the cytotoxicity of QDs.

Currently, γH2AX foci formation has been generally accepted as a sensitive indicator of DSBs. In this study, we used two methods, immunofluorescence and flow cytometry, to detect γH2AX foci formation. Both methods indicated that CdTe QDs can induce γH2AX foci formation, i.e., DNA damage in HUVECs. These results suggest that there may be serious issues concerning the use of QDs in DNA based assays or *in vivo* applications.

Green and Horman have demonstrated that water-soluble II–IV core/shell semiconductor quantum dots can nick DNA, and they attributed this to free radicals, both photogenerated and surface oxide generated.[12] To determine whether ROS are also involved in CdTe QD-induced DNA damage in HUVECs, we pre-incubated cells with the antioxidant NAC, followed by QD treatment. Our results showed that NAC pre-incubation decreased both intracellular ROS induction by QDs in HUVECs and the formation of γH2AX foci, indicating that ROS are involved in QD-induced DNA damage. The effectiveness of NAC could be explained by at least three mechanisms of action:

(i) NAC contains a mercapto group that could allow it to adsorb to the surface of QDs and thus stabilize them in the culture media,

(ii) its protective activity could be attributed to its antioxidant properties and its ability to enhance glutathione expression, and

(iii) NAC can activate key anti-apoptotic signal transduction pathways that lead to the transcription of genes which are involved in cell survival.[33]

However, the exact mechanism(s) of NAC in this study remains to be investigated. In addition, NAC can only partially decrease QD-induced γH2AX foci formation, implying that other mechanisms could also be involved in QD-induced DNA damage in HUVECs.

5. CONCLUSION

In conclusion, we have shown that CdTe QDs can decrease cell viability, enhance intracellular ROS level and induce DNA damage in HUVECs. Our results also demonstrate that ROS are, at least partially, involved in QD-induced DNA damage. Further investigations of the mechanisms of QD cytotoxicity are required, which could broaden their range of application in biological systems.

Acknowledgment: This work was supported in part by grants from the National Natural Science Foundation of China (no. 30771824 and 30800923), Natural Science Foundation of Zhejiang Province (no. Y206537), China Postdoctoral Science Foundation (no. 20070420226) and China Ministry of Science and Technology (no. 2009DFB30390). We thank Professor Wuli Yang (Institute of Macromolecular Science, Fudan University, Shanghai, China) for providing the CdTe quantum dots.

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Received: 31 October 2008. Accepted: 15 June 2009.