

Journal Pre-proof

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PII: S0048-9697(20)33972-3

DOI: <https://doi.org/10.1016/j.scitotenv.2020.140450>

Reference: STOTEN 140450

To appear in: *Science of the Total Environment*

Received date: 2 May 2020

Revised date: 20 June 2020

Accepted date: 21 June 2020

Please cite this article as: O. Al-Jamal, H. Al-Jighefee, N. Younes, et al., Organ-specific toxicity evaluation of stearamidopropyl dimethylamine (SAPDMA) surfactant using zebrafish embryos, *Science of the Total Environment* (2020), <https://doi.org/10.1016/j.scitotenv.2020.140450>

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Organ-specific Toxicity Evaluation of Stearamidopropyl Dimethylamine (SAPDMA) Surfactant Using Zebrafish Embryos

Ola Al-Jamal^{1†}, Hadeel Al-Jighefee^{1,2†}, Nadin Younes^{1†}, Roba Abdin², Maha A. Al-Asmakh^{1,2},
A. Bahgat Radwan³, Mostafa H. Sliem³, Amin F. Majdalawieh⁴, Gianfranco Pintus⁵, Hadi M.
Yassine^{1,2}, Aboubakr M. Abdullah^{3,6}, Sahar I. Da'as⁷, Gheyath K. Nasrallah^{1,2*}

¹*Biomedical Research Center, Member of QU Health, Qatar University, P.O. Box 2713 Doha, Qatar.*

²*Department of Biomedical Science, College of Health Sciences, Member of QU Health, Qatar University, P.O. Box 2713 Doha, Qatar.*

³*Center for Advanced Materials, Qatar University, P.O. Box 2713 Doha, Qatar.*

⁴*Department of Biology, Chemistry, and Environmental Sciences, College of Arts and Sciences, American University of Sharjah, P.O. Box 26666 Sharjah, United Arab Emirates.*

⁵*Department of Medical Laboratory Sciences, University of Sharjah P.O. Box 27272 Sharjah, United Arab Emirates.*

⁶*Department of Chemical Engineering, College of Engineering, Qatar University, P.O. Box 2713 Doha, Qatar.*

⁷*Department of Human Genetics, Sidra Medicine, P.O. Box 26999 Doha, Qatar.*

† Equal Contributions

*** Correspond to:** Gheyath K. Nasrallah

Department of Biomedical Science, College of Health Sciences, *Member of QU Health*, Qatar University, Doha, Qatar

Women's Science building, C01, Tel: +974 4403 4817, Fax: +974-4403-1351, P.O Box: 2713,

email:

gheyath.nasrallah@qu.edu.qa

Abstract

Surfactants are widely used in the industry of detergents, household products, and cosmetics. SAPDMA is a cationic surfactant that is used mostly in cosmetics, conditioning agents and has recently gained attention as a corrosion inhibitor in the sea pipelines industry. In this regard, literature concerning the ecotoxicological classification of SAPDMA on aquatic animals is lacking. This study aims to evaluate the potential ecotoxicity of SAPDMA using the aquatic zebrafish embryo model. The potential toxic effects of SAPDMA was assessed by different assays. This includes (i) mortality/survival assay to assess the median lethal concentration (LC_{50}); (ii) teratogenicity assay to assess the no observed effect concentration (NOEC); (iii) organ-specific toxicity assays including cardiotoxicity, neurotoxicity (using locomotion assay), hematopoietic toxicity (hemoglobin synthesis using o-dianisidine staining), hepatotoxicity (liver steatosis and yolk retention using Oil Red O (ORO) stain); (iv) cellular cytotoxicity (mitochondrial membrane potential) by measuring the accumulation of JC-1 dye into mitochondria. Exposure of embryos to SAPDMA caused mortality in a dose-dependent manner with a calculated LC_{50} of 2.3 mg/L. Thus, based on the LC_{50} value and according to the Fish and Wildlife Service (FWS) Acute Toxicity Rating Scale, SAPDMA is classified as “moderately toxic”. The No Observed Effect Concentration (NOEC) concerning a set of parameters including scoliosis, changes in body length, yolk, and eye sizes was 0.1 mg/L. At the same NOEC concentration (0.1 mg/L), no organ-specific toxicity was detected in fish treated with SAPDMA, except hepatomegaly with no associated liver dysfunctions. However, higher SAPDMA concentrations (0.8 mg/L) have dramatic effects on zebrafish organ development (eye, heart, and liver development). Our data recommend a re-evaluation of the SAPDMA

employment in the industry setting and its strictly monitoring by environmental and public health agencies.

Keywords: Stearamidopropyl Dimethylamine (SAPDMA) Surfactant; zebrafish; organ-specific toxicity; LC₅₀; NOEC.

1. Introduction

In comparison with conventional solvents, surfactants have attracted considerable attention in various applications due to their unique physico-chemical properties and abilities to be tailor-made to suit various applications (Al-Kandari et al., 2019; Radwan et al., 2017; OECD, 2013). In this regard, a very strong impulse on surfactants research is coming from their growing employment in many important practical and fundamental industries like petroleum oil recovery, water, environmental pollutions, and most importantly corrosion inhibition (Radwan et al., 2017). Corrosion inhibitors have always been considered to be the first line of defense against corrosion in the oil extraction and processing industries (Chilingar et al., 2008; Migahed and Al-Sabagh, 2009; Popov, 2015). Therefore, current and future challenges in this field are the findings of environmentally friendly “green”, cost-effective, organic corrosion inhibitors, which are of great interest to oil and gas operating companies associated with deep-sea excavation (Darling and Rakshpal, 1998). Recent studies have shown that surfactants employment is one of the best-known “green” methods of corrosion protection (El-Lateef, 2014; Malik et al., 2011; Radwan et al., 2017; Sliem et al., 2019; Zhu et al., 2017).

Nevertheless, whether these compounds are truly eco-friendly and free of environmental toxic effects especially towards aquatic organisms is a question that remains to be answered yet (Belanger et al., 2006; Olkowska et al., 2014; Rhein, 2007). Even though surfactants are usually labeled as “green”, a recent *in vivo* study explored the toxicity of three commonly employed

surfactants (sodium dodecyl sulfate, dodecyl dimethyl benzyl ammonium chloride, and fatty alcohol polyoxyethylene ether) revealing that two of them are very toxic to zebrafish embryos even at very low concentrations (1 $\mu\text{g}/\text{mL}$) (Wang et al., 2015). Therefore, the eco-friendly “fame” of many of these widely used compound need to be revisited and further *in vivo* studied need to be performed to better investigate the potential toxicity of both new and marketed compound to ultimately withdraw highly toxic and non-biodegradable compounds from commercial use and replace them with environment-friendly ones.

Stearamidopropyl dimethylamine (SAPDMA) is a widely used cationic surfactant and the most common surfactant used in hair conditioners and personal care products, usually at concentrations below 5% (Minguet et al., 2010). Recently, our collaborators reported that SAPDMA is a very efficient corrosion inhibitor of API X120 steel under extremely aggressive conditions (Radwan et al., 2017), a discovery that may prompt the wide employment of SAPDMA in the industry field of steel pipes-using companies. Given the potential environment implication associated with the massive employment of this surfactant, especially by sea oil companies, conducting a comprehensive toxicity and safety evaluation of SAPDMA in an aquatic animal model remains an essential aspect to be answered. Indeed, to the best of our knowledge, no studies are present in the literature reporting on this specific aspect of SAPDMA. According to the Environment Canada Domestic Substance List, SAPDMA is classified as a low hazardous ingredient and not expected to be potentially toxic or harmful (Canada.ca., 2014). However, according to PubChem and European Chemical Agency, SAPDMA is very toxic to the aquatic environment with long-lasting effects and can cause serious eye and skin damage (National Center for Biotechnology Information., 2020). Thus, the safety employment of SAPDMA in aquatic environments is still uncertain and needs to be addressed urgently. We

believe that answering this question may provide new insights concerning the mechanisms associated with surfactants toxicity in aquatic animals and provide useful information to local and international public health and environmental agencies concerning the actual SAPDMA toxicity.

The present study was undertaken to assess the general *in vivo* and organ-specific toxicity (cardiac, hepatic, and neuromuscular) to comprehensively evaluate any potential toxicity of SAPDMA using zebrafish (*Danio rerio*) embryo as a model for marine fauna. Zebrafish is recognized by the National Institute of Environmental Health Science (NIEHS, USA) and the Institute for Environment and Sustainability (IES, Europe) as an excellent system to study environmental toxicity and is accepted by the National environmental toxicity and is accepted by the National Institutes of Health (NIH, USA) as an alternative model for exploring human diseases (Bar-Ilan et al., 2009; Dooley and Zon, 2000; National Institutes of Health., 2016; Parng, 2005). Since no toxicity studies have been performed on SAPDMA, we investigated a wider range of concentrations (0.1, 1.0, 5.0, 10, 50, 100, 500, and 1000 mg/L) to find both the no observed effect concentration (NOEC) and the median lethal concentration (LC₅₀). In this regard, the selected concentrations were consistent with previously published work using surfactants (Wang et al., 2015) and within the toxicity rating scale provided by the U.S. Fish and Wildlife Service (USFWS)(El-Harbawi, 2014).

2. Materials and Methods

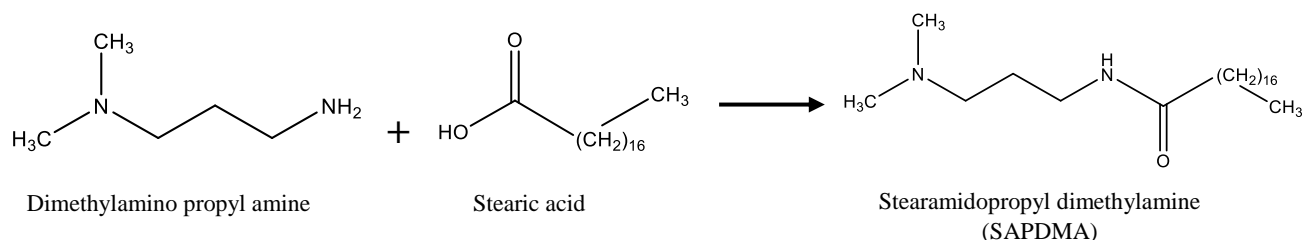
2.1 Chemicals

Zinc oxide (ZnO, catalog #721077-100G) dispersion (nanoparticles), of diameter <100 nm, was purchased from Sigma-Aldrich (St. Louis, MO, USA). ZnO nanoparticles, previously characterized by (Bai et al., 2010), is known to cause mortality and morphological deformities to

zebrafish embryos and was previously used as a positive control in toxicology studies (Bai et al., 2010; Choi et al., 2016; Kteeba et al., 2017). Thus, Zinc oxide was used as a positive control (PC) in all performed experiments. N-phenylthiourea (PTU) (Sigma, Germany) in egg water (E3 media) was used as a media to raise zebrafish embryos *in vitro*. In addition, it is used to inhibit pigment formation in the developing zebrafish embryos to facilitate their visualization under the microscope. All E3 media constituents including 5.0 mM sodium chloride (NaCl), 0.17 mM potassium chloride (KCl), 0.33 mM calcium chloride dihydrate (CaCl₂·2H₂O) and 0.33 mM magnesium sulfate heptahydrate (MgSO₄·7H₂O) were obtained from Sigma, USA. For the toxicity experiments, stock solutions such as ZnO, PTU, and E3 media were prepared as described in (Al-Kandari et al., 2019).

2.2 Preparation of SAPDMA

SAPDMA was prepared through amidation of stearic acid with dimethylamino propyl amine with a molar ratio of 1.2:1 to 0.8:1 (Maisonneuve et al., 2000). Most preferably, less than 5% excess of amine should be employed. The reactants were added to a reaction vessel in a nitrogen atmosphere under 90-105 psi pressure range at 40-210 °C. The temperature was held at 140 °C for almost 2 hr before additional ramping and then gradually increased to 180 °C (5 °C/30 min). The reaction's overall duration was about 10-24 hr to produce in the final SAPDMA product according to the following reaction:



The main stock was prepared by dissolving SAPDMA gel in distilled water using the ratio of 0.286 g/100 mL to obtain an aqueous solution of 2860 mg/L concentration. To prepare the desired concentrations, an intermediate stock was prepared by adding 1048.95 μ L of the main stock to 1951.05 μ L of PTU, and then it was vortexed to achieve homogenous suspension. From the intermediate stock, various dilutions (5 mL total volume) were prepared, in which PTU was used as the diluent to obtain final concentrations of 0.1, 0.5, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 5.0 mg/L.

2.3 Zebrafish embryos culture

Two types of zebrafish embryos were used in the toxicity experiments; wild-type AB strain and the naturally transparent Casper strain. Zebrafish embryos were maintained in an environmentally controlled lab (photoperiod: 14 hr light/10 hr dark cycle with a water temperature of 28 °C) (Korenbroet et al., 2013) using the Aquaneering system (San Diego, California, USA) in zebrafish laboratory in the Biomedical Research Center (BRC) at Qatar University, Doha, Qatar. Zebrafish were prepared for mating by placing two pairs of adult male and female fish in a single mating tank separated by a divider and left in the dark overnight. The next morning, spawning was triggered by removing the divider and the embryos were left to mate for 5 hr. After that, fertilized eggs were collected, and the healthy ones were selected and washed with PTU-E3 media in a new petri dish before conducting the experiments. All performed experiments on zebrafish were carried out following the animal protocol guidelines mandated by Qatar University Institutional Animal Care and Use Committee (IACUC) and policy on zebrafish research established by the Department of Research in the Ministry of Public Health, Doha, Qatar.

2.4 Acute toxicity (*acutotoxicity*) assays

Mortality and developmental toxicity of SAPDMA were investigated with an acute toxicity assay adopted by the Organization of Economic Co-operation and Development (OECD) guideline for testing chemical toxicity (N° 203 and 236) (OECD, 2013, 2019). As described in section 2.2, different concentrations of SAPDMA were prepared from the intermediate stock (0.1, 0.5, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 4.5 mg/L). Then, zebrafish embryos were dechorionated 24 hr post-fertilization (hpf) using 450 μ L of 1.0 mg/mL pronase (St. Louis, MO, USA) as described in (Rieger, 2013). Afterward, healthy dechorionated embryos were incubated in 5 mL of fresh PTU-E3 media containing (i) eleven different concentrations (0.1, 0.5, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 4.5 mg/L) of SAPDMA; (ii) positive control (PC) ZnO (1.5 mg/L); and (iii) negative control (NC) PTU-E3 media. The cumulative mortality and morphological deformities were observed and recorded for 3 consecutive days (48, 72, and 96 hpf) under a standard stereomicroscope microscope. The number of dead embryos and deformities were scored by gross microscopic examination of each embryo. The embryos that showed coagulated fertilized eggs, no somite formation, undetectable heartbeat, and undetached tail-bud from the yolk sac were considered dead. Embryos that showed defects or variations in body length, eye, heart and yolk size, and scoliosis were considered deformed (teratogenic phenotypes). LC₅₀ values were calculated using GraphPad Prism 7 software (version 7.01, San Diego, USA) by fitting a sigmoidal curve to mortality data at 95% confidence interval as described elsewhere (Nasrallah et al., 2018a; Nasrallah et al., 2018b). Variations in the body length, eye, and yolk sac size were captured using HImage software at 21x magnification and then measured using ImageJ software version 1.52a (NIH, Washington DC, USA) bundled with Java 1.8.0_172 (Nasrallah et al., 2019). Both mortality and teratogenicity percentage were used

to calculate the no observed effect concentration (NOEC) of SAPDMA. The NOEC is the highest concentration of SAPDMA that does not show a significant ($p < 0.05$) mortality or teratogenic defects compared to the negative control (PTU-E3 media) during the exposure period. Twenty-five embryos were used for each tested concentration of SAPDMA and both controls. The experiment was done 3 times.

2.5 Cardiotoxicity assay

The blood flow of two major blood vessels in zebrafish embryos was measured to assess the effect of SAPDMA on cardiac function. The posterior cardinal vein (PCV) and dorsal aorta (DA) were imaged at 96 hpf, which is the time where the heart usually fully develops (Denvir et al., 2008). At 96 hpf, embryos were placed on a depression slide with 1-2 drops of 3% methylcellulose. Embryos were positioned on their side with the same orientation for measurement of the DA and PCV. Videos of the tail were recorded for all embryos at the same site where the two major blood vessels were visible (Figure S1) using Zeiss SteREO Discovery V8 Microscope equipped with Hamamatsu Orca Flash high-speed camera and a workstation equipped with HImage software (Hamamatsu Photonics, USA). This camera can record image sequences with 100 frames per second (fps) speed.

By tracking RBC movements in the two major blood vessels using image analysis algorithms of MicroZebraLab Blood Flow software from ViewPoint (version 3.4.4, Lyon, France, three parameters were measured; blood flow velocity, vessel diameter, and heartbeat. After that, wall shear stress was calculated using the following formula:

Where μ is the blood viscosity (dynes/cm^2), V is the average blood velocity ($\mu\text{m/s}$) and D is the vessel diameter (μm) (Eisa-Beygi et al., 2018).

2.6 Hemoglobin staining

To evaluate the effect of SAPDMA on hemoglobin synthesis, a hematopoietic assay was performed by staining the embryos with o-dianisidine stain (Catalog #D9143-5G, Sigma, USA) based on a protocol previously described in (Leet et al., 2014). This assay is based on the oxidation of o-dianisidine by hemoglobin, which produces a dark red stain in hemoglobin containing cells. Casper strain was used for the staining. At 24 hpf, the embryos were dechorionated and incubated with three different concentrations of the surfactant (0.1, 0.5, and 0.8 mg/L) and with PTU-E3 media only as of the negative control. At 72 hpf, the stain was prepared by mixing 0.6 mg/mL of o-dianisidine, 0.65% hydrogen peroxide, 0.01 M sodium acetate at 4.5 pH, and 40% (v/v) ethanol solution (Leet et al., 2014). Following staining, embryos were post-fixed in 4% paraformaldehyde at 4 °C for at least 1 hr. 3% (w/v) methylcellulose was used to fix the embryos on the slide for imaging under a bright field microscope (Stemi 508 Zeiss, Oberkochen, Germany) at 50x magnification. Zeiss AxioCam ERc 5s professional digital camera was used for imaging. ImageJ software was used to quantify the intensity and size of the red-stained areas in the embryos' yolk sac.

2.7 Locomotion (neuromuscular toxicity) assay

For the locomotion assay, fertilized zebrafish embryos were collected in a petri dish containing E3 media. Abnormal and unfertilized embryos were discarded, and healthy embryos were incubated at 28.5 °C. At 96 hpf, healthy embryos were transferred to 12-well plates with E3 media containing SAPDMA solution at 0.1, 0.5, and 0.8 mg/L concentrations in addition to the positive (1.5 mg/L ZnO) and negative (E3 media) controls. Then, the embryos were additionally incubated for 24 hr at 28.5 °C. At 120 hpf, the embryos were transferred to a 96-well plate by placing one embryo per well to get a total of 12 embryos for each treated group. Neurotoxicity

was analyzed by locomotion assessment using ViewPoint ZebraLab technology as described previously (Nasrallah et al., 2018a; Younes et al., 2020). The 96-well plates containing the treated embryos were placed in a system chamber at 28.5 °C and irradiated for 20 min with white light for an acclimation period to allow the embryos to adapt to the environment. Then, the movement of the embryos was measured under the following conditions: an initial 10 min period of darkness accompanied by two repeated bright light cycles for 10 min, which was separated by 10 min of darkness. The neurotoxicity was determined through measurement of the average total distance moved after a cycle of 60 min and by assessing the response of the embryos by the dark-light cycles. The results were compared to the negative and positive controls using E3 media and 1.5 mg/L ZnO, respectively. Each embryo was analyzed individually in the 96-well plate.

2.8 Hepatotoxicity evaluation

The hepatotoxicity assays were performed using the Tg[cm1c: GRP] transgenic AB strain of zebrafish. This strain expresses the RFP in the hepatocytes thus allowing a good quality of liver imaging. To evaluate the toxic effect of SAPDMA on zebrafish liver, the following parameters were assessed: liver size (to measure necrosis and hepatomegaly) and yolk retention (to measure liver lipid metabolism) as previously described in (Abou-Saleh et al., 2019; Younes et al., 2020). At 96 hpf, embryos were incubated for additional 24 hr at 28 °C with the following treatments: (i) E3 media (NC) (ii) 1% EtOH (PC), and (iii) 0.1 mg/L SAPDMA.

2.8.1 Liver area analysis

For the liver size measurement, the fluorescent liver of the embryos was imaged with a fluorescence stereomicroscope (Olympus MVX10) using a digital camera (Olympus DP71). RFP filtered images of the liver were taken and their areas were analyzed using DanioScope software

(Noldus, Wageningen, Netherlands) for hepatomegaly and necrosis detection (Abou-Saleh et al., 2019; Younes et al., 2020).

2.8.2 Detection of yolk retention

Yolk retention was evaluated using Oil Red O (ORO) staining (Catalog #1320-06-5, Sigma-Aldrich, USA), which is a lysochrome, fat-soluble dye used to stain neutral triglycerides and lipids. At 24 hpf, healthy embryos were selected and allowed to develop normally until 96 hpf. At 96 hpf, zebrafish embryos were incubated in different SAPDMA concentrations along with the positive (1% ethanol) and negative control (PTU-E3 media). After 24 hr of incubation (120 hpf), which is the time at which the liver fully develops, embryos were stained as described in (Yoganantharjah et al., 2017). Briefly, 0.035 grams of ORO powder added to 7 ml of 100% isopropanol and stirred overnight on a magnetic stirrer at room temperature. Then, working ORO stain was prepared by mixing 1 part of ORO stock to 1 part 10% isopropanol (in MilliQ water). The treated embryos were washed from PTU-E3 media with 60% isopropanol and then replaced with 1 mL of ORO working solution for 75 min. ORO stain was discarded, and embryos were washed quickly (30 sec) with 60% isopropanol and then rinsed again for 3 min in 60% isopropanol, followed by a 30 sec wash in 1% PBS. After that, the ORO stain was extracted from the embryos for quantification. Sample sizes of 5 embryos were pooled together per eppendorf tube to extract an adequate volume of ORO stain. Replicates for each treatment group ranged from 5-6 eppendorf tubes (5 embryos each). PBS was removed from each eppendorf and then 250 mL of 4% ethanol made up in 100% isopropanol was added to each eppendorf tube. Samples were briefly vortexed and incubated overnight at room temperature to make sure that the ORO stain was completely extracted. Then, 200 mL of the solution that contained the extracted ORO stain was pipetted into respective wells of a 96-well plate and OD (absorbance) was read on a

Tecan GENios Pro 200 at 495nm (Yoganantharjah et al., 2017). Moreover, multiple reads per well (filled circle, 6×6) were performed.

2.9 Mitochondrial membrane potential assay ($\Delta\Psi_m$)

The mitochondrial membrane potential is one of the key parameters to look for when studying mechanisms related to cell health and when testing compounds. $\Delta\Psi_m$ is usually measured using cationic fluorescent dyes that accumulates in the anionic mitochondrial matrix. Such dyes can be used for qualitative measurement in fluorescence microscopy or quantitative measurement in flow cytometry or microplate spectrophotometry. Several mitochondrial membrane potential probes can be used such as TMRE, JC-1, and JC-10 probe (Sakamuru et al., 2016).

In this study, the JC1- mitochondrial membrane potential assay kit (Catalog #T3168, Sigma, USA) was used to measure the $\Delta\Psi_m$. This kit contains JC-1 fluorescent dye (tetraethylbenzimidazolocarboyanine iodide) that accumulates in the mitochondria based on its membrane potential. This dye is suitable for labeling the mitochondria in live cells of zebrafish embryos to study the effect of SAPDMA on mitochondrial health and apoptosis (Sakamuru et al., 2016). The procedure used for the preparation and treatment of the embryos was the same as that in the acute toxicity assay. At 24 hpf, 30 embryos per concentration were transferred to a 12-well plate with PTU-E3 media containing SAPDMA solution at 0.1, 0.5, and 0.8 mg/L concentrations in addition to the positive (1.5 mg/L ZnO) and negative (PTU-E3 media) controls. At 96 hpf, embryos were incubated with 5 μ M of JC-1 dye for 30 min in the dark at 28 °C and then washed 3 times with PTU-E3 media. Then, embryos were transferred to a 96-well black plate, 5 embryos were added per well with 100 μ L of PTU-E3 media. The green and red JC-1 signals were read using a microplate reader, Tecan GENios Pro. The working principle of the fluorescence plate

reader is similar to fluorescence microscopy, although the plate reader will only record the total fluorescence. The green and red JC-1 signals were measured at Ex 485 nm /Em 535 nm and Ex 535 nm /Em 590 nm respectively. Moreover, multiple reads per well (filled circle, 6×6) were performed. Subsequently, the ratio of green to red fluorescence was measured.

2.10 Statistical analysis

For the acute toxicity assessment, cumulative mortality was expressed as a percentage of dead embryos at 96 hpf. Descriptive statistics (DS) such as mean (m) and standard deviation (SD) were calculated for the cardiotoxicity assay, locomotion assay, cytotoxicity, hepatotoxicity, and o-dianisidine staining. Data were presented as mean \pm SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Dunnet test as compared to the negative control group. GraphPad Prism 7 software was used to remove all significant outliers. Significance (*) = $p < 0.05$; (**) = $p < 0.01$; (***) = $p < 0.001$.

3. Results and Discussion

3.1 SAPDMA can be classified as moderately toxic to the environment

At first, the potential adverse effect and the morality score of SAPDMA were examined at 24-96 hpf, which is the period where zebrafish embryos are most sensitive to external compounds and drugs (Olkowska et al., 2014). Measurement of the cumulative mortality percentage was done at 96 hpf, which is the recommended observation time (Cornet et al., 2017). According to the mortality data of ZnO nanoparticles (Figure 1A) that feed the mortality curve, the calculated LC₅₀ value for ZnO was 3.6 mg/L (Figure 1B). For SAPDMA, the mortality score of the treated embryos increased in a dose-dependent manner starting from 0.1 mg/L concentration reaching 100% at 3.5 mg/L (Figure 1A). In line with the mortality curve (Figure

1A), the calculated LC_{50} for SAPDMA was 2.3 mg/L (Figure 1B). According to the Fish and Wildlife Service Acute Toxicity Rating Scale that classify compounds toxicity based on the LC_{50} (Rasool et al., 2018), SAPDMA surfactant can be classified as “moderately toxic”

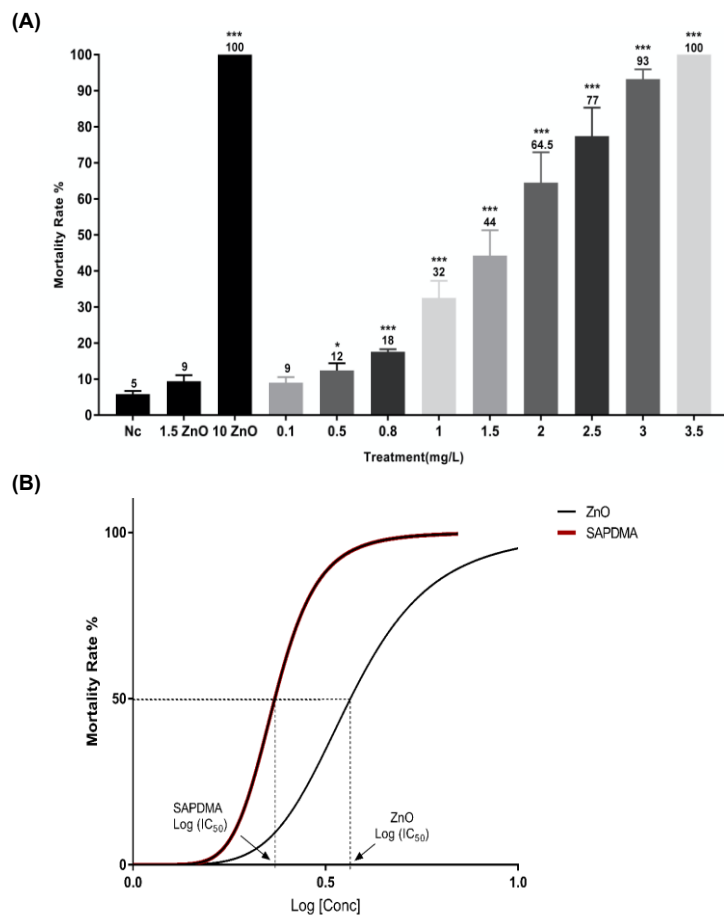


Figure 1. Embryos viability at different concentrations of SAPDMA and ZnO with the calculations of LC_{50} . **(A)** Mortality rate of zebrafish embryos exposed to different concentrations of SAPDMA compared to the negative and positive control groups. **(B)** Inhibitor dose-response curve for the calculation of LC_{50} for ZnO and SAPDMA. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ n = 75.

3.2 SAPDMA cause significant teratogenic phenotypes at very low concentrations

After assessing the survival rate, the morphological and developmental teratogenic effects of SAPDMA on zebrafish embryos were evaluated and recorded at 96 hpf of treatment to determine the NOEC, which is the concentration that shows no statistically significant mortality and teratogenicity ($p < 0.05$), within a stated exposure period compared to control (Nasrallah et al., 2018a; OECD, 2013; Suter et al., 1993). Thus, according to the mortality score of SAPDMA (Figure 1B), three concentrations of SAPDMA were selected to assess the teratogenic phenotypes; heart edema, scoliosis, body length, yolk and eye size (Figure 2B-E). As shown in Figure 2A-E, only 0.1 mg/L of SAPDMA, did not cause any teratogenicity. Thus, the NOEC of SAPDMA was chosen to be 0.1 mg/L where all treated embryos showed normal morphology compared to the negative control.

Yolk sac edema was the most significant and commonly observed teratogenic effect in embryos treated with 0.5 and 0.8 mg/L SAPDMA (Figure 2B, E). The significant increase in the yolk sac size strongly indicates that SAPDMA may have an impact on the normal metabolism and nutrients uptake of these embryos, leading to nutrients accumulation and fluid retention in the yolk sac. Therefore, in concordance with the acute toxicity findings (section 3.1), these results provide another line of evidence that SAPDMA surfactant can have a potentially toxic effect on the embryonic development of zebrafish embryos when tested at higher concentrations.

SAPDMA did not induce any significant effect on the body length and eye size compared to the negative control except at 0.8 mg/L concentration (Figure 2C, D). Hence, embryos treated with 0.8 mg/L SAPDMA presented a wide range of developmental defects in all measured parameters.

All organ-specific toxicities, including cardio, neuro, hepato, and hematopoietic toxicity were evaluated at the NOEC (0.1 mg/L). We also used 0.5 and 0.8 mg/L to test the dose-dependent effect of SAPDMA.

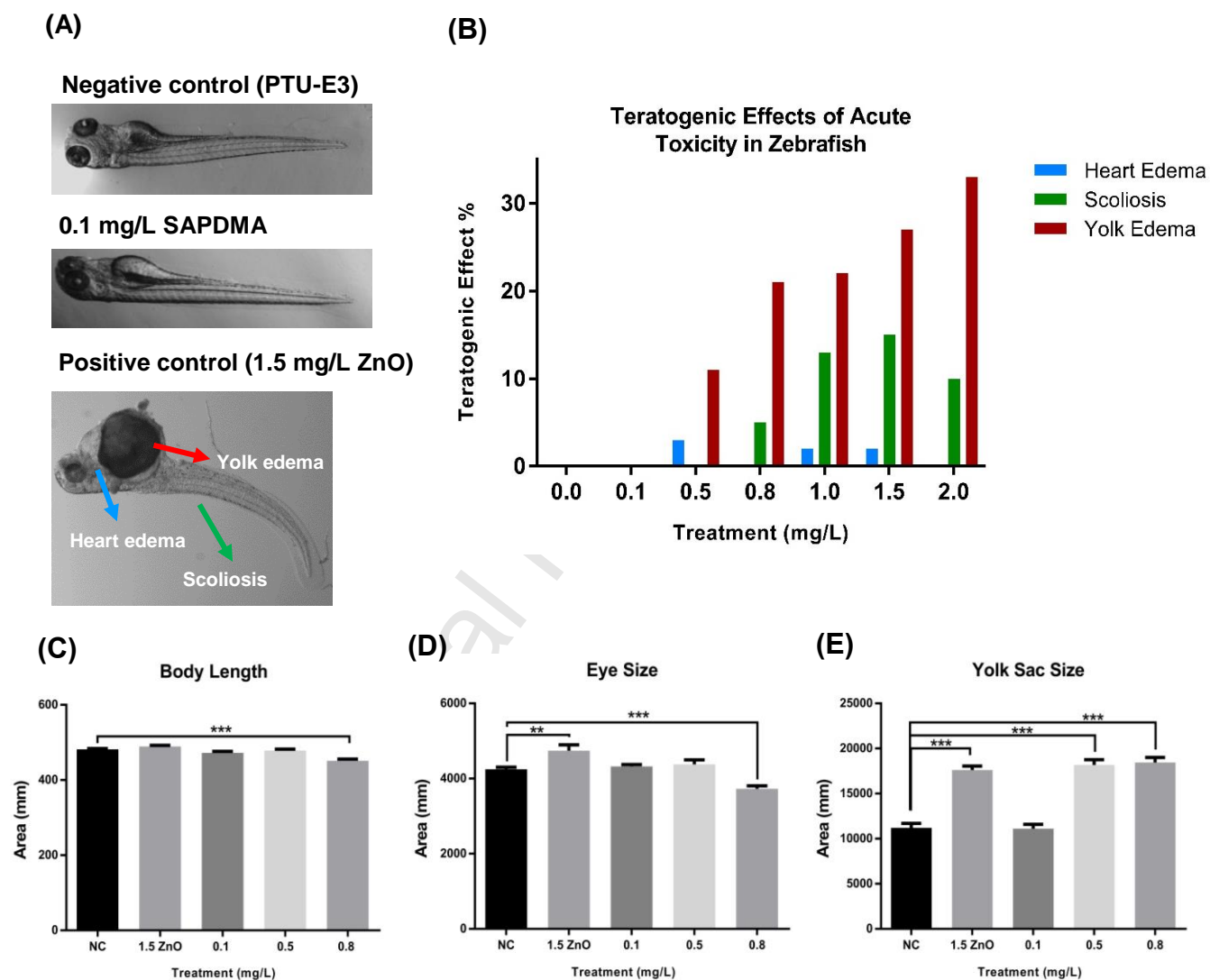


Figure 2. Teratogenic effects of acute toxicity in zebrafish embryos following treatment with SAPDMA. (A) Representative pictures (96 hpf) of acute toxicity experiments of ZnO nanoparticles-exposed embryos (PC), PTU-E3 media (NC), and SAPDMA surfactant. Note the deformed embryos at 1.5 mg/L ZnO (yolk (red arrow), scoliosis (green arrow), and cardiac edema (blue arrow)). Images were captured using ZeissStemi2000-C stereomicroscope (21X). (B) A graphical representation of the teratogenic effects observed in zebrafish embryos. Gross

microscopic examination was used (percentage of embryos with abnormalities was scored). (C-E) Specific teratogenic effects detected following treatment with three different SAPDMA concentrations compared to the NC and PC. (C) Average body length, (D) yolk, and (E) eye size were captured using HCImage software and analyzed using ImageJ software version 1.52a. One-way analysis of variance (ANOVA) followed by Dunnett test was used to compare the differences between the average of the imaged areas between groups, ** $p < 0.01$ and *** $p < 0.001$, $n = 25$.

3.3 SAPDMA does not affect cardiac parameters at the NOEC

Zebrafish is known to be an excellent model for studying the cardiotoxicity of drugs (Zakaria et al., 2018). In this study, two of the earliest developing blood vessel in the tail were examined; the posterior cardinal vein (PCV) and the dorsal aorta (DA) (Al-Kandari et al., 2019). Four parameters were assessed in the PCV and the DA including; blood flow velocity, vessel diameter, heart rate (pulse), and shear stress. Our findings showed that SAPDMA induces a dose-dependent reduction in most of the measured cardiac parameters [(Figure S1, Figure 3A-D (DA), and Figure 3E-H (PCV)]. The NOEC of SAPDMA (0.1 mg/L) did not affect any cardiac parameters except for a reduction in the DA diameter (Figure 3B).

The reduction in the blood vessel diameter was more prominent in the DA than the PCV in all tested concentrations. This could explain the significant induction of shear stress, caused by SAPDMA, in the DA (Figure 3D), but not in the PCV (Figure 3H). Moreover, SAPDMA did not induce a significant effect on the heart rate (pulse) in the PCV in all tested concentrations (Figure 3G). However, 0.8 mg/L of SAPDMA significantly decreased the heart rate in the DA (Figure 3C). We suggest that SAPDMA induces cardiotoxicity mainly by inducing vasoconstriction in the DA and PCV, which lead to the interruption and dysfunction of other cardiac parameters.

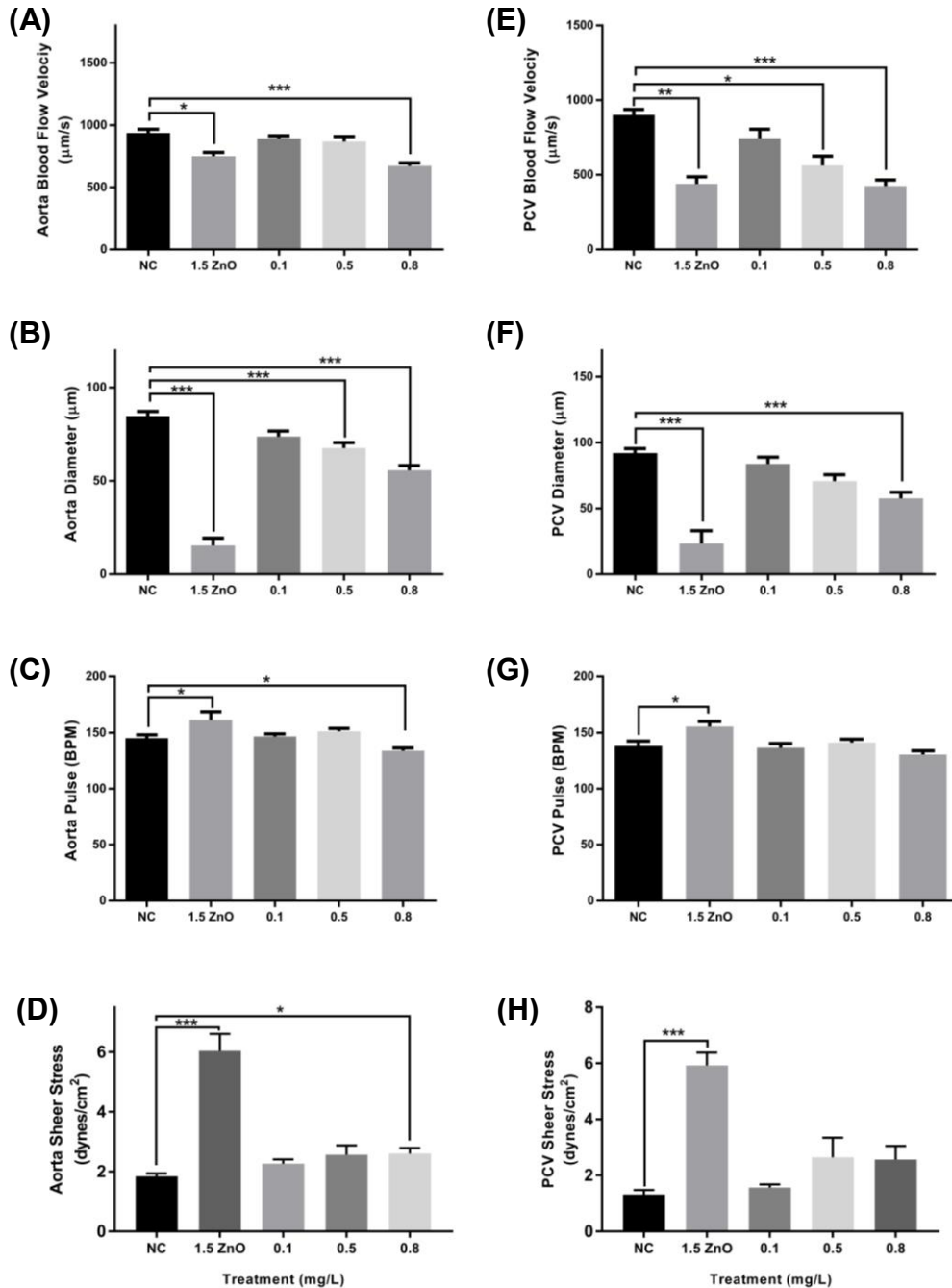


Figure 3. Effect of SAPDMA on the Dorsal Aorta (A) blood flow velocity, (B) diameter, (C) heart rate (pulse), (D) shear stress, and the Posterior Cardinal Vein (E) blood flow velocity, (F) diameter, (G) heart rate (pulse), (H) shear stress. Parameters were calculated from the DA and PCV of the embryos following treatment with each indicated concentration. Twenty-five embryos were used per concentration. One-way ANOVA was used to compare the differences between the averages of the imaged areas between groups. * $p < 0.05$ and ** $p < 0.01$, *** $p < 0.001$, $n = 25$.

3.4 SAPDMA does not induce hematopoietic toxicity at the NOEC

The o-dianisidine stain was used to study the effect of SAPDMA on hemoglobin synthesis in the red blood cells (RBCs) of zebrafish embryos. This is mainly done by detecting the peroxidase activity in erythrocytes through direct measurement of hemoglobin synthesis or indirect measurement of erythrocytes production by the bone marrow (erythropoiesis) (Al-Kandari et al., 2019). The o-dianisidine stain is taken up only by hemoglobin-positive cells, which means that the higher the hematopoietic activity, the larger the stained area in the embryos. The amount of hemoglobin in RBCs or the number of hemoglobin-positive RBCs is calculated by quantitative measurement of size and intensity of the red-stained areas in the embryos' yolk sac using ImageJ software. As shown in Figure 4A and 4B, no significant difference in the number of stained cells was detected in embryos treated at the NOEC (0.1 mg/L) of SAPDMA compared to the negative control. However, embryos treated with 0.8 mg/L SAPDMA were severely affected and showed a significant decrease in hemoglobin positive cells. This could be due to the reduced blood flow and vessel diameter as shown in Figure S1 and Figure 3A-F. Also, the reduced amount of hemoglobin could result from decreased RBCs production by the bone marrow and reduced hemoglobin synthesis in erythrocytes due to a blockage in the heme synthesis pathway (van der Vorm and Paw, 2017).

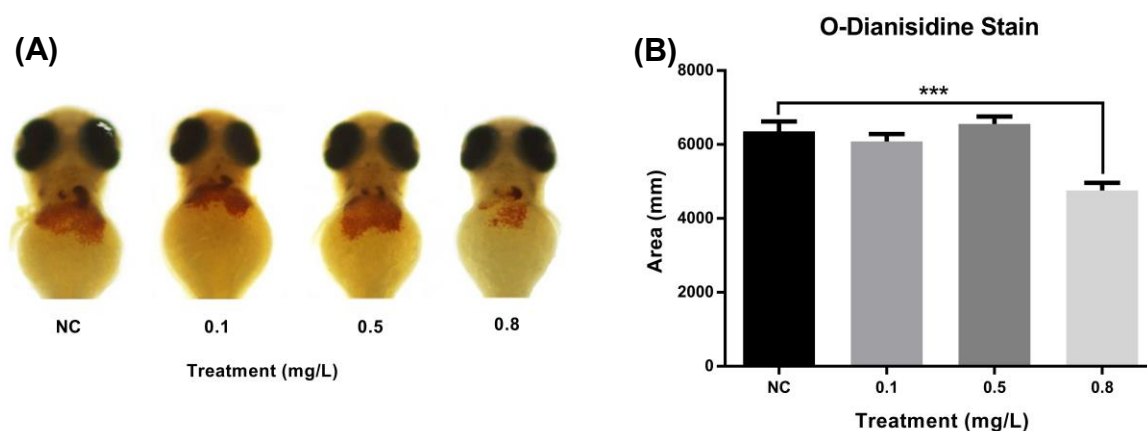


Figure 4. Distribution of hemoglobin-positive cells using o-dianisidine stain at 72 hpf. **(A)** Representative images of o-dianisidine stain concentrated in the yolk sac of the negative control, 0.1, 0.5 and 0.8 mg/L of SAPDMA. **(B)** The graph shows a quantification analysis difference in the number of erythrocytes stained by o-dianisidine. Embryos treated with 0.8 mg/L SAPDMA showed a significant reduction in the hematopoietic activity compared to the negative control. One-way ANOVA was used followed by the Dunnett test to compare the difference between the treated groups. *** $p < 0.001$, $n = 30$.

3.5 SAPDMA does not induce neuromuscular toxicity at the NOEC

Measurement of the locomotive behavior is very useful in assessing the effects of SAPDMA on the nervous system and muscle development of zebrafish embryos. In this study, locomotion and neurotoxicity were evaluated using ViewPoint ZebraLab technology by measuring the average total distance moved after a 60-min cycle and by assessing the response of the embryos to multiple dark/light phases. The results were compared to the negative and positive controls using E3 media and 1.5 mg/L ZnO nanoparticles, respectively. Each embryo was analyzed individually.

Consistently with the previous study (Chen et al., 2013), the neurotoxic positive ZnO nanoparticles triggered a significant increase in locomotive behavior compared to the negative control as shown in Figure 5. In addition, other studies reported an increase in locomotion behavior associated with different nanoparticles such as chitosan (Sakamuru et al., 2016; Soffker

et al., 2012). Most importantly, at the NOEC (0.1 mg/L), the locomotive behavior of SAPDMA-treated embryos slightly decreased. However, at higher concentrations of SAPDMA, starting from 0.8 mg/L, there was a significant reduction in the locomotive activity of the treated embryos. The reduction in total distance move of the 0.8 mg/mL-treated embryos could be due to different types of organ damage, in particular, the heart and muscle development and function. For instance, at 0.8 mg/L exposure, most of the heart function parameters was affected (see figure 3). There was also a delay/defect in muscle development. For example, as shown in Table S1, the average length of three somites in the 0.8 mg/L-treated embryos was significantly ($p < 0.001$) less than the NC. Furthermore, the average total number of the somites per specific area and also the distance between somites were significantly reduced ($p < 0.001$) in the 0.8 mg/L-treated embryos compared to NC (Table S1). More importantly, exposure to 0.8 mg/L of SAPDA caused severe mitochondrial damage (Figure 7), which is essential for ATP production that is required for muscle and heart functions. Collectively, all of these abnormalities could lead to a significant reduction in total distance move for the 0.8 mg/mL SAPMDA-treated embryos. In conclusion, the findings of the neurotoxicity assay suggest that SAPDMA may have a toxic effect on the neurons and muscle activity of zebrafish embryos at concentrations higher than the NOEC.

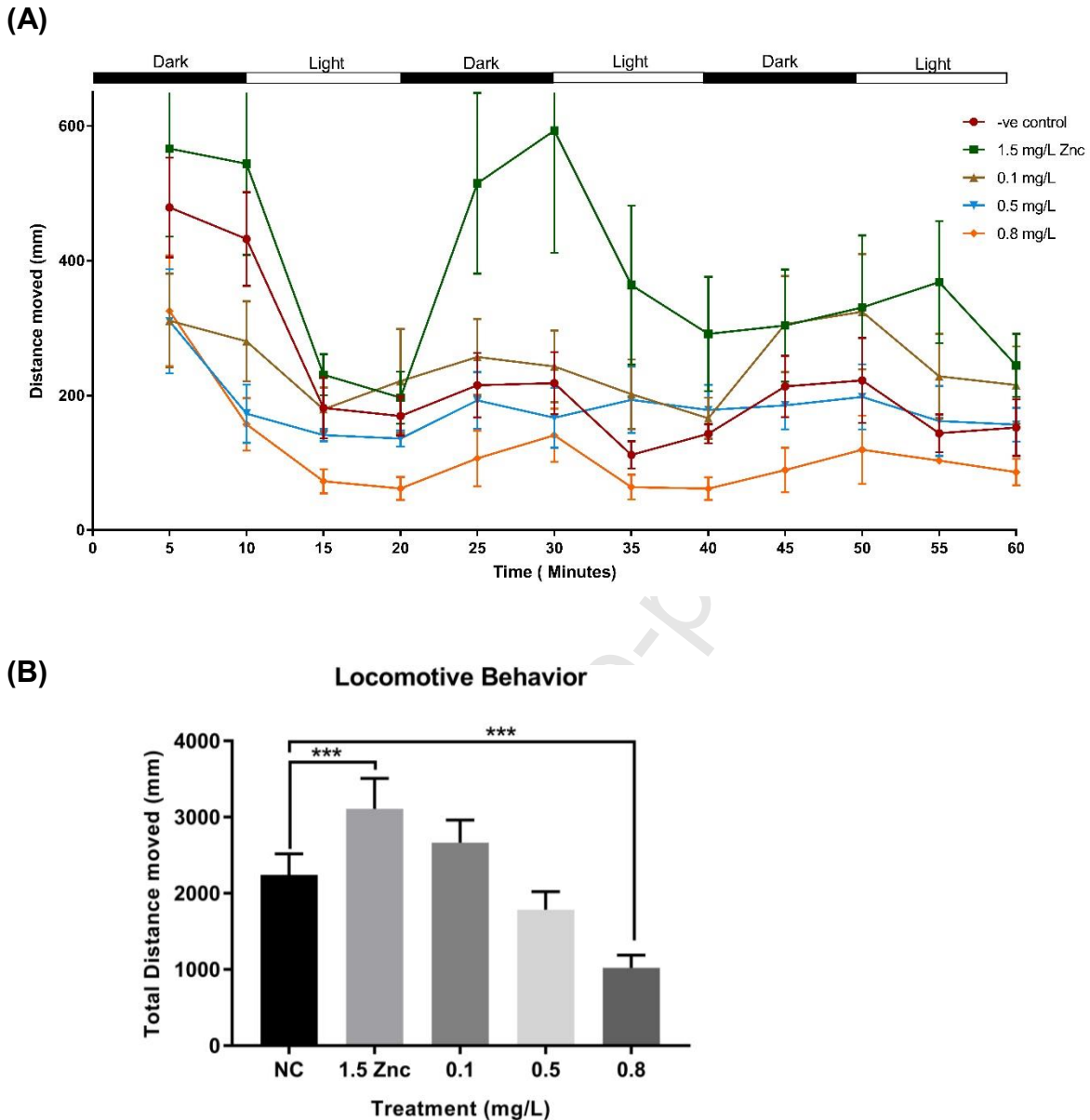


Figure 5. Locomotion and neurotoxicity assessment of zebrafish embryos after 24-hr exposure to different concentrations of SAPDMA. **(A)** Shows the average total distance moved (measured using ViewPoint, Micro lab system) every 5 min by the 120 hpf -old embryos treated with E3 media, 1.5 mg/L ZnO, 0.1, 0.5, and 0.8 mg/L of SAPDMA at 96 hpf. **(B)** Shows the average distance moved (mm) per hour under dark/light cycles of the same embryos in (A). *** $p < 0.001$, $n = 12$.

3.6 SAPDMA induces hepatomegaly without affecting liver functions at the NOEC

Previous studies have shown the robustness of zebrafish for hepatotoxicity prediction (Goessling and Sadler, 2015; Hill et al., 2005; Menke et al., 2011; Vliegenthart et al., 2014). This robustness is supported by a high degree of genetic conservation for the enzymes and pathways required in drug metabolisms, such as ARH receptors, CYP enzymes, or ADH isoenzymes, which are present and functional from early developmental stages, including our experimental window (Du et al., 2015; Klüver et al., 2014; Timme-Laragy et al., 2007). At 120 hpf, zebrafish embryos consume the entire yolk and start to seek food from exogenous sources (Chu and Sadler, 2009). At this point, the liver of the embryos should be fully functional to metabolize external nutrients from the environment. The yolk consists of 70% lipids, which are mainly metabolized by the liver (Jones et al., 2008). Therefore, an indirect indication of impaired liver function is lipid accumulation in the yolk sac of the embryo. To further elaborate, if the liver function is compromised, the metabolism and absorption of lipids will be delayed, which will result in lipid retention in the yolk (Huang et al., 2013; Vliegenthart et al., 2014). Hence, we performed two different hepatotoxicity assays. First, we assessed changes in the liver size in response to different treatments. As expected, 1% ethanol showed a significant decrease in liver size compared to the negative control, indicating liver necrosis (Abou-Saleh et al., 2019; Younes et al., 2020; Zhang et al., 2014). Unexpectedly, embryos treated with the NOEC (0.1 mg/L) of SAPDMA showed a significant increase in the liver size, indicating hepatomegaly, however, the normal morphology of the liver (crescent shape) was maintained. Thus, we suggest that 0.1 mg/L concentration of SAPDMA might cause hepatomegaly without significantly affecting the normal function of the liver.

Second, we assessed the neutral lipids accumulation and triglyceride content by staining the embryos with ORO stain to ensure that the liver function was not affected. ORO stain was extracted from zebrafish embryos and quantified via optical density (OD) analysis. Since ORO stains neutral lipids and triglycerides, OD measurements can be taken as a direct and correlative indicator for the present amount of these two components within zebrafish embryo compared to the control. As shown in Figure 6, embryos treated with 1% ethanol (PC) showed a significant increase in lipid accumulation by 47.6%, which is consistent with the previous studies (Abou-Saleh et al., 2019). However, SAPDMA-treated embryos at the NOEC (0.1 mg/L) showed a minor increase in lipid retention compared to the negative control, which was not significant. This means that although the NOEC of SAPDMA (0.1 mg/L) induces hepatomegaly, it does not affect the liver functions.

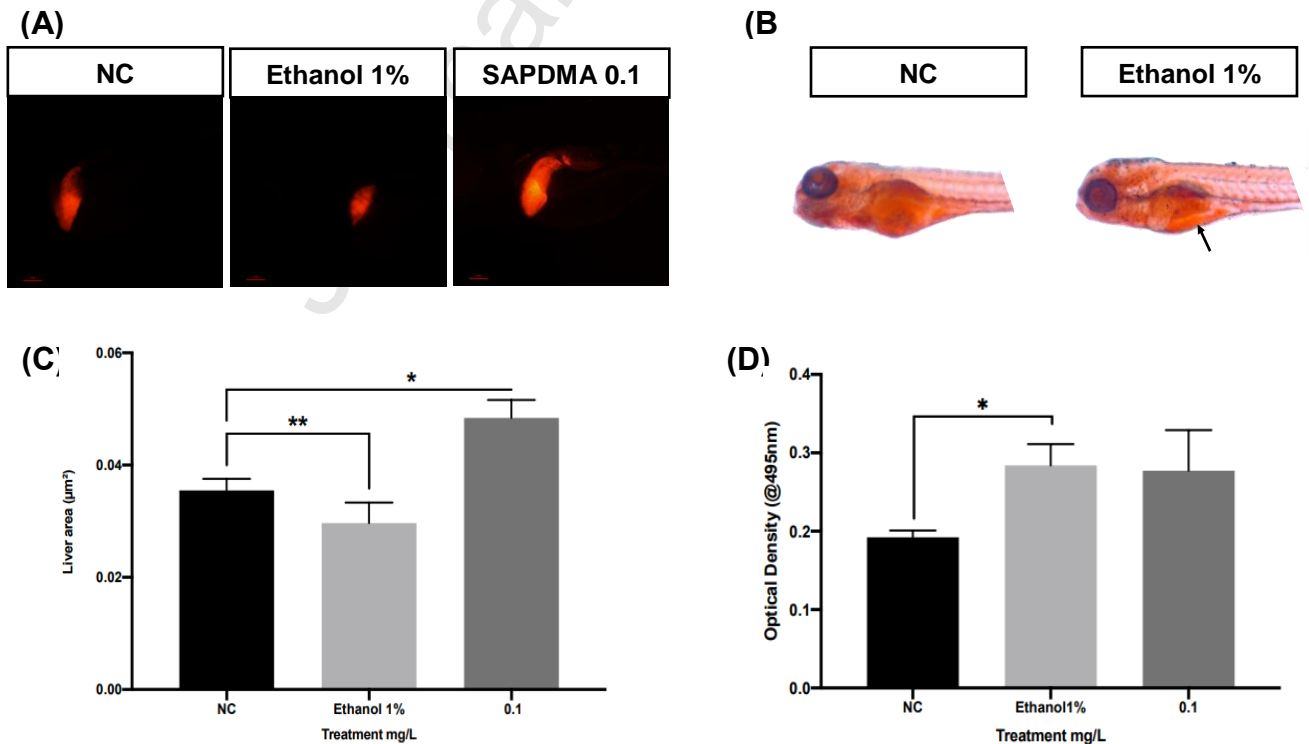


Figure 6. (A) Representative images for liver size. (B) Representative images of yolk retention. (C) Quantification and measurement of the RFP liver area (μm^2) for the controls and the tested compounds. (D) Staining of neutral triglycerides and lipids in wild-type embryos and quantification of ORO staining. Optical density analysis of ORO stained embryos using a microplate spectrophotometer. Quantification took into account the intensity and amount of ORO staining present within the whole zebrafish body. Paired two-tailed student t-test was performed to compare the difference between the treated group and the negative control. * $p < 0.05$, $n = 10$ embryos for liver size measurement, $n = 25-30$ embryos, 5-6 replicates for ORO stain measurement.

3.7 SAPDMA does not cause cytotoxicity at the NOEC

The mitochondrial membrane potential ($\Delta\Psi\text{m}$) is a critical parameter of mitochondrial function and it acts as an indicator of cell health. It is generated by proton pumps in the electron transport chain and is an important component of energy storage processes during oxidative phosphorylation (Vliegthart et al., 2014). It regulates ATP synthesis, ROS generation, and calcium influx into the mitochondria. Along with the proton gradient, $\Delta\Psi\text{m}$ generates a hydrogen ion transmembrane potential, which is harnessed to make ATP. The level of $\Delta\Psi\text{m}$ and ATP are usually kept stable, but sustained changes in $\Delta\Psi\text{m}$ and ATP may induce unwanted loss of cell viability leading to many mitochondrial dysfunctions (Olkowska et al., 2014). Therefore, the mitochondrial membrane potential is one of the key parameters to look for when studying mechanisms related to cell health and when testing compounds' toxicity.

JC-1 cationic dye was used to measure the $\Delta\Psi\text{m}$ in healthy and apoptotic cells. This dye naturally exhibits green fluorescence and can cross the mitochondrial membrane and accumulate inside forming reversible complexes known as J-aggregates (Yoganantharjah et al., 2017). Unlike JC-1 molecules that exhibit green fluorescence, these aggregates show excitation and emission in the red spectrum. Therefore, healthy cells with normal $\Delta\Psi\text{m}$ will form red

fluorescent J-aggregates as the dye accumulates inside the active negatively charged mitochondria (Yoganantharjah et al., 2017). On the other hand, the mitochondrial membrane in unhealthy and apoptotic cells will be less negative due to the disruption of mitochondria and changes in its membrane potential. Such alterations happen when the cell is undergoing early stages of apoptosis which leads to the opening of permeability transition pore (MPTP) and passage of ions and small molecules. The negativity of the membrane will decrease (depolarization) until equilibrium is reached which in turn leads to the decoupling of the respiratory chain and releasing of cytochrome C into the cytosol. This triggers the apoptotic cascade (Rasool et al., 2018; Yoganantharjah et al., 2017). Therefore, JC-1 dye will not be able to reach a concentration that is sufficient to enable the formation of J-aggregates and remains in the monomeric form with its natural green fluorescence.

Based on these premises, the green/red fluorescence ratio of JC-1 dye can be used as a direct indicator of the mitochondria's polarization status. SPADMA-treated embryos were stained according to the protocol mentioned in section 2.10. The intensity of green and red signals was measured, and then the ratio of green/red fluorescence was determined. As shown in Figure 7, there was no significant change in the green/red fluorescence ratio in ZnO-treated embryos or those treated with 0.1 mg/L and 0.5 mg/L SAPDMA. This indicates a normal polarization of the mitochondrial membrane, which means that the cells are healthy. In contrast, embryos treated with 0.8 mg/L SAPDMA showed a significantly elevated green/red fluorescence ratio indicating unhealthy or apoptotic cells. ZnO-treated embryos showed no significant change in the green/red fluorescence ratio, which could be due to cell necrosis or cells undergoing late stages of apoptosis (Wang et al., 2015), leading to the uptake of the JC-1 dye.

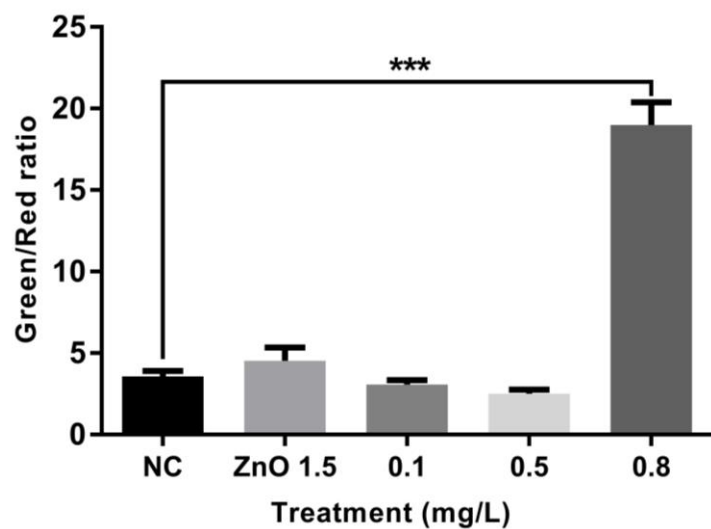


Figure 7. The green/red fluorescence ratio measured in zebrafish embryos treated with different SAPDMA concentrations. The ratio of J-monomers (green signal at 540 nm; dead cells) to J-aggregates (red signal at 590 nm; live cells) was calculated using GraphPad Prism 7 software. ***p < 0.001, n = 25.

4. Conclusions

Our study for the first time presents data that comprehensively investigates the organ-specific toxicity of the cationic surfactant SAPDMA *in vivo* in the zebrafish embryo model. The performed toxicity assays suggest that SAPDMA can cause adverse toxic effects in a dose-dependent manner on the embryonic development of zebrafish. Indeed, the mortality score at 96 hpf following exposure steadily increased, starting with 9% in embryos treated with 0.1 mg/L (NOEC) SAPDMA until it reached 100% in embryos treated with 3.5 mg/L SAPDMA. The LC_{50} and NOEC were found to be 2.3 and 0.1 mg/L, respectively. Thus, we concluded that SAPDMA is classified as “moderately toxic” according to the U.S. Fish and Wildlife Service (USFWS) acute toxicity rating scale (Table S2) (Rasool et al., 2018). Thus, we recommend that the use of SAPDMA in the industry should be re-evaluated and monitored by different environmental and public health agencies.

Author Contributions:

Conceptualization: GKN; Methodology: ABR, MHS, OAJ, HAJ, RA and NY; Software: OAJ, HAJ, RA and NY; Validation: GKN and GP; Formal Analysis: GKN, OAJ, HAJ, RA and NY; Investigation: GKN, OAJ, HAJ, RA and NY; Resources: GKN, GP, HMY, and MA; Data Curation: GKN; Writing – Original Draft Preparation: GKN, OAJ, HAJ, and RA; Writing – Review and Editing: GKN, AFM, OAJ, HAJ, NY, MA, HMY, and GP; Visualization: GKN, AFM, OAJ, HAJ, and NY; Supervision: GKN, GP and AMB; Project Administration, GKN and MA; Funding Acquisition: GKN and MA.

Acknowledgment:

This work is supported by Qatar University's internal grant QUCP-CHS-17-18-2 Given to MAA and Qatar University international research collaboration co-funds grant IRCC-2019-007 given to GKN and FP. This work was made possible by UREP grant # (UREP26-097-3-040) from the Qatar National Research Fund (a member of Qatar Foundation). The statements made herein are solely the responsibility of the author(s). We would also like to thank Qatar National Library (a member of Qatar Foundation) for sponsoring publication fees (gold open access) of this paper.

Conflict of interest:

Declarations of interest: none (all authors have no competing interests to declare).

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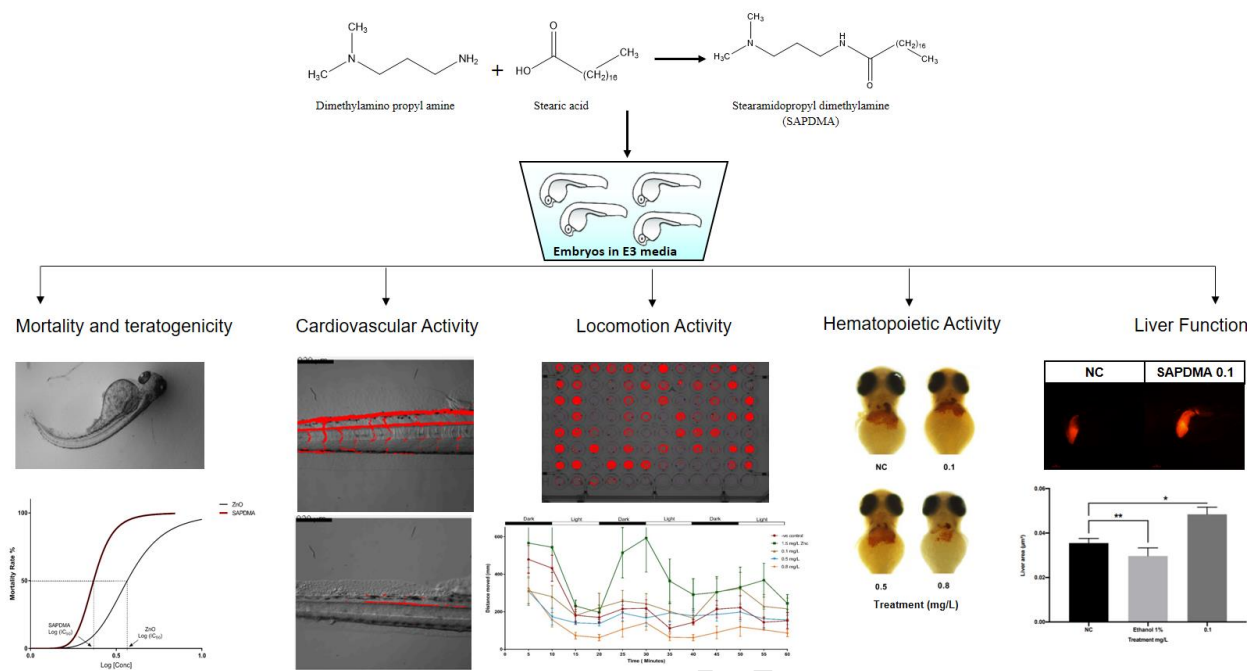
Credit Author Statement

Conceptualization: GKN; Methodology: ABR, MHS, OAJ, HAJ, RA and NY; Software: OAJ, HAJ, RA and NY; Validation: GKN and GP; Formal Analysis: GKN, OAJ, HAJ, RA and NY; Investigation: GKN, OAJ, HAJ, RA and NY; Resources: GKN, GP, HMY, and MA; Data Curation: GKN; Writing – Original Draft Preparation: GKN, OAJ, HAJ, and RA; Writing – Review and Editing: GKN, AFM, OAJ, HAJ, NY, MA, HMY, and GP; Visualization: GKN, AFM, OAJ, HAJ, and NY; Supervision: GKN, GP and AMB; Project Administration, GKN and MA; Funding Acquisition: GKN and MA.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Graphical abstract

Highlights:

- According to the U.S. Fish and Wildlife Service (USFWS) acute toxicity rating scale, SAPDMA is classified as “moderately toxic” surfactant.
- The exposure of the embryos to SAPDMA caused mortality in a dose-dependent manner with calculated LC50 of 2.3 mg/L and NOEC at 0.1 mg/L.
- SAPDMA induces cardiotoxicity by decreasing the Aortic pulse, stress shear, and blood flow velocity at concentration higher than the NOEC.
- Starting from 0.8 mg/L, SAPDMA severely affects the hematopoiesis process and locomotive activity.
- SAPDMA at the NOEC (0.1 mg/L) might cause hepatomegaly without significantly affecting the normal function of the liver.