JC-10 probe as a novel method for analyzing the mitochondrial membrane potential and cell stress in whole zebrafish embryos

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Abstract

Background A sensitive method to investigate cellular stress and cytotoxicity is based on measuring mitochondrial membrane potential. Recently, JC-10, was developed to measure mitochondrial membrane potential *in vitro* and used as an indicator for cytotoxicity. Yet, JC-10 has never been used *in vivo* (whole organism). In normal cells, JC-10 concentrates in the mitochondrial matrix, where it forms red fluorescent aggregates. However, in apoptotic/necrotic cells, JC-10 diffuses out of the mitochondria, changes to monomeric form, and stains cells in green. Here, we aimed to develop and optimize a JC-10 assay to measure cytotoxicity in zebrafish embryo. We also investigated the effectiveness of JC-10 assay by comparing it to common cytotoxicity assays.

Methods Zebrafish embryos were exposed to a toxic surfactant AEO-7 at no observed effect concentration (6.4 µg/L), and then cytotoxicity was measured using (i) JC-10 mitochondrial assay, (ii) acridine orange (AO), (iii) TUNEL assay, and (iv) measuring the level of Hsp70 by western blotting.

Results As compared to the negative control, embryos treated with NOEC of AEO-7 did not show significant cytotoxicity when assessed by AO, TUNEL or western blotting. However, when JC-10 was used under the same experimental conditions, a significant increase of green:red fluorescent ratio signal was detected in the AEO-7 treated embryos, indicating mitochondrial damage and cellular cytotoxicity. Noteworthy, the observed green: red ratio increase was dose dependent, suggesting specificity of the JC-10 assay.

Conclusion JC-10 is a sensitive *in vivo* method, thus, can be used as surrogate assay to measure cytotoxicity in whole zebrafish embryos.

Graphical Abstract
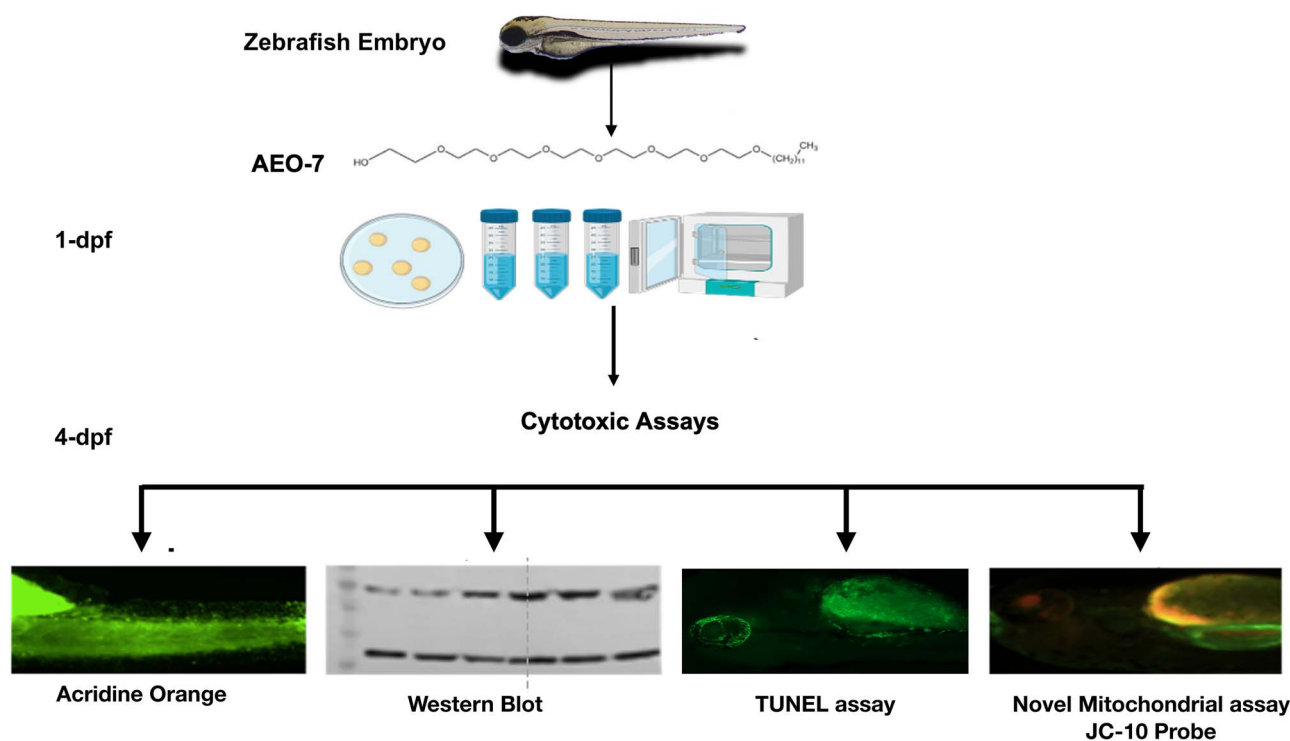
Keywords: JC-10 probe, mitochondria, cytotoxicity, zebrafish, AEO-7

Introduction

Mitochondria are essential organelles that regulate many critical biological processes including cell growth, ATP production, calcium homeostasis, apoptosis, and redox signaling [1, 2]. Mitochondrial dysfunction or mutations in the mitochondrial DNA (mtDNA) have been associated with many human diseases including cancer, cardiovascular, gastrointestinal, and neurological disorders [1, 3–7]. Additionally, mounting evidence suggests that environmental exposures cause substantial mitochondrial dysfunction due to the lack of mtDNA repair mechanisms that are essential to repair nuclear DNA damage, which leads to accumulation of mtDNA mutations over time. Most importantly, in response to environmental stressors, the mitochondria constantly change their quantity, morphology, and composition depending on cell type, developmental stage, and metabolic demands [2]. Thus, there is a need to examine the possible effects of environmental contaminants on mitochondrial function, particularly at the most sensitive, early stages of life as many of these contaminants may indeed be mitochondrial toxicants.

Although JC-1 probe is widely used in many labs to assess mitochondrial membrane potentials [8], its poor water solubility carries a great inconvenience. Even at very low concentration (1 μM), JC-1 precipitate in aqueous buffers. Thus, JC-10 was developed to be a superior alternative to JC-1 as JC-10 has a much better water solubility. As the mitochondrial membrane potential increases, JC-10 probe has the capability to selectively enter the mitochondria and reversibly changes its fluorescent color from green to orange. This property is due to the reversible formation of JC-10 aggregates upon membrane polarization that causes shifts in the emitted light from 520 nm (JC-10 monomeric form) to 570 nm (J-aggregate form). Upon JC-10 excitation at 490 nm, the color changes reversibly from green to greenish orange as the mitochondrial membrane polarization increase. Both colors can be detected using filters commonly mounted in all flow cytometers and fluorescent microscopes. In normal cells, JC-10 concentrates in the mitochondrial matrix where it forms red fluorescent aggregates. However, in apoptotic and necrotic cells, JC-10 diffuses out of the mitochondria and changes to a monomeric form, staining cells in green fluorescence. Therefore, the cationic lipophilic JC-10 dye can be employed to detect changes in the cellular mitochondrial membrane potential.

Zebrafish (*Danio rerio*) has emerged as a powerful animal model for environmental toxicity studies of vertebrates [9–12]. It provides an affordable and relatively rapid approach for cytotoxicity screening and probing the mechanism(s) of toxicity. In addition, it is accepted by the National Institutes of Health (NIH, USA) as a reliable model that replaces studies on laboratory rodents for examining human diseases and toxicity effects of different chemicals [13, 14]. Furthermore, zebrafish constitutes a bridge between vertebrate and invertebrate in developmental studies and environmental toxicity evaluations [15]. Zebrafish embryos serve as the most

competent candidate for such cytotoxicity experiments due to its small size that efficiently decreases housing space and cost. In addition, because it has been used for a long time in different research activities, the experimental protocols and keeping conditions of zebrafish are well determined [16].

In this study, we aimed to propose a novel protocol adapted for zebrafish embryogenesis that allows researchers to rapidly and reliably quantify the mitochondrial membrane potential in zebrafish embryos. We developed this method using wild-type (WT) zebrafish during early embryogenesis. We tested this method using Fatty Alcohol Polyoxyethylene Ether-7 (AEO-7), a nonionic surfactant [12]. In addition, we investigated the effectiveness of JC-10 assay compared to different cellular cytotoxicity assays; acridine orange (AO) and TUNEL assay to detect apoptosis and western blotting of heat shock protein 70 (Hsp70), a general stress marker).

Materials and Methods
Chemicals
Diethylamino benzaldehyde (DEAB) (Sigma–Aldrich, Steinheim, Germany) and zinc oxide (ZnO) nanoparticles (diameter < 100 nm) (catalog #721077-100G, Sigma–Aldrich) are known to cause morphological deformities and cellular stress, respectively, in zebrafish embryos [17]. Therefore, were used DEAB and ZnO nanoparticles as positive controls in our experiments. N-phenylthiourea (PTU) (Sigma–Aldrich) in E3 medium (buffered egg water solution used for cultivating zebrafish embryos) was used to raise zebrafish embryos *in vitro*. PTU-E3 is essential as it inhibits the pigmentation in zebrafish embryos by stopping melanogenesis, which facilitates their microscopic examination as the embryos will be transparent. AEO-7 surfactant was purchased from Shanghai Dejun Technology Co., Ltd, China (2 mg/L working solution was prepared using 5 µl of the stock in 4.995 ml PTU-E3 and homogenized by vortexing).

Zebrafish embryos culture
WT AB zebrafish (*D. rerio*) embryos were used in all performed experiments. The embryos were sustained in a 14 h light/10 h dark cycle with a water temperature of 28°C in the Biomedical Research Center (BRC) at Qatar University (QU). The collected embryos were produced by natural pairwise mating [18]. At 1 days post fertilization (dpf), unfertilized and dead eggs were removed whereas only healthy fertilized eggs were carefully chosen under a stereomicroscope and transferred to PTU-E3 medium. Then, the embryos were dechorionated by adding 1.0 mg/ml of pronase as performed in our previous studies [10, 19–21]. In all performed experiments, we followed the national and international guidelines for the use of zebrafish embryos in accordance with animal protocol guidelines required by Qatar University and the Policy on Zebrafish Research established by the Department of Research in the Ministry of Public Health in Qatar.

Acute toxicity analysis
AEO-7 acute toxicity assay was adapted from the guidelines for testing chemical toxicity formulated by the Organization for Economic Co-operation and Development (OECD) (N° 203, 210, and 236) [22–24]. After dechorionation at 1-dpf, deformed embryos (i.e. immotile, opaque, or embryos with undetectable heartbeats) were excluded. Healthy embryos were incubated in six-well plates containing prepared concentrations of AEO-7 (.4, .8, 3.2, 6.4, 12.8, 25 µg/L), PTU-E3 (negative control), and DEAB (10 and 100 µM) (positive control) [10, 12]. The survival rate of the embryos was assessed until 4-dpf. The median lethal concentration (LC_{50}) was calculated by fitting a sigmoidal curve to the data on mortality using the GraphPad Prism 8 software (version 8.2.1, San Diego, CA, USA), as previously described [21, 25, 26]. In addition, we used this curve to calculate the LC_{10}, LC_{20} and LC_{50} at a statistical significance of 95% ($P < .05$) [12, 27]. Moreover, we calculated the no observed effect concentration (NOEC), which is the highest concentration that does not cause a significant effect ($P < 0.05$) relative to the negative control (PTU-E3). For every control sample and tested concentration of AEO-7, 25 embryos were used.

AO staining of apoptotic cells
To assess AEO-7-induced apoptosis, AO fluorescent staining was used. AO is a metachromatic dye widely used to detect the presence of apoptotic cells. AO emits green fluorescence in its monomeric form when it binds to double-stranded DNA [28]. For detection, either qualitative assessment using fluorescence microscopy or quantitative measurement using microplate spectrophotometry or flow cytometry can be performed [29, 30]. At 1-dpf, after dechorionation, the embryos were treated with AEO-7 (6.4 µg/L and 12.8 µg/L), PTU-E3 (negative control), and ZnO nanoparticles (20 mg/L) (positive control). At 4-dpf, the embryos were washed with 1× phosphate buffer saline (PBS). Then, the embryos were incubated with 100 µl of 5.0 µg/ml of AO for 1 h in the dark. Subsequently, the embryos were washed 3× with 1× PBS and placed in 96-well plates (5 embryos/ well) with 100 µl 1× PBS. For quantification, the intensity of AO fluorescence, which reflects the presence of apoptotic cells, was measured using Tecan GENios Pro Microplate Reader to detect the green fluorescence at excitation wavelength 487 nm and emission wavelength 525 nm [27, 29, 30]. Representative images were taken by Zeiss Axiocam ERc 5 s camera attached to a fluorescence microscope (Zeiss Stereo). For every control sample and tested concentration of AEO-7, 30 embryos were used.

TUNEL assay for detection of apoptosis
Apoptosis was detected by Tunnel assay using the APO-DIRECT™ Kit [31] (Catalog no. 6536KK, BD Biosciences

Pharmingen). At 1-dpf, after dechorionation, the embryos were treated with AEO-7 (6.4 µg/L and 12.8 µg/L), PTU-E3 (negative control), and ZnO nanoparticles (20 mg/L) (positive control). At 4-dpf, the embryos were transferred to Eppendorf tubes and washed with 1× PBS. Then, the embryos were suspended for 30 min in 1% formaldehyde for fixation. Subsequently, the embryos were washed 3× with 1× PBS and 70% (v/v) ice cold ethanol was added to the embryos for 30 min for permeabilization. After that, the 70% ethanol was removed, and 1.0 ml of wash buffer was added to each Eppendorf tube twice for washing. Following the manufacturing protocol, we prepared the DNA labeling solutions by adding 120.00 µl of Reaction Buffer (green cap), 9.00 µl of TdT Enzyme (yellow cap), 96.00 µl of FITC dUTP (orange cap), and 387.00 µl of distilled water. We incubated the embryos with the DNA labeling solution for 60 min at 30°C. At the end of the incubation time, add 1.0 ml of Rinse Buffer. Finally, we transferred the embryos to 96 black well plate for imaging under BioTek Cytation 5 Imaging microscopy using the GFP filter. The area of fluorescence was measured using ImageJ software (version 1.52a, NIH, Washington DC, USA) in combination with Java 1.8.0_172 in the head region of the zebrafish embryos.

Western blot analysis (Hsp70 assay)

At 4-dpf, total protein was extracted from seven tested zebrafish groups (30 embryos for each condition) treated with AEO-7 (6.4 µg/L and 12.8 µg/L), PTU-E3 (negative control), ZnO nanoparticles (20 mg/L) (positive control). At 4-dpf, deyolking was performed by homogenizing the embryos and proteins were extracted from whole embryos using RIPA lysis and extraction buffer (ThermoFisher Scientific, USA) supplemented with 1X protease inhibitors cocktail (ThermoFisher Scientific). Protein quantification was done using the Pierce ™ BCA Protein Assay Kit (catalog #23225, ThermoFisher Scientific) according to the manufacturer's protocol. After that, 4× Laemmli sample buffer (ThermoFisher Scientific) supplemented with β-mercaptoethanol was used to dilute the protein samples. Then, 8% polyacrylamide gel was prepared and after loading the protein samples, the gel was subjected to 120 V for 1 h and 15 min at room temperature. The gel was electro-blotted using pure nitrocellulose membrane placed in 20% methanol for 10 min, and a semidry transfer using the sandwich method was done at 20 V current for 1 h. To prevent nonspecific binding of antibodies, the membranes were incubated in 5% nonfat milk using Tween 20 and Tris Buffered Saline (TBS-T) buffers. The membrane was then cut to separate the bands of Housekeeping GAPDH in the lower part and Hsp70 in the upper part of the membrane. Visualization of Hsp70 and GAPDH bands was achieved by incubation with mouse monoclonal anti-Hsp70 antibody at 1:1000 (ab5439, Abcam) and rabbit polyclonal anti-GAPDH antibody at 1:1000 (ab209856, Abcam), respectively, overnight at 4°C with shaking. The membranes were incubated with antimouse secondary antibody at 1:2500 (PAB0096, Abnova) to detect Hsp70 and antirabbit secondary antibody at 1:40 000 (A0545, Sigma–Aldrich) to detect GAPDH. Both secondary antibodies were conjugated with horse-radish peroxidase (HRP) for 1 h at room temperature on a shaker. Then, a substrate was prepared using the Western Blotting Detection kit (Abcam), a 1:1 ratio of reagent A and reagent B for each membrane and incubated for 5 min in the dark before reading, detection was then done using chemiluminescence ECL using GENE GENOME apparatus by SYNGENE. The band intensities were then measured through densitometry using ImageJ software (version 1.52a, NIH, Washington DC, USA) in combination with Java 1.8.0_172 and in comparison, with that of the negative control to identify the relative quantity of the protein. Band intensities were then quantified using ImageJ software (NIH Image Soft) to detect the relative quantity of Hsp70 protein compared that of GAPDH [27].

JC-10 mitochondrial membrane potential assay

The measurement of the mitochondrial membrane potential ($\Delta\Psi$m) is a key aspect of toxicity evaluation. The JC-10 probe (Catalog #ab112134, Abcam, USA) is a cationic fluorescent dye used to detect mitochondrial membrane potential disturbance. Although widely used in cell culture to assess $\Delta\Psi$m, JC-10 has never been used in zebrafish embryos. The principle of the dye is that in healthy cells, JC-10 forms red fluorescent aggregates as it concentrates in the mitochondrial matrix upon membrane polarization. When cells undergo apoptosis due to toxic effects, the mitochondrial membrane potential changes and the dye switches to its monomeric form that emits green fluorescence as it diffuses out of the mitochondria that are unable to keep the dye inside. It is worth noting that the JC-1 probe is widely used in many laboratories [8]. However, it has poor solubility in water, which makes JC-10 a superior alternative as it has a much better water solubility.

At 1-dpf, embryos were dechorionated and placed in six-well plates. The embryos were treated with AEO-7 (6.4 µg/L and 12.8 µg/L), PTU-E3 (negative control), and ZnO nanoparticles (20 mg/L) (positive control). At 4-dpf, a working solution of the dye was prepared using 100X JC-10 and assay buffer A in a ratio of 50 µl of 100X JC-10 (Component A): 5 ml assay buffer A (Component B) according to the manufacture's protocol [32]. Then, 50 µl of the dye was added to the embryos after placing them in Eppendorf tubes. After 30 min of incubation in the dark at 28°C, 50 µl assay buffer B was added to each group. The embryos were then transferred to black 96-well plates (10 embryos/well). Reading of red and green fluorescence was done using Tecan GENios Pro Microplate Reader at Ex/Em = 490/525 nm (cutoff at 515 nm) and 540/590 nm (cutoff at 570 nm) for ratio analysis (Abcam, 2019). Imaging was performed using BioTek Cytation 5 Imaging Reader using GFP and Texas Red filters [10].
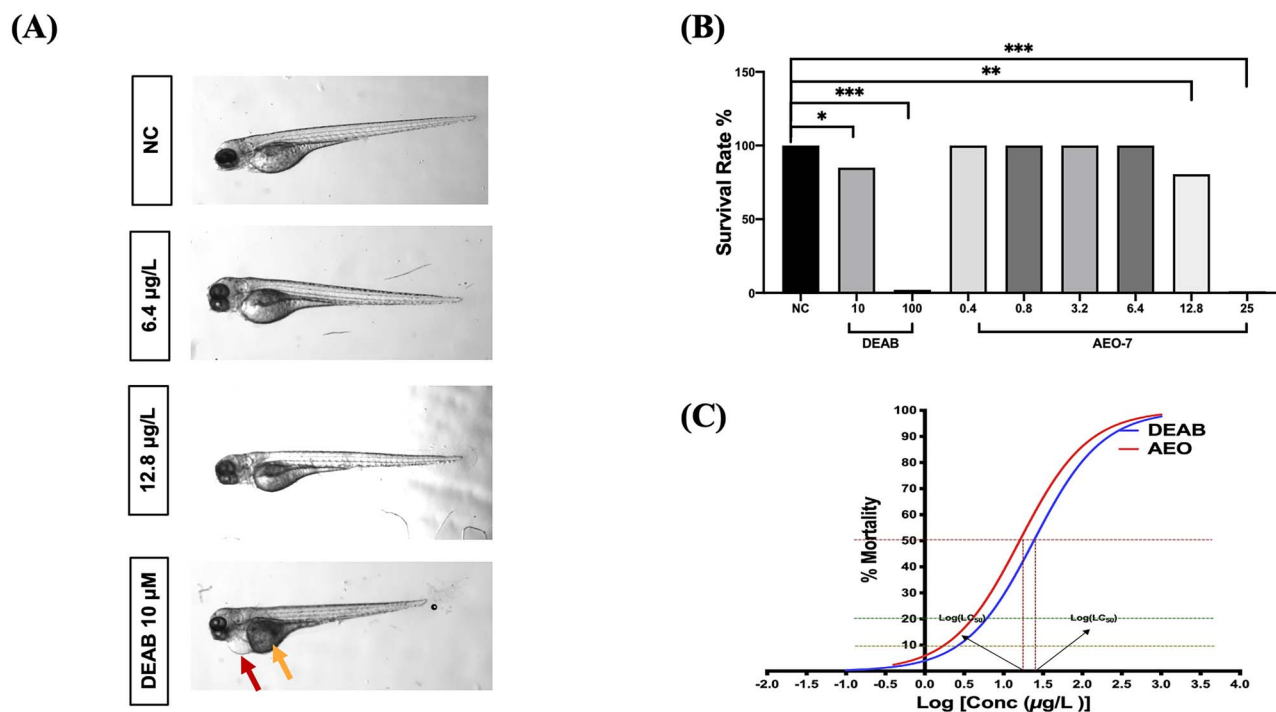
Figure 1. (A) Representative images of 96-dpf embryos treated with AEO-7, PTU-E3 (negative control), or DEAB (positive control). Note the edema in the yolk sac (yellow arrow) and heart (red arrow) after exposure to 10 µM DEAB. These images were captured with a ZeissStemi2000-C stereomicroscope (20×). **(B)** The survival rate of embryos exposed to different concentrations of AEO-7 and the positive and negative controls. **(C)** Dose–response curves were used to calculate the LC_{50} for AEO-7 and DEAB. One-way analysis of variance (ANOVA) followed by Dunnett test was used to compare the groups. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ ($n = 25$).

Statistical analysis

For the acute toxicity evaluation, collective mortality was presented as a rate of surviving embryos at 4-dpf. Calculation of descriptive statistics (DS) including the mean (m) and standard deviation (SD) was done for mitochondrial membrane potential examination, apoptosis detection and western blot analysis. All the values for the negative control and treated groups were compared statistically using one-way ANOVA, Dunnett test. Statistical significance is shown as *$P < .05$; **$P < .01$ and ***$P < .001$. All statistical analyses and removal of all significant outliers were performed using GraphPad Prism 8 software (version 8.2.1, San Diego, CA, USA).

Results

AEO-7 is extremely toxic toward zebrafish embryos

AEO-7 was tested for its toxicity between 1 and 4-dpf, a time frame whereby zebrafish embryos are most sensitive to toxicants [17, 33–35]. At 4-dpf, 10 µM DEAB (positive control) showed observable teratogenic effects like yolk edema, pericardial edema, heart abnormalities (Fig. 1A) and 85% survival rate (Fig. 1B), whereas 100 µM DEAB (positive control) showed 100% mortality rate (Fig. 1B). This resulted in an DEAB LC_{10} of 2.686 µM, an LC_{20} of 6.043 µM and LC_{50} of 24.17 µM (Fig. 1C). In addition, significant mortality rates resulted upon treatment with 12.8 and 25 µg/L AEO-7. From the mortality rate curve, AEO-7 showed an LC_{10} of 1.775, an LC_{20} of 3.995 µM

and an LC_{50} of 15.98 µM. According to the Fish and Wildlife Service Acute Toxicity Rating Scale [36], AEO-7 surfactant would be classified as "super toxic". The NOEC of AEO-7 is 6.4 µg/L, which is the highest concentration that showed no significant difference compared to PTU-E3 treatment (negative control). In the following experiments, we used the NOEC and a higher concentration of AEO-7 to assess its cytotoxicity in zebrafish embryos.

AO staining does not detect apoptosis at NOEC of AEO-7

At 4-dpf, Zebrafish larvae were stained with AO, which is one of the most common methods used for apoptosis detection in whole zebrafish embryos. The apoptotic cells are presented as bright fluorescent green spots (Fig. 2A). Only apoptotic cells can uptake the AO dye, which selectively binds to the double-stranded DNA and emit green florescence [28]. Quantification of fluorescence intensity of apoptotic cells showed no significant difference upon treating the embryos with AEO-7 (6.4 µg/L and 12.8 µg/L) compared to PTU-E3 treatment (negative control). However, when the embryos were treated with ZnO nanoparticles (20 mg/L), apoptosis increased significantly ($P < 0.001$) compared to PTU-E3 treatment (negative control; Fig. 2B).

TUNEL assay was not sensitive in detecting apoptosis in whole embryos

TUNEL assays are designed to detect apoptotic cells that undergo extensive DNA degradation during the
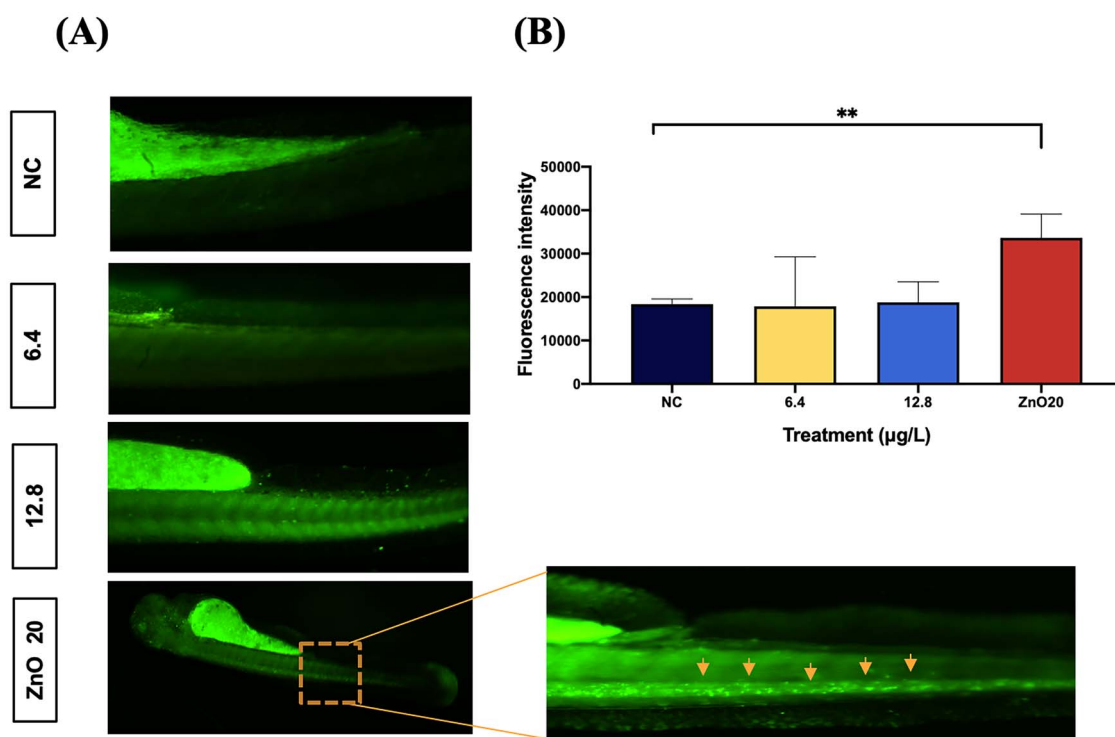
Figure 2. The detection of apoptosis using AO. (A) Representative images of 96-dpf embryos stained with AO (100 μl of 5 μg/ml of AO after 72-hour treatment). Bright green fluorescent at 100× magnification represent apoptotic cells. (B) The average fluorescence intensity of apoptotic cells after AO staining. One-way analysis of variance (ANOVA) followed by Dunnett test was used to compare the groups. **$P < 0.01$ ($n = 30$).

late stages of apoptosis in cell culture. In our study, we adapted the APO-DIRECT™ assay (Cat. No. 6536KK) protocol to be used in whole zebrafish embryos to detect cytotoxicity. About 20 embryos/per condition were treated with AEO-7 (6.4 μg/L and 12.8 μg/L), PTU-E3 (negative control), and ZnO nanoparticles (20 mg/L) (positive control). The apoptotic cells are presented as bright fluorescent green spots (Fig. 3A). The area of fluorescence was measured using Image J in the head region of the zebrafish embryos. As shown in Fig. 3B, no significant difference was observed upon treating the embryos with AEO-7 (6.4 μg/L and 12.8 μg/L) compared to PTU-E3 treatment (negative control). Although, when the embryos were treated with ZnO nanoparticles (20 mg/L), apoptosis increased significantly ($P < 0.001$) compared to PTU-E3 treatment (negative control; Fig. 3B).

Overexpression of Hsp70, a general stress marker, was not detected at NOEC of AEO-7

Hsp70 is one of the major chaperones that serves in protecting the nervous system from toxicity and protein aggregation [37]. There are several biochemical, genetic, and pharmacological studies that have associated Hsp70 with regulating misfolding and toxicity in some diseases [38]. Accordingly, Hsp70 expression level was used as a stress marker to evaluate whether the exposure to AEO-7 has any cell stress implications in zebrafish embryos. To this end, zebrafish embryos (30 embryos for each condition) were treated with AEO-7 (6.4 μg/L and 12.8 μg/L), PTU-E3 (negative control), and ZnO

nanoparticles (20 mg/L) (positive control). The western blot gel is shown in Fig. 4A. The results obtained from western blotting were analyzed and the relative levels of Hsp70 protein in the treatment and control groups were plotted (Fig. 4B). Treatment with ZnO nanoparticles (20 mg/L) significantly increased the expression of Hsp70 (Fig. 4). Nevertheless, Fig. 4 demonstrates that, similar to the AO apoptosis assay, we could not detect significant alteration for the Hsp70 expression. After AEO-7 treatment (6.4 μg/L and 12.8 μg/L), suggesting that no cell stress was elicited.

Only the novel JC-10 assay was capable of detecting cellular stress at NOEC of AEO-7

We tested the ability of the JC-10 probe to detect cell stress in zebrafish embryos. At 4-dpf, embryos (30 embryos per condition) were treated with AEO-7 (6.4 μg/L and 12.8 μg/L), PTU-E3 (negative control), and ZnO nanoparticles (20 mg/L) (positive control). As shown in Fig. 5, representative images obtained by BioTek Cytation 5 Imaging Reader suggest a dose-dependent increase in green fluorescence and a dose-dependent decrease in red fluorescence upon treating the embryos with AEO-7 and ZnO nanoparticles, in comparison to PTU-E3 treatment. By combining the images, the green fluorescence dominates in the embryos exposed to toxic concentrations (Fig. 5A). JC-10 assay was sensitive enough to detect the effect at the NOEC of AEO-7 compared to the other performed cellular assays (AO apoptosis assay and Hsp70 Western botting). In Fig. 5B, the green:red fluorescence
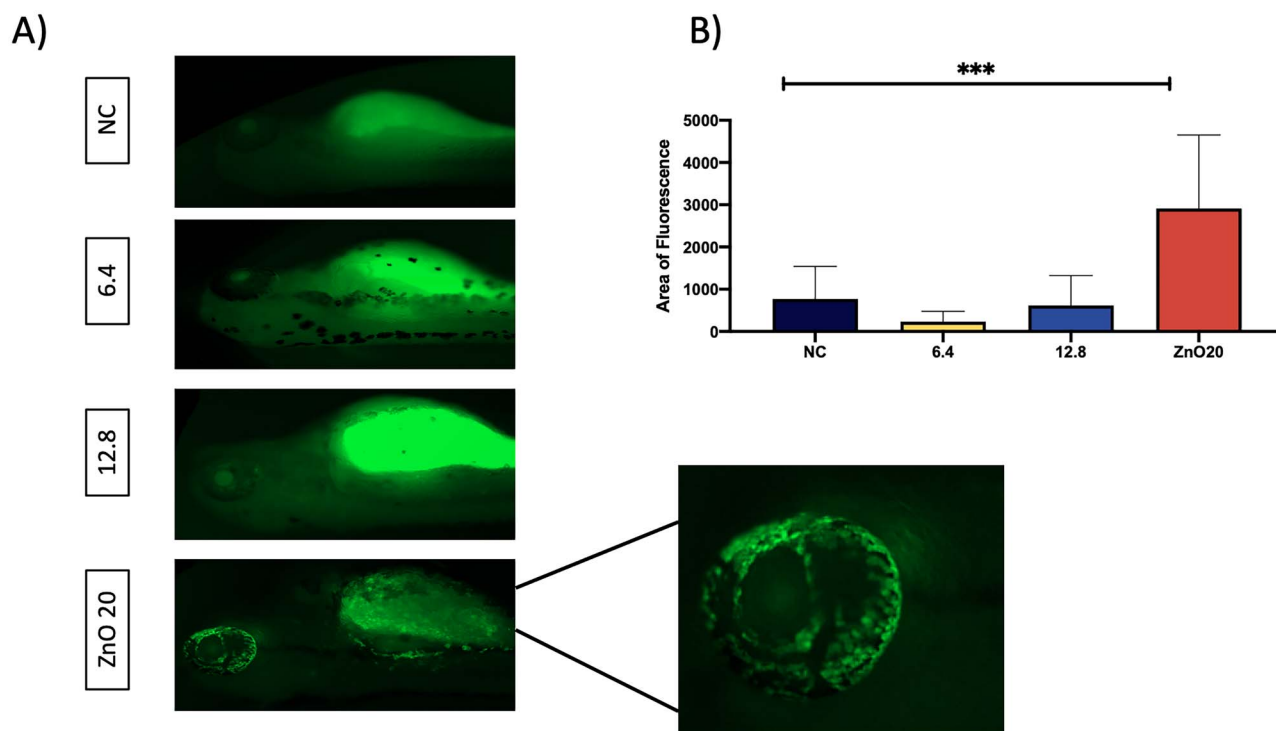
Figure 3. (A) Representative images of 4-dpf embryos stained with APO-DIRECT™ assay captured with BioTek Cytation 5 Imaging Reader. (B) The average area of fluorescence of apoptotic cells in the head region was measured using Image J. One-way analysis of variance (ANOVA) followed by Dunnett test was used to compare the groups. **$P < 0.01$ ($n = 10$).
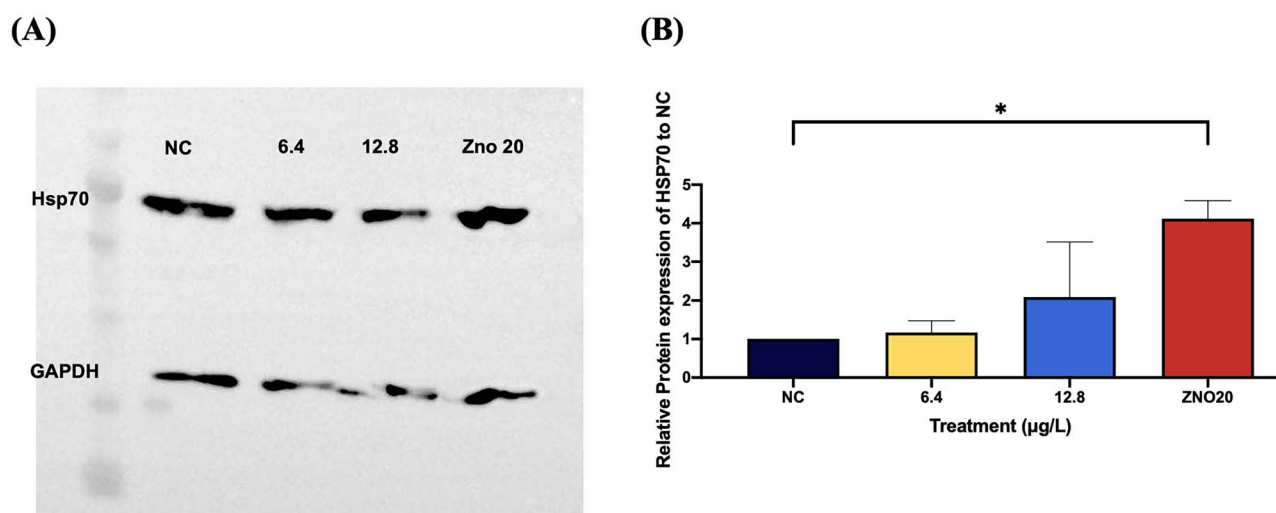

Figure 4. (A) Image of the western blot gel. (B) Relative quantity of Hsp70 protein upon the indicated treatment conditions compared to the PTU-E3 treatment (negative control). Data were analyzed by one-way (ANOVA) using GraphPad Prism 8 software. *$P < 0.05$ ($n = 30$).

ratio (J-monomers with a green signal at wavelength 540 nm representing unhealthy cells to J-aggregates with a red signal at wavelength 590 nm representing healthy cells) supported the representative images. Both treatment groups of 6.4 µg/L and 12.8 µg/L AEO-7 showed significant increase ($P < 0.05$) in the green:red ratio, indicating the accumulation of unhealthy mitochondria. In addition, treatment with ZnO nanoparticles (20 mg/L) showed a significant increase ($P < 0.01$) in the green:red ratio (Fig. 5B) confirming mitochondria dysfunction.

Discussion

To our knowledge, this is the first study intended to explore the potential cytotoxicity mechanism of AEO-7 exposure in zebrafish embryos. In this study, we developed a protocol using the JC-10 probe to detect changes in mitochondria membrane potential at cellular level in acute toxicity studies in zebrafish embryos. According to the acute toxicity rating scale by the Fish and Wildlife Service (USFWS), AEO-7 is considered a "super toxic" surfactant [12, 36].
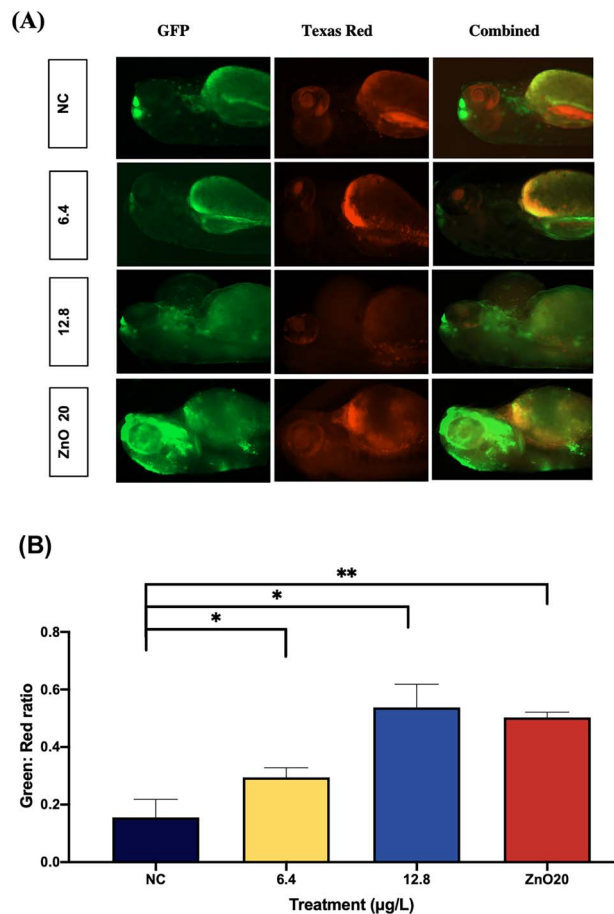
(A)

(B)

Figure 5. The green: red fluorescence ratio in zebrafish embryos subjected to different treatment conditions at 4-dpf. (A) Representative images of embryos stained with JC-10 fluorescent stain captured with BioTek Cytation 5 Imaging Reader. (B) The green:red ratio of green J-monomers (at wavelength 540 nm representing unhealthy cells) to the red J-aggregates (at wavelength 590 nm representing healthy cells) was calculated using GraphPad Prism 8 software. Data were analyzed by one-way (ANOVA) using GraphPad Prism 8 software. *$P < 0.05$ and **$P < 0.01$ ($n = 30$).

Apoptosis is programmed cell death. It consists of a cascade of biochemical changes and alterations in apoptotic cells that lead to specific morphological changes and eventually cell death [39]. For this reason, identifying the presence of apoptotic cells is a valuable element in studies aimed to assess cytotoxicity. There are numerous ways to detect apoptotic cells, one of which relies on the use of AO, a fluorescent dye [40]. The metachromatic properties of AO at 488 nm cause emission of green fluorescence when the dye is in its monomeric form and binds to the double-stranded DNA in cells undergoing apoptosis. Therefore, its behavior fluctuates expressively between viable and apoptotic cells. In viable cells, AO dye is retained in cellular compartments with low pH and does not interact with the double-stranded DNA of the undamaged living cells nor aggregates in other organelles like the mitochondria or Golgi apparatus [28]. Yet, when apoptosis is triggered, a loss in plasma membrane intactness and pH compartmentalization occurs. Consequently, AO outflows the acidic lysosomes

and selectively binds to the double-stranded DNA, resulting in a detectable and measurable green signal from smooth and shrunken nuclei remnants [28]. In our study, we investigated whether AEO-7 is capable of inducing cytotoxicity in zebrafish embryos using AO. According to our findings (Fig. 2), no significant difference was observed between embryos treated with AEO-7 and the negative control. Hence, AO was not sensitive enough to detect the stress induced by AEO-7.

One of the later steps in apoptosis is DNA fragmentation, a process which results from the activation of endonucleases during the apoptotic cascade [41]. These nucleases degrade the higher order chromatin structure into fragments of ∼300 kb and subsequently into smaller DNA pieces of about 50 bp in length. A method which is often used in cell culture to detect fragmented DNA utilizes a reaction catalyzed by exogenous TdT, often referred to as "end-labeling" or "TUNEL" [42]. The APO-DIRECT™ kit is a single-step method for labeling DNA breaks with FITC-dUTP, followed by flow cytometric analysis. In our study, we adapted the APO-DIRECT™ kit with the aim to detect apoptosis and cellular stress in whole zebrafish embryos. Similar to AO, no significant difference was observed between embryos treated with AEO-7 and the negative control (Fig. 3). Hence, the TUNEL assay was not sensitive enough to detect the stress induced by AEO-7 in whole zebrafish embryos.

Other biomarkers that can be studied when it comes to cytotoxicity evaluation are stress-associated proteins markers, one of which is Hsp70. Hsp70 is a ubiquitous protein with a molecular weight of 70 kDa that functions as a molecular chaperone in normal conditions [43]. Due to the fact that Hsp70 is overexpressed when cells are exposed to stress, we investigated whether AEO-7 is capable of inducing stress in zebrafish embryos. According to our findings, although there was an increase in Hsp70 expression when the embryos were treated with AEO-7 in comparison to the PTU-E3 treatment, this increase did not reach statistical significance (Fig. 4). Hence, Hsp70 overexpression was not sensitive enough to detect the stress induced by AEO-7.

Changes in the mitochondrial membrane potential ($\Delta\Psi m$) and permeability are key indicator demonstrating the depolarization of the transmembrane potential, release of apoptogenic factors and loss of oxidative phosphorylation leading to cell death. Therefore, used extensively in toxicity assays. Indeed, $\Delta\Psi m$ mediates and maintains hydrogen ion transport, which has a significant role in ATP synthesis [44]. In viable cells, $\Delta\Psi m$ and ATP levels are usually maintained at a stable rate. Prolonged changes in $\Delta\Psi m$ and ATP can induce a decrease in cell viability, which is due to a decrease in membrane negativity, leading to decoupling of the respiratory chain and consequently releasing cytochrome C into the cytosol, triggering apoptosis [45]. The lipophilic cationic dye JC-10 is used to indicate mitochondrial depolarization. In viable healthy cells, it

forms red fluorescence aggregates in the mitochondrial membrane, while when the membrane potential is disturbed, the original monomeric JC-10 remains in the cytosol, reflecting green fluorescence [46]. JC-10 has never been used in zebrafish embryos, which constitutes the novelty of this study. Indeed, we aimed to employ JC-10 to detect mitochondrial membrane potential disturbance as a possible mechanism of AEO-7-induced toxicity. Notably, the novel JC-10 assay displayed a dose-dependent dysfunctionality of the mitochondrial membrane potential even at the NOEC of AEO-7 (6.4 µg/L). In comparison, the AO apoptosis, western blot, and TUNEL assays failed to detect such effects at the NOEC of AEO-7 (6.4 µg/L). Although there is no clear evidence, the variations of sensitivity in detecting cytotoxicity in zebrafish embryos using these assays might be due to the fact that reduced $\Delta\Psi$m is considered in some apoptotic systems an early, initial, and irreversible event progressing toward apoptosis [44, 47].

The aim of our study is to find a suitable, easy, time-effective, and sensitive assay that can measure apoptosis and/or mitochondrial membrane damage in whole zebrafish embryos. The usefulness of JC-10 probes as a marker for assessing mitochondrial membrane potential was already validated and used in several cell culture studies [48, 49]. Disturbing the mitochondrial membrane potential in the zebrafish embryos and then measuring the fluorescence of their probe would be a perfect way to confirm our zebrafish finding. However, we believe that this is beyond the scope of our study because of the complex nature of the mitochondrial staining protocol in zebrafish. This can be done in the future as a separate study by following the same protocol of [50]. Their protocol is somehow very complicated as mitochondrial membrane potential needs to be specifically disrupted using particular drugs such as Troglitazone (an antidiabetic drug) and Tolcapone (an antiepileptic drug), both of which have been withdrawn from the market due to mitochondrial toxicity. The protocol also involves localizing neuromasts in zebrafish by staining with YoppPo1 stain, which stains the nuclei, but not the mitochondria, of neuromasts. The neuromasts are rich in mitochondria and only found in the head area. Subsequently, a colocalization study, which requires confocal microscopy imaging, should be performed. It is worth mentioning that the neuromast stating needs to be performed at 7-dpf, which needs specific IACUC approval. However, performing experiments until 5-dpf (such as JC-10) does not fall under the IACUC protection.

As can be seen in the JC-10 probe assay (Fig. 5), we were able to detect a significant difference in fluorescence intensity between the negative control and each drug treatment (AEO-7 6.4 and 12.8 µg/L) in a dose-dependent manner. However, using the AO assay (Fig. 2), we were only able to detect a significant difference only between the positive and negative control. Similarly, the TUNEL

assay (Fig. 3) failed to show any significant difference in the amount of apoptosis compared to the negative control. These data suggest that the JC-10 could be more sensitive in detecting early apoptosis than the other assays. Interestingly, the JC-10 probe was able to detect apoptotic cells mainly in the head area (including the eyes) and to a lesser degree in the different parts of the whole zebrafish embryos (Fig. 5). However, in the TUNEL assay (Fig. 3), we were able to detect apoptotic cells mainly around the eye region. Only, few apoptotic cells were detected in the head area and in the rest of the body. It should be mentioned that the AO has an advantage over the other two assays as the apoptotic cells can be seen homogeneously in the whole zebrafish embryos. Furthermore, the TUNEL assay used in this study, is time-consuming, needs a fixation process before the staining, and most importantly, the quantification of apoptotic cells cannot be done using a fluorescent plate reader. Thus, we believe that JC-10 probe is a novel and ideal method for the detection of pre and apoptotic cells and mitochondrial stress from whole zebrafish embryos at early developmental stages. However, the TUNEL assay could be more sensitive than the JC-10 when it is applied to cell culture. To increase the sensitivity of the TUNEL assay in zebrafish, a specific apoptotic marker, such as caspase-3 antibody, could be used [51]. The TUNEL assay sensitivity could also increase if zebrafish embryo tissue is permeabilized then stained. However, the fluorescent reading should be performed by flowcytometry after zebrafish cell dissociation.

Conclusion

The development of this method demonstrates that JC-10 probe can now be used as a consistent and reliable *in vivo* method of quantifying changes in $\Delta\Psi$m during zebrafish embryogenesis. In addition, this novel assay provides a visual representation, which ultimately saves time and resources. This method not only paves the way toward the application of JC-10 probe in measuring $\Delta\Psi$m in zebrafish during embryogenesis, but it also provides the benefit of being utilized in an *in vivo* model whereas in the past it was limited to *in vitro* studies [32]. Most importantly, our novel JC-10 assay is the most sensitive method among the tested cytotoxicity assays due to the fact that it allows detection of significant toxicity changes in the preapoptotic stage.

Authors' contributions

G.K.N. did the conceptualization; G.K.N., N.Y., B.S.A., A.J.A., and G.P. performed methodology; N.Y., B.S.A., and A.J.A. developed the software; G.K.N., N.Y., B.S.A., and A.J.A. did the validation; N.Y., B.S.A, and A.J.A did the formal analysis; G.K.N., N.Y., B.S.A., A.J.A., S.I.D., and A.F.M. did the investigation; G.K.N., S.I.D., and G.P. collected the resources; G.K.N. did the data curation; G.K.N., N.Y., B.S.A.,

Conflict of interest statement

None declared.

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