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RESEARCH PAPER

The evaluation of the cortex neurons viability in CdS nanoparticles induced toxicity

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ABSTRACT

Objective(s): Cadmium sulfur (CdS) is a type of quantum dot which is a unique light-emitting semiconductor nanocrystal. Quantum dots have wide applications in optoelectronics, solar cells, biology, and medicine fields.

Materials and Methods: Morphological properties and structural analysis for CdS were tested by using different methods (TEM, XPS and XRD). Cortical neuron cells were used for toxicity investigations. The cells were treated with different concentrations of CdS (100, 10, 1, 0.1, 0.01 µg/mL) and incubated for 24 h (5% CO_2; 37 °C). In vitro studies were done by examining cellular viability (MTT assay) and oxidative stress/status (TAC/TOS).

Results: According to our results, the increasing concentration of CdS resulted in decreased cell viability. Total antioxidant capacity (TAC) of neurons increased following exposure to the lowest concentrations of CdS. In addition, inverse to our TAC findings, total oxidant status (TOS) was decreased following exposure to lower concentrations of CdS.

Conclusion: Recently, because of advances in diagnostic and drug delivery systems ingestion rate of CdS by humans were increased. Hence, this study aimed to investigate the toxic effects of CdS on Cortex Neurons cell cultures. The production of CdS quantum dot particles was done by using the Viridibacillus arenosi K64 (biosynthesis method) which provides environmentally friendly, economical, reliable, and controlled production.

Keywords: *CdS, Cortex Neurons, TEM, XRD, XPS*

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INTRODUCTION

Quantum dots are one of the most expensive high-tech products in the world and are used in many products that we use in daily life from mobile phones to cancer diagnostic systems [1, 2]. It is believed that it is possible to perform better cellular and biomedical imaging with the use of CdS. However, toxicity problems of quantum dots are a major obstacle to medical practice in humans [3]. Therefore, the development of a non-toxic and environmentally friendly method for cadmium sulfide synthesis is extremely important. One way to overcome this problem is to use the biological synthesis of these particles using microorganisms [4]. Developing the synthesis methods in researches for biocompatible materials. The studies conducted on various organisms by studying the biological synthesis/biosynthesis method revealed that microorganisms were more useful. It is aimed to produce biocompatible,

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biodegradable, non-toxic, environmentally friendly materials with nano-size in the production of the desired material [5] [6]. For this purpose, the materials produced as a result of the production of nanoparticles are also commercially valuable. As biosynthetic particle producers, we found that the plants, plant extracts [7], bacteria [8], and human cells [9] are used in the literature. In addition, particle synthesis with microorganisms is also quite common [10], fungi [11], actinomycetes [12], lichens [13], and algae [14]. Many microorganisms, including S-layer bacteria, diatoms, and magnetotactic bacteria, have been found to have the ability to synthesize nanoparticles [15]. The synthesis of cellular systems is generally divided into two categories: intracellular and extracellular synthesis [16]. Bacteria from microorganisms are the most commonly used microorganisms in nanomaterial synthesis due to their inexpensive production and easy purification process [17]. CdS NPs synthesized as a result of incubation of salt solutions (CdCl2, Na2S) and Escherichia coli are obtained as more stable and colloidal dispersions at different pH values [16]. Sweeney and his colleagues Performed CdS NP synthesis by shaking Escherichia coli biomass with CdCl2 and Na2S solutions in a dark environment at 37 °C for 12 hr. Biomolecule-coated particles have been determined to have a more stable, hydrophilic, and larger surface area. The team heated the particles to 95 °C in Laemli buffer to remove biomolecules from the particle surface. The study revealed the stability and homogeneity of CdS NPs measured at different pH values in coated or coated biomolecules. There is currently little to no evidence about green synthesize of CdS using bacteria. Hence, it is essential to widen the range of biosynthesized CdS NPs toxicity investigations. Cortex Neurons receive input from the sensory neurons that help with spatial muscle control and is important for the control of motor movements. Motor movements usually involve fine movement and the Cortex Neurons coordinate voluntary movements. Changes in Cortex Neurons function can impact our posture, balance, coordination, and speech. Cell viability, antioxidant capacity and oxidant status are some of the parameters that need to be further investigated in the presence of CdS. Reduced cell viability in Cortex Neurons tissue results in the decreased number of synapses and an inability to control the finer motor movements associated with Cortex Neurons function [18] [16]. Total oxidant status (TOS) points to the level of free radicals while the total antioxidant capacity (TAC) represents antioxidant capacity of the cell. Collectively, these indices can point to the overall status of the cerebellum with regard to toxicity associated with exposure to CdS.

In the present study, after production of the CdS quantum dot particles by using the Viridibacillus arenosi K64 (biosynthesis method) Morphological properties and structural analysis of fluorescent particles were done by using TEM, XPS, and XRD techniques. we evaluate the toxicity of different doses of biosynthesized CdS NPs in Cortex Neuron cultures by using 3-[4,5 -dimethylthiazole-2-yl]-2,5 diphenyltetrazolium bromide (MTT), total antioxidant capacity (TAC), and total oxidant status (TOS) test. To our knowledge, this is the first study that investigates these parameters in Cortex Neurons culture in the presence of CdS NPs.

MATERIALS AND METHODS

Biosynthesis of CdS NPs

Chemicals and reagents

Dulbecco modified eagle's medium (DMEM), Fetal calf serum (FCS), Neurobasal medium (NBM), Roswell Park Memorial Institute (RPMI) 1640, phosphate buffer solution (PBS), antibiotic-antimycotic solution (100×), L-glutamine, and trypsin–EDTA was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Bacteria used for biosynthesis of CdS NPs

Gram-positive bacterium *Viridibacillus arenosi* K64 (GenBank Acceptance No: KR873397) obtained from stock production from Ataturk University East Anatolian High Technology Research and Application Center (DAYTAM) for biosynthesis was isolated from caves, the isolate was incubated by line seeding on the NA medium with aseptic techniques.

Biosynthesis of CdS NPs

Bacteria suspension (~ 0.5 × 108 CFU) 100 ml LB (Luria Bertani: yeast extract 5.0 g/L, peptone (casein) 10.0 g/L, NaCl 10.0 g/L) was transferred in medium and incubated in a shaker at 120 rpm for 32 hr at 32 °C. After centrifugation of Falcon tubes in an ultracentrifuge at 6.000 rpm for 10 min, the supernatant was incubated in a shaker at 120 rpm for 32 hr at 32 °C for the development of bacteria again for 36 hr. It was then diluted with sterile and fresh LB (by adding equal volume) and incubated

for 24 hr at 32 °C at 120 rpm with a shaker. As a result of the process, fluid in the upper phase (supernatant) was preserved and 20 ml of CdCl2 and 5 ml of Na_2SO_3 were added to the supernatant. The supernatant was placed in a water bath at 60 °C for 10-20 min until a yellow-white color was formed [19].

Isolation and purification of CdS nanoparticles

Centrifuge at 20 °C for 10 min at 10.000 rpm for obtaining the quantized quantum dots. The washing process was performed with hexane, ethanol, and ultra-pure water, and the supernatants were removed by centrifugation [20, 21].

Characterization of CdS nanoparticles

The biosynthesized CdS nanoparticles were characterized by TEM (Jeol 2100F 200kV RTEM), XPS (Specs-Flex), XRD (PANalytical Empyrean Inspect S50), USA. All analyses were made through the purchase of services from Atatürk University DAYTAM and ODTÜ University.

In vitro studies

Cortex neuron cells culture

Cortex Neurons cell cultures were obtained from the Department of Medical Pharmacology at Ataturk University (Erzurum, Turkey). The cells were thawed and briefly centrifuged to form a pellet. The pellet was resuspended in growth media and the cells were seeded into a 48-well culture plate at a density of 1×105 cells/ml. The cells were treated with increasing concentrations of CdS (0.01 – 100 µg/ml) and incubated for 24 h (5% CO_2; 37 °C). As a control, 150 µL NBM (Neurobasal medium) (Gibco, sigma, USA) only was added to one set of wells for 24 hr [19, 22].

MTT assay

The MTT assay was performed with a commercially available kit (Sigma Aldrich, USA). Briefly, an MTT reagent (10µL at a concentration of 5 mg/ml) was added to each well in the plated and then incubated for 4 hr (5% CO_2; 37°C). After applications, the medium was removed and 100 µL of dimethylsulfoxide (DMSO) (Sigma, USA) was added to each well to dissolve formazan crystals. Cell viability (%) was calculated by optical density read at 570nm using the Multiskan ™ GO Microplate Spectrophotometer reader (Thermo Scientific, Canada, USA) [23, 24].

TAC assay

The antioxidant capacity was determined by inhibition of the 2-2'-azinobis (3-ethylbenzothiazoline 6-sulfonate = ABTS +) radical cation in the TAC assay (Rel Assay Diagnostics® Company (Gaziantep, Turkey)). Briefly, to determine the TAC level, the wells are respectively; 30 µL sample and 500 µL Reagent 1 solution were added, the initial absorbance was measured at 660nm. Then, 75µL Reagent 2 solution was added to the same wells and after 10 min, the second measurement was made at 660 nm. Absorbance values were replaced according to the formula specified in the procedure and TAC values were calculated as Trolox Equiv / mmol L^{-1}.

A2-A1= ΔAbsorbance (Standard, sample or H_2O)

(H_2O ΔAbs - Sample ΔAbs)

$$\text{Result} = \frac{(H_2O\ \Delta Abs - \text{Sample}\ \Delta Abs)}{(H_2O\ \Delta Abs - \text{Standard}\ \Delta Abs)}$$ [19, 25].

TOS assay

TOS assay is called the evaluation of color density with spectrophotometric properties depending on the number of oxidants in the sample (from Rel Assay Diagnostics® Company (Gaziantep, Turkey)). For this purpose, to determine the TOS level, briefly; 500 µL of Reagent1 solution was added to the wells containing 75 µL of sample and the initial absorbance value was read at 530 nm. Then 25 µL of Reagent2 solution was added to the same well. After 10 min at room temperature, the second absorbance value was read. Absorbance values were replaced according to the formula specified in the procedure and TOS values were calculated as H_2O_2 Equiv/mmol L^{-1}.

A2-A1= ΔAbsorbance (Standard or sample)

$$\text{Result} = \frac{\text{Sample}\ \Delta Abs}{\text{Standard}\ \Delta Abs} \times 10$$ [26].

Ethical approval

This study was conducted at the Medical Experimental Research Center in Ataturk University (Erzurum, Turkey). The Ethical Committee of Ataturk University approved the study protocol (42190979-01—02/2411).

Statistical analysis

Statistical comparison between groups was calculated using One-way ANOVA and Tukey HSD method. All calculations were performed using

SPSS 20 software for statistical analysis, and $P<0.05$ was considered as a statistically significant difference in all tests. Results are presented as mean ± standard error.

RESULTS

TEM analysis

The physical appearance and particle size of biosynthetic CdS quantum dots were determined by TEM. Prepared sample CdS nanoparticles were observed to be around 4-12 nm in size (Fig 1). It is thought that clusters do not exhibit a homogenous distribution and nanoparticles may be in spherical structures.

XPS analysis

XPS is used for the analysis of elemental and chemical state information of the examined surfaces. The element and chemical state analyzed on the surface of the device and the sample examined is determined by chemical bonds. Five kinds of elements, including Cd, S, O, C and P, are observed on the surface. The surface of the synthesized nanoparticle was significantly oxidized, as indicated by the relatively high-density O-associated XPS (O1s). Here are the main Cd and S elements to be observed. the chemical states of the nanoparticles were also investigated by XPS (Fig 2).

XRD analysis

The crystallographic properties of CdS nanoparticles obtained by the biosynthesis method were determined by XRD (Xhenium diffraction) technique and were observed in the most severe peak (101) orientation. The XRD patterns showed 2θ = 27.6°, 31.9°, 45.6°, 54.1°, 56.6°, 66.4° and 75.4° (SCPDS Card No: 41-1049). It was determined that the observed peaks belong to the hexagonal CdS structure and these peaks correspond to the reflection planes (002), (101), (110), (201), (004), (203), and (105). Here, the peak at 2θ = 31.9º is more severe than the other peaks (Fig 3).

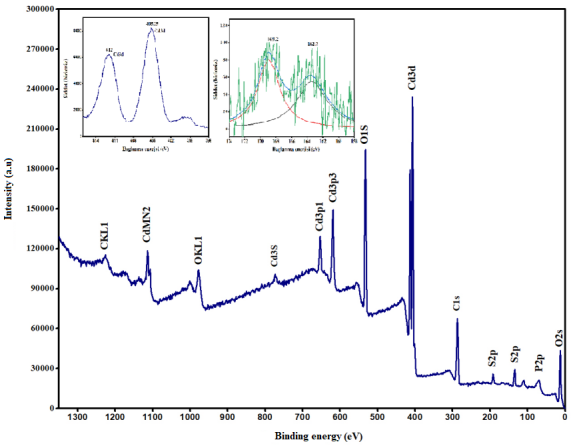

Fig 2. XPS general spectroscopy of CdS nanoparticles obtained by biosynthesis and Cd and S spectroscopy

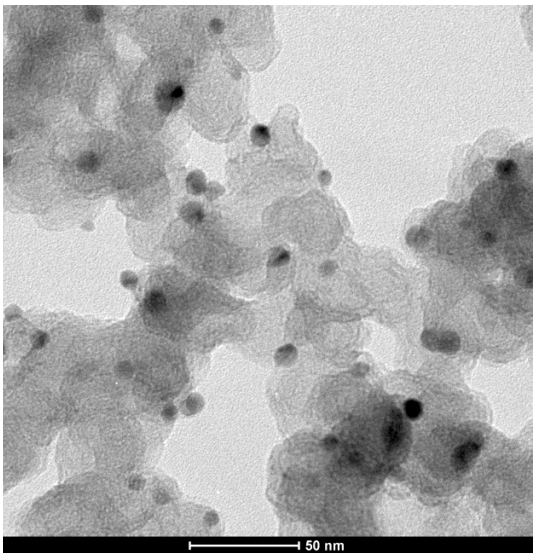

Fig 1. TEM images of CdS nanoparticles obtained by biosynthesis

Cell viability – MTT assay

To determine cell viability, the MTT assay

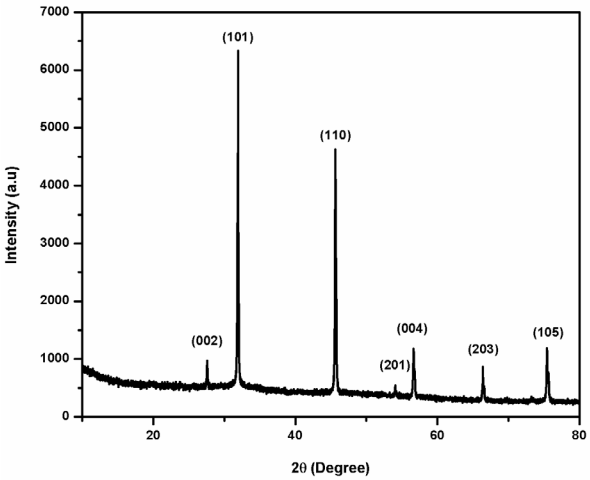

Fig 3. XRD diffraction pattern of CdS nanoparticles obtained by biosynthesis

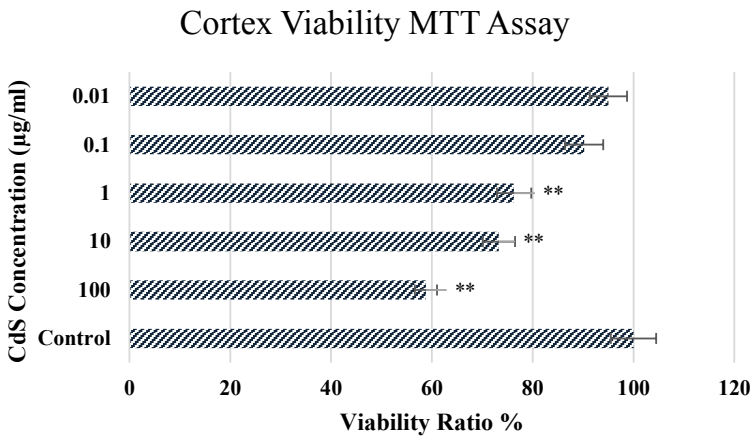

Fig 4. *In vitro* viablity ratio of CdS (0.01 – 100 µg/ml) on Cortex Neurons cells (n=6/group). * Significant differences at P < 0.05 compared to control group; ** Significant differences at the P<0.001 compared to control group

test was done after 24 h exposure of CdS NPs, and data were analyzed by Mann–Whitney test (Fig 4). According to our result, the viability ratio decreased with the increase in CdS concentration. According to the results, the highest viability rate was found as 95 and 90.1% (*P*> 0.05) in 100 and 10 µg/ml CdS doses. In the data obtained with vitality rate, the dose of 0.01, 0.1 and 1 µg/ml CdS levels were 58.8, 73.3, and 76.3%, respectively [19].

Total antioxidant capacity (TAC) assay

TAC assay was obtained after 24 h exposure of CdS NPs (Fig 5). The total antioxidant capacity was decreased by CdS concentration dose-dependently. According to the results, the highest antioxidant capacity was determined as 5.8 and 5.6 Trolox Equiv/mmol L^{-1} at 100 and 10 µg/ml CdS (*P* <0.05). The antioxidant capacity of CdS 0.01 and 0.1 µg/ml CdS was determined as 3.1 and 4.2 Equiv/mmol L^{-1}.

Total oxidant status (TOS) assay

TOS assay was obtained after 24 h exposure of CdS NPs (Fig 6). While the total oxidant levels were equivalent to H_2O_2 in the control group, this ratio decreased in a dose-dependent manner. According to the results, the survival rate of 0.01 and 0.1 µg/ml CdS was determined as 5.4 and 4.9 Equiv/mmol L^{-1}, while the highest oxidant capacity was determined as 3.7 and 3.9 Equiv/mmol L^{-1} at 100

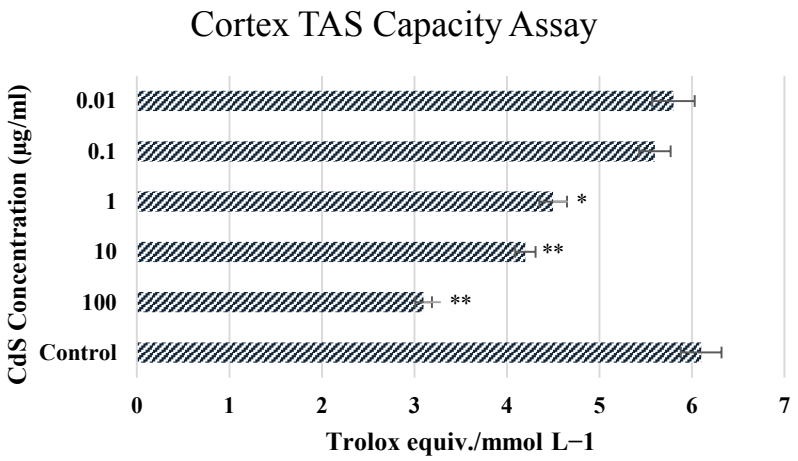

Fig 5. In vitro TAC capacity effects of CdS (0.01 - 100 µg/ml) on Cortex Neurons cells (n=6/group). * Significant differences at *P* <0.05 compared to control group; ** Significant differences at the *P* < 0.001 compared to control group

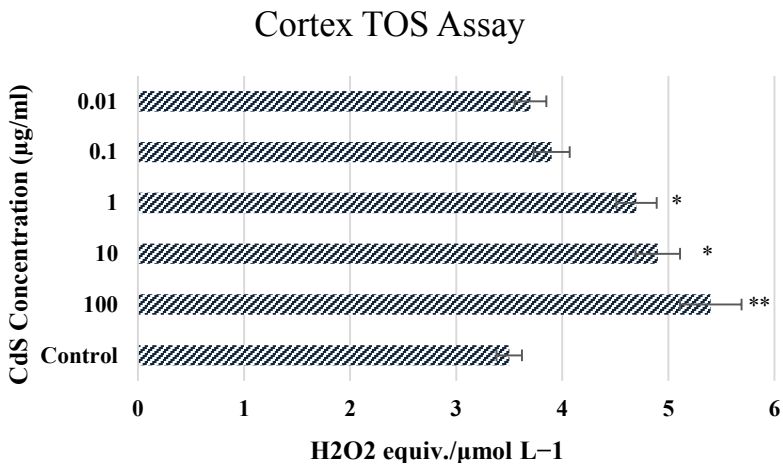

Fig 6. *In vitro* TOS status of CdS (0.01 – 100 µg/ml) on Cortex Neurons cells (n=6/group). * Significant differences at P <0.05 compared to control group; ** Significant differences at the P < 0.001 compared to control group

and 10 µg/ml CdS dose. In addition, 100 and 10 µg/ml CdS showed no significant difference when compared with control. In the data collected, the doses of 0.01 µg/ml CdS were significant with P <0.001, whereas 0.1 and 1 µg/ml CdS with P<0.05 were significant in the control group.

DISCUSSION

In this work, CdS were analyzed in an *in vitro* toxicity model using a Cortex Neurons neuron cell line and measure cell viability and oxidative stress status using MTT, TAC, and TOS assays. Through TEM imagery and in particular spectrophotometric and spectrofluorometric analysis, the resulting data is very effective in explaining the individual dimensions of the particles. When the physical appearance and particle size of the CdS quantum point synthesized by the biologically synthesized matrix (Fig 1) are examined, it is seen that small size and large size particles are scattered. The obtained nanoparticle sizes were measured at 4-12 nm. Based on the present studies, it was determined that they synthesized nanoparticles in the range of 4-10 nm [27] and smaller 2-4 nm [28]. The particle obtained by this study was shown as clear and non-clustered in an organic holder since it was viewed before washing. When the particle morphologies were examined carefully, it was observed that they were in a hexagonal structure. TEM images, and in particular spectrophotometric and spectrofluorimetric analysis results, provided very effective values for explaining the individual dimensions of the particles.

The surfaces were analyzed by XPS for content and binding structures. With XPS, elemental and chemical status analysis can be performed from the surface to a depth of 10 nm. The general XPS scanning curves of the CdS sample synthesized biologically are shown in (Fig 2). Six types of elements, including Cd(3d/2), O(1s), C(1s), P(2p), and S(2p) in the spectrum, were measured on both the surface and CdS nanoparticles. As indicated by the relatively high-density O associated XPS O(1s) property, the magnified CdS NP surface was significantly oxidized. Here are the main Cd and S elements to be observed. The broad spectral properties of the Cd(3d/2) spectrum can be separated from the 2 sharp peaks at the binding energy of 405.25 and 412 eV [4]. The S(2p) photoelectric line shown is divided into multiple complex peaks. The most severe peak is indicated by the binding energy in the 169.2 and 162.7 eV bonds. It is desired to observe Cd and S as a target. It was determined that the fluorescence property of the obtained particle was compatible with the CdS QD feature. The reason for the small amount of sulfur in the general spectrum is that it can be oxidized to the surface in sulfur. The other elements O and C are the two main macro elements found abundantly inorganic materials. The predominance of phosphate in XPS indicates that the synthesis is done by extracellular mechanisms [29]. In particular, adenosine phosphate plays a role in this process and phosphorylates existing structures, and this is a sign of the use of phosphate adenosine reductase enzymes [30]. It is possible to remove

or reduce the compounds completely by changing the procedures or by increasing the number of processes. Crystal phases were observed in many samples in XRD analysis [4]. After the parameters obtained from XRD patterns, library and existing sources were evaluated and it was determined that hexagonal crystals were formed in general. In this study, according to XRD measurements of CdS obtained by biosynthesis method (27.6°, 31.9°, 45.6°, 54.1°, 56.6°, 66.4°, and 75.4°), respectively (002), (101), (110), (201), (004), (203) and (105) coincide. The largest pick-up from the XRD results was measured as the lowest peak width of the plane (101) at approximately 6400.

Considering the intensity of these peaks and the order of peaks, the crystals we obtained according to and his colleagues the structure is very close to the hexagonal but shows that there is a tendency towards the cubic structure of the heights of some peaks [31]. According to the TEM images, hexagonal, according to the data obtained from XRD, hexagonal measurements were made. XRD results support the results of TEM. For the CdS quantum point obtained using bacteria, our XRD results were evaluated as a hexagonal crystal structure by the available sources [31-33]. This morphological structure and quantum dots can be used for different applications. In particular, it is possible to use CdS nanoparticles for applications to cells and the absorption of the incoming sunlight for the solar cells from the visible region to the close ultraviolet region. Cadmium sulfide nanoparticles (110-130 nm and 80-100 nm) were used to investigate liver tissue toxicity [34]. Data showing that small NPs cause more oxidative damage showed an inverse relationship between size and toxicity. We also used very small CdS nanoparticles in our study and 0.01 µg/ml did not show significant toxicity. Surface modification, also used by Nisha et al. (2015), is one of the most effective and simple techniques to reduce CdS toxicity [35]. The polyvinylpyrrolidone (PVP) and cysteine they used in their work were used to coat the nanoparticle surface, as well as to evaluate toxicity using Vero cells. The toxicity results with Vero cells revealed that surface coated NPs exhibit less toxic properties compared to unmodified NPs. The importance of surface coating reveals that the vitality in surface modified NPs is significantly higher than in non-surface modified NPs. Although Li et al. (2018) do not support our findings with their study (increased toxicity due to reduced size), Nisha et al. (2015) support the hypothesis that surface modification can significantly reduce CdS particle toxicity. According to Li et al. among the 80-100 nm and 110-130 nm groups, CdS toxicity was higher in the 80-100 group than the other. The author stated that the smaller particle size group had higher concentrations of Cd in the blood and liver, and that tissue damage was greater than the groups with large particles. Varmazyari et al. [19] showed that the highest viability rate was 93% in the CdS 0.01 µg/ml group with their study on both cortex and cerebellum cell lines. Consistent with the MTT results, they stated that the highest antioxidant capacity occurred in the CdS 0.01 µg/ml group with 5.2 Trolox Equiv/mmol L^{-1}. Similar results are observed at CdS 10 µg/ml (MTT: 90.1%; TAC: 5.6 Trolox Equiv/mmol L^{-1}) and 100 µg/ml (MTT: 95%; TAC: 5.8 Trolox Equiv/mmol L^{-1}) doses in our study performed on cortex neuron culture. Munari et al. (2014) in their study SAOS, HEK293T, and TOLEDO cells 10 nmol.L^{-1} 3 gun, 10 nmol.L^{-1} 3 and 5 days and 10 and 50 nmol.L^{-1} using 3-5-7 day groups compared with the control group [36]. According to the results obtained, the viability ratio is equal to the control group and CdS is determined as non-toxic at a concentration of 10 nmol.L^{-1}. The results of this study were correlated with our results of 0.00001 mg. Pujalte et al. used different concentrations of CdS NPs in IP15 (glomerular mesangial) and HK-2 (epithelial proximal) cell lines [37]. They observed that the NPs exhibited toxicity at concentrations of 5 µg/cm2 and 6.5 µg/cm2, whereas this toxicity was not observed at 1.4 µg/cm2 concentrations. It was found that the viability rates of cells at maximum toxicity seen at the highest concentration were 26 ± 2.5%. Although the results shown were similar to our findings, the doses of 0.01 and 0.1 µg/ml did not show any toxicity, but the viability rate at the 5 µg/cm2 and 6.5 µg/cm2 concentrations showed more toxic effects than our results. Due to factors such as general particle size, different production techniques or cell type, there is variation and difference in the data here and in Pujalte's study [38]. In a study conducted by Souto and his colleagues in 2017, they showed that aflatoxin B1 decreased the antioxidant capacity and caused cellular death. In our study, it was shown that the antioxidant capacity decreased with the concentration of nanoparticles used in our study. The decrease in antioxidant capacity has led to increased external stress by decreasing the cellular

defenses and increasing the mortality rate [39]. Ozyurt and his colleagues in their study in 2014 in the psychosis model of oxidative and antioxidant using melatonin capacity looked. According to this study, oxidative stress increases with the increase of symptoms and psychosis. In our study, cellular or neuronal stress increased with concentration increase due to CdS [40]. Wang and his colleagues in their study in 2017 were examined a Quantum dot of kidney and liver antioxidant capacity and ROS formation. According to this study, the increase in concentration depending on the dose showed that oxidative stress increased. In our study, the dose increase caused the formation of oxidants [41].

CONCLUSION

One of the main objectives of the study is to synthesize the quantum particles by having a low toxicity. Because for biological applications QD biologic toxicity is a critical factor. CdS are produced by biological synthesis to reduce the toxicity of QD. Quantum dots can be used in cellular and biomedical imaging, observation of intracellular activities, drug and gene targeting, (Nano) for biosensor purposes. As a result of many studies with quantum dots, some radioisotope labeled quantum dots are prepared in the field of "Nuclear Medicine" for imaging and therapy purposes. Long-term toxicity and biocompatibility tests involving quantum dot conversions are essential to assuring safety in the intended applications without compromising the risk of various environmental conditions, living or environmental health. If a concentration of CdS NPs is used that is low enough to be below the threshold of toxicity (0.01 µg/ml), there may utility for these NPs to be used in biological systems as a delivery agent, or a diagnostic tool. According to our results, 0.01 µg/ml CdS did not show significant toxicity in any of the assays performed and it can be used in both smart drug delivery and diagnosis at the same time. In conclusion, although CdS NPs may be used in the biological system there need future studies to evaluate chronic exposure of CdS toxicity conditions in a laboratory animal. In conclusion, CdS is proper to be used in a biological system but there need future studies to evaluate for acute and chronic exposure of CdS toxicity condition in the laboratory animal.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest.

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