Biomass Cellulose Nanoparticles Display Considerable Neurotoxicity in Zebrafish

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Research

Keywords:

Posted Date: June 11th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-34331/v1>

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Abstract

Background

The widespread use of nanomaterials poses a great threat to human living environments. Among them, biomass cellulose nanoparticle (CN) is one of the widely used nanomaterials. To date, the toxicity of CNs during embryonic development remains undetermined. In this study, we exposed zebrafish embryos to cellulose nanofibrils (CNFs) and cellulose nanocrystals (CNCs) to evaluate the toxicity of these CNs.

Results

Exposure to CNFs or CNCs below 30 mg/ml exhibited no dose-dependent increases in malformation and mortality in zebrafish embryos. Then we demonstrated that CNs were highly enriched in zebrafish embryo via imaging analysis of embryos treated with FITC-coupled CNCs. In addition, we found that CNF or CNC exposure resulted in compromised motor ability of zebrafish larva. Furthermore, it was revealed that the differentiation and the morphogenesis of motor neurons were significantly interrupted. While, blood vessels were normally patterned, suggesting the specific neurotoxicity of those materials. Transcriptome sequencing assay demonstrated that the neurotoxicity of CNs in the motor neurons might be attributed to the expression alteration of neural genes.

Conclusion

We found that the specific neurotoxicity of CNF and CNC using zebrafish model. CNF or CNC exposure interrupted the morphogenesis of motor neurons, which resulted in the compromised motor ability of zebrafish larva. In addition, the neurotoxicity of CNs in the motor neurons might be attributed to the expression alteration of neural genes.

Introduction

Nanomaterials possess unique small size effect, surface effect and quantum effect, making them widely applied in imaging [1], industry [2], communications [3, 4], medicine [5], food and other fields [6]. Nevertheless, it is inevitable that nanomaterials will be released into the environment directly or indirectly in the producing and applying process, resulting in diverse degrees of exposure of organisms [7]. Nanoparticles released into water, soil and atmosphere may act on organisms with unpredictable consequences. Consequently, while utilizing the advantageous properties of nanomaterials, the nanomaterials should be sufficiently evaluated to learn about their adverse effects on organisms. There have already been many researches on the toxicities of metal oxide nanoparticles and their underlying mechanisms. Nano-ZnO have previously reported to exert toxicities in RAW 264.7 and BEAS-2B cell lines, resulting in the generation of oxidative stress, inflammatory response, and cell death [8]. In recent researches, silver nanoparticles (AgNPs) have displayed multiple damages such as cell toxicity [9, 10], respiratory system injury [11, 12], dermal toxicity, kidney toxicity, reprotoxicity, genotoxicity, development toxicity [13, 14], and immunotoxicity [15] in cell and animal models. TiO₂ nanoparticles was reported to

penetrate through the skin, and reach major organs in mice, arousing oxidative stress induced pathological lesions [16].

Another widely used natural nanomaterial, cellulose nanoparticles (CNs) have entered people's life in diverse modalities. In recent years, many CNs with special attributes were prepared [17, 18]. CNs, such as cellulose nanocrystallines (CNCs) and cellulose nanofibrils (CNFs), are well applied in many fields benefiting from the improvement of their production process. In previous studies, though the quality of the available studies is improving over time, it is generally insufficient for risk assessment purposes [19]. Based on the results of toxicity studies of metal nanomaterials, we consider that the research on the toxicity of CNs should be carried out urgently. Till now, the toxicity of these biomass CNs were reported mainly in the inhalation toxicity to the lung in both cellular [20–22] and animal [20, 23, 24] models. These investigations demonstrated CN exposures resulted in inflammatory responses [19]. Some other toxicity studies of CNs, such as hepatotoxicity in rats [25], systemic toxicity exhibiting inflammatory and acute phase responses in mice [26], renal toxicity via the combined impacts of electrolyte imbalance and inflammation [27], and so forth, also reveal that CNs possess multiple toxicities in organisms.

The transparent zebrafish larvae facilitate both whole body and microcosmic viewing by *in vivo* imaging. And zebrafish models have been widely utilized in toxicity researches. Thus, we selected the zebrafish model to investigate the effects of nanocelluloses on organisms in this study. Moreover, the zebrafish model enables to achieve genetic profiles in a whole vertebrate by high-throughput sequencing analysis. So, we further investigated the potential mechanisms underlying the induction of zebrafish developmental defects by CNs.

Materials And Methods

Natural CNs

CNFs were purchased from Intelligent Chemicals, Ltd. (Tianjin, China) and used directly, with 1.46% solid content. CNCs were prepared from switchgrass cellulose powder with sulfuric acid, with 3% ~ 4% solid content. The CNs were deposited onto fresh cleaved mica. The morphology and size of prepared CNs were obtained by means of the winer802 nano-laser particle size meter (Jinan Microner Instrument Co., Ltd, China) and scanning electron microscopy (SEM; the Zeiss Dual Beam instrument, Germany).

Zebrafish Husbandry And Ethics

The wild type and transgenic zebrafish line *Tg(Kdrl:ras-mcherry::Hb9:GFP)* are provided by the Zebrafish Center of Nantong University. The breeding conditions were in accordance with the previously described procedure [28]. The study was conducted conforming to the local laws and the Chinese law for the Protection of Animals.

Cns Treatments And Development Status Recording

Stock solutions of CNFs and CNCs were dispersed in ultrapure water, and sonicated for 1 h at 900 w using JY92-IIDN Ultrasonic Cell Crusher (Scientz Biotechnology Ltd., Ningbo, China) prior to preparation of the working solutions. The CNFs and CNCs concentration of working solutions ranged between 30 mg/ml and 0.1 µg/ml. The embryos were exposed to CNFs or CNCs in a 96-well plate. The exposure was conducted in a 28 ± 0.5 °C incubator. The development status was recorded with a bright field microscope in according with the previous described procedure [28].

Locomotion Analyses

The swimming ability of control and CNs exposed zebrafish larvae were determined in accordance with the method described previously [29].

Imaging

The confocal imaging of blood vessels and motor neurons in *Tg(Kdrl:ras-mcherry::Hb9:GFP)* zebrafish embryos was handling according to the previous described procedure [29]. The trunk motor neurons rostral primary (RoP) motor neuron, middle primary (MiP) motor neuron, and caudal primary (CaP) motor neuron as shown in Fig. 5A were captured with a Leica TCS-SP5 LSM confocal imaging system.

Transcriptome Sequencing Analyses

The control and CNFs exposed zebrafish larvae were collected for total RNA extraction. Three independent replicates of each group were sequenced. The total RNA was extracted using Trizol kit (Thermo Scientific) according to the manufacturer's instructions. RNA quality was control by RNA integrity number (RIN). The purified mRNA from the total RNA was used to build cDNA libraries according to the Sample Preparation Kit instructions (Illumina). The cDNA libraries were sequenced by an Illumina Hiseq platform at GENEWIZ (Suzhou, China). The Cuffdiff software was used to estimate differential expression between samples at the transcript level. The R package cluster profiler was used for GO and KEGG pathway annotation and enrichment analysis of differential expression genes, and the network were visualized by Cytoscape software.

Statistics

Statistical analyses were performed using GraphPad Prism 5. All data were expressed as mean $\pm$ SEM. T-test was used for comparing 2 groups ($p < 0.05$). One-way ANOVA ($p < 0.05$) with Dunnett's tests was used for multiple comparisons.

Results

Characteristics of CNFs and CNCs

The size distribution of CNFs and CNCs was assayed (Fig. 1A and B). The diameter of the CNFs mainly distributed between 50 nm and 70 nm, while the diameter of CNCs mainly distributed in the range of 24–35 nm. SEM images showed that CNFs and CNCs were capable of dispersing on mica substrate without tendency for agglomerating (Fig. 1C and D). It should be mentioned that any surface aggregation of the nanoparticles was not observed. The shape of CNFs is mainly long fibrous, with the length between 1 ~ 2 μm and diameter 15–40 μm , whose aspect ratio varies from 15 to 40. With a rod-shaped structure, the CNCs length is between 250–400 nm, aspect ratio in 10–11.

Toxicities of CNFs and CNCs on zebrafish embryos at the whole animal level

To assess the potential toxicity of CNs, we treated the wild type zebrafish embryos with CNFs or CNCs during 8–96 hpf. The toxicological responses such as mortality, hatching, and abnormality were observed and recorded (Fig. 2). At the concentration between 30 mg/ml and 0.1 $\mu\text{g/ml}$, CNFs and CNCs showed no dosage-dependent toxicological responses in treated zebrafish embryos, respectively at 48, 72, and 96 hpf.

Enrichment Of Cns In Zebrafish Larvae

According to the scheme in Fig. 3A, the wild type zebrafish larvae were treated with FITC labelled CNCs (CNC-FITC). Fluorescence microscope imaging indicated that CNC-FITC was enriched in the whole embryo at both 10 $\mu\text{g/ml}$ and 1 $\mu\text{g/ml}$ (Fig. 3B). The enrichment of CNC-FITC also at both 10 $\mu\text{g/ml}$ and 1 $\mu\text{g/ml}$ was further observed by confocal microscope (Fig. 3C). FITC coupled CNCs treatments showed that CNs could be enriched in zebrafish larvae.

Cnfs Obviously Injured The Motor Ability

After treated with CNFs or CNCs, the motor ability of zebrafish larvae receded obviously. Thus, we carried out locomotion analyses in order to quantitatively obtain the injury of CNs. Here, we showed that CNFs of 30 mg/ml obviously inhibited the motor ability of zebrafish larvae through recording the swimming track (Fig. 4A) and retention time displayed with heat map (Fig. 4B). In addition, the average swimming velocities (Fig. 4C) and distances (Fig. 4D) in every minute were obviously decreased by CNFs treatments. Similarly, compared with control group, the average swimming accelerations (Fig. 4E) and decelerations (Fig. 4F) in every minute were obviously decreased by CNFs treatments. Moreover, we found CNF treatments significantly increased alteration of swimming direction through recording the angular velocity (Fig. 4G) and turning angle (Fig. 4H). These data demonstrated that CNF exposures significantly injured zebrafish larva motor ability.

Cnfs And Cncls Significantly Inhibited Motor Neuron Development

Judged from locomotion analyses, we deduced there must be some wrong with the nervous system in the CNs treated zebrafish larvae. So, we investigated the effects of CNs on the trunk motor neurons using transgenic zebrafish line *Tg(Hb9:GFP::Kdrl:ras-mcherry)*. Meanwhile, we detected the effects of CNs on the zebrafish trunk blood vessels (Fig. 5A). Comparing with control group, the number of CaP motor neurons and the length of their projections in the zebrafish larvae treated with 30 mg/ml of CNFs or CNCs were significantly reduced (Fig. 5B). In addition, the projections of these motor neurons were disorganized (Fig. 5B). While the blood vessels showed no obvious developmental defects in the CN treated zebrafish larvae (Fig. 5B). By calculating the missing percent of CaP motor neurons, both 30 mg/ml of CNFs and CNCs significantly resulted in the CaP motor neuron missing in the trunk of zebrafish larvae (Fig. 5C). Moreover, almost all CaP motor neurons showed developmental defects. And comparing with control group, the RoP and MiP motor neuron development was also obviously abnormal. These results suggest the motor neuron differentiation and morphogenesis was interrupted.

mRNA-seq analyses revealed CNs directly injured nervous system.

The injured swimming ability and motor neuron phenotype of CNs treated zebrafish larvae directed us to focus on the neurotoxicity. Here, we investigated the molecular alterations using mRNA sequencing. GO enrichment analyses demonstrated that CN treatments participated in the nervous system injury, including axon guidance, neuron projection morphogenesis, spinal cord motor neuron differentiation, axon extension, axonogenesis, synaptic signaling, and etc. biological processes (Fig. 6A). In addition, cellular component enrichment and molecular function enrichment analyses revealed that the different expression genes well matched with the regulated biological processes (Figure S1 and 2). The signaling pathway enrichment analyses showed that CN treatments acted on the multiple typical signaling pathways in neurons, such as calcium signaling pathway, neuroactive ligand-receptor interaction, MAPK signaling pathway, Notch signaling pathway, Wnt signaling pathway, regulation of actin cytoskeleton, and etc. (Fig. 6B).

Discussion

Nowadays, CNs were widely used in many fields due to the easy assessments to raw materials and improved manufacturing processes, such as paper, building materials, food, cosmetics, pharmaceutical products, hygiene and absorbent products, textiles for clothing, automotive composites, and many other applications [19, 26]. The toxicity evaluation of CNs is greatly lagging in the circumstance of enormous commercial applications. Especially in neurotoxicity evaluation of CNs, there are no reference data available till now. In present study, we investigated the neurotoxicity of CNs using zebrafish models. The zebrafish model is conveniently used in this exploratory research.

Our results demonstrated that CNs exerted their undisclosed neurotoxicity in the zebrafish larvae. The neurotoxicity was mainly manifested in the phenotypes of neurite extension obstruction, abnormal axon guidance, and loss of motor neurons in the CN treated zebrafish larvae (Fig. 5). These severe phenotypes of the motor neurons in CN treated zebrafish larvae correlated well with the impaired swimming ability (Fig. 4). And this is directly related to the enrichment of CNs in zebrafish larvae (Fig. 3). Although CNs resulted in motor neuron developmental defects, the vascular system was not apparently injured in zebrafish larvae (Fig. 5). We supposed that the normal development of vascular system may be the pivotal reason for maintaining the whole zebrafish embryo normal before 96 hpf. In other words, the gene altered by CN exposures mainly associated with the neuron development but not vascular system. This conclusion was further confirmed by bioinformatics analyses. Among the significantly changed genes, we selected the genes involved in the nervous system biological processes to establish the GO enrichment network. In the network, we found these genes showed complex interconnections (Fig. 6A). Both vascular system and nervous system related signaling pathway were enriched such as VEGF signaling pathway, Notch signaling pathway, and Wnt signaling pathway. And these pathways were not the insignificant nodes in the signal cascade network (Fig. 6B). Deduced from the phenotype and gene network analyses, we consider that exposures to CNs are likely to pose a potentially high risk to organisms.

In the previous review [30], the authors retrospected that no studies could be located in examining the neurotoxicity of CNs till that time. Although there are still no studies focusing on this region from then on, many neurotoxicity researches of other nanoparticles can be consulted. For example, ZnO nanoparticles and TiO₂ nanoparticles could significantly deposit in the central nerve system with resulting in the cerebral cortex and hippocampus histopathological injury [31]. And AgNPs can be deposited in brain to cause neuron, astrocytes and glial cell injuries *via* multiple molecular mechanisms [32, 33]. The application of bioinformatics analysis will also promote the neurotoxicity research of CNs. In view of the neurotoxicity of CNs in zebrafish larvae, more detailed researches, for example, experiments on adult zebrafish, researched on other animal models, and further verifications after systematic bioinformatics analyses are needed.

Conclusion

We found that the specific neurotoxicity of CNF and CNC using zebrafish model. CNF or CNC exposure resulted in the accumulation in zebrafish embryos and interrupted the morphogenesis of motor neurons, which resulted in the compromised motor ability of zebrafish larva. In addition, the neurotoxicity of CNs in the motor neurons might be attributed to the expression alteration of neural genes.

Declarations

Ethical Approval and Consent to participate

All animal experimentation was carried out in accordance with the NIH Guidelines for the care and use of laboratory animals (<http://oacu.od.nih.gov/regs/index.htm>). Meanwhile, our study complies with the rules of the Guidelines for the care and use of laboratory animals (<https://www.biomedcentral.com/getpublished/editorial-policies#research+involving+animals>) and ethically approved by the Administration Committee of Experimental Animals, Jiangsu Province, China.

Consent for publication

All authors agreed to this publication.

Availability of data and materials

All data generated or analysed during this study are included in this published article, and available from the corresponding authors on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by grants from the National Natural Science Foundation of China (81870359, 2018YFA0801004 to Dong Liu; 81970432 to Yunwei Shi), Natural Science Foundation of Jiangsu Province (BK20180048, 17KJA180008 and BRA2019278 to Dong Liu), and Nantong Science and Technology Project (JC2018017 to Guanyun Wei).

Authors' contributions

1. Liu and Y. Shi designed the experiments, analyzed the data, and prepared the manuscript. C. Liu, J. Zhao, X. Wang, and C. Yang did the experiments. G. Wei performed the bioinformatics analyses. The authors have no conflicts of interest to declare.

Acknowledgements

Authors wish to thank Dong Liu for the indispensable help and expertise in the present study.

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34. Declarations.

Figures

Figure 1

The size distribution assay and SEM image of CNFs and CNCs. A, Size distribution of CNF. B, Size distribution of CNCs. C, SEM image of CNFs. D, SEM image of CNCs.

Figure 2

The toxicological responses of CNFs and CNCs in zebrafish embryos. A, The percent of death, malformation and normality of zebrafish embryos exposed to CNFs (n = 30). B, The percent of death, malformation and normality of zebrafish embryos exposed to CNCs (n = 30).

Figure 3

The enrichment of FITC labelled CNCs (CNC- FITC) in wild type zebrafish larvae. A, The experiment scheme. B, The enrichment of CNC- FITC by fluorescence microscope imaging in the whole embryo at both 10 µg/ml and 1µg/ml. C, The enrichment of CNC-FITC at both 10 µg/ml and 1µg/ml observed by confocal microscope.

Figure 4

Locomotion analyses of control and CNFs treated zebrafish larvae. A, the swimming track of control and CNFs treated zebrafish larvae. B, Heat map of retention time of control and CNFs treated zebrafish larvae. C, The average swimming velocities of control and CNFs treated zebrafish larvae in every minute (n = 12). D, The average swimming distances of control and CNFs treated zebrafish larvae in every minute (n = 12). E, The average swimming accelerations of control and CNFs treated zebrafish larvae in every minute (n =

12). F, The average swimming decelerations of control and CNFs treated zebrafish larvae in every minute (n = 12). G, The angular velocity of control and CNFs treated zebrafish larvae in every minute (n = 12). H, The turning angle of control and CNFs treated zebrafish larvae in every minute (n = 12). Data were represented as mean $\pm$ SEM. **p < 0.01, and ***p < 0.001 versus control group.

Figure 5

Effects of CNFs and CNCs on motor neurons and blood vessels in zebrafish embryos at 48 hpf. A, The diagram of motor neurons and blood vessels in the trunk of zebrafish embryos at 48 hpf. Three kinds of motor neurons middle primary (MiP) motor neuron, rostral primary (RoP) motor neuron, and caudal primary (CaP) motor neurons, were drawn in green. The blood vessels dorsal longitudinal anastomotic vessel (DLAV), intersegmental vessel (ISV), dorsal aorta (DA), and posterior cardinal vein (PCV) were drawn in red. B, The confocal imaging of motor neurons and blood vessels in the trunk of Tg(Hb9:GFP::Kdr:ras-mcherry) zebrafish embryos at 48 hpf. C, the statistical analyses of CaP motor neurons missing percent in control, 30 mg/ml CNFs and 30 mg/ml CNCs treated groups (n = 6). Data were represented as mean $\pm$ SEM. ***p < 0.001 versus control group.

Figure 6

GO enrichment and KEGG signaling pathway enrichment analyses of control and CNF treated zebrafish larvae. A, Biological process enrichment analyses. B, KEGG signaling pathway enrichment analyses.

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