

## Toxicity of ceria nanoparticles to the regeneration of freshwater planarian *Dugesia japonica*

Xie, Changjian; Li, Xiaowei; Hei, Lisha; Chen, Yiqing; Dong, Yuling; Zhang, Shujing; Ma, Shan; Xu, Jianing; Pang, Qiuxiang; Lynch, Iseult; Guo, Zhiling; Zhang, Peng

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1                    ***Toxicity of ceria nanoparticles to the regeneration of freshwater***  
2                    ***planarian *Dugesia japonica*: the role of biotransformation***

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30 **Abstract**

31 Cerium oxide nanoparticles (n-CeO<sub>2</sub>) have wide applications ranging from industrial to  
32 consumer products, which would inevitably lead to their release into the environment. Despite the  
33 toxicity of n-CeO<sub>2</sub> on aquatic organisms has been largely reported, research on developing organisms  
34 is still lacking. In this study, we investigate the toxic effects of n-CeO<sub>2</sub> on the stem cells, tissue- and  
35 neuro-regeneration, using freshwater planarian *Dugesia japonica* as a model. Effects of bulk sized  
36 ( $\mu$ -) CeO<sub>2</sub> and ionic Ce (Ce<sup>3+</sup>) were compared with that of n-CeO<sub>2</sub> to explore the origin of the toxic  
37 effects of n-CeO<sub>2</sub>. No overt toxicity was observed in  $\mu$ -CeO<sub>2</sub> treatment. n-CeO<sub>2</sub> not only impaired  
38 the homeostasis of normal planarians, but also inhibited the regeneration processes of regenerated  
39 planarians, demonstrated by the inhibited blastema growth, disturbed antioxidant defense system at  
40 molecular levels, elevated DNA-damage and decreased stem cell proliferation. Regenerating  
41 organisms are more susceptible to n-CeO<sub>2</sub> than the normal ones. Ce<sup>3+</sup> exhibited significantly higher  
42 toxicity than n-CeO<sub>2</sub>, even though the total Ce uptake is 0.2% less in Ce<sup>3+</sup> than in n-CeO<sub>2</sub> treated in  
43 planarian. X-ray absorption near edge spectroscopy (XANES) analysis revealed that 12.8% of  
44 n-CeO<sub>2</sub> (5.95 mg/kg Ce per planarian) was transformed to Ce<sup>3+</sup> after interaction with planarian,  
45 suggesting that biotransformation at the nano-bio interface might play an important role in the  
46 observed toxicity. Since the biotransformation of n-CeO<sub>2</sub> is a slow process, it may cause long-term  
47 chronic toxicity to planarians due to the slow whilst sustained release of toxic Ce<sup>3+</sup> ions.

48 **Keywords:** n-CeO<sub>2</sub>, toxicity, planarian, XANES; Regeneration

49  
50 **1. Introduction**

51 The rapid development of nanotechnology promotes the applications of nanomaterials in  
52 various fields. Worldwide, about 10, 000 metric tons of cerium oxide nanoparticles (n-CeO<sub>2</sub>) are  
53 produced per year<sup>1</sup>. n-CeO<sub>2</sub> are used in numerous applications such as biomedical fields, fuel  
54 additives, gas sensors, polishing agent, and metal-oxide semiconductor devices<sup>2, 3</sup>. The increasing  
55 production and widespread application would inevitably lead to the release of n-CeO<sub>2</sub> into the  
56 environment, causing concerns for the potential toxicological risks to aquatic organisms as well as  
57 human health<sup>4</sup>. Developing organisms and tissues are often more susceptible to environmental  
58 pollutants including nanoparticles<sup>5</sup>.

59 The bio-effects of n-CeO<sub>2</sub> remain controversial. On one hand, n-CeO<sub>2</sub> is reported to have

60 inherent or acquired ability to scavenge reactive oxygen species (ROS)<sup>6-8</sup>, which was equivalent to  
61 the antioxidant enzymes in biological systems<sup>7,9</sup>. For example, n-CeO<sub>2</sub> can induce the proliferation  
62 of stem cells of mouse<sup>10</sup> and human<sup>11</sup> by regulating the intracellular ROS and the relative expression  
63 of genes responsible for the stem cell proliferation, migration and antioxidant defense system. Singh,  
64 et al.<sup>12</sup> also confirmed that n-CeO<sub>2</sub> can scavenge ROS accumulation and regulate the status of cell  
65 redox and thereby accelerating the growth in human keratinocytes (HaCaT cells). On the contrary,  
66 studies also showed that n-CeO<sub>2</sub> could exhibit adverse effects both *in vivo* and *in vitro*<sup>13-15</sup>. For  
67 instance, n-CeO<sub>2</sub> induced ROS accumulation and caused oxidative stress in *Caenorhabditis elegans*  
68 at environmental relevant concentration (1 nM), and ultimately led to a decreased lifespan<sup>13</sup>. The  
69 toxicity of n-CeO<sub>2</sub> depends on the species, dose, and the physiochemical property of n-CeO<sub>2</sub><sup>14, 16-20</sup>.  
70 The biological fates of n-CeO<sub>2</sub> are still unclear. Some studies suggest that the primary bioavailable  
71 form of n-CeO<sub>2</sub> in tissue is particulate form rather than the ionic (Ce<sup>3+</sup>)<sup>21</sup> as n-CeO<sub>2</sub> was considered  
72 as highly insoluble under environmental conditions<sup>22</sup>. Studies based on synchrotron radiation  
73 techniques have revealed the biotransformation of n-CeO<sub>2</sub> in plants and bacteria<sup>23-25</sup>. Is the  
74 transformed n-CeO<sub>2</sub> responsible for the toxicity? This issue needs to be urgent studied.

75 Developing organisms and tissues are often more susceptible to environmental pollutants than  
76 mature organisms<sup>26, 27</sup>. Stem cells, which undergo rapid cell division, are highly vulnerable to  
77 environmental chemicals, impairing their differentiation and development<sup>28</sup>. However, vertebrate  
78 experimental protocols need to be more prepared in terms of licensing and ethics. In fact, planarian  
79 as an alternative invertebrate organism has been proven to be an excellent stem cell model for more  
80 than 20 years<sup>29</sup>. Planarian has large numbers of pluripotent stem cells (20-30%) and can regenerate  
81 any body part including the whole head and the entire central nervous system<sup>30</sup>, which makes them a  
82 suitable model for studying developmental (neuro-) toxicity<sup>31</sup>. In addition, planarian can provide  
83 early warning of harmful substances even at very low concentrations, thus, it is widely used as an  
84 indicator for water quality<sup>5, 32</sup>. As a benthic animal in the water body, planarians use their motile cilia  
85 to glide over sediment surfaces<sup>33</sup> and interacted with the deposited nanoparticles (NPs), which may  
86 make them an excellent model for investigating the effects of NPs on aquatic animals.

87 Planarians have been used for assessing nanotoxicity of Ag nanoparticles<sup>5</sup>, polystyrene  
88 microplastics<sup>34, 35</sup>, and Fe<sub>3</sub>O<sub>4</sub> nanoparticles<sup>36</sup>. For instance, Kustov, et al.<sup>37</sup> found that Ag NPs was  
89 moderately toxic to the planarian with a dose-dependent effect. Compared with regeneration

90 planarians, the behavioral changes such as side-lying and screw-like movements, was only happened  
91 at the highest concentration (50 mg/L) in the homeostatic planarians, while the stem cell proliferation  
92 was not affected<sup>5</sup>. In a previous study, Fe<sub>3</sub>O<sub>4</sub> NPs had not affected the stem cell population dynamics,  
93 nor did they induce significant changes in either homeostatic or regenerative planarians<sup>36</sup>. Instead,  
94 Ermakov, et al.<sup>38</sup> found that CeO<sub>2</sub> NPs (an average size of 1-2 nm) may play an inorganic mitogens  
95 role in stimulating *Schmidtea mediterranea* (planarian) regeneration at 10<sup>-11</sup> M colloid solution, and  
96 CeF<sub>3</sub> NPs (F<sup>-</sup> can stabilize Ce<sup>3+</sup> that makes Ce<sup>3+</sup> less prone to oxidation,  $K_{sp}=8 \times 10^{-16}$  of CeF<sub>3</sub>) also  
97 did not show any toxic to the homeostatic and regeneration planarians<sup>39</sup>. In fact, the toxicity of  
98 n-CeO<sub>2</sub> is related to its physicochemical properties, such as size, shapes, concentrations, Ce<sup>3+</sup>  
99 released and species-specific. However, only little is known about the effects of n-CeO<sub>2</sub> sizes, doses,  
100 and biotransformation on the (neuro-) development toxicity of the aquatic organisms. In this study,  
101 we studied the toxicity of n-CeO<sub>2</sub> to freshwater planarian *Dugesia japonica* and compared their toxic  
102 effects to bulk-CeO<sub>2</sub> ( $\mu$ -CeO<sub>2</sub>), ionic Ce (Ce<sup>3+</sup>) and control treatment (CT) on stem cells, tissue  
103 regeneration and development *in vivo*. We also used X-ray absorption near-edge spectroscopy  
104 (XANES) to identify the chemical species to examine the role of biotransformation in the observed  
105 biological effects.

## 106 2. Materials and Methods

107 **2.1 Chemical and animals.** n-CeO<sub>2</sub> (nominal size < 25nm, determined by nitrogen sorption, purity  
108 99.9%, manufacturer's data) were purchased from Sigma-Aldrich (St. Louis, MO, USA). As  
109 previously reported<sup>40</sup>, the particles were 100% cubic ceria with diameters ranging from 6.2 nm up to  
110 48 nm, with the center of the distribution at 16.3 nm. The diameters of  $\mu$ -CeO<sub>2</sub> (nominal size <5  
111  $\mu$ m, 99.9% trace metal basis, Sigma-Aldrich) varied from 237.5 nm to 1320 nm, and the distribution  
112 center was 473.5 nm (**Fig. S1**). Hydrodynamic size of n-CeO<sub>2</sub> and  $\mu$ -CeO<sub>2</sub> in DI water and MW  
113 medium were  $138.2 \pm 31.8$  nm and  $2703.4 \pm 147.6$  nm,  $162.3 \pm 42.5$  nm and  $2863.2 \pm 258.7$  nm  
114 respectively. The zeta potential were  $13.8 \pm 4.0$  mV and  $11.5 \pm 5.7$  mV in DI water and  $11.3 \pm 2.9$   
115 mV and  $9.8 \pm 3.2$  mV in MW medium for n-CeO<sub>2</sub> and  $\mu$ -CeO<sub>2</sub> respectively. Cerium (III) chloride  
116 (CAS no. 7790-86-5, > 99.99%) was also purchased from Sigma-Aldrich.

117 Planarians *D. japonica* used in the study were collected from a spring in Yiyuan (Zibo, China),  
118 and acclimated in mineral water (MW, Lushan fountain water, Zibo, China) in a 12h/12h day/night

119 cycle at 20 °C for about 2 months to allow reproduce asexually in the laboratory. Then the  
120 planarians were maintained in our lab under the same cultural conditions for further studies. The  
121 planarians used in this study have been maintained in the lab for more than 1 year. Before exposure,  
122 the planarians were fed twice a week with bovine liver homogenate and the water was changed  
123 regularly (every two days) to ensure a clean environment. Planarians with lengths of 8 ~10 mm were  
124 selected for experiments and starved for at least a week before use.

125 **2.2 Exposure experiment.** Both homeostatic and regenerating planarians were used to examine the  
126 toxicity of n-CeO<sub>2</sub>. Regeneration was induced right before exposure by transversally cutting  
127 homeostatic planarians anterior to the pharynx (mouth part) using an ethanol-sterilized razor blade,  
128 creating a head-regeneration part. Tissue regeneration, such as new blastema formation was  
129 monitored over time, indicated as days post amputation (dpa). Stock of n-CeO<sub>2</sub> (10 mg/mL), μ-CeO<sub>2</sub>  
130 (20 mg/mL) and Ce<sup>3+</sup> (1000 mg/L) were prepared. *D. japonica* was exposed to different treatments  
131 for 7 days. MW medium was used as a control treatment. In brief, thirty planarians were exposed to  
132 10 mL of each Ce-based material suspension with 3 replicates for each treatment. A series of n-CeO<sub>2</sub>  
133 suspensions (0.01, 0.1, 1, 10, 50, 100, and 200 mg/L), μ-CeO<sub>2</sub> suspensions (1, 10, 50, 100, 200, 500,  
134 and 1000 mg/L), and Ce<sup>3+</sup> ions suspensions (0.01, 0.1, 0.5, 1, 5, 10, and 50 mg/L) were prepared in  
135 MW medium (the truth concentration was also detected by ICP-MS, **Table. S1**). The suspensions  
136 were sonicated in the MW medium for 15 mins before exposure. All experiments were performed in  
137 a 12h/12h day/night rhythm at 20 °C. No food was given during the experiment. For homeostatic and  
138 regeneration planarians, the number of live planarians was recorded every day. Besides, based on  
139 acute toxicity tests, 40 mg/L n-CeO<sub>2</sub>, 40 mg/L μ-CeO<sub>2</sub>, and 3 mg/L Ce<sup>3+</sup> were chosen for the  
140 follow-up studies as these concentrations were close to LC<sub>50</sub> based on our preliminary experiment  
141 (**Fig. S2**).

142 **2.3 Phenotype assay** For the regenerating planarians, the growth of the regeneration bud (blastema)  
143 was evaluated by measuring the size using a computer morphometry as described previously<sup>41</sup>. The  
144 planarians were photographed with a Nikon SMZ 1500 stereomicroscope (Nikon, Japan) at 7 dpa.  
145 The area of the blastema and the total area of the body were determined using Image J software  
146 (Version 1.8.0, Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). The quantitative  
147 measure of blastema growth was calculated as the values of new blastema size divided by the sizes  
148 of the whole body were used as. Data from 30 animals were averaged. All experiments at each time

149 point were repeated in triplicate. Each planarian was photographed at the same exposition and  
150 magnification. Details of the experiments are shown in the **SI**.

151 **2.3 Post-exposure locomotion velocity (*pLMV*) assay.** At 7 dpa, thirty planarians per treatment  
152 were individually placed in the center of a clear acrylic box (50.0 cm × 50.0 cm) positioned over  
153 graph paper (grid lines spaced 0.5 cm apart) with mineral water covering the bottom of the paper.  
154 The observation started 30 s after placement of the planarians at the center of the grid and lasted for  
155 8 min. *pLMV* was measured as the number of grid lines that each planarian crossed or re-crossed per  
156 min over an 8 min observation period.

157 **2.4 Whole-mount *in situ* hybridization (WISH).** After 2 days of exposure to Ce-based suspensions,  
158 the regenerated planarians (2 dpa) were used for *in situ* hybridizations. The experiments were  
159 performed to characterize the expression level of *Djpiwi-A*, which is the main stem cell marker  
160 gene<sup>42</sup> following a previously described method<sup>43</sup>. In brief, *Djpiwi-A* probe primers (F:  
161 5'-AAGAGAGATAGGAAGACTGCG-3' and R:  
162 5'-GATCACTAATACGACTCACTATAGGGAAGAGAGATAGGAAGACTGCG-3') were designed,  
163 synthesized and labeled with digoxigenin (DIG) using an *in vitro* labeling kit (Roche, Switzerland).  
164 Planarians were then analyzed using a stereomicroscope (Nikon, SMZ1500, Japan). Each planarian  
165 was photographed at the same exposition and magnification. Details of the experiments are shown in  
166 the **SI**.

167 **2.5 Whole-mount immunofluorescence.** At 2 dpa, 30 planarians were collected for  
168 immunochemistry following a previously described method<sup>44</sup>. Anti-phospho-histone-3 (H<sub>3</sub>P,  
169 Millipore, Billerica, MA, USA) was used at a 1:500 dilution to investigate the effects on the stem  
170 cell proliferation (*in vivo* mitotic activity of stem cells). Phospho-histone-3-positive cells were  
171 counted under an Olympus DP80 inverted microscope (Olympus, Tokyo, Japan). To analyze  
172 differences in the regeneration of the center nervous system (CNS), immunostaining was carried out  
173 using anti-SYNORF1 (3C11, mouse monoclonal antibody specific for synapsin, 1:500 dilution) to  
174 investigate the effects on the neurodevelopmental process, and nerve fiber density of  
175 SYNORF1-immunopositive neurons were measured at 10 dpa. The detailed method is shown in the  
176 **SI**.

177 **2.6 ICP-MS and XANES measurement.** To quantify the concentration of Ce in the regenerating  
178 planarians, the samples were collected at 2 dpa after a thorough wash using DI water. For ICP-MS

179 analysis, these samples were freeze-dried, ground into fine powders and digested with a 3:1 (v:v)  
180 mixture of HNO<sub>3</sub> (75%) and H<sub>2</sub>O<sub>2</sub> (25%) on a heating plate (80 °C for 1 h, 120 °C for 3 h, and  
181 160 °C for 0.5 h). Ce concentrations in the digestion solution were then analyzed by inductively  
182 coupled plasma-mass spectrometry (ICP-MS, 7900, Agilent Technologies). Ce standard solutions  
183 (0.5-50 mg/L) were used for calibration. The recovery rate of Ce was tested to be 99.75%.

184 XANES was used to quantify the Ce<sup>3+</sup> fraction in the regenerating planarians. At 2 dpa, the  
185 planarians were collected, freeze-dried, motor-homogenized, and pressed into circular slices with a  
186 diameter of 10 mm and a thickness of 1 mm. Fluorescence mode was applied for the collection of Ce  
187 *L<sub>III</sub>*-edge XANES spectra of the samples and reference standards. Athena software was used to  
188 perform linear combination fitting (LCF analysis) of XANES spectra<sup>45</sup>.

189 **2.7 Oxidative Stress responses.** At 2 dpa, 30 planarians were collected and homogenized in PBS on  
190 ice using a tissue grinder (Tiangen, OSE-Y20, Beijing, China) with a pestle (Tiangen, OSE-Y001,  
191 Beijing, China). The homogenates were centrifuged at 12,000 g at 4 °C for 10 min. The supernatant  
192 was collected for determination of activities of superoxide dismutase (SOD), glutathione peroxidase  
193 (GPx), and glutathione S-transferase (GST) and contents of malondialdehyde (MDA), glutathione  
194 (GSH), and glutathione disulfide (GSSG) following the manufacturer's instructions. Details of the  
195 analyses are provided in the **SI**.

196 At 2 dpa, 10 planarians for collected for real-time quantitative PCR analysis (qPCR). Total RNA  
197 was isolated using TRIzol (Invitrogen, USA). cDNAs were generated from total RNA with the  
198 reverse transcription system (Thermo, USA). The expression of oxidative stress-associated genes  
199 (*gpx*, *gst*, *p53* and *nak*) in planarians were quantified (LightCycler 480 II Real-Time PCR System  
200 (Roche, Basel, Switzerland)). The primer sequences are provided in **Table S3**. The detailed method  
201 is shown in the **SI**.

202 **2.8 ELISA for Caspase-3 and 8-OHdG activities.** At 2 dpa, 5-6 planarians were collected for  
203 ELISA. Cleaved cysteine-aspartic proteases-3 (caspase-3), an apoptotic marker, was quantified in  
204 planarian's tissue using a commercially available ELISA assay (Jiangsu Jingmei Biotechnology Co.,  
205 Ltd, Jiangsu, China). The content of 8-hydroxy-2 deoxyguanosine (8-OHdG) was measured to assess  
206 the oxidative damage to DNA using a DNA Damage ELISA kit (Jiangsu Jingmei Biotechnology Co.,  
207 Ltd, Jiangsu, China). Details of sample preparation and the analyses are shown in the **SI**.

208 **2.9 Statistical analysis.** All tests were repeated at least three times. Data were expressed as mean ±



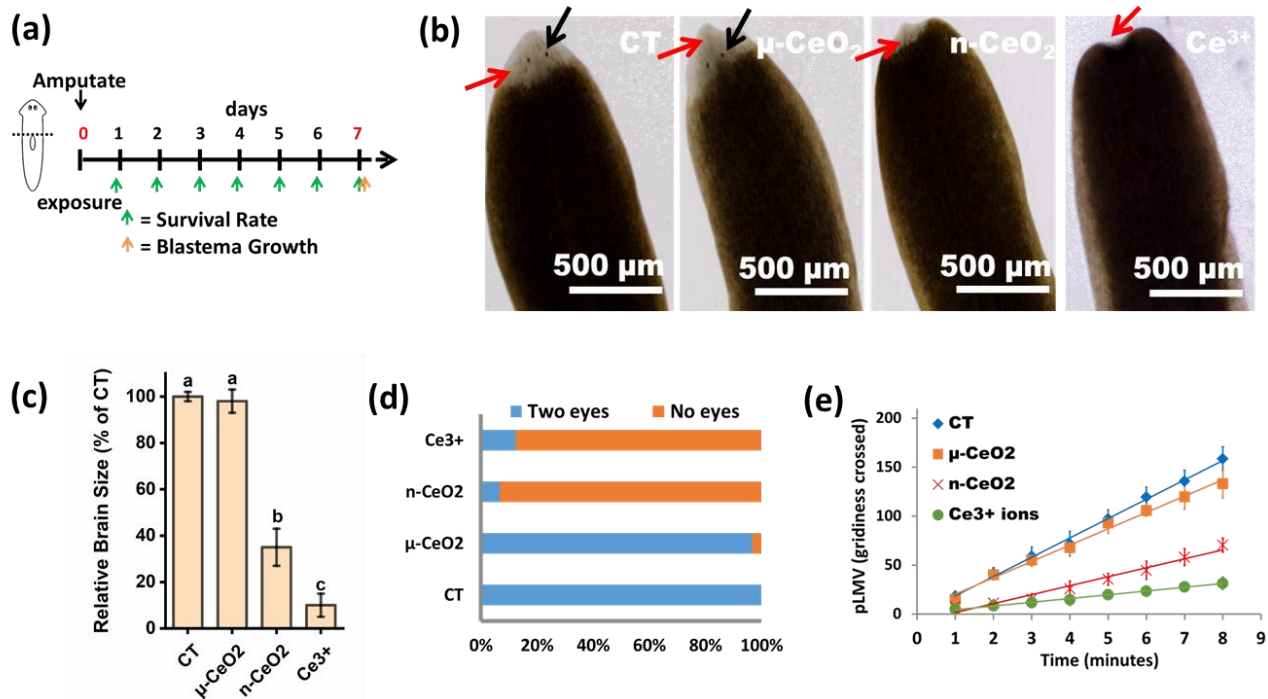
209 SD (standard deviation). Statistical significance was analyzed by ANOVA or student's t-test.  $p < 0.05$   
210 was considered as the level of significance.

### 211 3. Results and discussion

#### 212 3.1 Effect of n-CeO<sub>2</sub> exposure on tissue regeneration and mobility of regenerating planarian

213 A pilot experiment was carried out to identify the exposure concentration (**Fig. 1a**). As shown in  
214 **Fig. S2** and **Table. S4-S5**, the LC<sub>50</sub> values after 7-day n-CeO<sub>2</sub> exposure for homeostatic and  
215 regeneration planarians were 51.2 mg/L and 46.3 mg/L, respectively. The LC<sub>50</sub> of Ce<sup>3+</sup> for the  
216 homeostatic and regenerating groups were 3.5 mg/L and 3.2 mg/L, respectively (**Table. S4-S5**).  
217 Therefore, we chose 40 mg/L n-CeO<sub>2</sub>, 40 mg/L μ-CeO<sub>2</sub>, and 3 mg/L Ce<sup>3+</sup> which were close to the  
218 LC<sub>50</sub> values for the following studies.

219 After an injury, the stem cells (neoblasts cells) in the planarian would proliferate and migrate to  
220 the wound site to form a protective mass of new cells which called blastema<sup>46</sup>. We examined the  
221 influences on the tissue regeneration by measuring the size of newly growing blastema after  
222 decapitation. As shown in **Fig. 1b** and **1c**, the average anterior new blastema sizes in the n-CeO<sub>2</sub> and  
223 Ce<sup>3+</sup>-exposed planarians were significantly smaller than CT and μ-CeO<sub>2</sub> groups. In the CT and  
224 μ-CeO<sub>2</sub> groups, 100% planarians developed eyes at the 7 dpa, whereas only 12.5% and 6.7% of  
225 planarians reformed eyes under n-CeO<sub>2</sub> and Ce<sup>3+</sup> ions exposure, respectively (**Fig. 1d**). These results  
226 indicated that the n-CeO<sub>2</sub> and Ce<sup>3+</sup> treatment delayed planarian's regeneration. Locomotion velocity  
227 test result showed that n-CeO<sub>2</sub> and Ce<sup>3+</sup> reduced the mobility of the regenerating planarians (**Fig. 1e**).  
228 The data were plotted as the cumulative means of each group over an 8-min observation period at 7  
229 dpa. The slope of the plot represents the speed of movement across the gridlines. The planarians in  
230 the CT group displayed an average *pLMV* of about 18.3-24.3 gridlines per minute (the slope value is  
231 20.1), similar to that of the μ-CeO<sub>2</sub> group (the slope value is 17.8). n-CeO<sub>2</sub> and Ce<sup>3+</sup> ions reduced the  
232 slope of the *pLMV* to 8.9 and 3.8, respectively.



233

234 **Fig. 1** n-CeO<sub>2</sub> and Ce<sup>3+</sup> delay planarian regeneration and affect the mobility. **a)** Experimental time points of  
 235 Ce-based suspensions (40 mg/L n-CeO<sub>2</sub>, 40 mg/L μ-CeO<sub>2</sub>, and 3 mg/L Ce<sup>3+</sup>) exposure, amputation, and  
 236 morphological observation. **b)** Blastema reform at 7 dpa in different treatments (**Black Arrow:** eyespots; **Red**  
 237 **Arrow:** blastema). **c)** Average size of regenerated blastema in proportion to the body area of the planarian. **d)**  
 238 Percentage of planarians with eyespots regeneration in different treatments. **e)** Effect of Ce-based suspensions on  
 239 the regenerating planarians' motility at 7 dpa. PLMV was quantified as the number of gridlines crossed or  
 240 re-crossed over an 8-min. This was plotted as cumulative crosses vs. time. Thirty animals per experimental  
 241 condition were used, and each experiment was repeated three times. The results are presented as the mean ± SD,  
 242 one-way ANOVA.

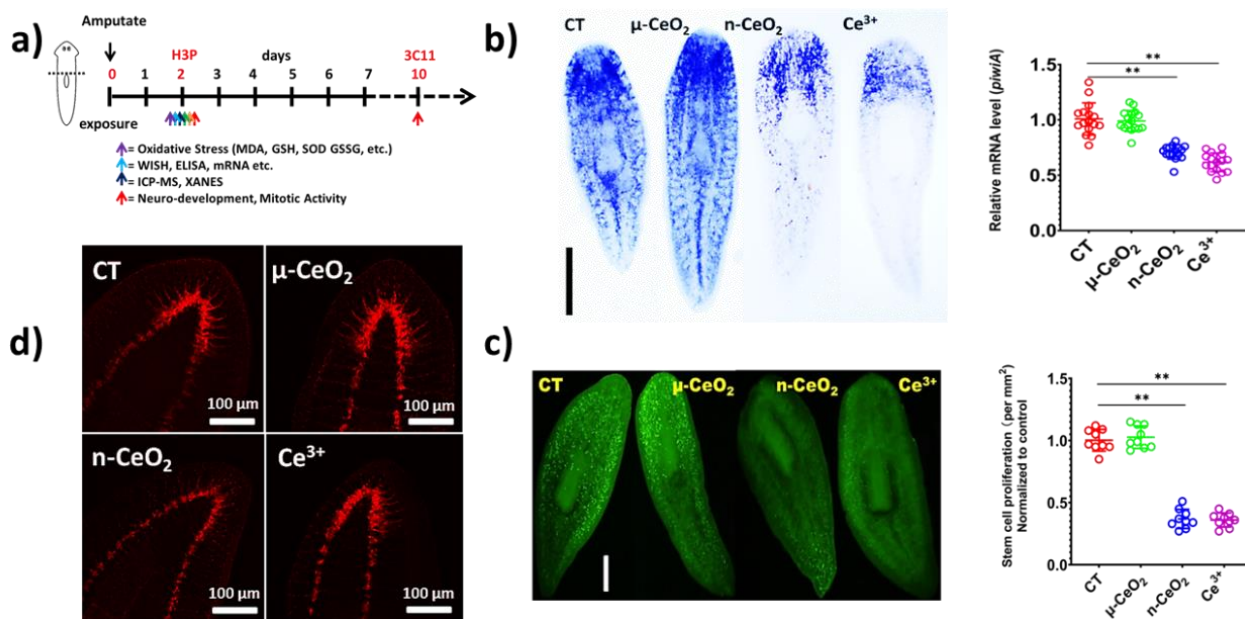
### 243 3.2 n-CeO<sub>2</sub> impaired stem cell dynamics and regeneration of the nervous system.

244 The stem cell proliferation promotes the tissue regeneration. Planarian has large number of  
 245 pluripotent stem cells for rapid cell division and regeneration. The decreased size of anterior new  
 246 blastema may arise from the disrupted cell division and regeneration. Stem cells are highly  
 247 vulnerable to environmental chemicals, which can lead to adverse effects on differentiation processes  
 248 and development<sup>47</sup>. The detailed experimental design was described in **Fig. 2a**. *DjpiwiA*, a main stem  
 249 cell gene marker<sup>44</sup>, was quantified using the WISH method. As shown in **Fig. 2b**, at 2 dpa, *DjpiwiA*  
 250 were extensively expressed (shown as the blue color) in the whole body except for the pharynx in CT  
 251 and μ-CeO<sub>2</sub> group, and the expression were reduced by 29.2% and 38.4% in n-CeO<sub>2</sub> and Ce<sup>3+</sup> group,  
 252 respectively.

253 During development, mitotic activity (cell proliferation) can be regarded as one of the primary

254 events of tissue remodeling and repair<sup>48</sup>. Anti-phospho-Histone H3 antibody can be used to label the  
255 nuclei of stem cells to examine the *in vivo* mitotic activity<sup>49</sup>. Compared to CT group, the number of  
256 labeled nuclei (green dots) significantly decreased by 63.1% and 65.2% after exposure to n-CeO<sub>2</sub> and  
257 Ce<sup>3+</sup>, respectively (**Fig. 2c**), while  $\mu$ - CeO<sub>2</sub> group did not show significant difference with CT. This  
258 suggested that n-CeO<sub>2</sub> and Ce<sup>3+</sup> treatment impaired the proliferation of stem cell.

259 A recent study found that Ag NPs can affect the locomotion of freshwater planarians *Schmidtea*  
260 *mediterranea* by inducing neurotoxicity and disorder in neurodevelopment<sup>5</sup>. These suggested that  
261 n-CeO<sub>2</sub> induced locomotive inhibition may be attributed to the neurotoxicity. Planarian is a unique in  
262 the animal kingdom as they can regenerate its brain, including a fully functional CNS<sup>50</sup>. To evaluate  
263 whether the reduced stem cell proliferation further impeded the regeneration of the CNS, the neuron  
264 morphologies were observed at 10 dpa using immunohistochemistry method. The anti-SYNORF1  
265 antibody was used as a biomarker, which allows visualization of the planarian CNS. The normal  
266 planarian brain has an inverted U-shaped structure comprised of a cortex of neural cells and a core of  
267 axons, which displays rich branches<sup>51</sup>. Based on the behavioral changes and regeneration defects, we  
268 observed that planarian could not regenerate its complete CNS. As shown in **Fig. 2d**, n-CeO<sub>2</sub> and  
269 Ce<sup>3+</sup> treatment significantly reduced the number of lateral branches, indicating that n-CeO<sub>2</sub> and Ce<sup>3+</sup>  
270 induced neural regeneration defects in the treated planarians.  $\mu$ -CeO<sub>2</sub> groups showed no significant  
271 effect. Overall, these results suggest that n-CeO<sub>2</sub> and Ce<sup>3+</sup> exposure impaired the stem cell activity  
272 and neurodevelopment, and thus the health and regeneration capacity of the CNS in the exposed  
273 planarians.



274  
 275 **Fig. 2** n-CeO<sub>2</sub> and Ce<sup>3+</sup> affects stem cell proliferation. (a) Experimental time points of Ce-based suspensions (40  
 276 mg/L n-CeO<sub>2</sub>, 40 mg/L μ-CeO<sub>2</sub>, and 3 mg/L Ce<sup>3+</sup> ions) exposure to regeneration planarians and detect biomarkers,  
 277 such as oxidative stress marker MDA and SOD, WISH and Immunochemistry. (b) **Left**, *in situ* hybridization to  
 278 detect *DjpiwiA* transcripts in planarians. **Right**, the gene expression of *DjpiwiA* in the regenerating planarians in  
 279 different treatment. (c) Immunohistochemistry using the mitotic marker Anti-H<sub>3</sub>P antibody. The **left** panel shows a  
 280 view of the mitotic cells in regenerating planarian in different treatments. **Right**, the number of mitotic events in  
 281 Ce-based suspensions treatment and control (CT) groups. The results are presented as the mean ± SD of a  
 282 minimum of 10 biological replicates per condition (1 planarian per point, asterisks indicate the level of significant  
 283 differences between treatment and CT groups of the planarian, one-way ANOVA, \**p* < 0.05 \*\**p* < 0.01). (d)  
 284 Whole-mount immunostaining of the nervous system with anti-SYNORF1 (3C11) in regeneration planarians after  
 285 exposure to Ce-based suspensions for 10 days. Scale bars represent 2 mm or 100 μm.

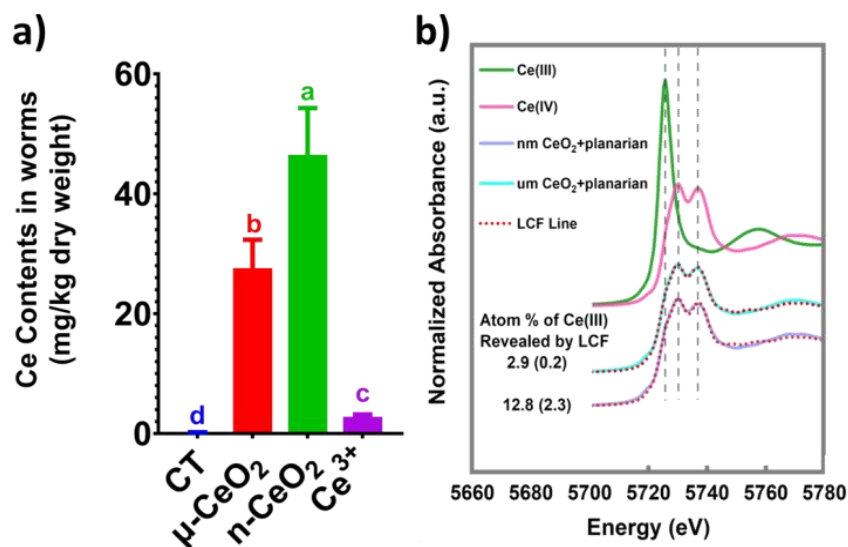
### 286 3.3 Chemical origin of the toxicity: role of bioaccumulation and biotransformation

287 Biotransformation and bioaccumulation determine the ultimate fate and toxicity of nanomaterials in  
 288 living organisms<sup>52, 53</sup>, such as *Lactuca plants*<sup>39</sup>, *Bacillus subtilis*<sup>24</sup>, and *Chlorella pyrenoidosa*<sup>14</sup>. *In*  
 289 *vivo* studies rarely consider physicochemical characteristics and actual exposure concentrations when  
 290 linking them to uptake, distribution patterns, and potential adverse effects. In the present study, the  
 291 actual size of the n-CeO<sub>2</sub> and μ-CeO<sub>2</sub> were considerably larger than their nominal reported size (**Fig.**  
 292 **S3**). In fact, n-CeO<sub>2</sub> and μ-CeO<sub>2</sub> changes in both concentration and particle size were observed  
 293 during the exposure concentration (**Fig. S4**, the hydrodynamic diameter from 162 nm and 2863 nm at  
 294 the beginning up to 312 nm and 3312 nm at 48 h by n-CeO<sub>2</sub> and μ-CeO<sub>2</sub> interacted with planarian,  
 295 respectively; **Table S6**). Sedimentation also depends on the surrounding environment<sup>5</sup>, such as the  
 296 production of organic material mucus, in which particles agglomerate (**Fig.S4-S5**). This way, the  
 297 bioavailability in the aquatic column is reduced, while increasing the risk for benthic animals.

298 Planarians inhabit the lowest water layer, and are able to take up NPs as they glide over the substrate<sup>5</sup>.  
299 Then we used ICP-MS to detect the Ce-based materials and found that Ce was significantly  
300 accumulated in the regeneration planarian after 2 days of exposure to different Ce treatments, and  
301 n-CeO<sub>2</sub> group showed the highest accumulation, followed by  $\mu$ -CeO<sub>2</sub> and Ce<sup>3+</sup> group (**Fig. 2b**). For  
302 the 40 mg/L  $\mu$ -CeO<sub>2</sub>, n-CeO<sub>2</sub>, and 3 mg/L Ce<sup>3+</sup> ions, Ce accumulations in the planarians were  $27.6 \pm$   
303  $4.7$ ,  $46.5 \pm 7.8$ , and  $2.8 \pm 0.4$  mg Ce kg<sup>-1</sup>, respectively. The percentages of the accumulation from the  
304 total applied Ce are 0.31%, 0.52% and 0.32% for  $\mu$ -CeO<sub>2</sub>, n-CeO<sub>2</sub> and Ce<sup>3+</sup>, respectively. Many  
305 factors would influence the bioaccumulation and bioavailable of Ce in planarian, such as sizes and  
306 culture environment. On the one hand, n-CeO<sub>2</sub> with smaller size is easier to move into the organism  
307 thus accumulate more than  $\mu$ -CeO<sub>2</sub>. For example, neither  $\mu$ -CeO<sub>2</sub> nor n-CeO<sub>2</sub> are ingested by  
308 planarian, however, the latter with a smaller size allows them to stay a longer time in body during  
309 this ingestion process. In addition, planarians use their motile cilia to glide across the sediment  
310 surface and come into contact with the deposited materials, which make them adsorb  $\mu$ -CeO<sub>2</sub> and  
311 n-CeO<sub>2</sub> in their body. Besides, planarian will secrete mucus after being stimulated, as shown in **Fig.**  
312 **S5**, large numbers of mucus proteins were present in the n-CeO<sub>2</sub> treatment group while a small  
313 amount these proteins were present in the  $\mu$ -CeO<sub>2</sub> group, which may promote n-CeO<sub>2</sub> adsorption by  
314 planarian. Once n-CeO<sub>2</sub> penetrated into glial and neurons cells, it would be directed to the lysosomes  
315 or persist in the cytoplasm and subsequently interact with other organelles<sup>54</sup>. Although  $\mu$ -CeO<sub>2</sub> also  
316 accumulated significant amount of Ce (equal to 60% of that in n-CeO<sub>2</sub> group), it did not show  
317 significant effects on locomotion, regeneration and neurodevelopment of planarian. Therefore, both  
318 the higher accumulation of Ce and higher reactivity of n-CeO<sub>2</sub> may contribute to its higher toxicity  
319 than  $\mu$ -CeO<sub>2</sub>. However, n-CeO<sub>2</sub> exposure resulted in 16.6-fold higher Ce accumulation but led to less  
320 toxicity than Ce<sup>3+</sup>, further demonstrating that Ce<sup>3+</sup> is more toxic than n-CeO<sub>2</sub>. It is well known that  
321 nanomaterials in environment and biota may undergo transformation either through chemical or  
322 biological processes, and the transformation process may determine their toxicity<sup>24, 55</sup>. We therefore  
323 hypothesize that the biotransformation of n-CeO<sub>2</sub> might contribute to the significant toxicity.

324 We firstly examined the release of Ce<sup>3+</sup> from  $\mu$ -CeO<sub>2</sub> and n-CeO<sub>2</sub> in the culture media. Only less  
325 than 26.5  $\mu$ g/L of Ce were detected, suggesting toxicity is unlikely caused by the Ce<sup>3+</sup> that were  
326 initially in the exposure solution. However, biotransformation is a continuous process once the  
327 nanomaterials contact the biota<sup>56</sup>. Biotransformation of n-CeO<sub>2</sub> may occur on or within the planarian

328 during the exposure. Therefore, we examined the real amount of transformation in the organism  
329 using synchrotron radiation based XANES technique (**Fig. 3b, Table. S7**). The spectrum of Ce in the  
330 planarians under treatments of  $\mu$ -CeO<sub>2</sub> at 40 mg/L shows a small feature of Ce(III) oxidation state  
331 ( $2.9 \pm 0.2\%$ ), suggesting a small extent of transformation. However, the feature of Ce(III) under  
332 n-CeO<sub>2</sub> (40 mg/L) account for  $12.8 \pm 2.3\%$  of the total Ce. This is equivalent to a release of 5.96  
333 mg/kg ( $46.5 \times 12.8\%$ ) Ce<sup>3+</sup> in planarian, which demonstrated our hypothesis that biotransformation  
334 contributed to the toxicity observed for n-CeO<sub>2</sub>. It should be also noted that the concentration of  
335 Ce(III) is twice of that in Ce<sup>3+</sup> treated group (2.8 mg/kg), while the toxicity is lower than Ce<sup>3+</sup>. This  
336 can be attributed to the fact that the biotransformation of nanomaterials is a time-dependent process.  
337 For example, it can take up to 7 days to show 20% of transformation of n-CeO<sub>2</sub> in plant shoot<sup>57</sup>.  
338 Depending on different species, the transformation rate can be lower or higher. Unlike Ce<sup>3+</sup> which  
339 instantly release all the Ce<sup>3+</sup> to organism and thus cause acute toxicity, n-CeO<sub>2</sub> released Ce<sup>3+</sup> slowly  
340 by continuously interact with the planarian thus the toxicity is more chronic rather than acute. As  
341 with all chemicals and bulk materials, the biological and/or toxicological effects of NPs will be  
342 affected by both their physicochemical properties and the biological environment around the  
343 organism. NPs interactions with biological environments result in the formation of a protein corona<sup>58</sup>,  
344 <sup>59</sup>. A corona-NPs complex may feature NPs undergoing different changes as a result of their  
345 adsorption behavior, agglomeration, accumulation, distribution, biotransformation, and dissolution. A  
346 protein corona complex and NPs represent a new entity in every biological environment which will  
347 inevitably define NPs toxicology, pharmacokinetics, and dynamics<sup>60, 61</sup>. In the present study, we  
348 found that n-CeO<sub>2</sub> induced a more mucus secreted by planarian and the higher biotransformation  
349 occurred with a higher toxicity in the homeostatic and regeneration planarians. In fact, n-CeO<sub>2</sub> would  
350 gain a negative charge due to the formation of a protein-corona-NPs complex and cause planarian  
351 absorbed a more n-CeO<sub>2</sub>, which may promote NPs' biotransformation and dissolution (**Fig. S7,**  
352 **Table. S4**). Thus, we hypothesized that the protein corona affects not only n-CeO<sub>2</sub>'s distribution, but  
353 also its toxicity. In summary, n-CeO<sub>2</sub> induced toxicity to planarian via both its nano-specific effect as  
354 well as the ions induced effects.



355

356 **Fig. 3 a)** Cerium contents in the regeneration planarians at 2 dpa in different treatments. Data are expressed as  
 357 mean  $\pm$ SD (n=6) an average of six replicates. Different letters stand for statistical differences between treatments at  
 358  $p < 0.05$ . **b)** XANES normalized Ce L<sub>III</sub>-edge spectra of reference compounds (CePO<sub>4</sub> and CeO<sub>2</sub>) and samples.  
 359  $\mu$ -CeO<sub>2</sub>, n-CeO<sub>2</sub> and Ce<sup>3+</sup> ions exposure to regeneration planarians for 2 days and the samples were detected by  
 360 XANES. Ce speciation in Ce<sup>3+</sup> treatment sample could not be detected, as the Ce signal was too low to obtain a  
 361 reliable spectrum.

### 362 3.4 n-CeO<sub>2</sub> disrupts the antioxidant system and damage DNA integrity

363 In previous studies, Ce<sup>3+</sup>/Ce<sup>4+</sup> fractions (two reversible states) on n-CeO<sub>2</sub> particle surfaces were  
 364 found to determine the particle's SOD mimicking activity<sup>62</sup>. Compared with the smaller n-CeO<sub>2</sub>,  
 365 n-CeO<sub>2</sub> with dimensions bigger than 5 nm exhibit a lower Ce<sup>3+</sup>/Ce<sup>4+</sup> ratio, and thus exhibit negligible  
 366 activity<sup>63</sup>. Once n-CeO<sub>2</sub> undergoes biotransformation and releases Ce<sup>3+</sup> ions, which plays a role of  
 367 pro-oxidative effects, and the toxicity would be exhibited<sup>24</sup>. In addition, a Fenton-like reaction could  
 368 be catalyzed by the released Ce<sup>3+</sup> ions with hydrogen peroxide, producing damaging oxygen radicals,  
 369 which may cause oxidative stress happened<sup>64</sup>. Oxidative stress has been considered a major  
 370 contributor to the toxicity of environmental pollutants including nanomaterials<sup>65-67</sup>. Changes in  
 371 activities of antioxidant enzymes and contents of MDA are the primary indicators of oxidative stress  
 372 and cell damage. The antioxidant enzymes such as SOD, GPX, and GST, are involved in the  
 373 protective mechanisms adapted by animals to scavenge ROS<sup>68</sup>. To examine whether the disorder of  
 374 redox balance underlies the toxicity of n-CeO<sub>2</sub>, we evaluated the expression levels of different  
 375 categories of antioxidative in regeneration planarians. In this study, the antioxidant defense system  
 376 including enzymatic (SOD, GST and GPx) and non-enzymatic (GSH, GSSG) and MDA levels were

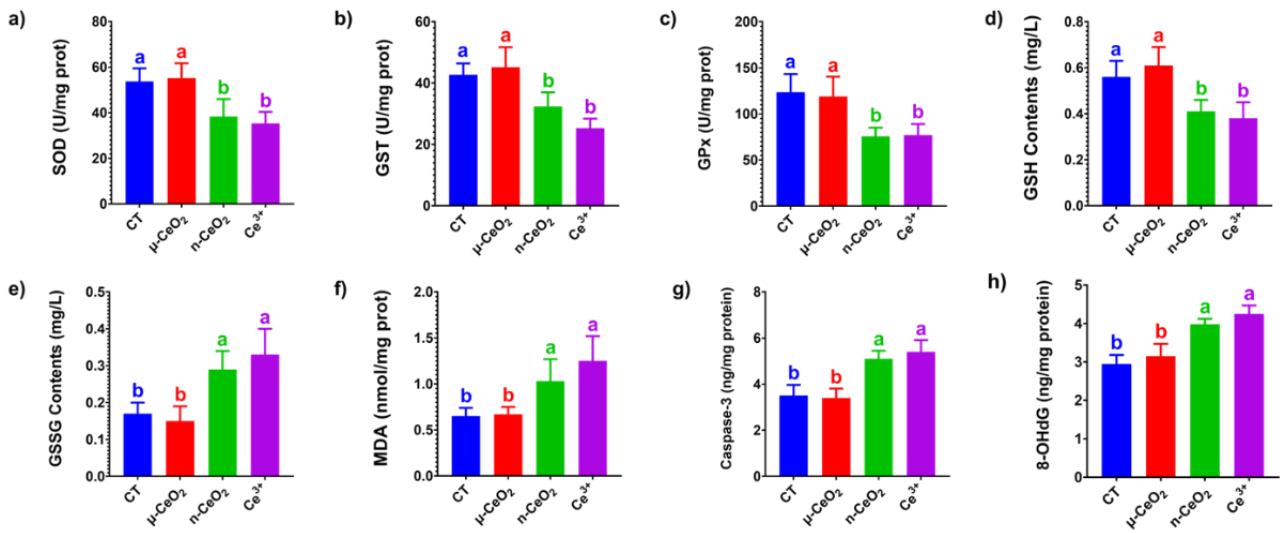
377 determined in the regenerating planarians. As shown in **Fig. 4a-c**, both n-CeO<sub>2</sub> and Ce<sup>3+</sup> treatments  
378 induced significant changes in the activities of SOD, GST, and GPx in planarians. n-CeO<sub>2</sub> and Ce<sup>3+</sup>  
379 decreased the activity of SOD by 28.6% and 34.4%, respectively, compared with CT. The activity of  
380 GST in planarians in n-CeO<sub>2</sub> and Ce<sup>3+</sup> treatment was significantly decreased as compared to that of  
381 CT. n-CeO<sub>2</sub> and Ce<sup>3+</sup> significantly decreased the GPx activity by 38.8% and 37.5%, respectively. The  
382 results of the present study indicate that both n-CeO<sub>2</sub> and Ce<sup>3+</sup> treatments disturbed the homeostasis  
383 of antioxidant defense system in regeneration planarians. A similar pattern of impacts on the  
384 non-enzymatic antioxidant system was also observed (**Fig. 4d** and **4e**). The GSH contents at 2 dpa  
385 decreased by 26.8% and 32.1 % after exposure to n-CeO<sub>2</sub> and Ce<sup>3+</sup>, respectively. Correspondingly, a  
386 significant increase of the GSSG content, an oxidized form of GSH, was observed in planarians after  
387 exposure to n-CeO<sub>2</sub> and Ce<sup>3+</sup>. n-CeO<sub>2</sub> and Ce<sup>3+</sup> decreased activities of SOD, GST, GPx and GSH in  
388 the regeneration planarians compared to control with a higher level of MDA and GSSG which  
389 suggests the treatments provoked lipid peroxidation.

390 We further examined whether the antioxidant system was impaired at the genetic level by  
391 examining the mRNA expression of key genes responsible for antioxidant production (*gpx*, *gst*), ATP  
392 synthesis (*nak*), and cell apoptosis (*p53*) (**Fig. 5**). Consistent with the above result, *gpx*, *gst*, and *nak*  
393 expression were significantly down-regulated after 2 days of exposure to n-CeO<sub>2</sub> and Ce<sup>3+</sup>.  $\mu$ -CeO<sub>2</sub>  
394 did not show significant difference with CT in all cases. *Gpx* and *gr* are key antioxidant defense  
395 genes involved in the glutathione metabolism. In planarians, *p53* has a wider range of functions than  
396 known tumor suppressor<sup>69</sup>. Pearson and Alvarado<sup>70</sup> found that *p53* involved in tumor inhibition and  
397 regulation of stem cell self-renewal in planarians. In this work, *p53* expression was significantly  
398 up-regulated under n-CeO<sub>2</sub> and Ce<sup>3+</sup> exposure when compared with CT. The apoptosis was affected  
399 in regenerating planarians, which could be due to the ability of Ce<sup>3+</sup> to promote programmed cell  
400 death.

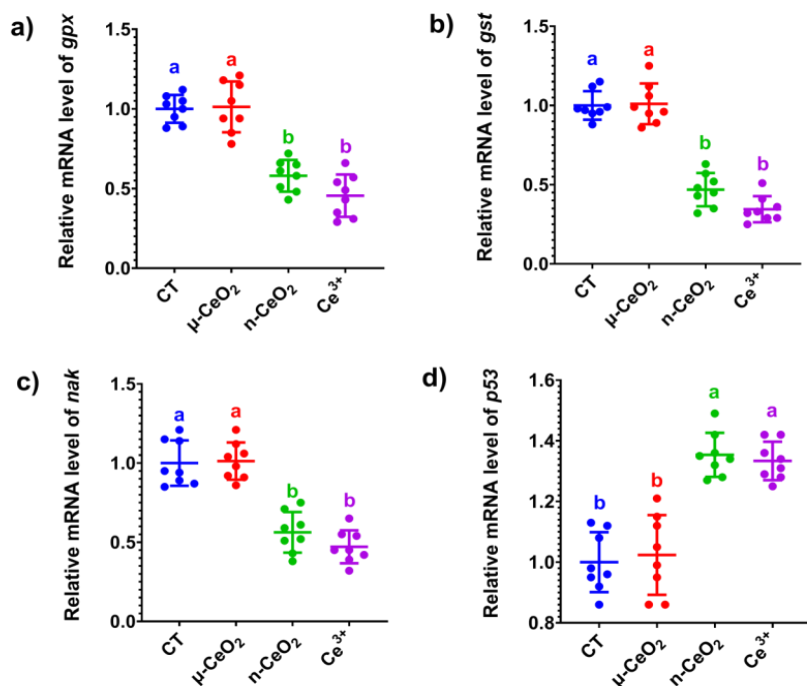
401 Oxidative stress can cause tissue alternations, trigger apoptosis and induce cell death. So we  
402 studied whether n-CeO<sub>2</sub> and Ce<sup>3+</sup> exposure can induce cell apoptosis (**Fig. 4g**) and DNA damage at  
403 the molecular levels (**Fig. 4h**). 8-OHdG, a maker for oxidative DNA damage, was increased after  
404 n-CeO<sub>2</sub> and Ce<sup>3+</sup> exposure (**Fig. 4h**), indicating n-CeO<sub>2</sub> and Ce<sup>3+</sup> exposure can cause genotoxic  
405 effects Similarly, Arslan, et al.<sup>71</sup> found 0.78 mg/L n-CeO<sub>2</sub> induced genotoxic effect *in vitro* using  
406 micronucleus and chromosome aberration tests.



407 The overall oxidative responses of planarian to n-CeO<sub>2</sub> and Ce<sup>3+</sup> are similar and to similar  
 408 extents on molecular level (**Fig. 5**), although the phenotypic effects caused by the two are different.  
 409 Since the phenotypic changes are essentially originated from the changes at molecular or genetic  
 410 levels and given that n-CeO<sub>2</sub> can release toxic Ce<sup>3+</sup> in a slow and sustained way, these findings give  
 411 implications that n-CeO<sub>2</sub> may cause longer-term adverse impacts to planarian, which might be worse  
 412 than that caused by acute exposure to Ce<sup>3+</sup>.



413  
 414 **Fig. 4** n-CeO<sub>2</sub> regulates planarian's antioxidant defense system and cell damage. SOD (**a**), GST (**b**), GPx (**c**), GSH  
 415 (**d**), GSSG (**e**), and MDA levels (**f**) in regeneration planarians after exposure to Ce-based suspensions for 48 h.  
 416 Contents of caspase-3 (**g**) and 8-OHdG (**h**) in planarians affected by Ce-based suspensions treatments. Different  
 417 letters stand for statistical differences between treatments at  $p < 0.05$ .



418

419 **Fig. 5** The effect of Ce-based suspensions on relative mRNA expression levels of gene *gpx* (a), *gst* (b), *nak* (c), and  
 420 *p53* (d) measured in regeneration planarians. Different letters stand for statistical differences between treatments at  
 421  $p < 0.05$ .

#### 422 4. Conclusion

423 In this study we compared the toxicity effects of n-CeO<sub>2</sub>,  $\mu$ -CeO<sub>2</sub>, and ionic Ce (CeCl<sub>3</sub>) in  
 424 planarian *D. japonica*. We not only showed that n-CeO<sub>2</sub> induced nano-specific toxicity to planarian  
 425 compared with  $\mu$ -CeO<sub>2</sub>, but also demonstrated that the biotransformation of n-CeO<sub>2</sub> contributed  
 426 significantly to the observed toxicity. Interestingly, although n-CeO<sub>2</sub> induced more Ce accumulation  
 427 in the planarians than Ce<sup>3+</sup>, the toxicity is actually lower which is attributed to that the release of  
 428 Ce<sup>3+</sup> from n-CeO<sub>2</sub> is a slow process. However, this in turn implicates that n-CeO<sub>2</sub> may cause  
 429 long-term adverse effects to planarian, especially to the regenerating ones which are more sensitive  
 430 to the n-CeO<sub>2</sub> toxicity. This knowledge gap should be addressed in the future. On the other hand,  
 431 interests in exploring the beneficial effects of n-CeO<sub>2</sub> to enhance regeneration of neural systems have  
 432 increasingly growing due to the ROS capturing activity of n-CeO<sub>2</sub>. However, such effects are only  
 433 reported at very low concentrations and particles with extremely small size (usually less than 10 nm).  
 434 Our results suggest that a threshold between an inhibitor and an enhancer should be established, and  
 435 caution should be made for this type of application.

436 **CRedit authorship contribution statement**

437 **Changjian Xie:** Conceptualization, Methodology, Investigation, Formal analysis, Writing-original  
438 draft, Funding acquisition. **Xiaowei Li:** Methodology, Resources. **Lisha Hei:** Resources, review &  
439 editing. **Yiqing Chen:** Resources, review & editing. **Yuling Dong:** Investigation, Funding  
440 acquisition. **Shujing Zhang:** Resources. **Shan Ma:** Investigation. **Jianing Xu:** Formal analysis,  
441 Funding acquisition. **Qiuxiang Pang:** Conceptualization, Methodology, Formal analysis,  
442 Writing-review & editing, Resources. **Iseult Lynch:** Funding acquisition, Writing-review & editing.  
443 **Zhiling Guo:** Conceptualization, Methodology, Formal analysis, Writing-review & editing,  
444 Resources. **Peng Zhang:** Writing-review & editing, Resources.

445 **Declaration of competing interest**

446 The authors declare that they have no competing interests.

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