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Research paper

Fluorescence resonance energy transfer-based sensor zebrafish for detecting toxic agents with single-cell sensitivity

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ABSTRACT

Zebrafish are widely used for detecting toxic agents because of their unique advantages. The conventional zebrafish-based tests use lethal rates and morphological changes as criteria to evaluate the toxicity. To increase the sensitivity of using zebrafish to detect toxic agents, a fluorescence resonance energy transfer-based apoptotic biosensor was introduced into zebrafish genome to generate transgenic sensor zebrafish. Seven chemicals including heavy metals, nanomaterials and DNA-damaging agents were used to treat the sensor zebrafish to determine the sensitivity of the sensor zebrafish. The results showed that sensor zebrafish can detect the toxicity of the tested agents with single-cell sensitivity. Using the sensor zebrafish, we found that, at 100 nM, heavy metal cadmium (Cd) induced apoptosis of zebrafish cells, while no obvious morphological or behavioral changes were observed from the sensor zebrafish. Even at 44.5 nM (the maximum allowable concentration in drinking water), Cd induced a significant increase of apoptosis in sensor zebrafish. ZnO nanoparticles caused apoptosis in sensor zebrafish at a very low concentration of 100 ng/mL. DNA-damaging agents induced the apoptosis of many cells in sensor zebrafish. The sensor zebrafish are much more sensitive than the conventional zebrafish-based tests and can serve as a powerful tool for detecting toxic agents.

1. Introduction

Effective toxic agent detection methods are essential for environmental monitoring and human health. Traditional chemical methods can detect the presence of toxic agents but cannot reflect their toxic effects in animals and humans. In contrast, biological methods can provide direct evaluation of the risks of the toxic agents (Jia et al., 2015; Kuklina et al., 2013; Ma et al., 2014). Zebrafish are widely used for toxicity studies (Dai et al., 2014; Scholz et al., 2008; Song et al., 2020) as they have several unique advantages: zebrafish are vertebrates and share 70% coding genes with humans (Howe et al., 2013); zebrafish have a transparent body which benefits the observation of morphological and physiological changes after treatments (Jia et al., 2019); zebrafish are easy to handle and can produce hundreds of embryos in every breeding, which gives a large sample size (Spence et al., 2008); there is no need to feed zebrafish before 5 days post fertilization (dpf) (Clift et al., 2014), which can rule out additional factors during the tests.

Sensitivity is critical for toxic agent detection. To increase the sensitivity of zebrafish-based tests, in recent years, transgenic zebrafish have been generated for detecting different kinds of toxic agents. For example, the gene of green fluorescent protein (GFP) was driven by the upstream sequence of *ddit3* gene to generate a transgenic zebrafish line which can sensitively detect the stress signals induced by heavy metals in river water samples (Lee et al., 2014). Transgenic zebrafish Tg (*hsp70*:GFP) were used to detect the toxicity of gold nanoparticles at low concentrations (Pan et al., 2013). Using the Tg(*fli1*:EGFP) zebrafish, the effects of fipronil, a common pollutant in the aquatic environment, on angiogenesis during development were revealed (Park et al., 2020). These transgenic zebrafish broaden the application areas and sensitivity of zebrafish-based tests. It will be of great significance if we can develop transgenic zebrafish which enable the detection of toxic agents with single-cell sensitivity.

Apoptosis is the programmed death of cells when they receive endogenous death signals or are exposed to exogenous stress. Traditional apoptosis detection methods such as terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Crowley et al., 2016) and annexin V staining (Rieger et al., 2011) require complicated experimental steps and much time. To overcome these drawbacks of apoptosis detection, in our previous study, we developed a fluorescence resonance energy transfer (FRET)-based biosensor, sensor C3, which

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enabled the detection of apoptosis through a color change from green to blue in a real-time manner (Anand et al., 2015; Luo et al., 2001; Tian et al., 2007). We further generated transgenic sensor zebrafish expressing sensor C3 in zebrafish skin, which can be used to detect apoptosis of skin cells in real-time and at single-cell resolution (Jia et al., 2020).

In the present study, we generated new transgenic sensor zebrafish Tg(*ubi*:sensor C3), which can ubiquitously express sensor C3 and be used to detect toxic agents. The cells of Tg(*ubi*:sensor C3) zebrafish quickly changed from green to blue, within 5 min, when they underwent apoptosis after UV irradiation. Using the sensor zebrafish, we built a new toxicity detection system, in which FRET imaging, morphological analysis and behavioral tests were integrated to evaluate the toxicity of the agents. Seven kinds of toxic agents, including heavy metals, nanomaterials and DNA-damaging agents, were tested on the sensor zebrafish. We found that the sensor zebrafish can detect the apoptosis induced by these toxic agents with single-cell sensitivity by FRET imaging. Because of this high sensitivity, we can reveal the toxicity of these toxic agents at very low concentrations, which cannot be detected by conventional zebrafish methods such as lethal rate measurement, morphological analysis and behavioral tests. For example, we found that, at an environmentally relevant concentration of 100 nM, heavy metal cadmium (Cd) induced apoptosis of sensor zebrafish cells. Even at 44.5 nM, which is the maximum allowable concentration in drinking water in European Union, USA and China, Cd induced a slight but significant increase of apoptosis in sensor zebrafish. We revealed the toxicity of ZnO nanoparticles (ZnO NPs) at the very low concentration of 100 ng/mL with the sensor zebrafish. We also found that the sensor zebrafish are very sensitive to agents that can damage DNA. The transgenic sensor zebrafish generated in this study can be used as an ultrasensitive in vivo tool for detecting toxic agents.

2. Materials and methods

2.1. Maintenance of zebrafish

AB strain zebrafish were used in the present study. The zebrafish were raised in a ZebTEC multilinking rack system at 28 ± 1 °C and a photoperiod of 14 h light to 10 h dark. All animal experiments were approved by the Animal Research Ethics Committee of University of Macau (Approval ID: UMARE-032-2016).

2.2. Zebrafish transgenesis

The *Tol2* transposon system-based transgenesis was used to generate the sensor zebrafish (Jia et al., 2020; Kawakami, 2007). A 3.5 kb *ubiquitin* (*ubi*) promoter (Mosimann et al., 2011) was amplified from the wide-type (WT) zebrafish genome (forward primer, CCGCTCGAGACCAGCAAAGTTCTAGAATTTGTCG; reverse primer, CCCAAGCTTCTGTAAACAAATTCAAAGTAAGATTAGC). The amplified *ubi* promoter was placed between the *Xho* I and *Hind* III sites in front of the sensor C3 gene to generate the *Tol2* construct pSK-ubi-C3. Prior to the microinjection, the *Tol2* construct pSK-ubi-C3 and the *Tol2* transposase mRNA were mixed (final concentration of the plasmids, 50 ng/μL; final concentration of the mRNAs, 100 ng/μL). Around 2–4 nL of the mixed plasmids and mRNAs were injected into fertilized WT zebrafish embryos at one-cell stage with a MPPI-3 microinjector. One day after the microinjection, the embryos with green fluorescence were screened and cultured as founder zebrafish. When the founder zebrafish reached the adulthood, they were crossed with WT zebrafish to generate the transgenic sensor zebrafish.

2.3. UV irradiation of the zebrafish

Live zebrafish were placed in a Petri dish with fish water and then exposed twice to UV rays (wavelength, 254 nm; dosage, 200 mJ/cm^2), which was produced by an Ultraviolet Crosslinker CL-1000 (UVP). After UV ray exposure, the live zebrafish were cultured for several hours and then imaged using a Carl Zeiss LSM 710 confocal microscope.

2.4. FRET imaging to detect apoptosis in live sensor zebrafish

According to the design, sensor C3 gene was expressed in Tg(*ubi*:sensor C3) zebrafish as a fusion protein of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), between which is the cleavage site of caspase-3, DEVD. Prior to the FRET imaging, live zebrafish were mounted in 1% low-melting agarose gel or 3% methyl cellulose containing 0.016% tricaine. The sensor zebrafish were excited using a 458 nm laser in a Carl Zeiss LSM 710 confocal microscope. The emission fluorescence was collected at the same time using two channels: the emissions of 460–500 nm was named as CFP image and colored blue; the emissions of 520–550 nm was named as YFP image and colored green. The FRET image is generated by merging the CFP image and YFP image. Live sensor cells appear green in the FRET image because of the energy transfer between CFP and YFP. When caspase-3 is activated during apoptosis, the sensor proteins are cleaved at the DEVD site, which stops the energy transfer, and the sensor cells appear blue in the FRET image. The details of the imaging procedures can be found in our previous publication (Jia et al., 2020).

2.5. Application of toxic agents to sensor zebrafish

All toxic agents were dissolved or dispersed in fish water. Prior to the treatment, the ZnO NPs dispersion was sonicated for 1 h, and then, the hydrodynamic diameter of the nanoparticles was measured using dynamic light scattering with a Brookhaven BI-200SM laser light scattering spectrometer. The details of the toxic agents tested in the study can be found in Tables S1 and S2. Sensor zebrafish at 48 h post fertilization (hpf) were dechorionated with fine forceps and seeded in 24-well plates (20 zebrafish per well) containing different toxic agents diluted at different concentrations in 2 mL of fish water. The treatment lasted for 48 h. After treatment, the number of sensor zebrafish that survived at each concentration was counted. The 50% lethal concentration (LC_{50}) values of the tested toxic agents were calculated with IBM SPSS Statistics Version 25 software. Then, the toxic agents at individual LC_{50} concentration were used to treat sensor zebrafish at 48 hpf for 48 h. After treatment, FRET imaging was used to detect the apoptotic effect of each toxic agent. Toxic agents at much lower concentrations than the LC_{50} were used to treat the sensor zebrafish at 48 hpf. The treatment lasted for 72 h. After treatment, FRET imaging was used to detect apoptotic cells.

2.6. Zebrafish behavioral tests

A DanioVision observation chamber (Noldus) was used to monitor the swimming ability of the sensor zebrafish. After treatment with toxic agents, sensor zebrafish (120 hpf) were seeded in 96 square well plates (GE Healthcare, #7701-1651, one zebrafish per well), and the locomotion of each sensor zebrafish was tracked in the observation chamber. The zebrafish were subjected to a 10 min dark and 10 min light cycle while being tracked. During tracking, the temperature was maintained at 28.5 °C with the DanioVision temperature control unit. The zebrafish were tracked for 70 min, and the data from the first cycle were not used for analysis because the zebrafish needed to adapt to the test environment. The data were analyzed using EthoVision XT 14 software (Noldus).

2.7. Statistics

In the figures, all data are shown as the means ± SD, except the behavioral tests, which are shown as the means ± SEM. For two-group comparisons, statistical tests were performed using the *t*-test. For multiple comparisons, one-way ANOVA with Tukey's post hoc test was used. Both *t*-test and one-way ANOVA were performed with GraphPad Prism 7.

3. Results and discussion

3.1. Expression of sensor C3 in Tg(ubi:sensor C3) zebrafish

An *ubi* promoter (Mosimann et al., 2011) was used to drive the ubiquitous expression of sensor C3 in the Tg(*ubi*:sensor C3) zebrafish. After we obtained the transgenic sensor zebrafish, time-lapse tracking of sensor C3 expression in the same Tg(*ubi*:sensor C3) zebrafish embryo was conducted within 120 hpf. Green fluorescence was observed as early as 4 hpf and mainly in the blastodisc region of the embryo, which contains embryonic stem cells (Kimmel et al., 1995), but not in the yolk sac region. Similarly, from 6 to 12 hpf, much stronger fluorescence signals of sensor C3 were observed, mainly at the outer layer of the embryo, where the developing zebrafish embryo was located. The sensor C3 fluorescence signals were strongly and stably expressed during the developmental process (Fig. 1A). Sensor C3 fluorescence could still be detected in 10-month-old adult Tg(*ubi*:sensor C3) zebrafish (Fig. 1B).

3.2. Detection and real-time tracking of apoptosis in live sensor zebrafish

Sensor C3 is a fusion protein of CFP and YFP, between which is the cleavage site of caspase-3, DEVD. Intact sensor C3 proteins appear in green because of the energy transfer between CFP and YFP. When cells undergoing apoptosis, the sensor C3 proteins are cleaved at the DEVD site. Then, the FRET effect will be abolished, and the sensor C3 proteins appear in blue (Fig. 2A). To validate that the Tg(*ubi*:sensor C3) zebrafish can be used to detect apoptosis, we used UV rays to treat the sensor zebrafish at 72 hpf. After UV irradiation, FRET imaging was performed on the sensor zebrafish, and we found many apoptotic (blue) cells in different regions of the sensor zebrafish (Fig. 2B). The three-dimensional distribution of apoptotic cells in the tail region is shown in Video S1. Then, we performed real-time tracking of apoptosis after UV irradiation (Fig. 2C and Video S2). Initially, all three cells indicated here were green. Five min later, cell #1 changed from green to blue, indicating caspase-3 was activated inside of that cell. Another 5 min later, cells #2 and #3 also turned blue. These results showed that the Tg(*ubi*:sensor C3) zebrafish can be used not only to detect apoptosis in live zebrafish but also to track apoptosis in real-time and at single-cell resolution. In addition to displaying apoptotic cells after UV irradiation, Tg(*ubi*:sensor C3) zebrafish could also reveal the sporadic apoptotic cells in different parts of fish body, during normal zebrafish development, especially in the early stage (Fig. 2D).

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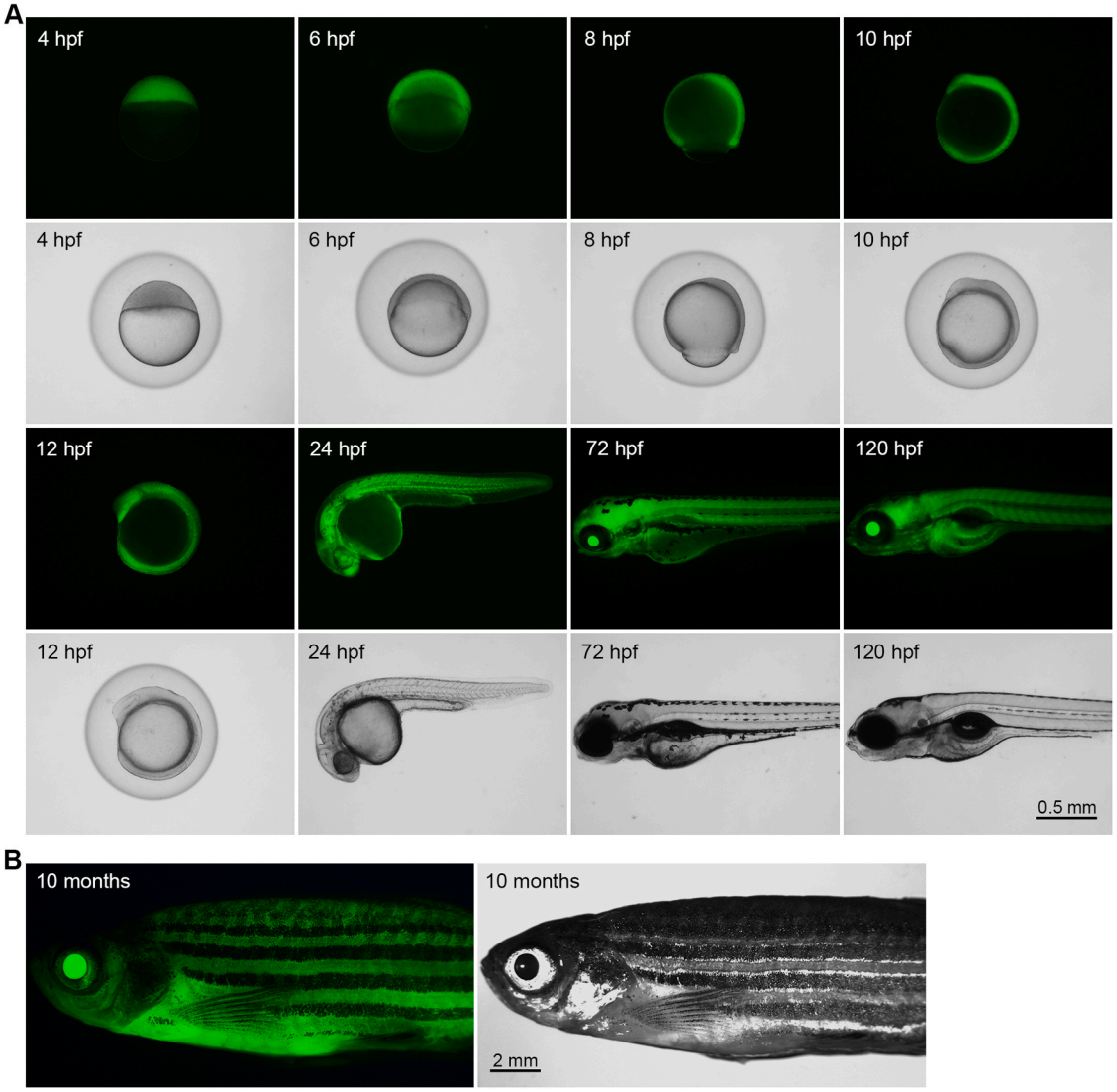

Fig. 1. Expression of sensor C3 in the Tg(*ubi*:sensor C3) zebrafish. (A) Sensor C3 was driven by the *ubi* promoter, and its expression pattern was tracked in the same live Tg(*ubi*:sensor C3) zebrafish within 120 hpf. (B) Sensor C3 expression could be observed ubiquitously in the 10-month-old adult Tg(*ubi*:sensor C3) zebrafish. The fluorescence images were taken using a GFP filter.

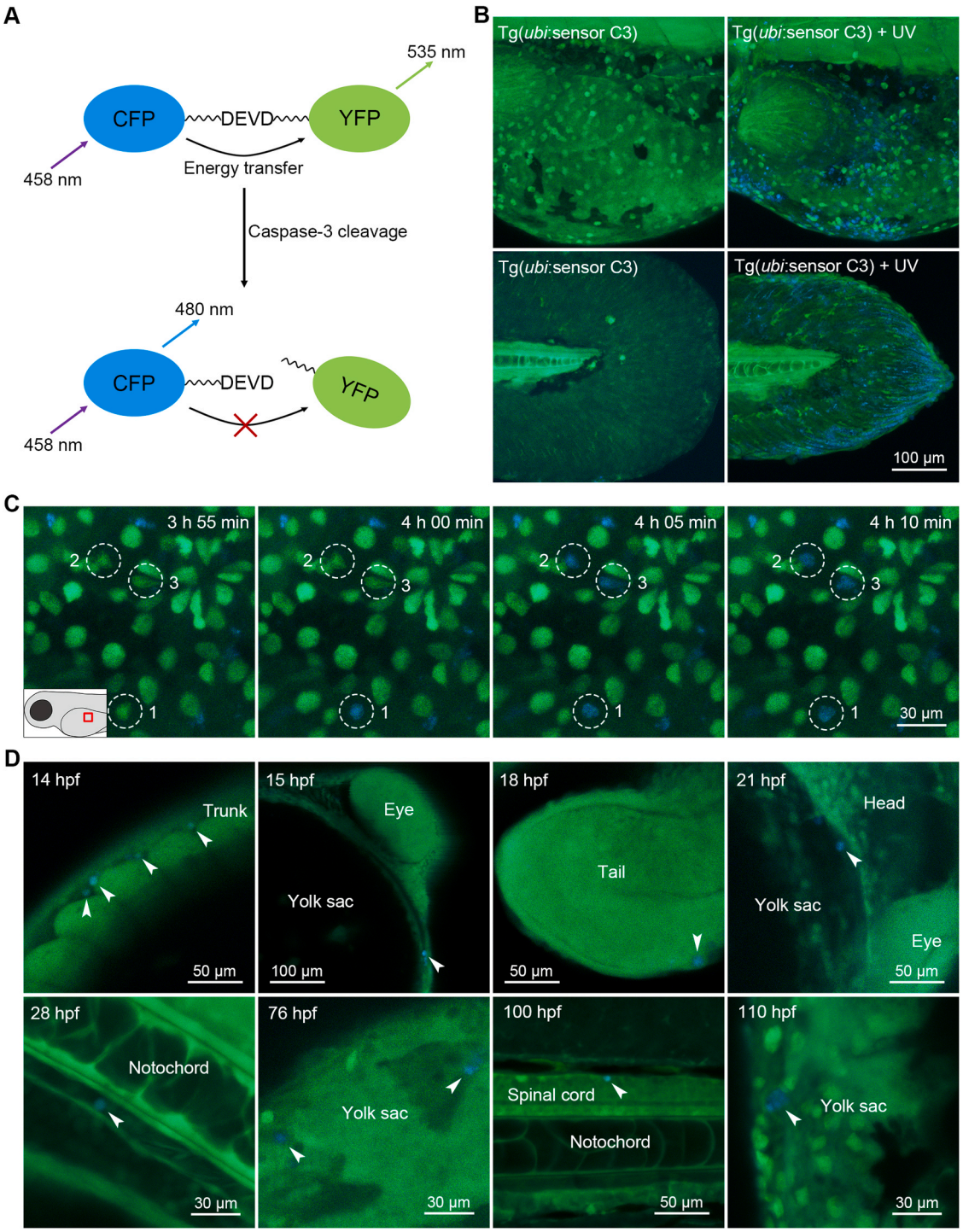

Fig. 2. Detection and real-time tracking of apoptosis in live sensor zebrafish. (A) The principle of sensor C3. (B) Tg(*ubi*:sensor C3) zebrafish at 72 hpf were irradiated with UV rays. FRET images showing apoptotic cells in the yolk sac region (left two images, 6 h after UV irradiation) and in the tail region (right two images, 8 h after UV irradiation). (C) Tg(*ubi*:sensor C3) zebrafish at 72 hpf were irradiated with UV rays. After UV irradiation, FRET images were taken every 5 min to show the fast dynamics of apoptosis. Three cells are indicated here, and the imaging region is shown in the zebrafish cartoon. The time after UV treatment is labeled in each image. (D) Developmental apoptosis was examined by FRET imaging in the same Tg(*ubi*:sensor C3) zebrafish within 5 dpf. Some blue apoptotic cells were observed in different parts of the sensor zebrafish during development especially during the early stage. The apoptotic cells were indicated by arrowheads. The developmental time and anatomical structures were indicated in each image.

3.3. Cd can induce apoptosis in sensor zebrafish at the maximum allowable concentration in drinking water

Human activities have caused a gradual increase in heavy metals in the atmosphere, water and soil (Tchounwou et al., 2012). Heavy metals are difficult to degrade in the environment and pose a large risk to human health. For example, heavy metals in wastewater can accumulate in algae and sediments, even when the initial concentration is low, and then can be internalized by fish through the food chain, which can directly threaten higher organisms, including humans (Jitar et al., 2015;

Ju et al., 2017; Zhang et al., 2018). Heavy metals can also bioaccumulate in crops. For example, Cd from wastewater can enter rice through irrigation. Long-term consumption of rice containing Cd can cause severe pain and diseases in humans (Mlangeni et al., 2020; Rai et al., 2019).

To test whether heavy metals can induce apoptosis in zebrafish, Tg (*ubi*:sensor C3) zebrafish at 48 hpf were treated with heavy metals at different dilutions for 48 h. After treatment, LC_{50} values were calculated based on the survival rates (Table S1). Then, the heavy metals were used to treat sensor zebrafish at 48 hpf at the LC_{50} for 48 h. At the end of the treatment, FRET imaging was used to measure the apoptotic effect of each tested heavy metal in Tg(*ubi*:sensor C3) zebrafish (Figs. 3A and S1). The quantified results showed that all three heavy metals exhibited significant apoptotic effects in Tg(*ubi*:sensor C3) zebrafish by causing 29–31 apoptotic cells per sensor zebrafish. Among these heavy metals, cadmium chloride ($CdCl_2$) produced the highest apoptotic effect as the LC_{50} of $CdCl_2$ was only 1/2 and 1/30 of that of lead chloride ($PbCl_2$) and cobalt chloride ($CoCl_2$) respectively. These results indicate that $CdCl_2$ is much more toxic than the other two heavy metals.

Then we used much lower concentrations of $CdCl_2$: 100 nM and 1 μM to treat Tg(*ubi*:sensor C3) zebrafish at 48 hpf for 72 h. The results showed that 1 μM $CdCl_2$ induced the apoptosis of many cells in the Tg(*ubi*:sensor C3) zebrafish. Even at the lower concentration of 100 nM, a significant increase of apoptosis was observed, and these apoptotic cells could be detected by the sensor zebrafish (Fig. 3B). Interestingly, we found that two

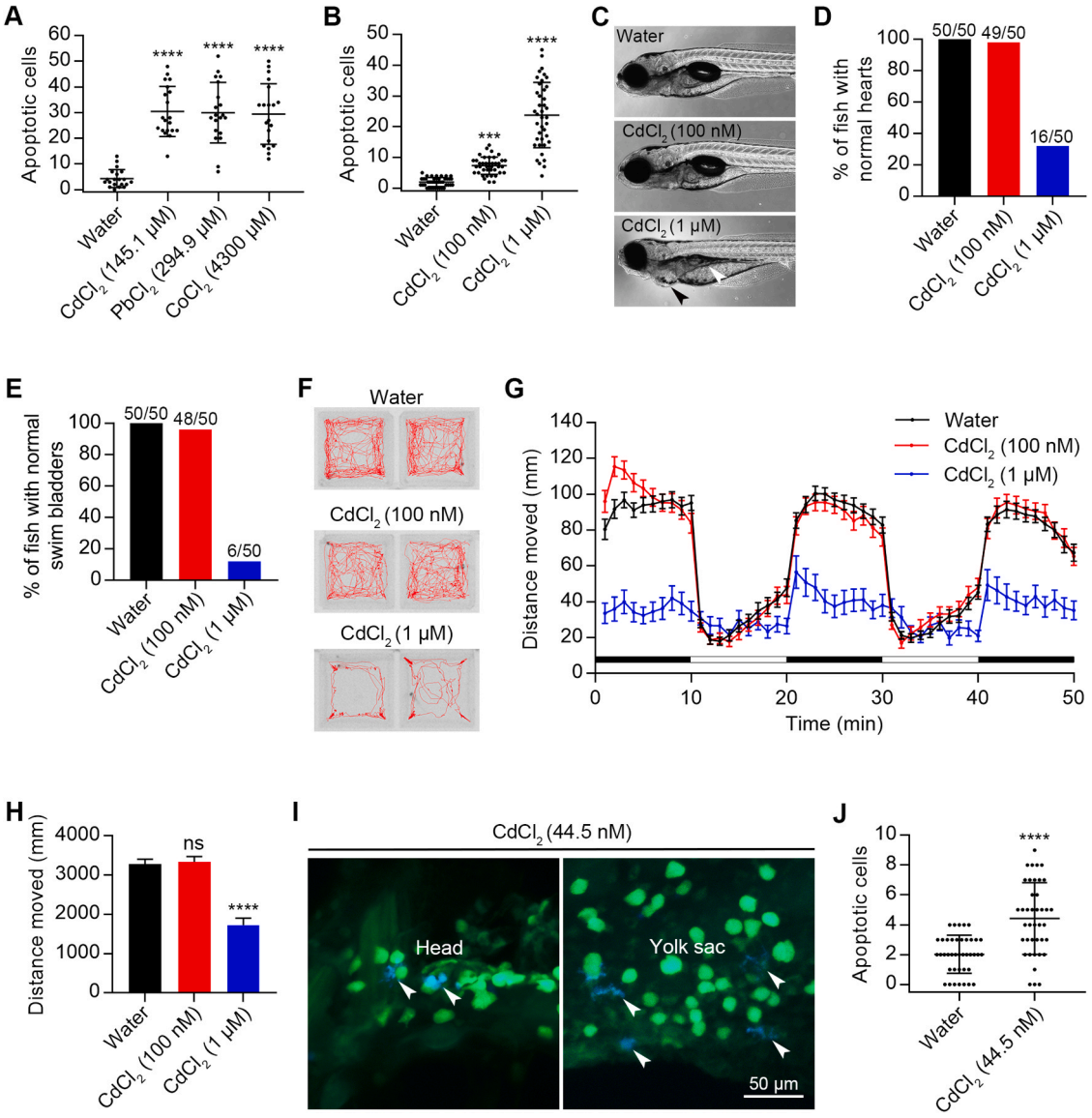

Fig. 3. Application of sensor zebrafish to detect heavy metals. (A) $CdCl_2$, $CoCl_2$ and $PbCl_2$ induced substantial number of apoptotic cells in the Tg(*ubi*:sensor C3) zebrafish (n = 20 for each group). (B) $CdCl_2$ induced significant apoptosis at both 1 μM and 100 nM in the Tg(*ubi*:sensor C3) zebrafish (n = 40 for each group). (C) $CdCl_2$ induced heart (black arrowhead) and swim bladder (white arrowhead) deformation in the Tg(*ubi*:sensor C3) zebrafish at the concentration of 1 μM but not at 100 nM. (D) The percentage of Tg(*ubi*:sensor C3) zebrafish with normal hearts after $CdCl_2$ treatment (n = 50 for each group). (E) The percentage of Tg(*ubi*:sensor C3) zebrafish with normal swim bladders after $CdCl_2$ treatment (n = 50 for each group). (F) $CdCl_2$ reduced the swimming ability of the Tg(*ubi*:sensor C3) zebrafish at the concentration of 1 μM but not at 100 nM. The track of each zebrafish during the final 5 min of the recordings is shown here. (G) Results from tracking Tg(*ubi*:sensor C3) zebrafish swimming for 50 min in a dark and light cycle (n = 48 for each group). (H) Statistical data of the swimming distances show that $CdCl_2$ reduced the swimming ability of the Tg(*ubi*:sensor C3) zebrafish at the concentration of 1 μM but not at 100 nM. (I) FRET imaging shows that 44.5 nM $CdCl_2$ induced apoptosis in the sensor zebrafish. The apoptotic cells are indicated with arrowheads. (J) Statistical data show the significant increase in the number of apoptotic cells in the Tg(*ubi*:sensor C3) zebrafish as induced by $CdCl_2$ at 44.5 nM (n = 40 for each group). Significant differences between the control group and treatment groups were determined using *t*-test or one-way ANOVA with Tukey's post hoc test. ns: not significant, ***$p < 0.001$, and ****$p < 0.0001$.

zebrafish organs, the heart and swim bladder, were very sensitive to toxic stress. The morphological changes in these organs were obvious and can be used as criteria to evaluate the toxicity. The results showed severe deformation in the zebrafish heart and swim bladder after treatment with $CdCl_2$ at the concentration of 1 μM but not at 100 nM (Fig. 3C–E). We also measured the swimming ability of the sensor zebrafish after treatment. Consistent with the morphological changes, the sensor zebrafish showed a significant decrease in swimming ability after treatment with $CdCl_2$ at the concentration of 1 μM but not at 100 nM (Fig. 3F–H). These results indicate that the sensor zebrafish-based detection method, that is, counting the number of apoptotic cells by FRET imaging, is more sensitive than the morphological analysis-based and behavioral test-based methods used in toxicological studies.

The concentration of Cd is one of the important indicators of drinking water quality (Hanhauser et al., 2020). To test whether the sensor zebrafish can be used in water quality monitoring, we used 44.5 nM (5 μg/L) $CdCl_2$ to treat the Tg(*ubi*:sensor C3) zebrafish; this treatment used the maximum allowable concentration of Cd in drinking water according to the directives of the European Union (Council Directive 98/83/EC), USA (EPA 822-F-18-001) and China (GB5749–2006). After treatment with 44.5 nM $CdCl_2$ for 72 h, we found a small but significant increase in the number of apoptotic cells in the Tg (*ubi*:sensor C3) zebrafish (Fig. 3I and J).

The toxicity of Cd at high concentrations has been well studied (Blechinger et al., 2002; Chow et al., 2008). However, much less is known about the in vivo toxicity of Cd at environmentally relevant concentrations, which are at scales from nanograms to micrograms per liter (Guo et al., 2018; Lacave et al., 2020). There is also a lack of suitable zebrafish models for the detection of Cd at such low concentrations. Our results indicated the power of the sensor zebrafish for water quality monitoring; that is, FRET imaging can detect the small number of cells induced to undergo apoptosis by Cd because of the single-cell sensitivity of the sensor zebrafish. Besides, the sensor zebrafish use ratio imaging to indicate apoptosis which can avoid the interference of autofluorescence. Comparing with other zebrafish-based methods, sensor zebrafish can detect the toxicity by FRET imaging without post-acquisition processing which is more user-friendly (Table 1). In addition, water samples can be applied on sensor zebrafish directly without dilutions and pre-treatments to evaluate the toxicity, which cannot be achieved using other biological detection methods such as cell culture-based tests.

3.4. Sensor zebrafish can reveal the apoptosis induced by ZnO NPs at very low concentrations

The widespread applications of nanomaterials have changed human life extensively but have also caused many environmental problems. Graphene oxide (GO) is a nanomaterial with excellent physical and chemical properties and is widely used in the field of optoelectronics, sensors and biomedical engineering (Huang et al., 2015). The release of GO in the aquatic environment causes significant toxicity (Jeong et al., 2015; Zhao et al., 2017). ZnO NPs are one of the mostly produced nanomaterials and are commonly used in sunscreen, cosmetics and antibacterial agents (Sirelkhatim et al., 2015). The toxicity of ZnO NPs is gradually receiving attention. For example, the cardiorespiratory toxicity of ZnO NPs was found in freshwater fish *Catostomus commersonii* (Bessemer et al., 2015). An in vitro study showed that ZnO NPs induced endoplasmic reticulum stress in endothelial cells and caused apoptosis (Chen et al., 2014a).

Here, we used sensor zebrafish to detect the toxicity induced by GO (thickness, 0.8–1.2 nm; size, 1–10 μm) and ZnO NPs (average particle diameter, 50 nm; average hydrodynamic diameter, 173.7 nm ± 7.4 nm). We first measured the LC_{50} of GO and ZnO NPs in Tg(*ubi*:sensor C3) zebrafish (Table S1). Then, the sensor zebrafish were treated with GO and ZnO NPs at their respective LC_{50} for 48 h. Both GO and ZnO NPs induced a significant increase in apoptosis in the sensor zebrafish. There were many more apoptotic cells per sensor zebrafish in the ZnO NPs group than in the GO group (Figs. 4A and S2). We then treated the sensor zebrafish for 72 h with ZnO NPs at much lower concentrations than the LC_{50}. ZnO NPs at a concentration of 1 μg/mL induced approximately 20 cells to undergo apoptosis in sensor zebrafish and impaired the development of heart and swim bladder in 34% and 64% zebrafish respectively. ZnO NPs at a concentration of 100 ng/mL did not impair the development of the heart or swim bladder but induced a significantly detectable number of cells to undergo apoptosis (Fig. 4B–D). For the behavioral tests, we found a similar pattern: ZnO NPs at the concentration of 1 μg/mL, but not at 100 ng/mL, decreased the swimming ability of the sensor zebrafish (Fig. 4E).

Previous studies from different research groups showed that ZnO NPs induced apoptosis in zebrafish at concentrations from 10 μg/mL to 120 μg/mL (Nasrallah et al., 2019; Verma et al., 2017; Zhao et al., 2016). Because of the single-cell sensitivity of our sensor zebrafish, we found that ZnO NPs can induce a significant increase in apoptosis at 1 μg/mL and 100 ng/mL. The sensor zebrafish were much more sensitive than other zebrafish-based methods to detect the toxicity of ZnO NPs (Table 2). This suggests that the sensor zebrafish can serve as a very good model for the detection of nanotoxicity.

3.5. DNA-damaging agents induced massive apoptosis in sensor zebrafish

The integrity of DNA is critical to the normal physiological activities of the cells. DNA-damaging agents are dangerous because they can cause many diseases, including cancer (Seplyarskiy et al., 2019). In this study,

Table 1
Comparation of sensor zebrafish and other zebrafish-based tests for cadmium.

Fish used	Treatment duration	Methods used	Toxic concentrations revealed	Ref.
Sensor zebrafish embryos to larvae	**72 h**	**FRET-based apoptosis detection**	**44.5 nM and above**	
Transgenic huORFZ larvae[a]	96 h	GFP fluorescence	22.2 nM and above	Lee et al. (2014)
Tg(*hsp70*:EGFP) larvae	96 h	GFP fluorescence	200 nM and above	Blechinger et al. (2002)
Tg(*hsp70*:EGFP) larvae	96 h	GFP fluorescence	500 nM and above	Matz and Krone (2007)
WT embryos to larvae	96 h	Hatching and behavioral analysis	1 μM and above	Jin et al. (2015)
WT embryos	48 h	Mortality and morphological analysis	2.2 μM and above	Hallare et al. (2005)
WT embryos	9 and 23 h	DNA damage measurement	3 μM	Ho et al. (2019)
WT embryos to larvae	72 h	Hatching and behavioral analysis	9 μM	Capriello et al. (2019)
WT embryos to larvae	96 h	Gene expression analysis	37 μM	Sonnack et al. (2018)
WT embryos	20 h	Morphological and immunohistochemical analysis	100 μM	Chow et al. (2008)
WT adults[b]	21 days (d)	Mortality and DNA damage measurement	17 nM and 85 nM	Cambier et al. (2010)
WT adults	21 d and 6 months	Mortality and histopathological analysis	89 nM	Lacave et al. (2020)
WT adults	48 h	DNA damage measurement	445 nM	Doganlar et al. (2016)
WT adults	7 d	Mortality and ultrastructure analysis	1.8 μM	Guo et al., (2018)
WT adults	24 and 96 h	Gene expression analysis	8.9 μM	Zheng et al. (2016)

[a] Autofluorescence interference and semi-quantitative analysis.
[b] Long test duration.

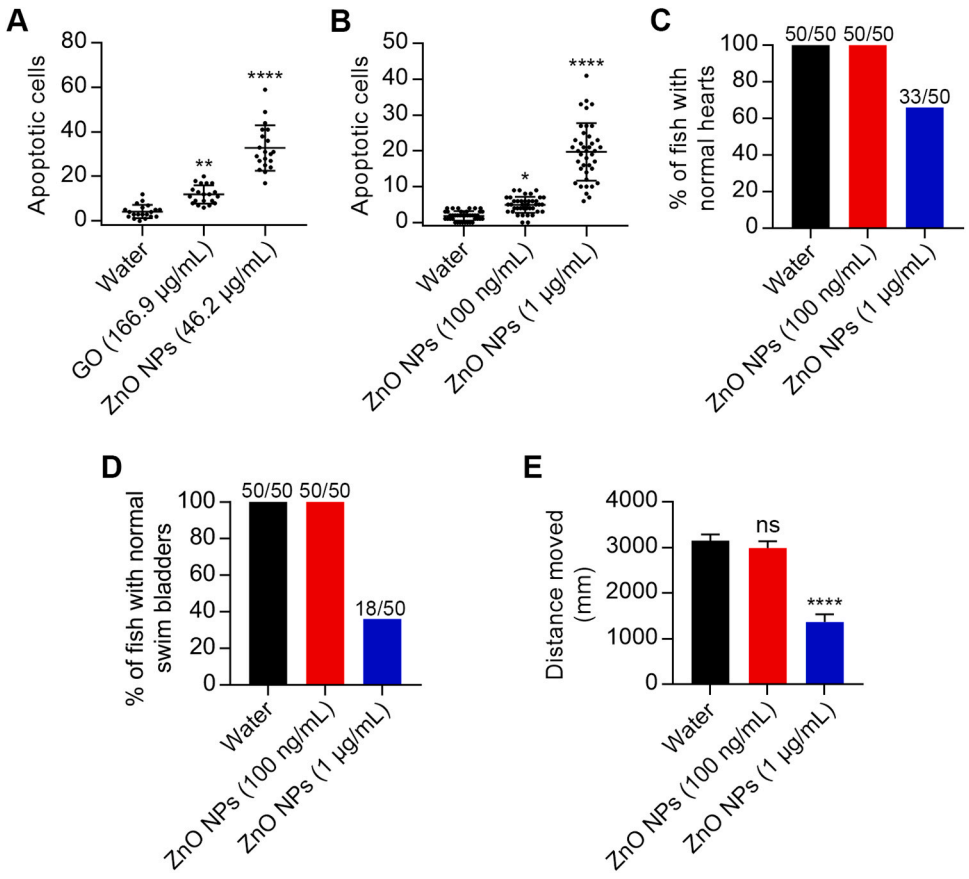

Fig. 4. Application of sensor zebrafish to detect nanotoxicity. (A) Both GO and ZnO NPs induced a significant increase in apoptosis in the sensor zebrafish (n = 20 for each group). (B) ZnO NPs induced significant apoptosis at both 1 μg/mL and 100 ng/mL in the Tg(*ubi*:sensor C3) zebrafish (n = 40 for each group). (C) The percentage of heart damage after ZnO NPs treatment (n = 50 for each group). (D) The percentage of swim bladder damage after ZnO NPs treatment (n = 50 for each group). (E) Statistical data show that ZnO NPs at the concentration of 1 μg/mL, but not at 100 ng/mL, reduced the swimming ability of the sensor zebrafish (n = 48 for each group). Significant differences between the control group and treatment groups were determined using one-way ANOVA with Tukey's post hoc test. ns: not significant, $*p < 0.05$, $**p < 0.01$, and $****p < 0.0001$.

Table 2
Comparation of sensor zebrafish and other zebrafish-based tests for ZnO NPs.

Fish used	Treatment duration	Methods used	Toxic concentrations revealed	Ref.
Sensor zebrafish embryos to larvae	**72 h**	**FRET-based apoptosis detection**	**100 ng/mL and above**	
WT embryos to larvae	96 h	Hatching and gene expression analysis	200 ng/mL and above	Brun et al. (2014)
WT embryos to larvae	144 h	Hatching and behavioral analysis	500 ng/mL and above	Chen et al. (2014b)
WT embryos to larvae	144 h	Morphological and gene expression analysis	1 μg/mL and above	Zhao et al. (2013)
WT, Tg(*isl2B*:GFP) and Tg(*fli1*:EGFP) embryos to larvae	91 h	Morphological and immunohistochemical analysis	1 μg/mL and above	Kteeba et al. (2018)
WT embryos to larvae	96 h	Morphological and gene expression analysis	1 μg/mL and 10 μg/mL	Choi et al. (2016)
WT embryos to larvae	93 h	Morphological and hatching analysis	5 μg/mL and above	Bai et al. (2010)
WT embryos to larvae	96 h	Hatching, gene expression and apoptotic analysis	10 μg/mL and above	Zhao et al. (2016)
WT embryos to larvae	96 h	Mortality, gene expression and apoptotic analysis	10 μg/mL and above	Verma et al. (2017)
WT embryos to larvae	96 h and 30 d	Hatching and mortality	10 μg/mL and 50 μg/mL	Kteeba et al. (2017)
WT adults	96 h	Mortality and gene expression analysis	2 μg/mL and above	Xiong et al. (2011)

doxorubicin (DOX), an anticancer drug, and methyl methanesulfonate (MMS), an alkylating agent, both of which are DNA-damaging agents (Feng et al., 2020; Tacar et al., 2013), were used at their respective LC_{50} (Table S1) to treat sensor zebrafish for 48 h. The results showed that treatment by each of them induced the apoptosis of many cells in the sensor zebrafish (Figs. 5A and S3). We then treated the sensor zebrafish with MMS for 72 h at a much lower concentration than the LC_{50}. The results showed that MMS induced a significant increase in apoptosis in sensor zebrafish, especially at the concentration of 40 μM (Fig. 5B). At the concentration of 40 μM, there was no obvious damage to the zebrafish heart or swim bladder (Fig. 5C and D). At this concentration, there was also no decrease in swimming ability in the sensor zebrafish (Fig. 5E). These results showed that the sensor zebrafish can serve as a good model for detecting DNA-damaging agents and can greatly benefit human health.

4. Conclusions

In this study, we generated sensor zebrafish that expressed a FRET-based apoptotic sensor using *Tol2* transposase-based transgenesis. The cells of the sensor zebrafish changed their color from green to blue when undergoing apoptosis. We showed that the sensor zebrafish can detect toxic agents with single-cell sensitivity. More importantly, FRET imaging-based apoptosis is more sensitive than morphological changes

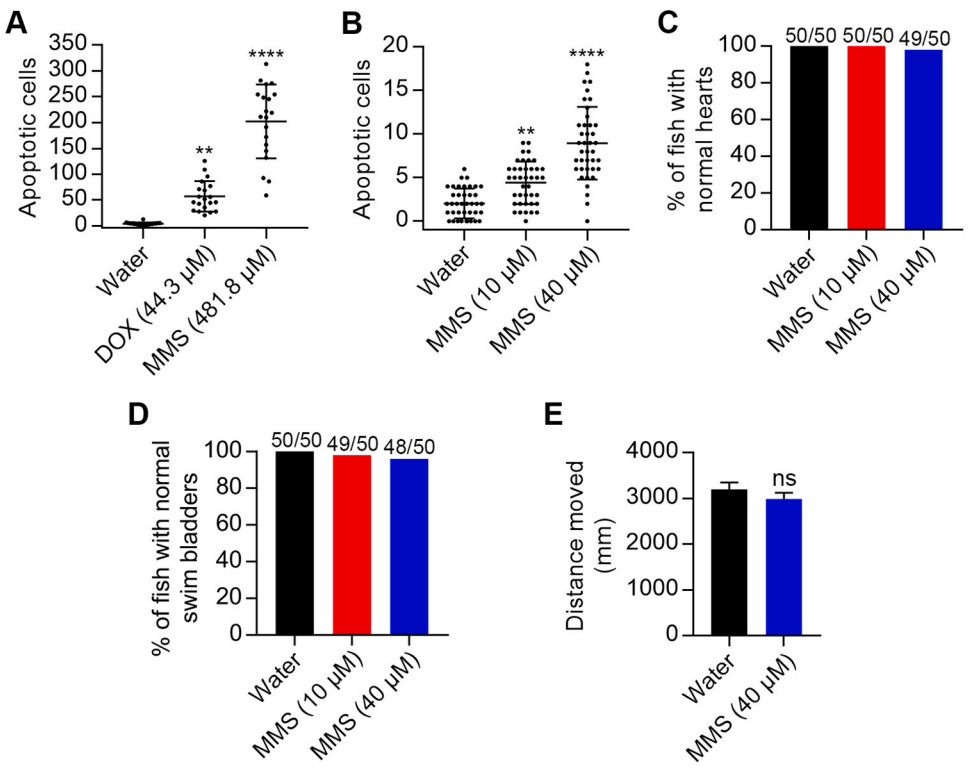

Fig. 5. Application of sensor zebrafish to detect the toxicity of DNA-damaging agents. (A) Both DOX and MMS induced many cells to undergo apoptosis in the Tg(*ubi*:sensor C3) zebrafish (n = 20 for each group). (B) At both 10 μM and 40 μM, MMS induced significant apoptosis (n = 40 for each group). (C, D) MMS treatment at 10 μM and 40 μM did not affect the development of zebrafish heart and swim bladder (n = 50 for each group). (E) Statistical data show that MMS did not reduce the swimming ability of the sensor zebrafish at the concentration of 40 μM (n = 48 for each group). Significant differences between the control group and treatment groups were determined using *t*-test or one-way ANOVA with Tukey's post hoc test. ns: not significant, *,**$p < 0.01$, and ****$p < 0.0001$.

and behavioral tests. Using the sensor zebrafish, we found that heavy metal Cd at the maximum allowable concentration in drinking water induced a significant increase of apoptosis in sensor zebrafish. Our results showed that two widely used nanomaterials, GO and ZnO NPs, especially ZnO NPs, can induce apoptosis in sensor zebrafish. We also found that these sensor zebrafish are very sensitive to agents that can damage DNA. The sensor zebrafish can serve as a powerful in vivo tool for toxic agent detection.

CRediT authorship contribution statement

Hao Jia: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft. **Kathy Qian Luo**: Conceptualization, Methodology, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare no competing financial interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2020.124826.

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