© by PSP Volume 29 – No. 07A/2020 pages 6102-6110 Fresenius Environmental Bulletin

TOXICITY OF CADMIUM BASED AND CADMIUM FREE QUANTUM DOTS IN PRIMARY CULTURED ZEBRAFISH HEPATOCYTES

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ABSTRACT

Quantum Dots (QDs) are one of the most important nanomaterials in nanotechnology owing to their unique physicochemical properties. A conventional QD contains Cd^{2+} in its core and in order to reduce its toxicity Cd-core/ZnS-shell and Cd-free QDs were produced. However, ZnS shell was not found as protective as predicted. Also, still not much is known about the toxicity of Cd-free QD. In this paper, we evaluated and compared the cytotoxicity and genotoxicity of three types of QDs (Cd-based core, Cd-based core/shell, and Cd-free QDs) in primary cultured zebrafish hepatocytes. We show that Cd-based QDs significantly decreased cell viability whereas Cd-Free QDs caused a significant increase in cell viability in 24h. Moreover, ZnS shell reduced Cd^{2+} ion release into the cell but did not effectively prevent the cell from cytotoxic or genotoxic effects of the QD. Although the effects of Cd-based and Cd-Free QDs on cell viability were different, they both induced reactive oxygen species (ROS) production, DNA strand breaks and Rad51 downregulation in primary cultured zebrafish hepatocytes, revealing that they induce oxidative stress in these cells. To the best of our knowledge, this is the first report on the comparative toxicity evaluation of Cd-based with core and core/shell type, Cd-free QDs and $CdCl_2$ by assessing ROS production, DNA damage and Rad51 expression.

KEYWORDS:
Cadmium free quantum dots, Zebrafish, Cadmium, COMET, Rad51

INTRODUCTION

Nanomaterials, which are in the dimensions of 1 to 100 nanometer, are the basis of nanotechnology. Nanomaterials are classified as carbon-based, metal-based, composite nanomaterials, dendrimers and quantum dots (QDs) by the U.S. Environmental Protection Agency (EPA) [1]. Among them, QDs are one of the most important nanomaterials both in nanotechnology and nanotoxicology owing to their unique physicochemical properties resulting from their different structures [2]. QDs are semiconductor metalloid crystal nanostructures within the diameter of 2-10 nm [3]. They have unique optical properties with a broad range of excitation and narrow emission bands. Besides, tunability of their size during synthesis enables to adjust their emission wavelength [4]. Their unique semiconducting and optical properties make QDs applicable for several different fields such as bioimaging, cancer diagnosis, solar cells or lasers leading to an increase in QD production [5-8]. Increased QD production could bring along ecological risks, such as contamination of aquatic environment [9].

Cd-based QDs' core contain the highly reactive form of Cd^{2+} in high amounts, therefore it is considered to be toxic [10]. Since Cd-based QDs are semiconductor and nano-sized, they are capable of inducing reactive intracellular oxygen species (ROS) production both *in vivo* and *in vitro*, which may play an important role in their cytotoxicity [11-17]. Moreover, increased intracellular ROS production leads to DNA damage in both aquatic and terrestrial organisms [18,19]. In addition to inducing intracellular ROS production, metals can also induce DNA damage by inhibiting DNA repair processes [20].

In order to reduce the toxicity caused by the reactive form of Cd^{2+}, a core/shell type QDs was produced by wrapping the Cd^{2+} containing core with a ZnS shell (ie:CdSe/ZnS). Previous studies reported that although the ZnS shell reduced the Cd^{2+} release it failed to effectively eliminate the cytotoxicity of Cd-based QDs [21, 22]. A possible explanation for this observation may be that Cd^{2+} is sufficient to induce cytotoxicity even in reduced amounts. Therefore, Cd-Free QDs were synthesized in effort to completely eliminate the Cd^{2+} dependent toxicity [23]. However, further studies investigating the toxic effects of Cd-Free QDs are required, since our knowledge on the toxicity of the Cd-Free QDs is still very limited.

The Zebrafish (*Danio rerio*) is a widely accepted model for toxicological studies in both *in vivo* and *in vitro*, owing to its easy culture conditions

[24]. Moreover, the conservation of more than 50% of metabolic enzymes between zebrafish and humans, makes the zebrafish a great model for human studies [25,26]. The aim of this study was to evaluate and compare the toxicities of the Cd-based (both core and core/shell type), Cd-Free QDs and $CdCl_2$ in primary cultured zebrafish hepatocytes. For this purpose, we established primary hepatocyte culture from zebrafish and investigated the effects on the cell viability, Cd accumulation, ROS production, DNA damage and alterations in a DNA repair protein, Rad51 recombinase levels in response to these materials. To the best of our knowledge, this is the first report on the comparative toxicity evaluation of Cd-based with core and core/shell type, Cd-free QDs and $CdCl_2$ by assessing ROS production, DNA damage and Rad51 expression.

MATERIALS AND METHODS

Quantum Dots. All QDs used in this study were carboxyl groups (-COOH) terminated. CdTe QDs (2,55 nm diameter, emission wavelength at 550 ± 5 nm) were purchased from PlasmaChem GmbH (Berlin), CdSe/ZnS QDs (4,0 nm diameter, emission wavelength at 540 ± 10 nm) and CuInZnS/ZnS QDs (3,6 nm diameter, emission wavelength at 540 ± 10 nm) were purchased from Nano Optic Materials (Carson, California). The ζ- potentials were -14.0, -23.5 and -25.4 mV for CdTe, CdSe/ZnS and CuInZnS/ZnS QDs, respectively.

Zebrafish Maintenance. Zebrafish were purchased from a commercial supplier (Dogasan Aquarium, Istanbul, Turkey) and acclimatized for two weeks in laboratory. Fish of both sexes (mean weight, 0,72 ± 0,1 g and length, 4,7 ± 0,1 cm) maintained under 14L:10D photoperiod and 28± 1°C heated, dechlorinated and aerated city water in glass aquaria at the pH of 7.5-8.1 ± 0,1 with dissolved oxygen level was 8,4 ± 0,7 mg/L, the nitrite was < 0,4 mg /L and ammonium was < 0,07 mg/L [27]. An equal number of fish from both sexes were randomly selected to establish a primary cell culture.

Primary Cell Culture and Treatment. Zebrafish (n =6) were anesthetized with MS-222 (Ethyl 3-aminobenzoate methanesulfonate, Sigma-Aldrich) and sterilized with 70% ethanol. Liver was dissected under stereomicroscope, transferred into antibiotic containing and Ca^{2+} and Mg^{2+} free PBS on ice. Then, minced with razor blades and stainless-steel forceps to facilitate enzymatic digestion. Minced liver tissues were taken into 0,5 % trypsin – EDTA solution (GIBCO) and incubated at 30°C for 20 minutes. Trypsin-EDTA was neutralized with Leibovitz's L-15 (GIBCO) media supplemented with 1% antibiotic and antimycotic (final concentrations: streptomycin, 0.1 µg ml^{-1}; amphotericin B, 2.5 ng ml^{-1}; penicillin, 100 U ml^{-1}) and 5% foetal bovine serum. This cell suspension was sieved using 70 µm-nylon mesh (CORNING) in order to separate possible cell clusters. Then, sieved cell suspension was spun down and washed with complete L-15 media. Cells were counted via automated cell counter (TC20 Cell Counter, BIORAD) to place in flasks in desired amounts. To investigate the toxicity of QDs and $CdCl_2$, cells were treated with 100 µg/mL QDs or $CdCl_2$ for 24 h at 28°C.

Measurement of Intracellular Cd^{+2} Concentration. Intracellular Cd^{2+} contents were evaluated by using a micro cadmium electrode and a micro reference electrode (ISM-146 Cd^{2+} Micro Cadmium Electrode and DJM-146 Micro-Reference Electrode (Lazar Research Laboratories) according to manufacturer's instructions. Cells were plated in 12 well flasks at 1x 10^6 cells per well and treated with 100 µg/mL of Cd based-QDs or $CdCl_2$ for 24 h. After incubation, media was removed, cells were washed with Ca^{2+} and Mg^{2+} free PBS and collected using a cell scraper. Then, cells were homogenized with ultrasonic probe (Bandelin Sonopuls UW 50, Bandelin Electronics, Berlin, Germany), homogenates of all samples were spun down and supernatants were collected. The samples from three independent experiments were measured and analyzed via Arrow ion software from Lazar Research Laboratories, Inc. The calibration curve range was 0.02– 6 mM $CdCl_2$ in a Ca^{2+} and Mg^{2+} free PBS solution [28-30].

Cell Viability Assay. The cell viability tests were performed by the WST-1 (water-soluble tetrazolium salt) assay, according to the user manual (Water Soluble Formazan, ROCHE). 5 x 10^4 cells per well plated onto 96 well culture plates. After incubation with 100 µg/mL of QDs or $CdCl_2$ for 24 hours, 10 µl of WST-1 dye was added to each 100 µl of media and cell containing well. Absorbance was determined at 450 and 630 nm (background) with a Synergy HT Multi-Mode Microplate Reader (Bio-Tek). Percentages of viable cells were determined by normalizing the absorbance values to control. Average percentage of cell viabilities of three independent experiments was plotted with standard deviation. To eliminate the absorbance of QDs, WST-1 free but QD containing wells were used as blank.

Intracellular Reactive Oxygen Species (ROS) formation. ROS level was analyzed by monitoring the fluorescent intensity of DCF, using the DCFDA-Cellular Reactive Oxygen Species Detection Assay Kit (Abcam) with slight modifications. Briefly, zebrafish hepatocytes were incubated in phenol red free L15 media containing 10 µM H_2DCFDA for 40 minutes at 28°C. Cells were washed in PBS and resuspended in complete L15 media. 5 x 10^4 cells per well plated onto 96 well black plates. QDs or $CdCl_2$ containing complete L15

media was added to each well at final concentration of 100 µg/ml. Fluorescence signal was monitored at 24 h (excitation at 485 nm and emission at 535 nm) on a Synergy HT Multi-Mode Microplate Reader (BioTek). Relative ROS levels of three independent experiments were calculated and graphed with standard deviation. To eliminate fluorescence signal caused from all QDs, H_2DCFDA free but QD-containing wells were used as blank.

Alkaline Comet Assay. DNA strand breaks were detected using the method of alkaline comet assay as described previously with slight modifications [31]. Briefly, *D. rerio* hepatocytes were plated in 6 well plates (1 x 10^6 cells per well) and cells were exposed to the QDs or $CdCl_2$ as described above. After incubation for 24 h, cells were collected, washed and re-suspended in ice-cold PBS. Cell suspension (5 µl) was mixed with 120 µl low-melting-point agarose (0.5% in PBS at 37 °C) and poured onto agarose-coated (1.5% in PBS) and dried slides. Slides were immersed into cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl, and 1% Triton X, pH = 10) for 1 h. For unwinding of DNA, slides were immersed into cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) for 20 minutes. Next, slides were electrophoresed (0.8 V/cm 300 mA) for 30 min at 4 °C. Following the electrophoresis, the slides were neutralized three times for 15 minutes in total and stained with ethidium bromide (10 µg/ml). Stained slides were evaluated using a fluorescence microscope and the Comet Score software (TriTek Comet Software v2.0.0.38). At least 100 cells were analyzed per group and data were expressed as % Tail DNA (the intensity of the comet tail relative to the head). For comet assay, all chemicals were purchased from Sigma-Aldrich.

Western Blot Analysis. After 1 × 10^6 cells per well exposed to 100 µg/ml QDs or $CdCl_2$ for 24 h; cells were harvested with a cell scraper. Proteins were isolated using RIPA buffer (Santa Cruz) with a mixture of protease inhibitors (Sigma-Aldrich), quantified using a nanospectrophotometer (Nanodrop, Thermo Scientific). Then, denaturated at 95°C for 5 min in protein loading dye (10% Tris–HCl, 50% glycerol, 1% sodium dodecyl sulfate, and 0.01% bromphenol blue, 2% mercaptoethanol). Then, electrophoresis, immunoblotting, visualization and analyzing were performed according to Atalay and colleagues' study with slight modifications [32]. 50 µg of protein samples were loaded onto 12% SDS-PAGE gel and separated with electrophoresis. Next, samples were transferred to nitrocellulose membranes (Trans-Blot SD, Semi-Dry Electrophoretic Transfer cell, Bio-Rad). Membranes were blocked with 5% skimmed milk powder predissolved in PBS for 90 min at room temperature. After blocking, membranes were incubated overnight with primary antibodies against β-actin (Cell Signaling), and Rad51 (Cell Signaling) at 4°C. Following incubation, membranes were washed with PBS three times and incubated with HRP-labeled anti-rabbit Ig (Sigma-Aldrich) as secondary antibody. Membranes were washed three times and the ECL substrate (WesternBright™ Sirius, Advansa) was used for detecting the signals. Membranes were exposed to MXBE blue film (Carestream) and manually developed. Films were photographed on a light box and Rad51and β-Actin band intensities from two independent experiments were quantified using the ImageJ software (Scion Corp, Bethesda, Maryland, USA). The intensity of Rad51 bands were normalized to β-actin intensity, which was used as loading control and average of Rad51 fold induction of two independent experiments was plotted.

Statistical analysis. One Way Analysis of Variance (ANOVA) and student t-test were performed to determine statistical differences among the groups. All analyses were conducted in Graphpad V. Data represent mean and standard deviation (SD) or standard error of the mean (SEM). Differences were accepted statistically significant when $p<0.05$.

RESULTS

Effects on Cell Viability. In response to Cd-based (CdTe and CdSe/ZnS) QD treatments, cell viability ratios were significantly decreased compared to untreated control ($p<0.001$) (Figure 1). Contrary to Cd-based QDs, relative cell viabilities were significantly increased when cells exposed to Cd-Free (CuInZnS/ZnS) QDs (111,58%) and $CdCl_2$ (128,68%) compared to untreated cells ($p<0.001$). Although average of the viability of cells exposed to Cd-core/shell QDs (54.32%) was higher than that of Cd-core QDs (51.31%), the difference between groups was not statistically significant ($p>0.05$).

Intracellular Cd^{+2} Accumulation. Intracellular Cd^{2+} concentrations were calculated after cells were exposed to 100 µg/mL of Cd-based QDs or $CdCl_2$ for 24 h (Figure 2). Our data revealed that both Cd-based QDs and $CdCl_2$ led to a significant Cd^{2+} accumulation in primary cultured zebrafish hepatocytes ($p<0.001$). Cd-based QDs resulted in significantly higher intracellular Cd^{2+} than that of $CdCl_2$. Moreover, Cd^{2+} accumulation of cells exposed to Cd-core QDs was significantly higher compared to Cd-core/shell QD treated cells ($p<0.001$).

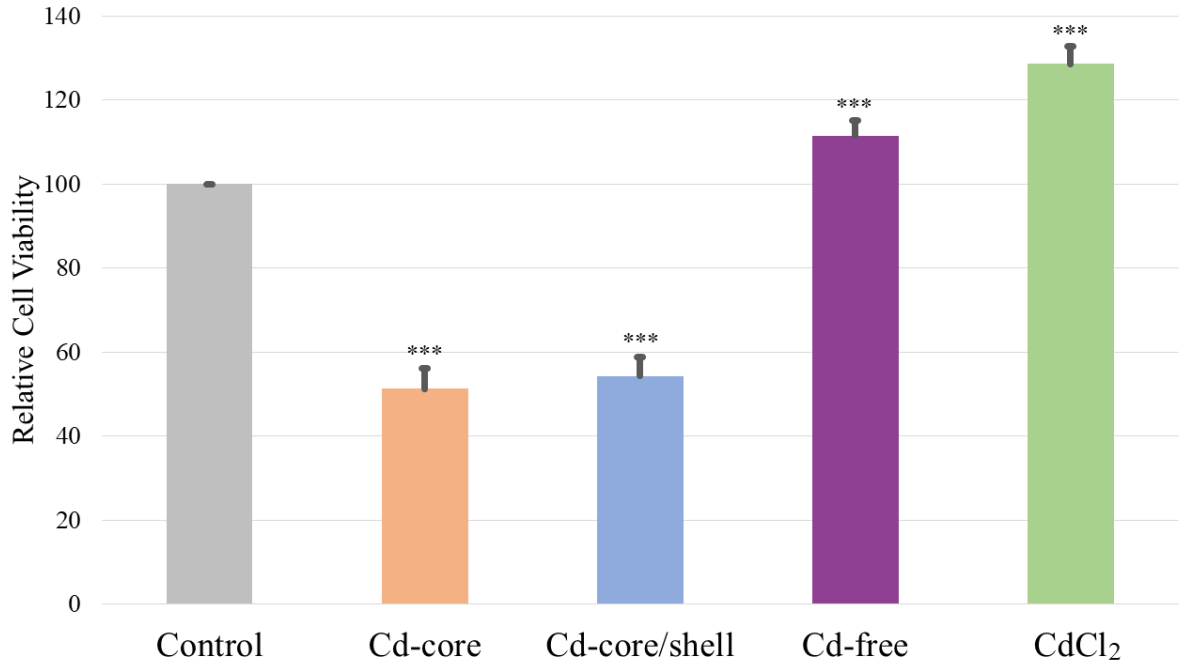

FIGURE 1

Relative cell viability ratios of cells exposed to Quantum Dots (QDs) and $CdCl_2$ for 24 hours. Primary cultured *D. rerio* hepatocytes were treated with 100 µg/mL Cd-core, Cd-core/shell, Cd-free QDs and $CdCl_2$ for 24 hours. Data represent mean and standard deviation of three independent experiments. (* $p < 0.001$, one way-ANOVA, compared to control).**

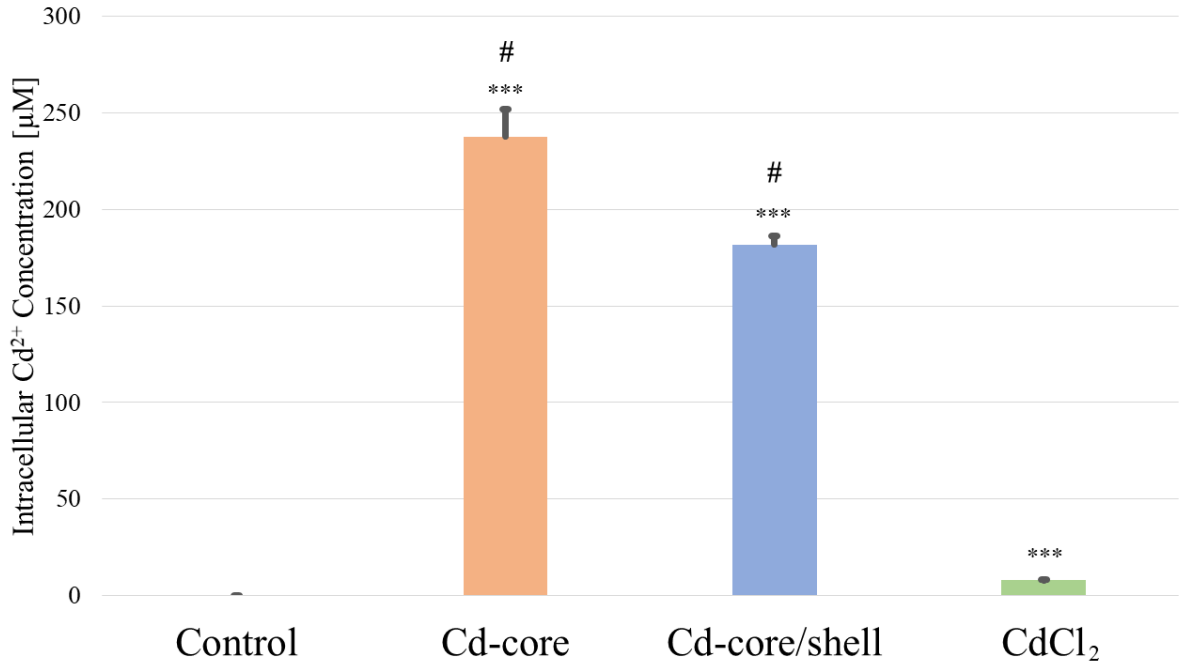

FIGURE 2

Intracellular Cd^{2+} accumulation in response to Cd-based QDs or $CdCl_2$ treatment. Cd^{2+} accumulation was detected in all groups. Comparison between intracellular Cd^{2+} content of Cd-core and Cd-core/shell was significant Data represents average of three independent experiments with standard deviation (#: p<0.001, student t-test; ***: p<0.001, one way-ANOVA, compared to control).

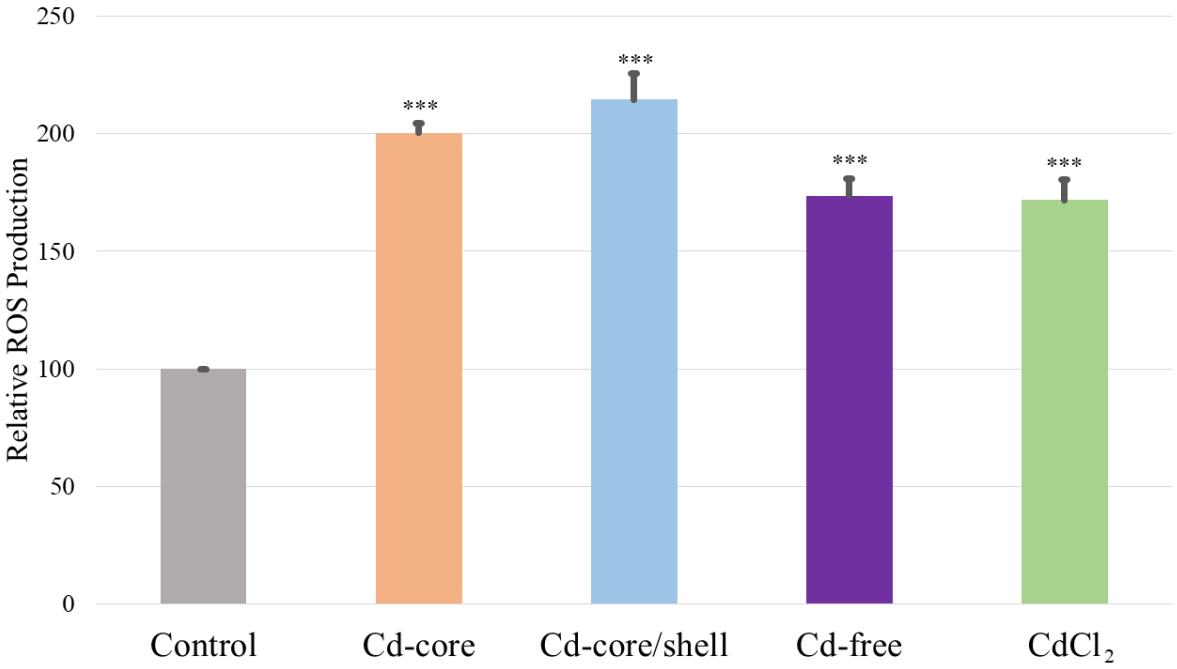

FIGURE 3

Relative reactive oxygen species production in cells exposed to 100 ug/mL QDs or $CdCl_2$ for 24 hours. Both QDs and $CdCl_2$ caused a significant ROS generation in primary cultured *D. rerio* hepatocytes. Data represent averages of three independent experiments with standard deviations (* $p < 0.001$, one way-ANOVA).**

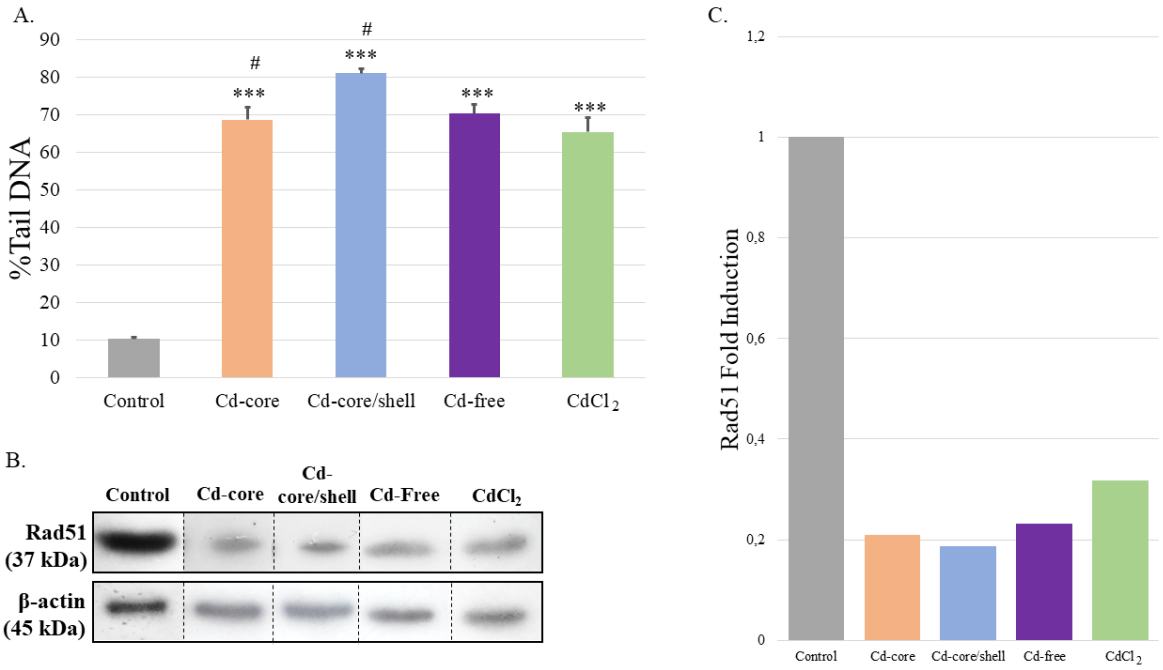

FIGURE 4

Quantum Dots and $CdCl_2$ induced DNA strand breaks and inhibited DNA double strand break repair by downregulating Rad51. A, DNA strand break induction in cells after exposure to 100 µg/mL QDs or $CdCl_2$ for 24 h. %Tail DNA of the cells was assessed by alkaline comet assay and data was expressed as mean and standard error of the mean of at least 100 cells (*: $p < 0.001$, one way-ANOVA, compared to control). B, Representative western blot analysis of two independent experiments showing that downregulation of Rad51 in cells exposed to QDs and $CdCl_2$ (β-actin was used as the loading control). C, The bar graph represents the Rad51 fold induction in cells exposed to 100 µg/mL QDs and $CdCl_2$.**

Reactive Oxygen Species Formation. ROS production in cells exposed to QDs or $CdCl_2$ for 24 hours was examined by DCF fluorescence intensity. Relative ROS levels were determined to be 200.36%, 214,49% 173.70% and 171.89% in Cd-core, Cd-core/shell, Cd-Free QDs and $CdCl_2$ respectively. As shown in Figure 3, both QDs and $CdCl_2$ treatments resulted in a significant increase in intracellular ROS levels compared to control ($p<0,05$). Although ROS formation in Cd-core/shell QD treated cells was higher than Cd-core QD treated cells, the difference between these groups was not significant ($p>0.05$).

DNA Strand Breaks and Rad51 expression. We investigated the DNA strand breaks associated with exposure to 100 µg/mL of QDs or $CdCl_2$ in primary cultured zebrafish hepatocytes by the alkaline comet assay (Figure 4). A significant increase in DNA migration was detected in response to both QDs and $CdCl_2$ treatment after 24 h of exposure. Percentage of DNA in tail (%Tail DNA) was significantly higher than the control ($p<0.001$). %Tail DNA ratios were determined to be 10.23%, 68.60%, 80.95%, 70.21% and 65.35% for untreated control, Cd-core QDs, Cd-core/shell QDs Cd-Free QDs and $CdCl_2$, respectively. Although the relative cell viability ratios of Cd-core and Cd-core/shell QDs was not statistically significant, the difference between %Tail DNA of the two groups was found to be significant ($p<0.001$). However, %Tail DNA was not significantly different compared to Cd-based QDs and Cd-Free QDs ($p>0.05$).

DNA double-strand breaks (DSBs) are the most deleterious type of DNA damage and they can be repaired by homologous recombination, via Rad51 recombinase [33]. For evaluation of double strand break repair biochemically, Rad51 levels in cells exposed to QDs or $CdCl_2$ were examined by western blot analysis (Figure 4B and 4C). Our data showed that exposure to QDs or $CdCl_2$ resulted in a dramatic downregulation of Rad51 protein compared to control. Rad51 fold induction ratios were plotted in Figure 4C.

DISCUSSION

Cd-based QDs dramatically reduced the number of viable cells, consistent with the previous studies performed in different mammalian and fish cell lines. [17, 15, 34, 35]. We observed that although the ZnS shell structure significantly reduced the amount of intracellular Cd^{2+} ($p<0.001$), there was no significant difference in cell viability in exposure to these materials. Additionally, ROS levels in cells did not show a significant difference which were treated with Cd-core QDs or QDs wrapped with ZnS shell. This could indicate that Cd^{2+} might not be responsible alone from this toxicity. This might be the result of the difference in QD size, core/shell chemistry, as well as ζ-potentials. Having a greater ζ-potential in absolute value is one of the important factors in nanomaterial toxicity [36]. Moreover, zinc ions which could be released from the nanomaterial could also generate ROS which was shown in a previous study conducted with a zinc-containing nanomaterial [37].

In contrast to Cd-based QDs, Cd-Free QDs resulted in a significant increase in cell viability, ($p<0.001$). The increase might be due to the copper leakage from Cd-Free QDs' core, as it was previously shown that low concentrations of Cu^{2+} increases cell proliferation [38, 39]. Moreover, both Cu^{2+} from Cd-free QDs' core and Zn^{2+} release from the QD's shell might be a reason for the increase in ROS levels [37, 39]. Similar to Cd-free QDs, percentage of cell viability was increased in cells exposed to 100 µg/mL $CdCl_2$ ($p<0,05$). Cd^{2+} accumulation in $CdCl_2$-exposed cells was significantly lower than the Cd-based QD treated cells ($p<0.001$) however, the accumulation was statistically significant compared to untreated cells ($p<0.001$). Concomitant to our data, previous studies also reported that low Cd^{2+} concentrations stimulate DNA synthesis and cell growth [40] and affect cell proliferation, apoptosis and proto-oncogene activation in zebrafish liver cells [21].

Cd^{2+} concentrations in cells exposed to Cd-based QDs were significantly higher than $CdCl_2$-treated cells ($p<0.001$). Because the ionic radii of Ca^{2+} and Cd^{2+} are similar, Cd^{2+} can possibly be taken up by Ca^{2+} channels, as well as through facilitated diffusion or active transport [41]. On the other hand, nanomaterials can actively take up by cells via endocytic pathways [42]. The significant difference between the intracellular Cd^{2+} contents of Cd-based QDs and that of $CdCl_2$ should be the result of "lysosome-enhanced Trojan horse effect" identified by Sabella and colleagues. In this effect, when metal nanoparticles endocytosed into the cell, acidic environment of endosomes results in corrosion of nanoparticles and metal ion release increases [43].

Nanomaterials can induce genotoxicity by two known ways; via entering the nuclear envelope (directly) [44] or oxidative stress (indirectly) [45]. Nanoparticles do not necessarily have to reach the nucleus; they can induce genotoxicity via oxidative stress; which induces DNA strand breaks in cells [45]. Our data imply that the QD-genotoxicity is not through direct binding of the QDs to the DNA. Nanoparticles with negative ζ-potentials are not considered as efficient in binding to the DNA [46]. Since the QDs used in our study have negative ζ-potentials, it is unlikely that this genotoxicity was induced by directly binding of these nanomaterials to the DNA. Furthermore, we investigated Rad51 levels in cells exposed to QDs and $CdCl_2$ to determine whether there was a link between genotoxicity and DNA double strand break repair. To the best of our knowledge, it is the first study examining the expression of

Rad51 protein in cells exposed to QDs. Our results revealed that both QDs and $CdCl_2$ caused downregulation of Rad51 protein, consistent with the previous studies reporting that Cd^{2+} or Cu^{2+} resulted in reduction of rad51 gene expression as well as other genes involved in DNA repair pathways *in vitro* and *in vivo* [47,48]. Thus, we conclude that as well as ROS production, inhibition of DNA double strand repair by downregulation of Rad51 might also be important in the QD-induced genotoxicity.

CONCLUSIONS

In this study, we investigated and compared the efficiency of two approaches in effort to reduce the Cd-core QD- induced toxicity; 1) wrapping a QD with ZnS shell 2) Cd-Free QDs instead of Cd-based QDs, by evaluating their effects on cell viability, ROS formation, DNA strand breaks and Rad51 regulation in primary cultured zebrafish hepatocytes. We report for the first time that both Cd-core/shell or Cd-free QDs are harmful to primary cultured zebrafish hepatocytes. Moreover, capping a QD with ZnS shell reduces Cd^{2+} leakage from QDs' core; but this reduction is not sufficient to protect cells from QDs' toxic effects. Even if Cd-free QDs did not result in cell death at 24 h; ROS generation, DNA DSBs and downregulation of Rad51 showed that Cd-free QDs are toxic to primary cultured zebrafish hepatocytes; similar to Cd-based QDs. These nanomaterials are needed to be used in a controlled manner and waste management must be implemented properly. It should be noted that non-targeted organisms can be affected as much as the targeted organisms. Further *in vivo* studies are needed to be performed to elucidate possible effects on the whole organism.

ACKNOWLEDGEMENTS

This work was supported by the Marmara University (FEN-A-200716-037). The authors gratefully thanks to Pinar Buket DEMİREL, PhD. and Maltepe University Cancer and Stem Cell Research Center (MUKKAM) for participating laboratory facilities. The author NK kindly thanks to TUBITAK for 2211-C Domestic Priority Areas PhD scholarship program.

Conflict Of Interest. The authors declare that there is no conflict of interest regarding the publication of this article.

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Received: **06.01.2020**
Accepted: **24.04.2020**

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