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Biocompatibility and toxicity of novel iron chelator Starch-Deferoxamine (S-DFO) compared to zinc oxide nanoparticles to zebrafish embryo: An oxidative stress based apoptosis, physicochemical and neurological study profile☆

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ABSTRACT

Objectives: Clinically approved iron chelators are effective in decreasing significant transfusional iron accumulation. Starch-Deferoxamine (S-DFO), a novel high molecular weight iron chelator, was produced to increase binding capacity to iron and reduce toxicity. Although its efficacy was established in one small cohort clinical trial, its potential adverse effect was not adequately addressed.

Methods: We utilized zebrafish model to assess S-DFO toxicity using following assays: mortality, teratogenicity, hatching rate, tail flicking, Acridine Orange staining for apoptosis detection, *o*-dianisidine staining for hemoglobin synthesis, and the level of Hsp70 as a general stress indicator. Embryos were exposed to different concentrations of S-DFO, Zinc Oxide nanoparticle (ZnO) (positive control), along with untreated control (UC).

Results: S-DFO showed no significant mortality nor deformities at all tested concentrations (0.0–1000 μM). Thus, the LC50 is expected to > 1000 μM. 100 μM S-DFO treatment did not affect embryo development (as judged by hatching rate); neuromuscular activity (as judged by tail flicking); and hemoglobin synthesis. Neither apoptosis, nor increase in Hsp70 level was noticed upon S-DFO treatment.

Conclusion: Our assays demonstrate that S-DFO does not induce cellular or biochemical stress and has no adverse effect on organ development of zebrafish embryos, suggesting its safe use as an iron chelator.

1. Introduction

Blood transfusion is considered a life-saving therapy in many acute and chronic diseases associated with blood loss or ineffective erythropoiesis like thalassemia major, sickle cell anemia, prematurity, and malignant disorders. Although, chronic blood transfusion therapy effectively increases survival rate and improves quality of patient's life, it is associated with many complications, especially transfusional iron overload. Excess iron in human body can accelerate the production of highly reactive oxygen species (ROS), thus increasing susceptibility of organs to be oxidatively injured (Ozment and Turi, 2009). ROS destroys cellular elements like proteins, and nucleic acids, and also induces peroxidation of lipids and de-polymerization of polysaccharides. Further, because iron is a vital element for almost all living organisms, excess iron also triggers growth of pathogenic organisms leading to increase vulnerability of transfusion-dependent patients to a broad range of infections (Flora, 2013). To decrease morbidity rate associated with significant iron accumulation, patients, who receive blood chronically, are given iron chelators (Ozment and Turi, 2009). Although clinically approved iron chelators are effective, one of the major

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problems, is poor compliance with therapy, which affects disease prognosis and outcomes (Parody et al., 2007).

Deferoxamine (DFO) the first clinically approved high molecular weight (HMW) iron chelator showed an efficient role in maintaining iron stores and preventing oxidative damage (Ozment and Turi, 2009; Kontoghiorghes, 2006). Due to short plasma half-life (5–10 min) and oral inactivity, DFO has to be continuously infused intravenously to achieve maximum benefit, thus reducing patients' adherence to the course of medical treatment (Kontoghiorghes, 2006; Poletti et al., 2000). Deferiprone (L1; 1, 2 dimethyl 1-3-hydroxypiridin-4-one), another clinically approved low molecular weight (LMW) iron chelator (Poletti et al., 2000), is administered orally and proven successful in removing iron from cardiac tissues (Cohen, 2006). However, L1 treatment efficacy is weaker than DFO, with a number of toxic side effects such as arthropathy, gastrointestinal problems, and also develops severe undesired consequence such as neutropenia/agranulocytosis (Kontoghiorghes, 2006; Poletti et al., 2000).

Starch-Deferoxamine (S-DFO) is a novel high molecular weight (260 kDa) iron chelator, in which DFO molecules are conjugated to starch polymer (40SD02, Biomedical Frontiers, Inc., Minneapolis, MN, USA). Interestingly, conjugation of DFO to starch has no alteration to the specificity and affinity to iron binding capacity of DFO. S-DFO is an improved version of HES-DFO (Hydroxyethyl starch deferoxamine) with prolonged half-life in blood circulation and 50% increase in number of DFO molecules bound to starch, which in turn increases the affinity of this polymeric drug to iron (Fig. 1) (Harmatz et al., 2007; Hallaway et al., 1989). S-DFO efficacy as a potent iron chelator was established in one small cohort clinical trial (consists of four β-thalassemic patients' groups). Harmatz et al. (2007) showed that single intravenous infusion of S-DFO into β-thalassemia transfusion-dependent patients at doses of 150, 300, 600 and 900 mg/kg, induced clinically significant urinary iron excretion. Iron was eliminated through urine in dose-dependent manner, with the highest dose resulting in 1.31 mg of iron/kg (range 0.79–1.90 mg/kg) being excreted, and residual iron-binding capacity remaining in the plasma over 1 week. Interestingly, the authors stated that S-DFO related adverse effect was only limited to urticarial (allergic) reaction manifested by some patients, where they require anti-histamine administration, but without termination of treatment. Although the authors claimed safe use of S-DFO over three weeks, this study included only small cohort (16 patients), and long term side effect was not adequately addressed. Further, although the previous mentioned studies and others potential polymer based iron chelator drugs (Kontoghiorghes, 2006; Poletti et al., 2000; Rossi et al., 2009; Imran et al., 2013; Gehlbach et al., 1993; La and Hamilton, 2015; Li et al., 2015; Hom et al., 2000; Hamilton and Kizhakkedathu, 2015) added a lot to the field of iron chelation, there is a lack of detailed data on their toxicological effects in literature, especially during early developmental stages (pregnancy and childhood).

Toxicity of clinically approved iron chelators have been evaluated in the literature using mice and zebrafish models (Hamilton et al., 2014; Bosque et al., 1995; Nasrallah et al., 2018). Recently, zebrafish becomes an ideal *in vivo* vertebrate model for developmental and drug toxicity screening studies due to many advantages [reviewed in (Teraoka et al., 2003; Ali et al., 2011; Hill, 2013; Mccollum et al., 2011)]. Thus, the objective of this study was to illustrate the potential drug related adverse effect of a promising modified iron chelator S-DFO using zebrafish embryos model. For this purpose, here, we utilized a number of widely used zebrafish toxicity assays to address this issue (Mccollum et al., 2011; Truong et al., 2011; Basnet et al., 2017; Chen et al., 2011; Hu et al., 2011).

2. Materials and methods

2.1. Materials

Starch-Deferoxamine (S-DFO) was manufactured by Biomedical Frontiers, Inc., Minneapolis, MN, USA (ready-made stock with concentration of 3.8462 mM). Zinc oxide (ZnO) nanopowder, < 100 nm particle size (known to generate toxic effects on zebrafish) (Hu et al., 2011; Choi et al., 2016; Hu et al., 2016; Zhao et al., 2013); E3 media (used to cultivate zebrafish embryos) constituents including sodium chloride (NaCl), potassium chloride (KCl), magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$) and calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$); *N*-Phenylthiourea (PTU); 3% methyl cellulose, 4-*N*, *N*-diethylaminobenzaldehyde (DEAB); ferric ammonium citrate, and Acridine Orange (AO) hydrochloride solution 10 mg/ml in H_2O; were all purchased from (Sigma-Aldrich, Germany). Methylene blue (added to E3 media to prevent fungal, bacterial, or viral contamination) was obtained from Kordon (USA). 10× Phosphate buffer saline (pH 7.4) was purchased from Gibco by Life Technologies (USA).

2.2. Zebrafish husbandry and embryos preparation

AB Zebrafish (*Danio rerio*) were maintained in recirculating Pentair aquatic system in the Biomedical Research Center (BRC) at Qatar University (QU). For experiments, all embryos collected from the same spawns were selected for all treatment groups to ensure valid statistical comparison between control and treated groups. At 3 hours post fertilization (hpf), only fertilized healthy embryos were selected under Ziess Stemi 2000-C stereomicroscope and placed in either E3 or PTU media (for imaging purposes) and maintained at 28.0 °C throughout the treatment period. 450 mg of PTU was added to 250 ml of Reverse Osmosis (RO) water to prepare 60× stock of PTU. Then, to make the working solution used as a media to contain fish embryos, 13.2 ml of E3 and 13.2 ml of 60× PTU were added together and completed to 1 l with (RO) water with 50 µl of Methylene Blue solution added at the end. All chemicals were water soluble, freshly prepared and diluted in either E3 or PTU media just prior to assay on zebrafish embryos and embryos incubated in only E3 or PTU media were used as untreated control (UC). The study experiments were carried out according to the local and international regulations and complied with animal protocol guidelines required by the Qatar University in laboratory animal and Policy on Zebrafish Research established by Department of Research in the Ministry of Public Health, Qatar.

2.3. Mortality scoring and LC50 calculation

To determine a suitable range of concentrations for testing, we performed dose-inhibition response assay. For S-DFO viability (survival) rating, triplicates of 10 embryos were exposed to 4 concentrations of S-DFO (50, 100, 500 and 1000 µM) along with UC. Zebrafish embryos were treated as well with ZnO nanoparticles, which was used as a positive control in this study. Five different concentrations of ZnO were prepared as triplicates of 10 embryos for each concentration (10, 20, 40, 80 and160 µg/mL). Embryos were distributed in 12 well plates, placed in S-DFO and ZnO for 3 days from 72 to 144 hpf, counted and evaluated for lethality every 24 h. Embryos were recorded as dead if their hearts stopped beating, color of embryo tissues changed to opaque or were no longer motile. Mortality and deformity rates were recorded using Ziess Stemi 2000-C stereomicroscope and to be used later in the determination of median lethal dose (LC50), no observed effect concentration (NOEC) and low observed effect concentration (LOEC). NOEC is the concentration at which the mortality score is < 20%, whereas LOEC is when the death rate is equal to or > 20%. A sigmoidal S-shaped curve was graphed to illustrate the dose-inhibition response in which the percentage of mortality represents the y-axis and Log [Concentration] in the x-axis. Then, the sigmoid function equation was determined and LC50 was estimated from the curve using Graph pad prism software.

2.4. Toxicity assays

2.4.1. Teratogenic phenotypes (TP)

We explored if S-DFO at the therapeutic dose could cause any

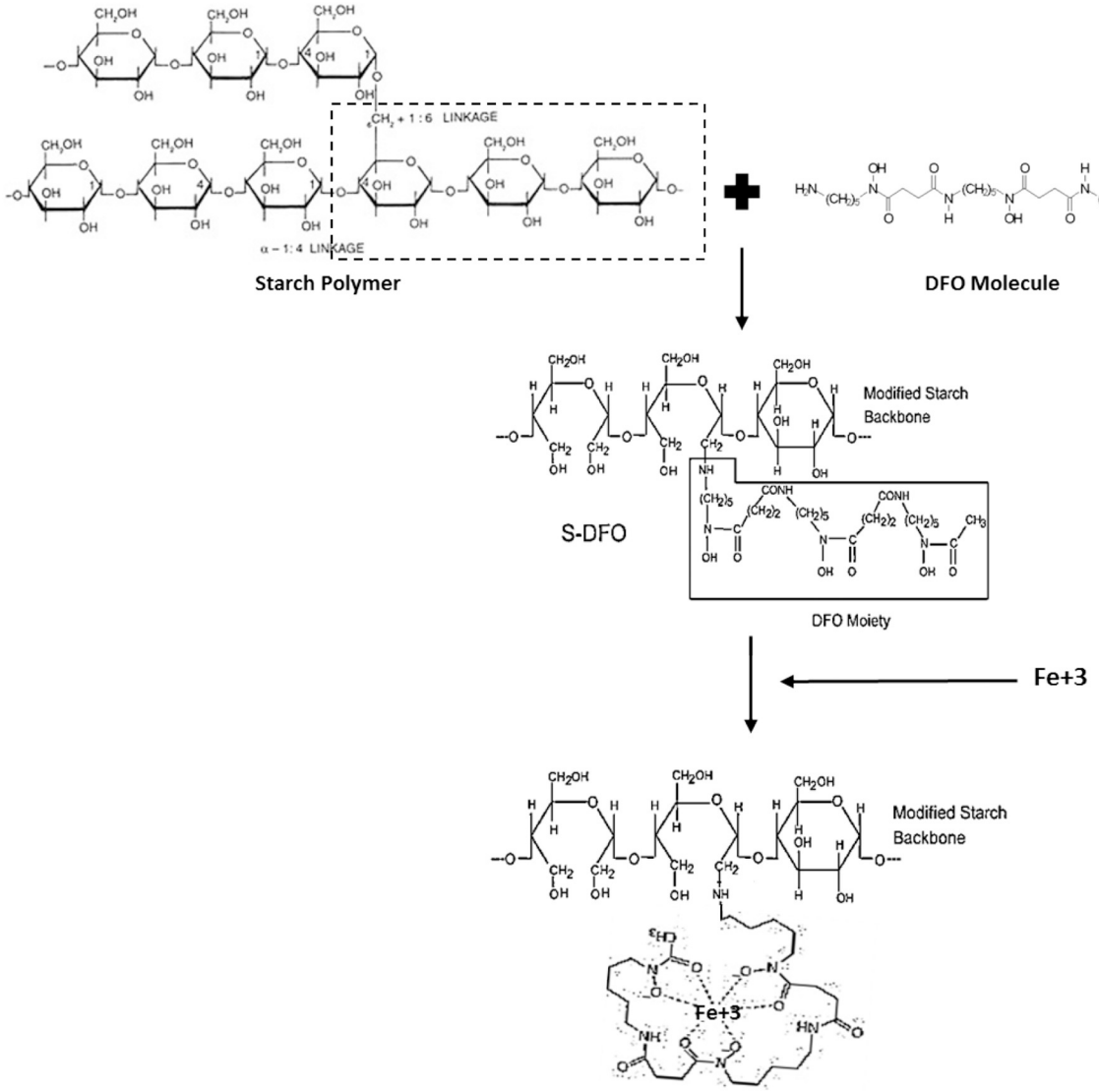

Fig. 1. Schematic diagram of the chemical structure of novel iron chelator Starch-Deferoxamine (S-DFO). DFO molecules attach to a modified starch backbone by covalent bonds. Each hexadentate DFO molecule conjugated to starch polymer forms six bonds with a central iron atom. The resulting modified HMW chelator has maintained the DFO specificity and affinity for iron (Harmatz et al., 2007; *Chelators for Iron Overload*, 1999; Manisha, n.d.; Bogdan et al., 2016).

developmental and behavioral malformations or cardiac dysfunction. Zebrafish embryos were incubated with 100 µM S-DFO from 72 to 144 hpf at 28.0 °C and assessed every 24 h for morphological changes. Embryos were visualized under Zeiss Stereo Lumar. V12 microscope was used to detect any morphological and teratogenic defect, which included: deformities in embryo body like malformed tail or bent trunk, abnormal movement, heart or yolk edema, irregular pigmentation and escoliosis (snaps captured at 16× by DMK 22BUC03 Y800 camera) and analyzed using Image J Software. After exposure to drug, heart rate was also measured and videos were acquired at 150× using Micromanager Software. Videos were converted later by Image J Software from stack of files to AVI format and heart beats per 1 min were counted manually by VLC media player. During imaging, embryos were positioned on side and mounted using 3% methyl cellulose (MC). Three trials of this experiment were conducted and included three treatment groups: PTU only as UC, 100 µM S-DFO, 20 µg/mL ZnO (positive control) with 10 embryos per well and 2 wells per treatment ($n = 20$). From images and videos, each individual embryo was evaluated and any abnormal phenotype was observed and scored as described in (Ozment and Turi, 2009). The percentage of each abnormal phenotype of embryo was calculated and the average of the 3 independent experiments were determined for each deformity being examined, in addition to any other types of anomalies would also be noted if observed.

2.4.2. Hatching rate (HR)

On the day of collection, after 2–3 hpf, healthy fertilized embryos were selected and loaded with drug in 12 well plate. Then, every 24 h at (24, 48, 72, 96 hpf) embryos in the wells were observed for hatchability. Hatching rate was calculated = No. of hatched embryos at 96 hpf/total original number of embryos treated ×100%. Group preparation was as follows: UC in E3 media, 100 µM S-DFO, and 20 µg/mL ZnO (positive control) in 3 independent experiments with 20 embryos for each group.

2.4.3. Tail flicking (TF)

After 3 hpf, 30 healthy embryos in each group were exposed to the following chemicals: UC in PTU media, 100 µM S-DFO, and positive controls of 20 µg/mL ZnO and 1 µM of DEAB (another positive control). To track embryos activity and tail flicking within chorion at 24 hpf, Zeiss Stereo Lumar. V12 microscope and Micromanager Software were used to take videos at 15× magnification. Analysis was done using DanioScope Software (Noldus, Wageningen, the Netherlands) and

locomotor activity was expressed as total number of tail being flicked in 1 min (burst/min). In order to compare between tested groups, the number of spontaneous head-tail contraction of each individual embryo in the two independent experiments was counted and the average for each group was calculated. Experiment was performed in duplicate of a total of 60 embryos per treatment.

2.4.4. Acridine Orange (AO) staining of apoptotic cells

To determine whether S-DFO could induce damage and death at cellular level, a widely used AO staining was performed to detect presence of apoptotic cells and tissue viability. Dead cells have disturbed plasma membrane which are permeable to AO stain, unlike intact healthy live cells. AO is a metachromatic dye and when entering the cells, it binds selectively with nucleic acids and emits green fluorescent color (Chen et al., 2011). Five groups, each with 12 embryos, were treated (as indicated above) in 12 well plate for 3 days, from 72 to 144 hpf. All working solutions were prepared using PTU media and groups were as follows: UC, 100 µM of ferric ammonium citrate (FAC), 100 µM of S-DFO, 500 µM of deferiprone (L1), 20 µg/mL ZnO. High concentration of L1 (Hamilton et al., 2014), ZnO (known to cause apoptosis) and FAC (as an excess iron) positive controls, causes oxidative damage. At the end of the treatment period, embryos were washed 3 times with dye-free media 1× phosphate buffer saline (PBS). Each group of embryos was stained with 100 µl of 5.0 µg/ml of AO staining solution (Chen et al., 2011). Embryos were incubated for ½ h in dark. At the end of the incubation, embryos were rinsed 3 times with 1× PBS and prepared for imaging. Images were captured using Zeiss Axiocam ERc 5s camera under fluorescence microscopy (Zeiss Stereo Lumar. V12) (GFP filter) at 125×.

2.4.5. o-Dianisidine staining

The *o*-dianisidine staining was performed as described elsewhere (Fernández-Murray et al., 2016). At 72 hpi, the 100 µg/ml S-DFO, 20 µg/ml ZnO nanoparticles positive and PTU negative control-treated embryos (10 in each group) were decorionated and anesthetized with 0.02% Tricaine. Then, embryos were stained in the dark for 30 min in *o*-dianisidine solution [(0.6 mg/ml) containing 40% ethanol, 10 mM sodium acetate (pH 4.5) 0.65% hydrogen peroxide], fixed in 4% paraformaldehyde in PBS saline overnight at 4 °C, and then embedded into 3.0% (w/v) methyl cellulose for imaging. Images were taken using Zeiss Axiocam ERc 5s camera under bright field microscopy (Stemi 508 Zeiss) at 50×. The intensity and the size of red colored areas (*o*-dianisidine stained areas) in the yolk sac of each embryo were measured using the ImageJ software.

2.4.6. SDS-PAGE and western blot technique

Total protein were extracted from 25 embryos treated with 100 µg S-DFO, 10 µg ZnO nanoparticles (positive control) and, PTU negative control at 72 hpf. Protein was extracted from whole embryos using cell lysis RIPA buffer (ThermoFisher, USA) supplemented with 1× protease inhibitors cocktail (ThermoFisher, USA). In order to determine the total protein concentration in each sample, the BCA assay using Micro BCA™ Protein Assay Kit (ThermoFisher, USA) was used based on the kit protocol. The protein was diluted with 4× Laemmli sample buffer (Novex, CA) supplemented with β-mercaptoethanol so that 20 µg of the proteins were loaded per well into an 8% SDS-PAGE gel. The gel was subjected to electrophoresis at 110 V for 100 min at room temperature. The gel was then electro-blotted onto a pure nitrocellulose membrane (Novex, CA) using Towbin blotting buffer supplemented with 20% methanol. In order to evaluate transfer efficiency, the blotted membrane was stained by Ponceau-S for 5 min. Furthermore, the membrane was incubated with 5% nonfat dry milk in Tris-buffered saline with tween 20 (TBS-T) in order to block the non-specific protein binding for 1 h at room temperature followed by cutting the membrane into an upper part (Hsp70) and the lower part (GAPDH). Visualization of Hsp70 and housekeeping GAPDH protein was achieved by application of the Mouse monoclonal Hsp70 (1:1000, ab5439, Abcam) and the Rabbit poly clonal GAPDH (1:1000, ab209856, Abcam), respectively overnight at 4 °C with shaking. After washing again with TBS-T, the membranes' two parts were incubated with anti-Mouse at (1:2500, PAB0096, Abnova) to detect the Hsp70 and with the anti-rabbit immunoglobulin secondary antibody (1:40,000, A0545, Sigma) to detect the GAPDH protein; both secondary antibodies are conjugated with horse-radish peroxidase (HRP) for 1 h at room temperature on a shaker. The membrane had a final wash with TBS-T followed by detection with the chemiluminescence ECL Western Blotting Detection kit (Abcam). The band intensities were then quantified using ImageJ software (NIH Image Soft.) to detect the relative quantity of Hsp70 Protein compared to the PTU control.

2.5. Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparison was performed to compare the significant differences between groups using SPSS program [Version 23 statistical software]. Results were expressed as the mean ± standard error of the mean with 95% confidence interval. Chi-square test was used to analyze data in percentage (%) and compare different treatments with control groups. Data were obtained from at least two independent experiments and p-value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Embryos viability and determination of LC50

Our results showed that S-DFO was not lethal at all doses applied and did not cause any abnormality in embryo body up to 1000 µM when compared with UC. However, ZnO toxicity started to appear at 20 µg/mL dose (causing 5% mortality of the embryos) (Fig. 2A). Thus, 20 µg/mL ZnO considered as the NOEC, because it killed < 20% of the embryos. The LOEC concentration (mortality score > 20%) for ZnO was at 40 µg/mL, as this concentration leads to mortality score of 30% of the embryos. Thus, 20 µg/mL of ZnO (NOEC) was used as positive control for all toxicity assays carried out in this study. The LC50, the median lethal dose at which 50% of treated-embryos were dead, for ZnO was calculated as shown in Fig. 2B to be at 118.5 µg/mL (1482 µM). Since S-DFO did not show any lethal, sub-lethal or toxic effects at all tested concentrations, the LC50, NOEC and LOEC cannot be calculated. The expected LC50 for S-DFO was very high (hypothetical LC50 = 3.012×10^{7}) (Fig. 2B).

3.2. Detailed description of teratogenic and developmental effects of S-DFO

Morphological alterations and developmental teratogenic effects of S-DFO on zebrafish embryos were examined and recorded after 72 h of treatment. Seven developmental phenotypes were assessed including: malformation in body, embryo movement, cardiac function particularly heart rate, pericardial edema, yolk sac edema, pigmentation and abnormal curvature of the spine. Images of embryos were taken under a stereomicroscope and screened for each phenotype, which if absent scored as zero, while if present recorded as 1 and percentage of each observable anomaly for each group was calculated. 100 µM S-DFO did not induce any toxicity in zebrafish embryos and shared similar results with UC in all parameters tested. Some embryos showed irregular heartbeats, but it was statistically insignificant (Fig. 3A & 3B). Embryos treated with 20 µg/mL ZnO presented with a wide range of developmental defects including bent bodies, damaged head, eye and mouth, deformed tail, altered movement, abnormal heart rate, and enlarged heart with significant p value < 0.05. About 26.1% of ZnO-treated embryos noted to have yolk edema, but was insignificant when compared with UC. Further, ZnO-treated embryos did not show significant defects observed in pigmentation and no embryos presented with abnormal curved spine (Fig. 3A & 3B).

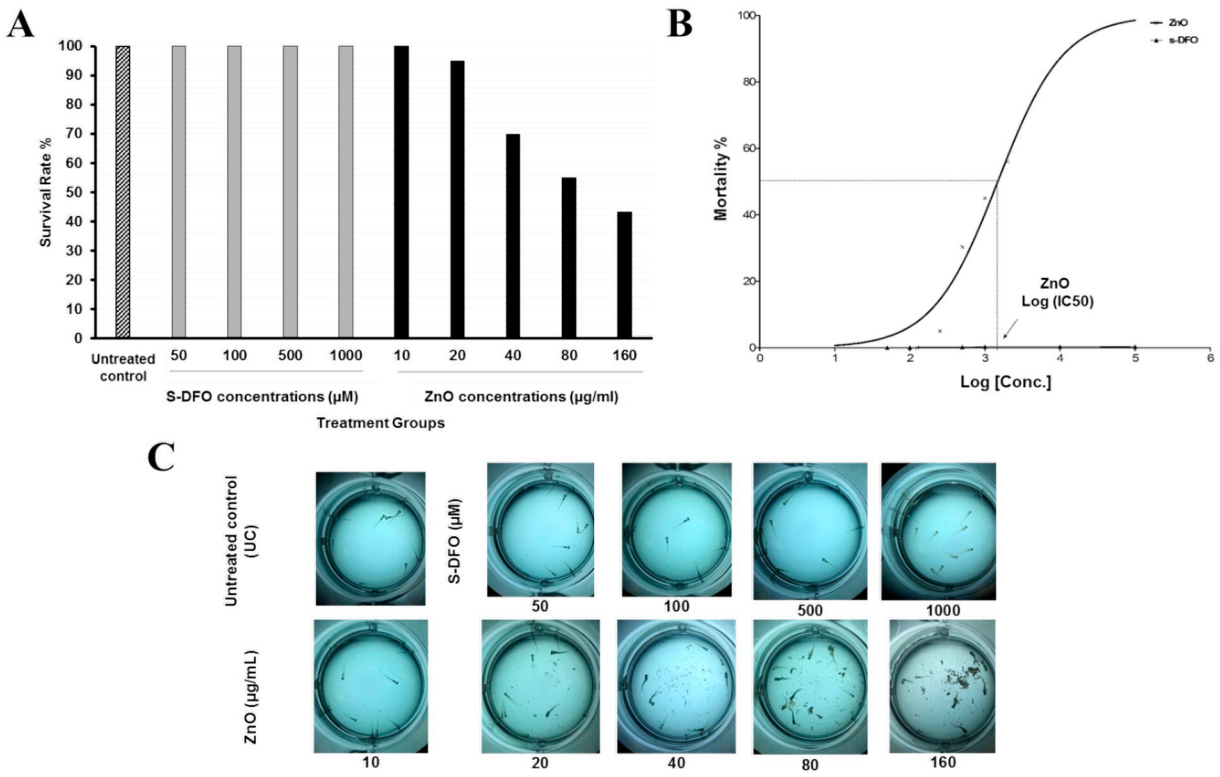

Fig. 2. Embryos viability at different concentrations of S-DFO and ZnO and determination of LC50. (A) Survival rate of zebrafish embryos treated with different concentrations of S-DFO and ZnO compared to control group. (B) Inhibitor dose response curve for the determination of LC50 of ZnO and S-DFO (see the Materials and methods section for more details). (C) Morphology of zebrafish embryos treated with different concentrations of S-DFO and ZnO. Representative optical images of zebrafish embryos after 3 days of treatment. Note the dead and deformed embryos in ZnO positive control starting from 20 µg/mL and the healthy embryos in the S-DFO treatments of different concentrations. Images were captured from 12 well plates (0.65×) using Ziess Stemi 2000-C stereomicroscope.

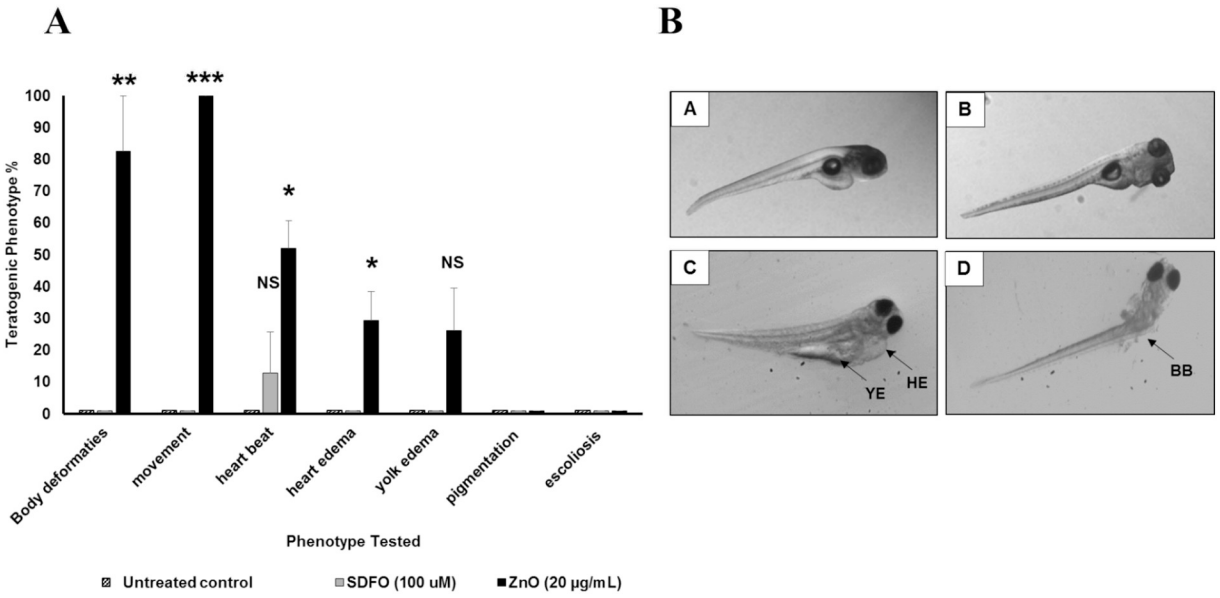

Fig. 3. Teratogenicity of S-DFO and ZnO. A) Teratogenic phenotype analysis at 100 µM of S-DFO compared to normal and abnormal controls. A set of 3 experiments performed with 20 embryos tested in each group. Embryos in ZnO had deformities in their bodies either in head, eyes, body, or tail or more than one part. They showed abnormal motility with abnormal heart beats. 26.1% of embryos had yolk edema and 29% had heart edema but no irregular pigmentation or escoliosis were noted. Note that, NS $p > 0.05$ (not significant), $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$. (B) Images of teratogenic phenotypes of zebrafish larvae after 72 h of exposure to different treatments. (A) UC. (B) S-DFO (100 µM). (C–D) ZnO (20 µg/mL). Larvae images were captured at 16× magnification using Zeiss Stereo Lumar. V12 microscope). YE: yolk edema. HE: heart edema. BB: bent body.

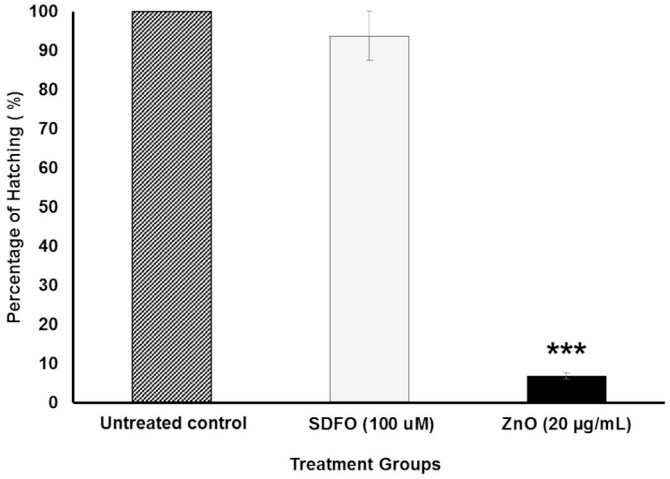

Fig. 4. Hatching rate of zebrafish embryos exposed to 100 µM S-DFO compared to control groups recorded after 96 hpf. Embryos treated with 100 µM S-DFO hatched successfully with 93.75%, compared to ZnO (positive control), $^{***}p \leq 0.001$.

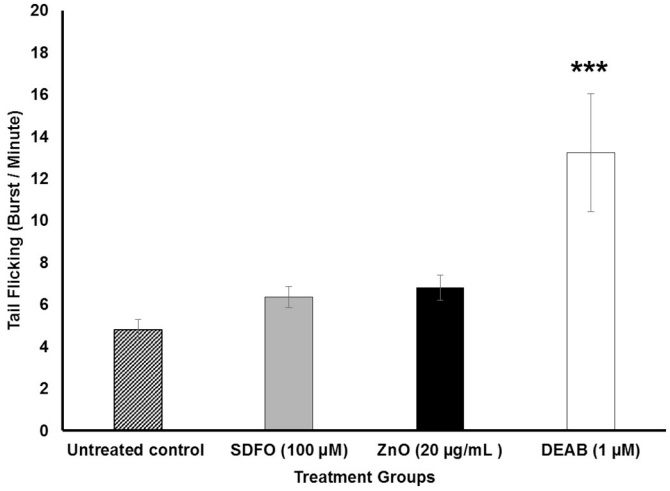

Fig. 5. Spontaneous head-tail coiling of embryos quantified after 24 h of exposure to various chemicals. Embryos in S-DFO and ZnO showed normal movement. However, embryos in DEAB had abnormal locomotive behavior. $^{***}p \leq 0.001$. The assay was performed in duplicate and average of burst/min was calculated.

3.3. Effect of S-DFO on zebrafish hatchability

Ability of zebrafish embryos to successfully hatch after exposure to certain chemicals has been utilized as a tool to screen for developmental toxicity at early stages. At 96 hpf, S-DFO treated embryos were able to hatch similar to UC. On the other hand, 93.16% of embryos placed in 20 µg/mL of ZnO were inhibited from hatching. Embryos hatching rate in S-DFO-treated embryos differ significantly ($p = 0.001$) from those of ZnO (Fig. 4).

3.4. Analysis of zebrafish embryos locomotor activity after exposure to S-DFO

Measuring the number of head-tail contractions of zebrafish embryos could be a useful tool to evaluate the effects of certain chemicals on the nervous and muscular systems development at early phases of zebrafish life. Normal embryos have a spontaneous tail flicking behavior with 3–5 burst (head-tail coil) per min at 24 hpf (Basnet et al., 2017). In our experiment, videos of embryos within their chorions were captured at 24 hpf in order to count number of spontaneous movements in 1 min, which were analyzed by DanioScope Software and expressed as number of burst/min. Locomotor behavior of embryos in S-DFO and ZnO-treated embryos did not show significant difference compared to UC (4.8 burst/min). For that reason, another positive control [1 µM 4-*N*, *N*-diethylaminobenzaldehyde (DEAB)] was included to support the experiment. DEAB is a competitive aldehyde dehydrogenases inhibitor known to exhibit toxic effects on the developing zebrafish embryos (Begemann et al., 2004; Yoganantharajah et al., 2016). As shown in Fig. 5, DEAB triggered an increase in the rate of spontaneous tail flicking (neurotoxic) and showed a clear statistical significance ($p = 0.0001$) between all other treated groups. S-DFO did not cause any movement defect and suggesting that S-DFO is not a neuromuscular toxic drug.

3.5. Detection of cellular death using Acridine Orange staining (AO)

To investigate the toxic role of S-DFO in apoptosis, AO staining of live embryos was performed. Only dead cells will take up the stain, while viable cells are not permeable. At the end of the treatment period of 72 h, embryos were stained immediately and visualized under fluorescence microscopy (Zeiss Stereo Lumar. V12) to determine whether exposure to S-DFO would lead to an increase in cellular death. No apoptotic cells were noted in the UC and S-DFO treated embryos (Fig. 6A). Whereas, embryos exposed to 100 µM FAC, 500 µM L1 and 20 µg/mL ZnO were severely affected at cellular level and showed significant ($p < 0.001$) increase in percentage of apoptotic cells (Fig. 6B). At the end of the treatment period, all embryos placed in media containing 500 µM L1 showed abnormalities and had bent bodies and yolk edema which proved as previously mentioned that L1 iron chelator causes toxic effects at high concentration (Hamilton et al., 2014).

3.6. S-DFO has no adverse effect on hemoglobin synthesis by RBCs

Next, we tested whether S-DFO treatment could have an adverse effect of hemoglobin synthesis by the red blood cells (RBC) of the zebrafish using the *o*-dianisidine staining. This staining could be used also as indirect measurement for the number of the erythrocytes synthesized by the bone marrow (erythropoiesis), as only hemoglobin positive cells will take up the stain. No significant difference in the amount of hemoglobin (measured in the yolk area) was noted between the negative control and the S-DFO treated embryos (Fig. 7A). Whereas, embryos exposed to 20 µg/mL ZnO nanoparticles were severely affected and showed a significant ($p < 0.001$) decrease of hemoglobin level (Fig. 7B), suggesting that SDF-O has no harmful effect on hemoglobin synthesis or normal RBCs production by the bone marrow.

3.7. S-DFO has no adverse effect on protein expression of the zebrafish embryo

Next, we wanted to be more confident about the safe use of S-DFO, thus we tested if the S-DFO treatment might have an adverse effect on the level of gene/protein expression. For this reason, we used the heat should protein Hsp70, that is Hsp70 expression is ubiquitous and also could be used as marker for general stress or genotoxicity. As shown in Fig. 8, there was no significant difference on Hsp70 protein expression compare to negative control, suggesting that S-DFO does not alter the level gene/protein expression or induces general stress in zebrafish after treatment with S-DFO.

4. Discussion

Patients who are in need for chronic blood transfusion require continuous administration of iron chelators to maintain a negative iron balance between iron intake, transfusional accumulated iron, and

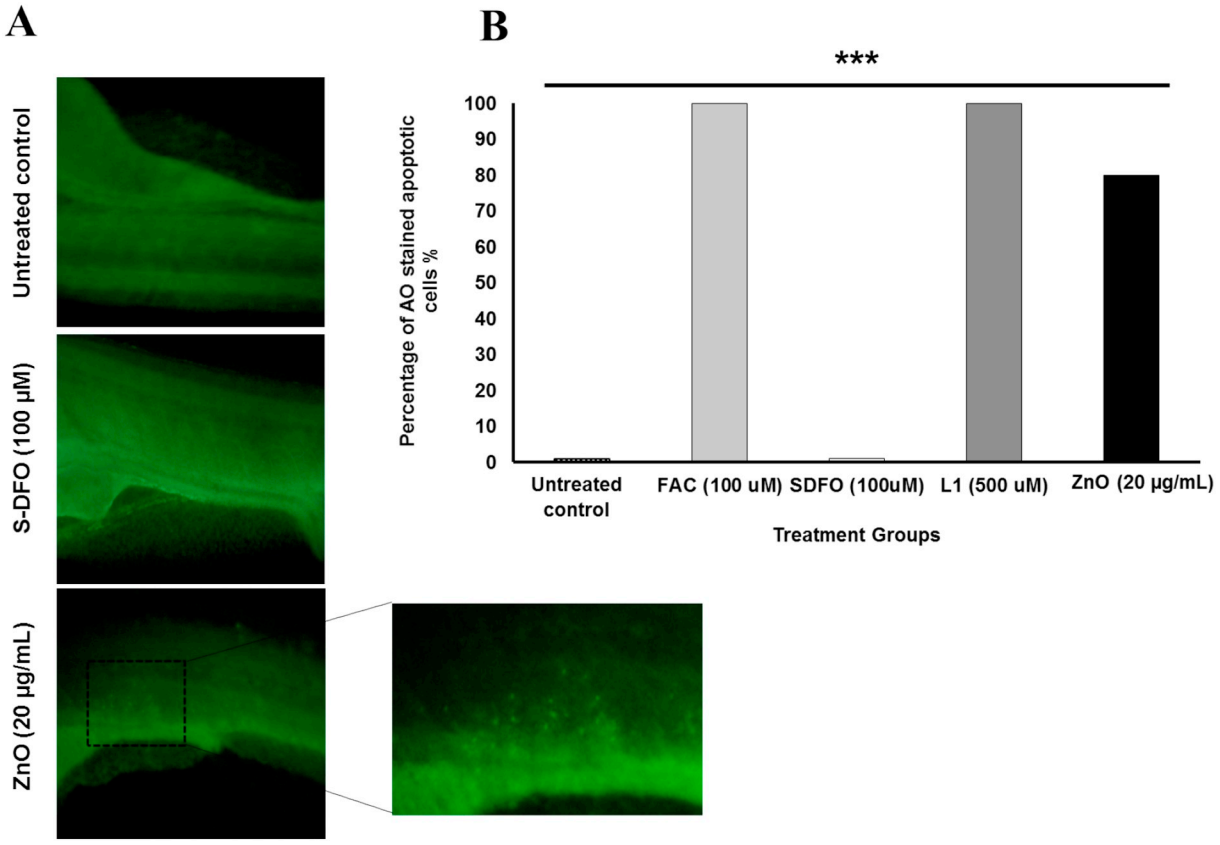

Fig. 6. Cellular death assay for the detection of apoptosis. A) Representative images of AO staining. Embryos were stained with 100 µL of 5 µg/ml of AO after being treated for 3 days from 72 to 144 hpf for every treatment condition. Bright green patches within the body area of the zebrafish embryos (magnification × = 125) indicate the presence of apoptotic cells. (A) Untreated and S-DFO showed no apoptosis. ZnO 20 µg/mL showed apoptosis (boxed area). B) Percentage of AO stained apoptotic cells in zebrafish embryos at 72 h post treatment. ***$p \leq 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

chelation therapy. Clinically approved iron chelators must be infused 2–3 times subcutaneously in case of DFO, or given orally three times for L1 or one time for deferasirox (ICL-670) every day, to reduce toxic iron and extend life expectancy of transfusional iron overloaded patients. In certain circumstances, dose of chelation therapy is increased to produce the maximum desirable outcome, which results in poor adherence and tolerance of patients to the course of treatment and increasing the probability to experience a wide range of toxic complications (Flora, 2013; Kontoghiorghes, 2006; Harmatz et al., 2007; Hamilton and Kizhakkedathu, 2015). In order to overcome this dilemma, effective long-acting and less toxic iron chelator would ensure rapid removal of excess iron and minimize the possible side effects of iron overload and chelation drugs (Kontoghiorghes, 2006; Rossi et al., 2009; Hamilton and Kizhakkedathu, 2015). The primary goal of this study was to demonstrate whether the polymeric chelator S-DFO would serve as a promising and safe drug in mobilizing iron with better performance and lower toxicity. A number of toxicity screening assays were conducted on zebrafish embryos to show that S-DFO could be intended for further experimental and clinical trials.

Toxicity assessment of substances using zebrafish has many advantages over other vertebrate models particularly evaluation of developmental morphology. Zebrafish has small size, high fecundity with early and rapid development and visual lucidity that make it favorable model for toxicological screening and characterization (Hill et al., 2005).

Our findings showed that S-DFO did not induce any death nor morphological malformations, or developmental delay to zebrafish embryos, even when exposure time is prolonged up to 72 h and concentration reached to 1.0 mM. Hamilton and coworkers investigated the toxicity of 3 clinically approved iron chelators, documented that no significant mortality was observed in zebrafish embryos treated with DFO and L1 from 0.015 to 1.0 mM after 72 hpf of exposure, however ICL-670 started to cause significant mortality at 1 mM after 24 hpf and at 96 hpf with 0.25 mM (Hamilton et al., 2014). In the current study, S-DFO leads to zero % mortality in all concentrations tested, while other chelators caused mortality with varying rates. Therefore, the approximately calculated LC50 (lethal concentration, 50%) was very high supporting that S-DFO has less toxic effects to human.

Natural ability of zebrafish embryos to hatch from their chorion at around 48–72 hpf is an important endpoint to be screened in developmental toxicological studies. The chemical compounds that most likely to influence hatchability could either delay, accelerate, or inhibit completely the process of hatching (Mccollum et al., 2011; Basnet et al., 2017). S-DFO treated embryos succeeded to hatch into larvae with no difference noted between S-DFO group and normal embryos. S-DFO effect on hatching shared similar findings with Hamilton study for DFO and L1. On the other hand, embryos in ICL-670 iron chelator had reduced hatching rate due to increased lethality (Hamilton et al., 2014).

Zebrafish exhibit a unique behavioral locomotive activity, which results from a network of signals between neural system (brain and spinal cord) and muscular system. Zebrafish embryos begin a powerful spontaneous pattern of movement within their chorions early at around 19–26 hpf. Later on after hatching, larvae start to swim (Basnet et al., 2017). Zebrafish central nervous system shared a conserved molecular, cellular and morphological phylogenetic background with other vertebrates including human. Thus, zebrafish has been used as an *in vivo* model for behavioral and pharmacological characterization of many chemicals and drugs and how they affect the neuromuscular function

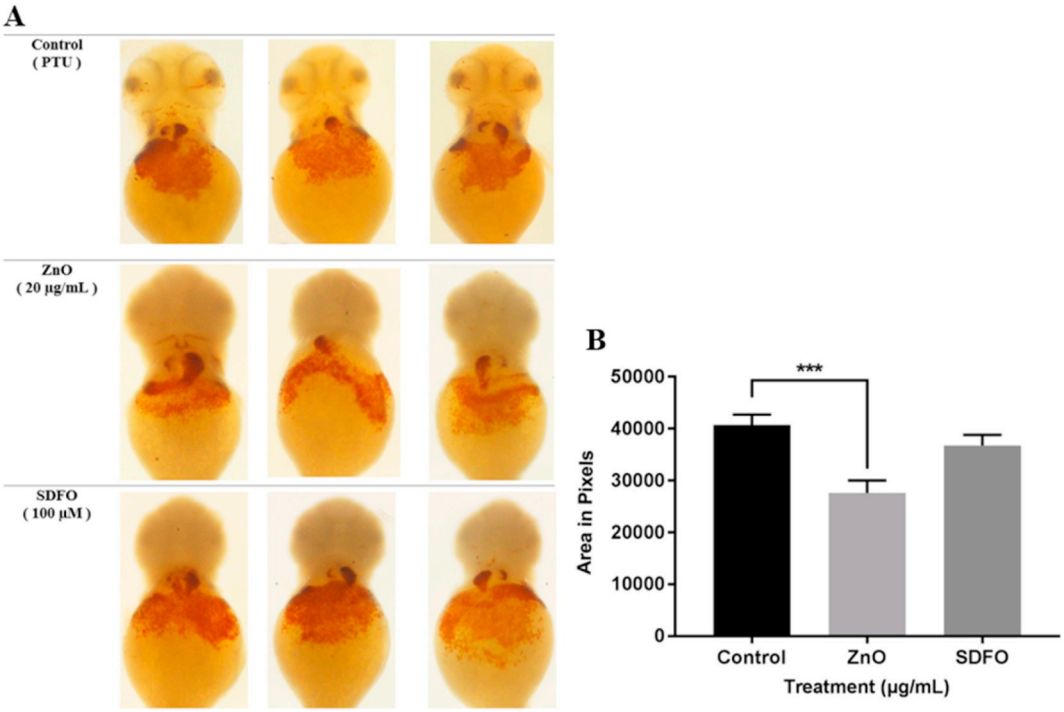

Fig. 7. The amount of hemoglobin synthesized by the hemoglobin-positive RBCs was measured by *o*-dianisidine staining at 72 hpf post treatment. (A) Representative images of o-dianisidine stain concentrated on the yolk sac of the negative control (PTU), positive control ZnO nanoparticles and the S-DFO. The images were captured using a bright field microscopy, at 50×. (B) The graph demonstrates the difference in the number of hemoglobin-positive RBCs with regards to difference in the areas stained by *o*-dianisidine. No significant difference was observed between the PTU negative control and S-DFO treated groups. 10-embryos were used per each treatment.

(Cario et al., 2011). Several studies evaluated the impact of certain drugs and chemicals on zebrafish movement at different time points using variety of analysis techniques (Mccollum et al., 2011; Basnet et al., 2017; Chen et al., 2011; Duan et al., 2013). In the current study, we utilized DanioScope Software to analyze tail flicking behavior of zebrafish embryos at 24 hpf, and we found that S-DFO did not alter the embryos functional locomotor capability. Polson and his colleagues utilized DFO on rheumatoid arthritis patients, who are not responding to conventional treatments, to chelate iron that may be a cause of the oxidative damage and inflammation occurring in synovial membranes. Among some patients involved in their study, DFO had shown a neurotoxic effects including nausea, vomiting and visual disturbance when it was in free form. However, when it was bound to iron, the toxicity decreased (Polson et al., 1985). For that reason, it is very crucial to

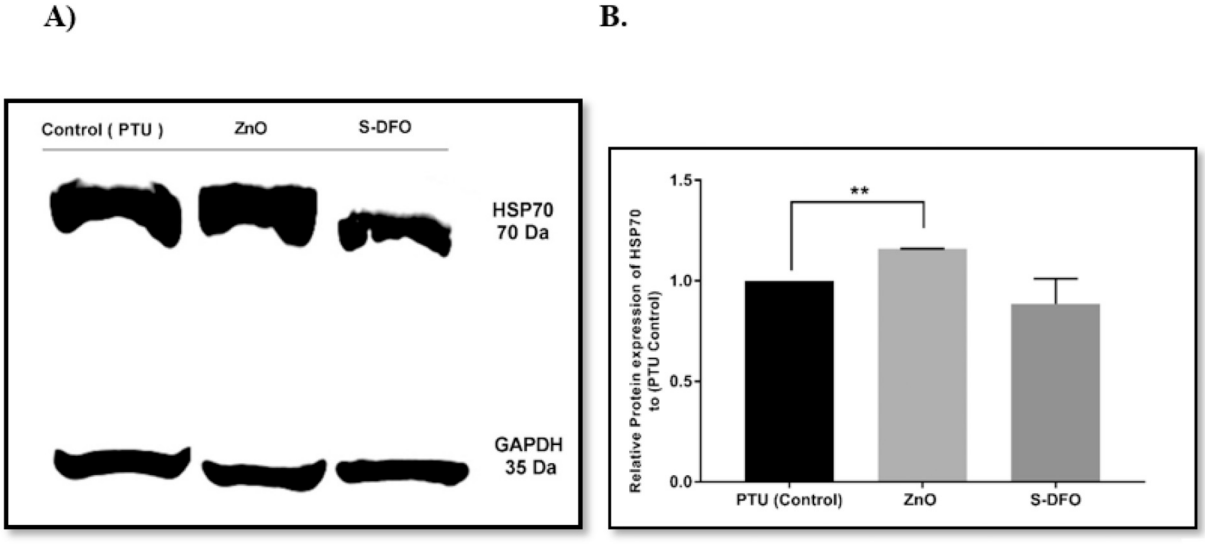

Fig. 8. Heat Shock Protein 70 (Hsp70) after treatment with S-DFO. A) Level Hsp70 expression by western blot from whole embryo embryos treated with PTU (negative control), ZnO (positive control), and S-DFO (tested compound). glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the loading control. B) Representative densitometry analysis of western blot for the expressions of Hsp70 in different zebrafish embryo treated samples and normalized with GAPDH loading control levels. Densitometry analysis of expressed Hsp70 was performed using ImageJ software and normalized by the PTU zebrafish-treated control level (equal to 1). Data were represented as the mean ± SD of three bands. One representative band from each treatment was shown. $^{**}p < 0.001$.

characterize if the toxicity manifested by organism is because of the drug itself, the mechanism of how iron is chelated, chelation of other vital trace elements, or formation of toxic compound when being iron bounded (Cuatrecasas et al., 1995).

Exposure to certain levels of toxic substances can disturb normal physiology of cells and accelerate cellular death (Sorrells et al., 2013). Different assays and tools have been introduced and established for the detection of cells undergoing apoptosis. DNA diffusion assay is one of the molecular technique that uses YOYO- 1 fluorescent dye to stain DNA (Singh, 2000). Another assays that use whole mount analysis of zebrafish are TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) and Caspase3 immunofluorescence assay (Cuatrecasas et al., 1995). A third whole mount technique is AO staining, which is considered a simple and straightforward assay that stains live embryo to easily detect apoptotic cells (Hu et al., 2011; Duan et al., 2013; Sorrells et al., 2013). We demonstrated the advantages of S-DFO as it did not stimulate progression of cell apoptosis. This is suggested to be due to its high molecular weight (HMW), which prevented it from being taken up by many cells unlike low molecular weight (LMW) compounds. Consequently, it will minimize access to other essential trace elements that could be un-specifically chelated by S-DFO. It is well documented that LMW chelators exerted more toxic effects compared to HMW drugs (Hamilton and Kizhakkedathu, 2015). L1 treated embryos in the present study exhibited high percentage of apoptosis at 500 μM after 72 h of exposure, which is consistent with the previous information mentioned above.

Market withdraw of a drug because of unexpected toxic adverse effects is every pharmaceutical executive's nightmare. Current protocols for toxicity assessment a drug candidate during development include histopathological assessments and biochemical measurements for different organ, particularly the liver. However, the disadvantage with these methods is that they are not sensitive enough. Therefore, a more sensitive molecular approach is essential to investigate whether a potential drug would be toxic at early developmental process. It is known that toxic drugs could cause variations in biochemical parameter values. Selection sets of biomarkers carefully allow an accurate assessment of drug-induced toxicity (Collins et al., 2012). Measuring the level of gene expression at RNA or protein level in response to drug should provide comprehensive picture about particular drug toxicity. In our study, we measured the level of Hsp70 protein as a general stress indicator. We also assessed the level of hemoglobin as biochemical marker and as an indirect indicator of RBC activity. We could have used other organs specific marker to assess organ specific toxicity. For example, in addition to heart rate assessment, measuring the level of vascular endothelial growth factor receptor 2 (VEGFR2) expression by western blot could provide a complete picture about cardiotoxicity (Zakaria et al., 2018). Imaging using, specific reporter zebrafish line, could be use as another approach to study toxicity at molecular level. For example, lever toxicity assessment, which is an essential tool to study drug-induce toxicity, cytochrome p450 reporter line, Tg(cyp1a:nls-gfp), could be used for this purpose (Kim et al., 2013).

5. Conclusion

This study clearly identified that lethal dose of polymeric drug S-DFO cannot be exactly determined and characterized that it had no toxic impacts on developmental morphology, hatchability, locomotor activity and cellular death of zebrafish embryos. S-DFO did not cause any movement defect and consequently it was not a neuromuscular toxic drug. Our findings in the zebrafish model provided assurance of safety and exhibited no developmental toxicity of S-DFO drug especially during early stages of development (during pregnancy and fetal development). This will allow accurate evaluation and management of transfusional iron overload and provide safe alternative to patients treated with iron chelators.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Conflict of interest and sources of funding

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ntt.2019.01.004.

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