Original Article

Polyethylene glycol modification decreases the cardiac toxicity of carbonaceous dots in mouse and zebrafish models

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Aim: Carbonaceous dots (CDs), which have been used for diagnosis, drug delivery and gene delivery, are accumulated in heart at high concentrations. To improve their biocompatibility, polyethylene glycol-modified CDs (PEG-CDs) were prepared. In this study we compared the cardiac toxicity of CDs and PEG-CDs in mouse and zebrafish models.

Methods: Mice were intravenously treated with CDs (size: 4.9 nm, 5 mg·kg⁻¹·d⁻¹) or PEG-CDs (size: 8.3 nm, 5 mg·kg⁻¹·d⁻¹) for 21 d. Their blood biochemistry indices, ECG, and histological examination were examined for evaluation of cardiac toxicity. CDs or PEG-CDs was added in incubator of cmlc2 transgenic Zebrafish embryos at 6 hpf, and the shape and size of embryos’ hearts were observed at 48 hpf using a fluorescent microscope. Furthermore, whole-mount in situ hybridization was used to examine the expression of early cardiac marker gene (cmlc2) at 48 hpf.

Results: Administration of CDs or PEG-CDs in mice caused mild, but statistically insignificant reduction in serum creatine kinase (CK) and lactate dehydrogenase (LDH) levels detected at 7 d, which were returned to the respective control levels at 21 d. Neither CDs nor PEG-CDs caused significant changes in the morphology of heart cells. Administration of CDs, but not PEG-CDs, in mice caused marked increase of heart rate. Both CDs and PEG-CDs did not affect other ECG parameters. In the zebrafish embryos, addition of CDs (20 μg/mL) caused heart development delay, whereas addition of CDs (80 μg/mL) led to heart malformation. In contrast, PEG-CDs caused considerably small changes in heart development, which was consistent with the results from the in situ hybridization experiments.

Conclusion: CDs causes greater cardiac toxicity, especially regarding heart development. Polyethylene glycol modification can attenuate the cardiac toxicity of CDs.

Keywords: carbon-based nanomaterials; carbonaceous dots; cardiac toxicity; polyethylene glycol modification; zebrafish

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Introduction

Novel nanomaterials are continually emerging to satisfy the requirements of use for various applications, and they have been used in multiple applications, including drug/protein/nucleic acid delivery, contrast probe delivery, disease diagnosis and analysis. However, although many nanomaterials have been developed with unique characteristics and functionalities, only a few of them have been approved for clinical use in humans, because of their potential toxicity. Therefore, toxicity should be paid greater attention when novel nanomaterials are developed.

Carbonaceous dots (CDs), one of the most extensively used carbon-based nanomaterials, have attracted increasing attention because of their excellent properties, such as high fluorescent quantum yield, low photo bleaching and absence of optical blinking. Accordingly, various methods have been developed for constructing CDs, and many applications have been evaluated, especially in tumor cell imaging and drug delivery. Although most of these studies have reported that CDs are quite biocompatible, no systemic toxicity evaluations have been performed.

The heart is one of the most important organs in humans.
Thus, toxicity in the heart should be paid particular attention\textsuperscript{[14]}. Our previous study showed that CDs accumulate in the heart at high concentrations, even higher than those in the liver, spleen, kidneys and lungs\textsuperscript{[15, 16]}. Although polyethylene glycol modification (PEGylation) decreases the CDs concentrations in the heart, the concentrations still remains higher than those of most other types of nanomaterials\textsuperscript{[16]}. Therefore, heart toxicity must be carefully evaluated before employing CDs in drug delivery and disease diagnosis.

Currently, the most widely used models for toxicity evaluation are the mouse and zebrafish. Mouse models can be used for whole body toxicity evaluation. The zebrafish model has shown many superior attributes, including easy maintenance, fast embryonic development and transparent bodies that can be observed using a light microscope\textsuperscript{[17, 18]}. Although zebrafish have been used for the evaluation of the toxicity of other nanomaterials\textsuperscript{[19, 20]}, this report is the first that used zebrafish as a model to determine the heart toxicity of CDs and PEGylated CDs (PEG-CDs). We used a mouse model to determine the toxicity in the heart and a zebrafish model to determine the effects of toxicity on heart development.

Materials and methods

Materials

Amino poly-(ethylene glycol) (NH\textsubscript{2}-PEG, MW=5000) was obtained from Seebio Biotech Inc (Shanghai, China). Glutamic acid was obtained from Sinopharm Chemical Reagent (Shanghai, China). A DIG RNA Labeling Kit (SP6/T7) was purchased from Roche (Basel, Switzerland). MEGAClear kit was purchased from Life Technologies (Grand Island, NY, USA). Hydrochloride (EDC) and N-hydroxy-succinimide (NHS) were obtained from Keddia Reagent (Chengdu, China). The other chemicals and reagents were all of analytical grade. Kun-ming mice (male, 4-5 weeks old, 18-22 g) were obtained from Dashuo Biotechnology Co Ltd (Chengdu, China) and were maintained under standard housing conditions.

Preparation and characterization

The CDs were prepared using a heat-treated method\textsuperscript{[10]}. After a flask was heated to 280 °C, 1 g of glutamic acid was added, and the flask was kept on a heater for 1 min. Then, 5 mL of deionized water was added after the flask cooled to 70 °C. The CDs were harvested after sonication and centrifugation. For conjugation with PEG, EDC and NHS were introduced into the CDs (20 mg/mL) to activate carboxyl units. Then, NH\textsubscript{2}-PEG (80 μg/mL) was added and stirred for 2 h, and the PEG-CDs were harvested after dialysis (cutoff size=10 kDa). The hydrated diameter was determined by a Malvern Zetasizer (Malvern, NanoZS, UK). The morphology was determined using a transmission electron microscope (TEM) (JEOL, JEM-2100, Japan) operated at 200 kV.

In vivo heart toxicity in mice

In vivo heart toxicity was determined by blood biochemistry indices and H&E staining. All of the animal experiments were performed in accordance with protocols evaluated and approved by the Ethics Committee of Sichuan University.

Thirty-six mice were divided into 3 groups: a saline group, a CDs (5 mg/kg) group and a PEG-CDs (5 mg/kg) group. Mice received the corresponding formulations every day through the tail vein. Waveform electrocardiography (ECG) and ECG-related parameters were obtained separately at 0, 7, 14, and 21 d. All of the results were acquired with a Data Acquisition & Analysis System (Chengdu Technology & Market Co, Ltd., Chengdu, China). Seven days and 21 d after the first administration, serum was collected from 6 mice from each group for hematology biochemistry analyses using an MEK-6318K Automated Hematology Analyzer (Nihon-kohden, Shinjuku-ku, Tokyo, Japan). Then, the heart was sampled and H&E staining was applied\textsuperscript{[21]}. Toxicity in the heart function of zebrafish

Cardiac myosin light chain 2 (cmlc2) transgenic zebrafish [Tg (cmlc2:EGFP)] were obtained from the zebrafish aquarium of the Joint Laboratory of Reproductive Medicine, Sichuan University-The Chinese University of Hong Kong and were staged according to standard procedures. Briefly, zebrafish strains were raised in an incubator at 28 °C in a 14 h light and 10 h dark cycle using UV-treated and aerated water\textsuperscript{[22]}. Fifteen 6-h post-fertilization (hpf) zebrafish embryos were added to each well of a 24-well plate, and this was followed by addition of CDs and PEG-CDs at different concentrations (20 μg/mL and 80 μg/mL). The shape and size of the hearts in each group were determined at 48 hpf using a fluorescent microscope (Nikon, Japan).

Three independent experiments were performed.

Zebrafish whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as previously described\textsuperscript{[23]}. After lineage by appropriate restriction enzymes, antisense RNAs for in situ hybridization were synthesized using the DIG RNA Labeling Kit (SP6/T7) and were purified with a MEGAClear kit. Then, images were obtained using a microscope (Nikon, Japan).

Statistical analysis

Significant differences were evaluated with Student’s t test. P values less than 0.05 were considered to be statistically significant.

Results

Characterization of CDs and PEG-CDs

The particle size of the CDs was 4.9 nm, and the size increased to 8.3 nm after PEGylation (Figure 1), which was consistent with our previous studies\textsuperscript{[24, 25]}. According to TEM, the CDs were all spherical and were well dispersed with an average size of 3-4 nm (Figure 1C); in comparison, the TEM size of PEG-CDs was similar to that of the CDs, which was consistent with results from our previous study\textsuperscript{[26]}. The zeta potential of the CDs was -5.8 mV, and the zeta potential of the PEG-CDs was -11.6 mV, contributing to PEG modification. The PEG concentration of the PEG-CDs was 4.04 μg/mg according to our previous study\textsuperscript{[25]}.
Biochemistry indices evaluation

After six administrations of CDs and PEG-CDs at doses of 5 mg/kg in mice, both the creatine kinase (CK) and the lactate dehydrogenase (LDH) levels showed mild, but statistically insignificant reduction compared to the controls (Figure 2A and 2B), thus indicating the CDs and PEG-CDs could cause somewhat toxicity in the heart. Two weeks later, both indices were almost the same in the control and treatment groups, thus suggesting that CDs and PEG-CDs could not cause long-term toxicity in the heart.

H&E staining

H&E staining was used to explore further the toxicity of CDs and PEG-CDs to the heart in mice (Figure 2E). Cells in all of the heart slices of the three groups were normal, and there were no significant differences among the three groups. These results suggest that neither CDs nor PEG-CDs cause considerable organic toxicity in the heart, consistently with their low cytotoxicity with results from other studies\(^{16, 27}\).
Effects of CDs and PEG-CDs on ECG

Next, ECG records were obtained from mice treated with saline, CDs and PEG-CDs from 0 d to 21 d (Figure 3A). Accordingly, the heart rate, PR intervals, QRS intervals and QT intervals of each group were recorded. As shown in Figure 3B, the heart rate was significantly increased in the CDs group at 21 d, showing a significant difference compared with the saline group. However, the PEG-CDs group showed the same trend as that in the saline group. In addition, other ECG parameters among the saline group, CDs group and PEG-CDs group presented no significant differences (Figure 3C-3E).

Heart development of zebrafish

cmlc2 transgenic zebrafish, which express GFP in the heart [Tg(cmlc2:EGFP)], were used to examine the effects of CDs and PEG-CDs on heart development because the fluorescent images directly reflected the functional development of the heart. The shape and size of the heart were observed using

![Figure 3](image-url)

Figure 3. Effects of CDs and PEG-CDs on ECG data. (A) Typical time course of ECG waveforms of mice treated with saline, CDs and PEG-CDs. (B-E) Comparison of heart rate (B), PR intervals (C), QRS intervals (D) and QT intervals (E) in the saline group, CD group and PEG-CD group (n=3). *P<0.01 between saline group and CDs group.
a fluorescent microscope (Figure 4). The morphology of the hearts in the group treated with CDs (80 μg/mL), compared to the control group, showed obvious malformations, suggesting that high concentrations of CDs could cause significant developmental toxicity in the heart. Reducing the concentration to 20 μg/mL considerably attenuated the malformation, although it was still smaller than in the controls, indicating that heart toxicity is concentration dependent, consistently with other studies\cite{17}. Comparatively, the heart development of PEG-CDs-treated zebrafish was better than that of CDs-treated fish, whereas zebrafish treated with low concentration (20 μg/mL) of PEG-CDs showed no obvious differences compared with the control group, demonstrating that PEGylation could significantly reduce the toxicity of CDs to the heart.

**In situ hybridization**

To examine early cardiac marker gene (clml2) expression of zebrafish, whole-mount in situ hybridization was used. Hearts of the zebrafish treated with CDs at 20 μg/mL or 80 μg/mL presented delayed development, compared with the control group (Figure 5). Moreover, the 80 μg/mL CDs group showed a malposition between the atrium and ventricle, which failed to loop or showed an inverse loop, thus further demonstrating that CDs, especially at high concentrations, inhibited the development of the heart and caused heart abnormalities. In contrast, the groups treated with PEG-CDs at 20 or 80 μg/mL had hearts of the same size and shape as those of the control group, indicating that PEGylation could indeed attenuate the toxicity of CDs to heart development, consistently with the above study.

**Discussion**

CDs have been extensively used in various fields, including diagnosis, drug delivery and gene delivery, showing excellent properties\cite{10, 28, 29}. However, toxicity concerns should be addressed before employing nanomaterials in humans. Because CDs have emerged so recently, only a few studies have evaluated the toxicity of CDs, and their cardiac toxicity has not been evaluated until now\cite{26, 27}. Therefore, several experiments were performed to evaluate the cardiac toxicity of CDs.

Myocardial cells contain a large number of enzymes, including CK and LDH, which can be released into the blood after myocardial cell damage\cite{30}; thus, blood biochemistry indices, specifically CK and LDH, were determined to reflect the in vivo toxicity in the heart. Although both the CK and LDH of CDs-treated mice were decreased at 7 d after the first administration, they remained at normal levels, and the number increased to almost the same as in the controls at 21 d. These results suggested that CDs do not cause permanent harm to the hearts of mice, as also demonstrated by H&E staining. The low systemic toxicity of CDs to the heart was consistent with

![Figure 4](https://www.chinaphar.com/ChenJT et al/Acta Pharmacologica Sinica/npg/www.chinaphar.com)

**Figure 4.** Heart function observation in zebrafish (Tg(clmlc2:EGFP)) upon treatment with CDs and PEG-CDs (48 hpf). Scale bar represents 100 μm.
results from other studies\cite{26,27}, which have shown that CDs cause low toxicity in the main organs of mice and low cytotoxicity in several types of cell lines.

As a more sensitive method, ECG was also used to evaluate the heart toxicity of CDs in mice. Our study showed that the heart rate at 21 d in the CDs group was significantly higher than that of the saline group, indicating that CDs had some toxicity in the heart, a result similar to other groups' findings\cite{31,32}. However, the heart rate of the PEG-CDs group was similar to that of the saline group, suggesting the PEGylation could considerably attenuate the heart toxicity of CDs.

Zebrafish were used to evaluate developmental cardiac toxicity further because zebrafish provide a quick and convenient model for this purpose\cite{17}. High concentrations (80 μg/mL) of CDs caused significant toxicity in heart development. The morphology of the heart in treatment groups compared to controls showed obvious malformations, and early cardiac marker gene (clml2) expression was reduced. Decreasing the concentration to 20 μg/mL attenuated the developmental cardiac toxicity. These studies suggested that CDs indeed caused toxicity in heart development, although they had low cardiac toxicity in adult mice. It is interesting that even 1 mg/mL CDs had low cytotoxicity when cell viability was used as an index\cite{26}. This contradiction indicated different sensitivities to CDs among different types of cells. The cells used in the cytotoxicity study were cell lines that were easy to adapt to the environment, whereas the primary cells, especially primary stem cells, were more sensitive to the environment. Thus the application of CDs in young humans should be paid greater attention.

PEGylation has been widely used in nanomaterials because it can improve the biocompatibility and reduce the clearance by the reticuloendothelial system\cite{33,34}. PEG-CDs had lower concentrations in the hearts of mice than bare CDs. Thus, we also evaluated the cardiac toxicity of PEG-CDs. Especially in the zebrafish model, PEG-CDs caused considerably lower toxicity in heart development, which further demonstrated that PEGylation can improve the biocompatibility of nanomaterials. The reduction in toxicity might have been caused by the reduced contact between the nanomaterials and cells.

In this study, both in vitro and in vivo experiments were utilized to evaluate the heart toxicity of CDs and PEG-CDs, and although some indices, such as heart rate, were abnormal, most of the indices of both the CDs and PEG-CDs groups were normal; thus, the heart toxicity of CDs to adult animals was low. However, as demonstrated in the zebrafish model, the developmental toxicity in the heart was strong and could not be ignored, although PEGylation attenuated this toxicity. Nevertheless, the reason for the heart toxicity of CDs remains unclear and will require further evaluation. The signal pathways involved in heart toxicity must also be elucidated.

In conclusion, CDs and PEG-CDs were prepared and characterized. Both in vivo biochemistry indices and H&E staining showed the low toxicity of CDs and PEG-CDs in heart function and in cells, and ECG showed that the toxicity of CDs in the heart was higher than that of PEG-CDs. Furthermore, in zebrafish, CDs showed significant toxicity in heart development, causing heart malformation (high concentration) and developmental delay (low concentration). Comparatively, PEG-CDs showed considerably lower toxicity in heart development.

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Author contribution
Hui-le GAO, Jun QIAN and Qin HE designed the study; Jian-tao CHEN and Hua-qin SUN performed the experiments and wrote the paper with assistance of Wei-liang WANG, Wen-ming XU and Shun SHEN.

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