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Aryl phosphate ester-induced pericardial edema in zebrafish embryos is influenced by the ionic composition of exposure media

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Abstract

Pericardial edema – fluid accumulation within the pericardium – is a frequently observed malformation in zebrafish embryo-based chemical toxicity screens. We recently discovered that the severity of triphenyl phosphate (TPHP)-induced pericardial edema was dependent on the ionic strength of exposure media. TPHP is an aryl phosphate ester (APE) widely used as a plasticizer and flame retardant. APEs are characterized by having one or more aryl groups bound to a phosphate center, with TPHP containing only unsubstituted aryl groups. Therefore, the objective of this study was to begin investigating whether, similar to TPHP, pericardial edema induced by other structurally related APEs is dependent on the ionic composition of exposure media. We first mined the peer-reviewed literature to identify other APEs that 1) induced pericardial edema in zebrafish embryos within a minimum of three peer-reviewed publications, and 2) demonstrated a statistically significant induction of pericardial edema in at least 70 % of the studies evaluated. Based on this meta-analysis, we identified four other APEs that caused pericardial edema in zebrafish embryos: isopropylated triphenyl phosphate (IPTPP), cresyl diphenyl phosphate (CDP), tricresyl phosphate (TMPP), and 2-ethylhexyl diphenyl phosphate (EDHPHP). Using TPHP as a positive control and pericardial edema as a readout, we developed concentration-response curves

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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.

CRediT authorship contribution statement

John Hoang: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Jenna Wiegand:** Methodology, Investigation. **Zoe Mersman:** Methodology, Investigation. **Kevin Michalicek:** Methodology, Investigation. **Nicholas Jimenez:** Methodology, Investigation. **David C. Volz:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Supplementary materials

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for all four APEs based on static exposure from 24 to 72 h post-fertilization (hpf). We then conducted co-exposures with D-Mannitol (an osmotic diuretic) and exposures within reverse osmosis (RO) water determine whether the ionic composition of exposure media mitigated APE-induced pericardial edema at 72 hpf. Using pericardial edema as an endpoint, the approximate EC_{50}s for TPHP (positive control), IPTPP, CDP, TMPP, and EDHPHP were 6.25, 3.125, 3.125, 25, and 100 μM, respectively, based on exposure from 24 to 72 hpf. Interestingly, similar to our findings with TPHP, co-exposure with D-Mannitol and exposure within ion-deficient water significantly mitigated IPTPP- CDP-, TMPP-, and EDHPHP-induced pericardial edema in zebrafish embryos, suggesting that chemically-induced pericardial edema may be 1) dependent on the ionic composition of exposure media and 2) driven by a disruption in osmoregulation across the embryonic epidermis. Therefore, similar to other assay parameters, our findings underscore the need to standardize the osmolarity of exposure media in order to minimize the potential for false positive/negative hits in zebrafish embryo-based chemical toxicity screens conducted around the world.

Keywords

Zebrafish embryos; Pericardial edema; Osmoregulation; Aryl phosphate ester

1. Introduction

Organophosphate esters (OPEs) are a class of chemicals characterized by the presence of a central phosphate moiety with alkyl or aryl substituents bound to the center with ester bonds (Greaves and Letcher, 2017; van der Veen and de Boer, 2012). Within OPEs, there are three broad groups of phosphate esters that are categorized by their structural features (Greaves and Letcher, 2017; van der Veen and de Boer, 2012; Blum et al., 2019). OPEs containing one or multiple halogens are classified as halogenated OPEs (Greaves and Letcher, 2017; van der Veen and de Boer, 2012; Blum et al., 2019). Chlorine is a common halogen found within halogenated OPEs, so the class is alternatively referred to as chlorinated phosphate esters (Blum et al., 2019). Some common chlorinated phosphate esters include tris(1-chloro-2-propyl) phosphate (TCIPP) and tris(1,3-dichloro-2-propyl) phosphate (TDCIPP). The other two categories of OPEs are defined by substituents bound to the phosphate center (Greaves and Letcher, 2017; van der Veen and de Boer, 2012; Blum et al., 2019). If moieties connected to the phosphate center are only alkyl groups, these OPEs are known as alkyl phosphate esters (Blum et al., 2019). Tributyl phosphate (TBP) and tris(2-butoxyethyl) phosphate (TBEP) are notable examples of the alkylated subclass. The final class of OPEs is characterized by having one or more aryl groups bound to the phosphate center (Blum et al., 2019). This class is known as aryl phosphate esters (APEs), and triphenyl phosphate (TPHP) is a representative chemical of the class due to the compound containing only unsubstituted aryl groups (van der Veen and de Boer, 2012; Blum et al., 2019). Some other examples of APEs include cresyl diphenyl phosphate (CDP), 2-ethylhexyl diphenyl phosphate (EDHPHP), isopropylated triphenyl phosphate (IPTPP), and tricresyl phosphate (TMPP). Halogenated phosphate esters are primarily used as flame retardants, whereas non-halogenated phosphate esters are also used as plasticizers and lubricants in addition to

flame retardants (Greaves and Letcher, 2017; van der Veen and de Boer, 2012; Blum et al., 2019).

Zebrafish is a widely used model for developmental toxicology due to its transparency during early *ex utero* development, providing a means to monitor and track embryogenesis in real time using microscopy (Roper and Tanguay, 2018). Due to these advantages, zebrafish embryos have emerged as frequently used model organisms in chemical toxicity screens. Within zebrafish embryo-based chemical screens, pericardial edema – fluid accumulation within the pericardium region – is a commonly observed and quantified phenotype (Hill et al., 2004; McGee et al., 2013; Yozzo et al., 2013). Despite being frequently measured, the mechanisms underlying pericardial edema in zebrafish embryos are still not fully understood (Hill et al., 2004). Prior studies have proposed a range of possible initiating events that lead to pericardial edema, such as renal failure, internal ion disturbances, and membrane permeability abnormalities (Hill et al., 2004). Other studies have suggested that pericardial edema is driven by a stress response with no underlying initiating event (Brotzmann et al., 2022).

Previous studies from our lab found that TPHP – an APE commonly used as a plasticizer and flame retardant – reliably induces pericardial edema formation in zebrafish embryos in a concentration-dependent manner (McGee et al., 2013; Isales et al., 2015; Mitchell et al., 2018; Reddam et al., 2019; Wiegand et al., 2022, 2023a, 2023b). Interestingly, the severity of TPHP-induced pericardial edema within zebrafish embryos was significantly mitigated when co-exposed with D-Mannitol – an osmotic diuretic that increases the osmolarity of the surrounding media – or exposed in ion-deficient water (Mitchell et al., 2018; Wiegand et al., 2022, 2023a, 2023b). Relative to exposures conducted in system water or embryo media, measured embryonic doses of TPHP were identical when co-exposed with D-mannitol or exposed in ion-deficient water, suggesting that 1) TPHP partitioning into embryos was not affected by the ionic composition of exposure media and 2) edema may related to disruption of osmoregulation across the embryonic epidermis (Wiegand et al., 2022, 2023a, 2023b). Prior to differentiation of gills at ~14 days post-fertilization (dpf), zebrafish embryos and larvae rely on the epidermis to maintain homeostasis and facilitate water and ion exchange through aquaporins and ionocytes localized to the skin (Rombough, 2007; Guh et al., 2015). Zebrafish ionocytes are analogous to mammalian kidney cells with respect to structure, function, and developmental processes, highlighting their importance and applicability as a model to study kidney physiology and development (Guh et al., 2015; Chang and Hwang, 2011). As such, the epidermis, especially during early embryonic development, plays an important role in maintaining the internal ionic and water balance due to the presence of ionocytes and aquaporins (Guh et al., 2015; Chang and Hwang, 2011).

The objective of this study was to determine whether OPE-induced pericardial edema may, similar to our prior findings with TPHP, be driven by disruption of osmoregulation across the embryonic epidermis. To accomplish this objective, we first conducted a literature search and meta-analysis to identify OPEs that reliably induce pericardial edema in zebrafish embryos. Second, we confirmed whether OPEs identified from the meta-analysis induced pericardial edema in zebrafish embryos in a concentration-dependent manner following a 48-h static exposure from 24 to 72 h post-fertilization (hpf). Finally, we determined

whether D-mannitol and ion-deficient media mitigated OPE-induced pericardial edema. To our knowledge, this is the first study to examine how modulation of the ionic composition of exposure media may impact a phenotype commonly measured in chemical screens involving zebrafish embryos on a chemical subclass basis. As such, our study further investigates the potential need for standardization of the ionic strength of exposure media, a variable that is commonly overlooked in zebrafish embryo-based chemical toxicity screens.

2. Materials and methods

2.1. Animals

Based on previously described procedures (Mitchell et al., 2018), wild-type adult (5D strain) zebrafish were maintained and bred on a recirculating system according to Institutional Animal Care and Use Committee (IACUC)-approved animal use protocols (#20210027 and #58) at the University of California, Riverside.

2.2. Identification of APEs

Based on a literature review of journal articles in the peer-reviewed literature, a meta-analysis and tiered strategy was employed to identify OPEs that reliably induce pericardial edema in zebrafish embryos (Fig. 1A). The criteria of the meta-analysis and tiered strategy included 1) OPE was tested in a chemical screen involving zebrafish embryos, 2) OPE induced pericardial edema following exposure, 3) OPE was examined in a minimum of three experiments, 4) OPE reproducibly induced pericardial edema in 70 % of experiments, and 5) OPE was an APE.

2.3. Chemicals

TPHP (99.5 % purity), TMPP (99 % purity), and D-mannitol (>98 % purity) were purchased from ChemService, Inc. (West Chester, PA, USA), Thermo Scientific Chemicals (Fairlawn, NJ, USA) and Bio-Techne Corp. (Minneapolis, MN, USA), respectively. CDP (96 % purity), EDHPHP (90 % purity), and IPTPP (40–70 % purity) were purchased from Toronto Research Chemicals (North Yolk, ON, CA). Using previously described procedures (Wiegand et al., 2023a), stock solutions of APE (TPHP, TMPP, CDP, EDHPHP, and IPTPP) were prepared by dissolving each chemical within liquid chromatography-grade dimethyl sulfoxide (DMSO) in 2-mL glass amber vials with polytetrafluoroethylene-lined caps. EDHPHP and IPTPP stock solutions were stored at 4 °C while TPHP, TMPP, and CDP were stored at room temperature. Based on previously described procedures (Wiegand et al., 2023a), working solutions were prepared by spiking vehicle (100 % DMSO) or stock solutions of APE (TPHP, CDP, EDHPHP, IPTPP, or TMPP) into particulate-free water from our recirculating system (pH and conductivity of ~7–8 and ~900–1000 μS, respectively). Final concentrations of working solutions were 0.1 % DMSO across all treatments. D-mannitol was stored at room temperature in the original container from the vendor. Solutions with D-mannitol were prepared on the day of the exposure by dissolving powder into water from our recirculating system.

2.4. APE concentration-response exposures

Using previously described procedures (Wiegand et al., 2023a), freshly spawned and fertilized eggs were collected and incubated in groups of approximately 100 per 100 × 15 mm polystyrene petri dish until 24 h post-fertilization (hpf) within a light- and temperature-controlled incubator. Viable embryos were then transferred to 100 × 15 mm polystyrene petri dishes containing 10 ml of a working solution of either vehicle (0.1 % DMSO) or APE (TPHP, CDP, EDHPHP, IPTPP, or TMPP at 3.125, 6.25, 12.5, 25, or 50 μM), resulting in 30 initial embryos per dish (three replicate dishes per treatment). Additional concentrations were required for CDP (0.78 and 1.56 μM), EDHPHP (100, 200, and 300 μM), and IPTPP (0.78 and 1.56 μM) to quantify the severity of pericardial edema formation. Higher concentrations were required for EDHPHP as lower concentrations did not induce pericardial edema formation. As all tested concentrations induced pericardial edema for CDP and IPTPP, lower concentrations were necessary to identify the threshold that did not result in a significant increase in pericardial area. All dishes were covered with a lid and incubated at 28 °C with a 14-h:10-h light-dark cycle until 72 hpf under static conditions. At 72 hpf, embryos were pooled into 1.5-mL microcentrifuge tubes (one tube per treatment replicate), fixed overnight at 4 °C in 4 % paraformaldehyde in 1X phosphate-buffered saline (PBS), transferred to 1X PBS, and stored at 4 °C for no longer than one month until imaging using previously described procedures (Wiegand et al., 2023a). Embryos were then transferred to individual 0.17-mm wells in black 384-well glass-bottom microplates (Matrice Bioscience, Spokane, WA, USA), centrifuged for 5 min at 150 rpm, and imaged under transmitted light on our ImageXpress Micro XLS Widefield High-Content Screening (HCS) System within MetaXpress 6.0.3.1658 (Molecular Devices, Sunnyvale, CA, USA). Body length, pericardial area, and yolk sac area were manually quantified within MetaXpress using images acquired by the ImageXpress Micro XLS Widefield HCS System (Figure S1). We only quantified body length, yolk sac area, and pericardial area from embryos that were in focus and positioned in right or left lateral recumbency, resulting in varying sample sizes.

2.5. D-mannitol co-exposures

Using procedures described above, 24-hpf embryos were exposed to 10 ml of either vehicle (0.1 % DMSO), 250 mM D-mannitol, APE (6.25 μM TPHP, 3.125 μM CDP, 100 μM EDHPHP, 3.125 μM IPTPP, or 25 μM TMPP), or APE + 250 mM D-mannitol in 100 × 15 mm polystyrene petri dishes until 72 hpf under the same conditions described above. D-mannitol concentrations were chosen based on previously published studies (Mitchell et al., 2018; Reddam et al., 2019; Wiegand et al., 2022, 2023a, 2023b). All tested APE concentrations (~EC_{50}) were based on nominal concentrations that induced pericardial edema formation in zebrafish embryos at a similar frequency and severity. 72-hpf embryos were processed and imaged/quantified for body length, pericardial area, and yolk sac area as described above. Using a handheld conductivity meter, we also quantified the conductivity of system water and system water + 250 mM D-mannitol (Figure S2).

2.6. Ionic strength exposures

Using the procedures described above, 24-hpf embryos were exposed to 10 ml of either vehicle (0.1 % DMSO) or APE (6.25 μM TPHP, 3.125 μM CDP, 100 μM EDHPHP, 3.125

μM IPTPP, or 25 μM TMPP) in either reverse osmosis (RO) water or 2X embryo media (EM) within 100 × 15 mm polystyrene petri dishes until 72 hpf under the same conditions described above. Media types were chosen based on previously published studies (Wiegand et al., 2023a). Based on previously established protocols (Wiegand et al., 2023a), 2X EM was prepared by diluting a stock concentration of 60X EM within RO water. 60X EM was made in-house by dissolving 17.2 g NaCl, 0.76 g KCl, 2.9 g $CaCl_2{\cdot}2H_20$, and 4.9 g $MgSO_4{\cdot}7H_20$ into 1 L of RO water and then autoclaving the final solution prior to long-term storage at 4 °C (Brand et al., 2002). 72-hpf embryos were processed and imaged/quantified for body length, pericardial area, and yolk sac area as described above. Using a handheld conductivity meter, we also quantified the conductivity of RO water and 2X EM (Figure S2).

2.7. Statistics

All data were analyzed within IBM SPSS Statistics 29.0.1.1 using a general linear model (GLM) analysis of variance (ANOVA) ($\alpha = 0.05$) and Tukey-based multiple comparisons. A GLM ANOVA was performed as it does not require the data to be normally distributed nor have equal sample sizes and variances between treatment groups. In addition, a GLM ANOVA provides the ability to analyze raw data without the need for data transformation. Within each GLM ANOVA, we set treatment as a fixed factor and replicate as a random factor since individual embryos were coded by treatment and replicate.

3. Results

3.1. Aryl phosphate esters are more likely to induce pericardial edema in zebrafish embryos

A tiered strategy was employed to identify OPEs that reliably induce pericardial edema (Fig. 1A). Based on studies published in the peer-reviewed literature, 34 initial OPEs were identified from zebrafish embryo chemical screens that measured chemically induced pericardial edema (Figure 1A; Table S1). Beginning with Stage 2 of the meta-analysis, 17 OPEs were found to induce pericardial edema formation within zebrafish embryos based on the peer-reviewed literature (Fig. 1A). 13 OPEs were examined in a minimum of three experiments within Stage 3 of the meta-analysis (Fig. 1A). When further examining the occurrence of pericardial edema in zebrafish embryos following exposure to OPEs, a total of 110 experiments were performed within the literature in which pericardial edema was a recorded endpoint (Fig. 2). Within these experiments, there were 58 incidences where pericardial edema was observed (Fig. 2A). Of the 58 positive occurrences, 43 were found to be a result of APE exposure, with the last 15 derived from alkyl and chlorinated phosphate ester exposures (Fig. 2B). With the negative occurrences, 32, 16, and 14 were derived from chlorinated phosphate ester, alkyl phosphate ester, and APE exposures, respectively (Fig. 2C).

Of the 13 OPEs, seven OPEs reproducibly induced pericardial edema in at least 70 % of the chemical exposures: triphenyl phosphate (TPHP), isopropylated triphenyl phosphate (IPTPP), 2-ethylhexyl diphenyl phosphate (EDHPHP), tricresyl phosphate (TMPP), tert-butylphenyl diphenyl phosphate (BPDP), cresyl diphenyl phosphate (CDP), and tris (1,3-dichloro-2propyl) phosphate (TDCIPP) (Fig. 2). Of these seven OPEs, TDCIPP (a

chlorinated phosphate ester) was the only chemical not within the same subclass, whereas the other six were APEs (Figs. 1B and 2). As such, we decided to proceed with testing these six APEs. However, BPDP was not commercially available, so we were only able to proceed with testing TPHP, IPTPP, EDHPHP, TMPP, and CDP.

3.2. CDP, EDHPHP, IPTPP, and TMPP induces pericardial edema formation in a concentration-dependent manner

To determine whether our previously observed results with TPHP are consistent with other APEs, it is important to confirm whether the APE will induce pericardial edema formation in zebrafish embryos following exposure from 24 to 72 hpf, and at what concentrations the phenotype would be observed. Using TPHP as a positive control, CDP, EDHPHP, IPTPP, and TMPP induced pericardial edema formation in a concentration-dependent manner following exposure from 24 to 72 hpf (Fig. 3). TPHP, CDP, EDHPHP, IPTPP, and TMPP induced pericardial edema formation beginning at 3.125 μM, 0.78 μM, 25 μM, 1.56 μM, and 12.5 μM, respectively (Fig. 3). TPHP, CDP, IPTPP, and TMPP also induced a significant concentration-dependent decrease in body length beginning at 6.25 μM, 6.25 μM, 3.125 μM, and 25 μM respectively (Figure S3). EDHPHP induced a significant increase in body length at 6.25 μM, 12.5 μM, and 200 μM as well as a significant decrease in body length at 300 μM (Figure S3). TPHP, CDP, IPTPP, and TMPP induced yolk sac edema beginning at 12.5 μM, 50 μM, 100 μM, 25 μM, and 50 μM – most of which were the highest concentrations in which apical measurements could be performed (Figure S4). EDHPHP induced a significant increase in yolk sac area in two of the three highest concentrations (100 μM and 200 μM) (Figure S4).

3.3. APE-induced pericardial edema is mitigated in the presence of D-mannitol

As all five APEs induced pericardial edema formation, we then determined whether D-mannitol mitigated pericardial edema. To ensure that experiments were comparable, chemical concentrations were selected based on pericardial edema severity relative to TPHP at approximately the EC_{50}, providing the ability to quantify an increase or decrease in edema formation in the presence of D-mannitol. Final concentrations for TPHP, CDP, EDHPHP, IPTPP, and TMPP were 6.25 μM, 3.125 μM, 100 μM, 3.125 μM, and 25 μM, respectively (Fig. 3). Relative to vehicle-exposed embryos, there were no significant changes in the yolk sac area across any of the treatment groups (Fig. 4). Consistent with our prior results, D-mannitol did not mitigate a TPHP-induced decrease in body length (Wiegand et al., 2023a) (Fig. 4). A TMPP-induced decrease in body length was also not mitigated in the presence of D-mannitol (Fig. 4). Interestingly, similar to TPHP-exposed embryos, co-exposure with 250 mM D-mannitol blocked APE-induced pericardial edema for all four APEs, resulting in pericardial areas that were similar to vehicle-exposed embryos even though the conductivity of system water vs. system water + *D*-mannitol was 912 μS and 757 μS, respectively (Figs. 4 and S2).

3.4. Decreasing the ionic strength of exposure media reduces the severity of APE-induced pericardial edema

Following D-mannitol co-exposures, zebrafish embryos were exposed to APEs at the same concentrations as the previous co-exposures in reverse osmosis (RO) water and 2X embryo

media (2X EM). TPHP and TMPP induced a significant decrease in body length regardless of the media type, whereas CDP, EDHPHP, and IPTPP did not alter body length in either media type (Fig. 5). There were no significant changes in the yolk sac area regardless of media type, except for TMPP-exposed embryos in RO water (Fig. 5). Previous results found that exposure to 5 μM TPHP from 24 to 72 hpf in RO water blocked pericardial edema formation compared to media containing ions (Wiegand et al., 2023a). In the current study, zebrafish embryos exposed to 6.25 μM TPHP, 3.125 μM IPTPP, and 25 μM TMPP in RO water resulted in a significant increase in pericardial area relative to vehicle-treated embryos in RO water, whereas zebrafish embryos exposed to 3.125 μM CDP and 100 μM EDHPHP in RO water were not significantly different relative to vehicle-treated embryos in RO water (Fig. 5). Embryos exposed to APEs in 2X EM resulted in a significant increase in pericardial area compared to vehicle-treated embryos in 2X EM (Fig. 5). Interestingly, when comparing pericardial area between the APE exposures in RO water vs. 2X EM, embryos exposed to APEs in RO water all resulted in a significant decrease in pericardial area compared to embryos exposed to APEs in 2X EM, a finding that was likely driven by a nearly 7.6-fold increase in conductivity in 2X EM (1747 μS) relative to RO water (230 μS) (Fig. 5 and S2).

4. Discussion

The results from this study are consistent with our prior results showing that TPHP induces pericardial edema following exposure from 24 to 72 hpf (McGee et al., 2013; Isales et al., 2015; Mitchell et al., 2018; Reddam et al., 2019; Wiegand et al., 2022, 2023a, 2023b). Interestingly, all five APEs tested within this study induced pericardial edema, suggesting that other APEs and aryl group-containing chemicals may also have the potential to induce pericardial edema in zebrafish embryos. When comparing potencies of the five APEs based on pericardial edema, EDHPHP, TMPP, TPHP, IPTPP, and CDP significantly induced pericardial edema at ≥25 μM, ≥12.5 μM, ≥3.125 μM, ≥1.56 μM, and ≥0.78 μM, respectively, suggesting that EDHPHP was the least potent and CDP was most potent based on static exposure from 24 to 72 hpf. Interestingly, EDHPHP only contains two aryl groups, whereas TMPP, TPHP, IPTPP, and CDP contain three aryl groups, suggesting that the number of aryl groups may be associated with potency. Moreover, all of the APEs tested within this study have similar octanol-water partitioning coefficients (4.51–5.73) (Renberg et al., 1980; Rosenberger et al., 2021), suggesting that differences in the rate of uptake from exposure media across the embryonic epidermis – and therefore differences in embryonic doses from 24 to 72 hpf – likely do not account for differences in APE-specific potencies.

APE-induced pericardial edema formation may be directly related to dysregulation of osmoregulation in the epidermis, suggesting that APEs may also impact osmoregulatory organs such as kidneys. In our studies, we found that co-exposure to D-mannitol, an osmotic diuretic, completely mitigated the formation of APE-induced pericardial edema across all five APEs tested. These data are consistent with previously published results with TPHP, TCDD, and isomer-specific TMPP (Mitchell et al., 2018; Wiegand et al., 2022, 2023a, 2023b; Hill et al., 2004; Yi et al., 2024). Moreover, our previous studies found that exposure of embryos to TPHP in RO water lacking ions mitigated TPHP-induced pericardial edema (Wiegand et al., 2023b). Similarly, in this study, the severity of TPHP-induced pericardial edema in RO water was significantly decreased relative to TPHP-induced pericardial

edema in 2X embryo media. This trend was consistent across the other four APEs, where pericardial area was significantly higher in 2X EM compared to RO water.

Our experiments with D-mannitol, 2X EM, and RO water suggest that, similar to TPHP, the ionic composition of exposure media is strongly associated with the formation and severity of pericardial edema induced by EDHPHP, TMPP, IPTPP, and CDP. Interestingly, the conductivity of 2X EM was approximately 7.6-fold higher than RO water, suggesting that higher concentration of ions within 2X EM was associated with an increase in the severity of pericardial edema. However, the conductivity within system water vs. system water + *D*-mannitol was similar even though D-mannitol completely mitigated APE-induced pericardial edema, suggesting that, similar to its mechanism within mammalian renal proximal tubules, D-mannitol inhibited reabsorption of water and/or sodium within the pericardial region in the absence of differences in conductivity within exposure media. Overall, these findings support the conclusion that APE-induced pericardial edema was a result of a disruption in osmoregulation across the embryonic epidermis. Other studies have also found that ions and salinity may influence chemically induced toxicity. For instance, increasing salinity induced greater developmental toxicity of silver nano colloids and cadmium on embryonic medaka and zebrafish, respectively (Kataoka and Kashiwada, 2016; Santos et al., 2021). Ion composition may be another factor that can impact chemically induced toxicity. Previous results demonstrated that chloride-containing salts in embryo media had the most significant effects on TPHP-induced pericardial edema (Wiegand et al., 2023a). Other studies have indicated that high calcium content within media induced developmental toxicity within Japanese medaka and fathead minnows (Kupsco et al., 2017; Goodfellow et al., 2000).

Previous studies have suggested that APEs are more toxic to zebrafish embryos than other subclasses of OPEs (Du et al., 2015; Shi et al., 2021; Truong et al., 2020). Within zebrafish embryos, these studies found that APEs induce severe cardiotoxicity, increase acetylcholinesterase inhibition, and are more bioactive compared to chlorinated and alkylated phosphate esters. Similarly, our meta-analysis and tiered strategy to prioritize OPEs for chemical testing revealed that APEs induced pericardial edema more frequently compared to chlorinated and alkylated phosphate esters. Moreover, based on the meta-analysis and our exposures within this study, we observed that six out of seven OPEs that reliably induced pericardial edema were APEs. Collectively, previous studies and our results suggest that the presence of aryl groups on APEs may be associated with increased developmental toxicity and frequency of pericardial edema formation (Du et al., 2015; Shi et al., 2021).

Increased developmental toxicity and pericardial edema formation in zebrafish are common phenotypes observed following exposure to zebrafish embryos with other aryl group-containing compounds. For example, numerous studies have found that polycyclic aromatic hydrocarbons (PAHs) such as benz[a]anthracene (BaA) and benzo[a]pyrene (BaP) reproducibly induce pericardial edema formation in zebrafish embryos (Incardona et al., 2006, 2011; Fang et al., 2022). Similarly, 2,3, 7,8-tetrachlorodibenzo-p-dioxin (TCDD), β-naphthoflavone, α-naphthoflavone, and polychlorinated biphenyl 126 (PCB-126) have been shown to induce developmental toxicity and increase the frequency of pericardial

edema in zebrafish embryos (Henry et al., 1997; Prasch et al., 2003; Billiard et al., 2006; Liu et al., 2016). Interestingly, chemically-induced pericardial edema by aryl group-containing compounds such as BaP and TCDD is often driven by activation of the aryl hydrocarbon receptor (AHR), where knockdown of AHRs lead to mitigation of pericardial edema (Incardona et al., 2006, 2011; Fang et al., 2022; Henry et al., 1997; Prasch et al., 2003; Billiard et al., 2006). Although the mechanism of chemically-induced pericardial edema is largely unknown, a potential reason why AHR activation may lead to edema is through increased vascular permeability following activation in endothelial cells (Prasch et al., 2003).

Contrary to studies with TCDD, we previously showed that TPHP-induced pericardial edema and cardiotoxicity were not mitigated by AHR2 knockdown (McGee et al., 2013). However, TPHP-induced pericardial edema was mitigated through pretreatment and/or co-exposure with fenretinide (a retinoic acid receptor agonist), suggesting that retinoid-mediated wound repair may be involved in rescuing the effects of TPHP (Mitchell et al., 2018; Reddam et al., 2019; Wiegand et al., 2023b). Indeed, as there are multiple initiating events that may lead to pericardial edema with different aryl group-containing chemicals, pericardial edema may result from epidermal injury due to aqueous exposure and chemical uptake across the embryonic epidermis (Wiegand et al., 2023a). The embryonic epidermis serves as the primary site of osmoregulation for embryonic zebrafish up until 14 dpf – the stage when ionocytes and aquaporins localize to the gills (Guh et al., 2015). Therefore, direct injury to the epidermis may prevent a rapidly developing zebrafish embryo from properly maintaining homeostasis, resulting in fluid accumulation and, ultimately, edema. However, limited studies are available that have determined whether the embryonic epidermis is the primary site of injury following exposure to aryl group-containing chemicals (Wiegand et al., 2023b). Alternatively, all of the tested chemicals within this study have been previously shown to disrupt lipid homeostasis within zebrafish embryos (Jin et al., 2024; Reddam et al., 2019; Xu et al., 2023; Yi et al., 2024; Zhang et al., 2024). Membrane permeability abnormalities has been a proposed mechanism related to pericardial edema occurrence and alterations in lipid homeostasis has the potential to interfere with membrane stability (Hill et al., 2004).

Currently, laboratories around the world rely on exposure media with varying ion compositions for zebrafish embryo-based chemical toxicity screens – a source of variability that has the potential to result in over- or underestimation of toxicity depending on the media used. Therefore, our results underscore the need to standardize exposure media utilized in zebrafish embryo-based chemical screens. While efforts have been made to standardize zebrafish embryo-based chemical screens and identify sources of variability within these screens, many of these efforts overlooked exposure media composition and ionic strength as areas to harmonize within chemical screens. For example, the OECD Fish Embryo Acute Toxicity (FET) test includes general water quality parameters that must be maintained throughout the exposure, such as water temperature and dissolved oxygen concentration (OECD, 2013). However, the only recommendation for exposure media and ionic composition is to reduce the hardness of maintenance water to levels between 100 and 300 mg/L $CaCO_3$ (OECD, 2013). Moreover, various zebrafish experts across the United States highlighted sources of variability within zebrafish embryo chemical screens and

did not identify the ionic composition and strength of exposure media as an important parameter for standardization (Hamm et al., 2019). In a follow-up study to this paper which compared toxicological endpoints between laboratories with varying exposure scenarios, the exposure media differed between the labs, which could be an additional factor that accounts for differences between results observed across all three labs (Hamm et al., 2024). Within the literature we analyzed for the meta-analysis, a variety of exposure media were used, ranging from embryo media to maintenance water to Hank's buffer. Collectively, our data demonstrate a critical need for embryo media composition and ionic strength to be considered during the standardization process of zebrafish embryo-based chemical screens.

It is important to also note the limitations within this study. For example, the wide range of purities of IPTPP (40–70 %) within this study may potentially misrepresent IPTPP's true toxicity. We found with 40–70 % purity that IPTPP induces pericardial edema formation in a concentration dependent manner beginning at 1.56 μM. However, a higher purity may result in increased toxicity due to the presence of isopropyl moieties attached to the aryl rings which is only found on IPTPP. As such, it will be important to repeat our experiments using a higher purity of IPTPP to confirm our results found here. Additionally, the sample size of this study is limited, and the results may not be fully applicable to all APEs or other fish models. As such, it will be important to reproduce our experiments, expand into other pericardial edema-inducing chemicals, and test within other fish models to confirm whether these results can be extrapolated to other chemicals and/or fish models.

5. Conclusions

To our knowledge, this is the first study that investigated how the ionic composition of exposure media may alter chemically-induced edema on a chemical class basis. First, we discovered that APEs have a higher potential to induce pericardial edema in zebrafish embryos compared to other subclasses of OPEs. Second, similar to TPHP, we found that CDP, EDHPHP, IPTPP, and TMPP all induced pericardial edema in a concentration-dependent manner. Third, we found that APE-induced pericardial edema was completely mitigated in the presence of D-mannitol, which increases the osmolarity of exposure media. Finally, we found that the severity of APE-induced pericardial edema was decreased when exposures were performed in ion-deficient media. Overall, our findings suggest that chemically-induced pericardial edema may be influenced by the ionic composition of exposure media and, as such, driven by disruption in osmoregulation across the embryonic epidermis. Therefore, similar to other assay parameters, our findings underscore the need to standardize the osmolarity of exposure media in order to minimize the potential for false positive/negative hits in zebrafish embryo-based chemical toxicity screens conducted around the world.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

Data will be made available on request.

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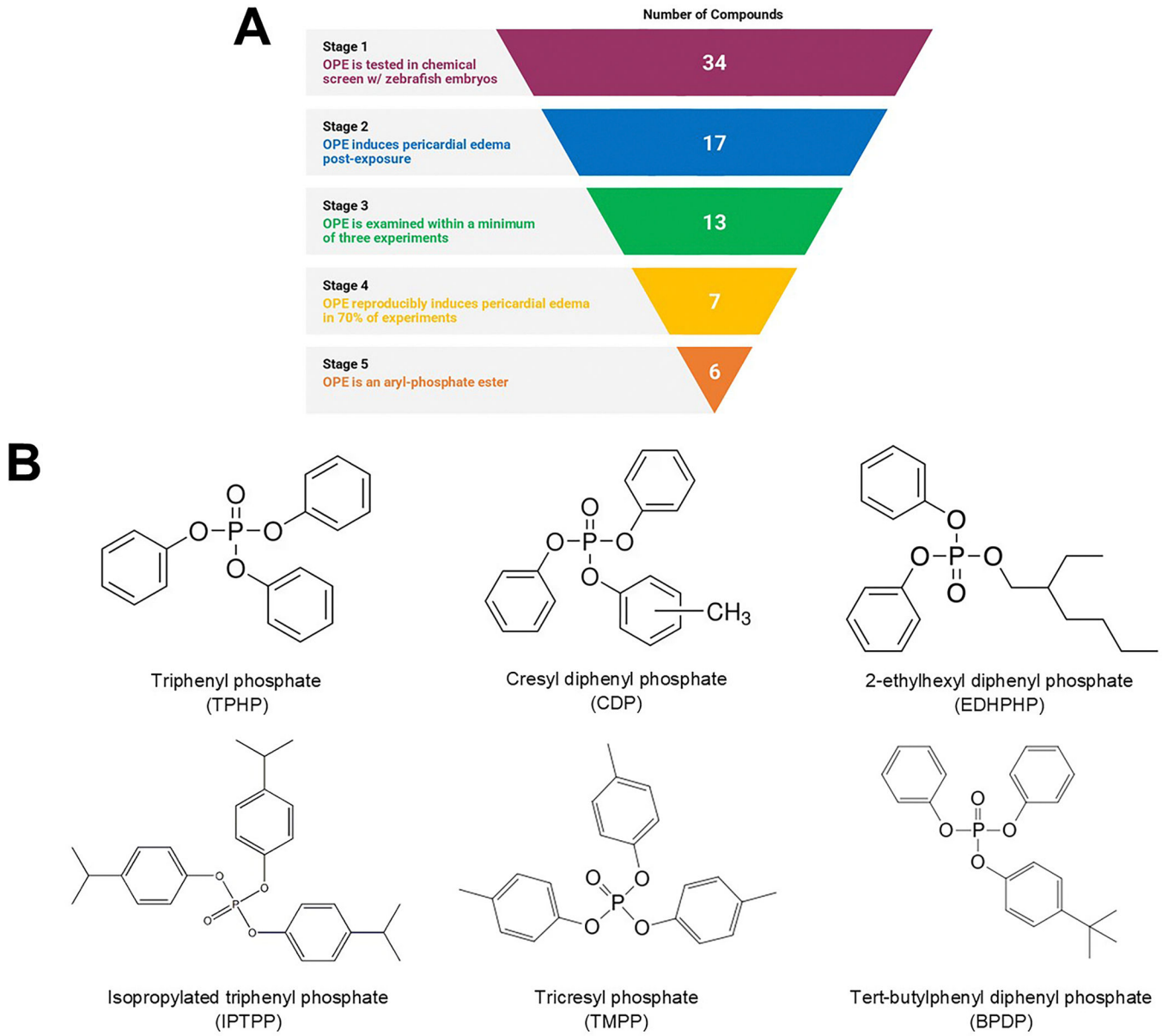

Fig. 1.

A) Tiered strategy for identification of organophosphate esters (OPEs) that reliably induce pericardial edema. 34 initial OPEs were identified from the peer-reviewed literature, and a series of stages were applied to prioritize OPEs that reliably induce pericardial edema. Based on these stages, six APEs were selected for additional testing. B) Chemical structures and names of the six APEs selected for additional testing based on Stage 5 of the tiered strategy.

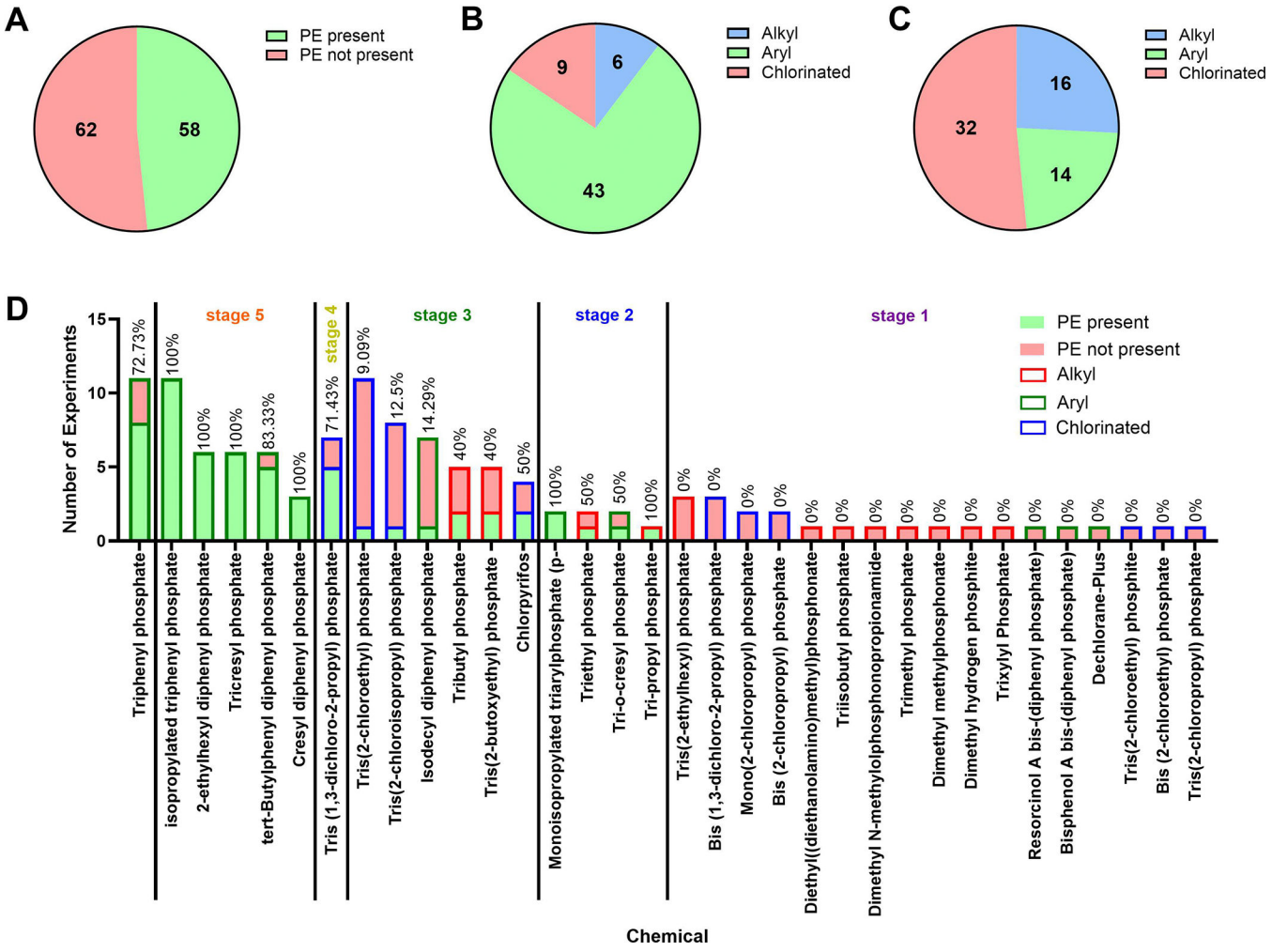

Fig. 2.

Meta-analysis of curated peer-reviewed papers focused on OPEs that reliably induce pericardial edema (PE). A) Total number of experiments that resulted in the occurrence or absence of pericardial edema in zebrafish embryos following OPE exposure. B) Total number of experiments that resulted in the occurrence of pericardial edema separated by OPE class. C) Total number of experiments that resulted in the absence of pericardial edema separated by OPE class. D) Chemical-organized representation of the number of experiments that resulted in either the occurrence or absence of pericardial edema separated by stages applied in Fig. 1. Stage 5 chemicals, including triphenyl phosphate (TPHP, the reference chemical in our experiments), were identified as APEs that reliably induce pericardial edema for additional testing.

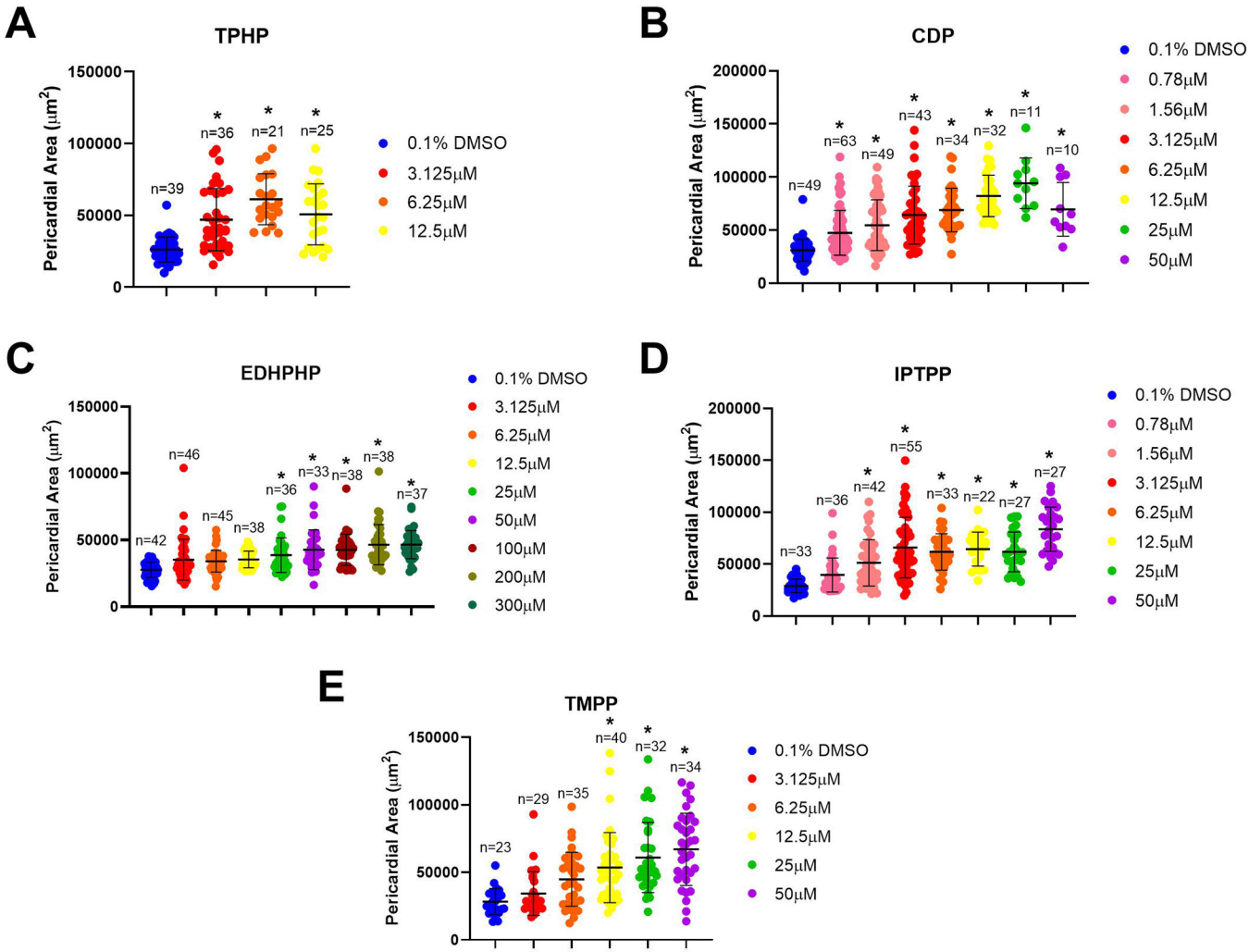

Fig. 3.

Mean pericardial area (± standard deviation) of embryos exposed to A) TPHP (3.125 μM - 12.5 μM), B) CDP (0.78 μM - 50 μM), C) EDHPHP (3.125 μM - 300 μM), D) IPTPP (0.78 μM - 50 μM), or E) TMPP (3.125 μM - 50 μM). All chemical treatments included embryos exposed to vehicle (0.1 % DMSO). Asterisk (*) denotes a significant difference ($p < 0.05$) relative to embryos exposed to vehicle controls.

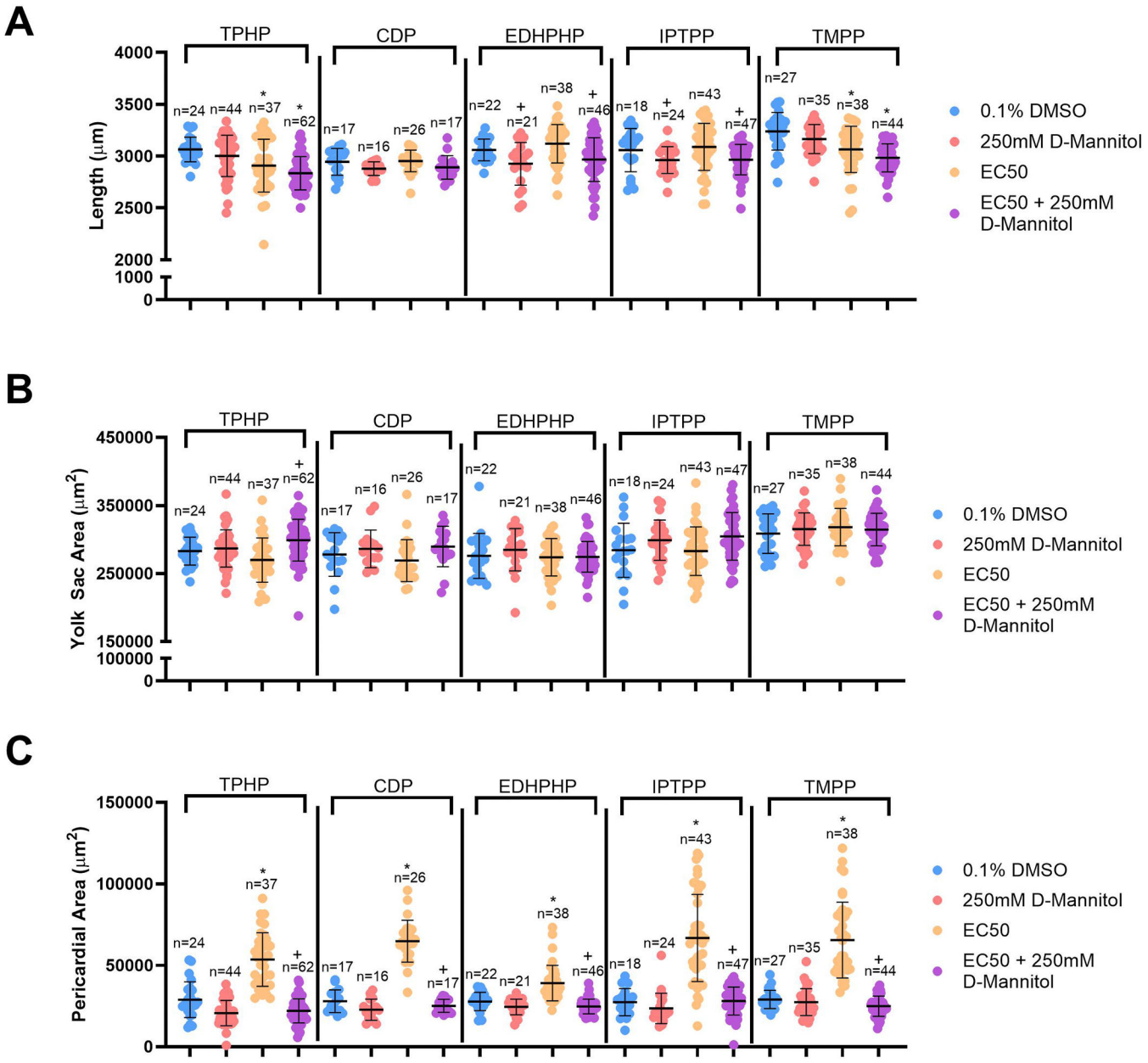

Fig. 4.

Mean (± standard deviation) of length (A), yolk sac area (B), and pericardial area (C) of embryos exposed to vehicle (0.1 % DMSO), 250 mM D-Mannitol, the approximate pericardial area-based EC_{50} of the respective APE (6.25 μM TPHP, 3.125 μM CDP, 100 μM EDHPHP, 3.125 μM IPTPP, or 25 μM TMPP), or the approximate pericardial area-based EC_{50} + 250 mM D-Mannitol. Plus sign (+) denotes a significant difference ($p < 0.05$) relative to the pericardial area-based EC_{50}, whereas asterisk (*) denotes a significant difference ($p < 0.05$) relative to embryos exposed to vehicle (0.1 % DMSO).

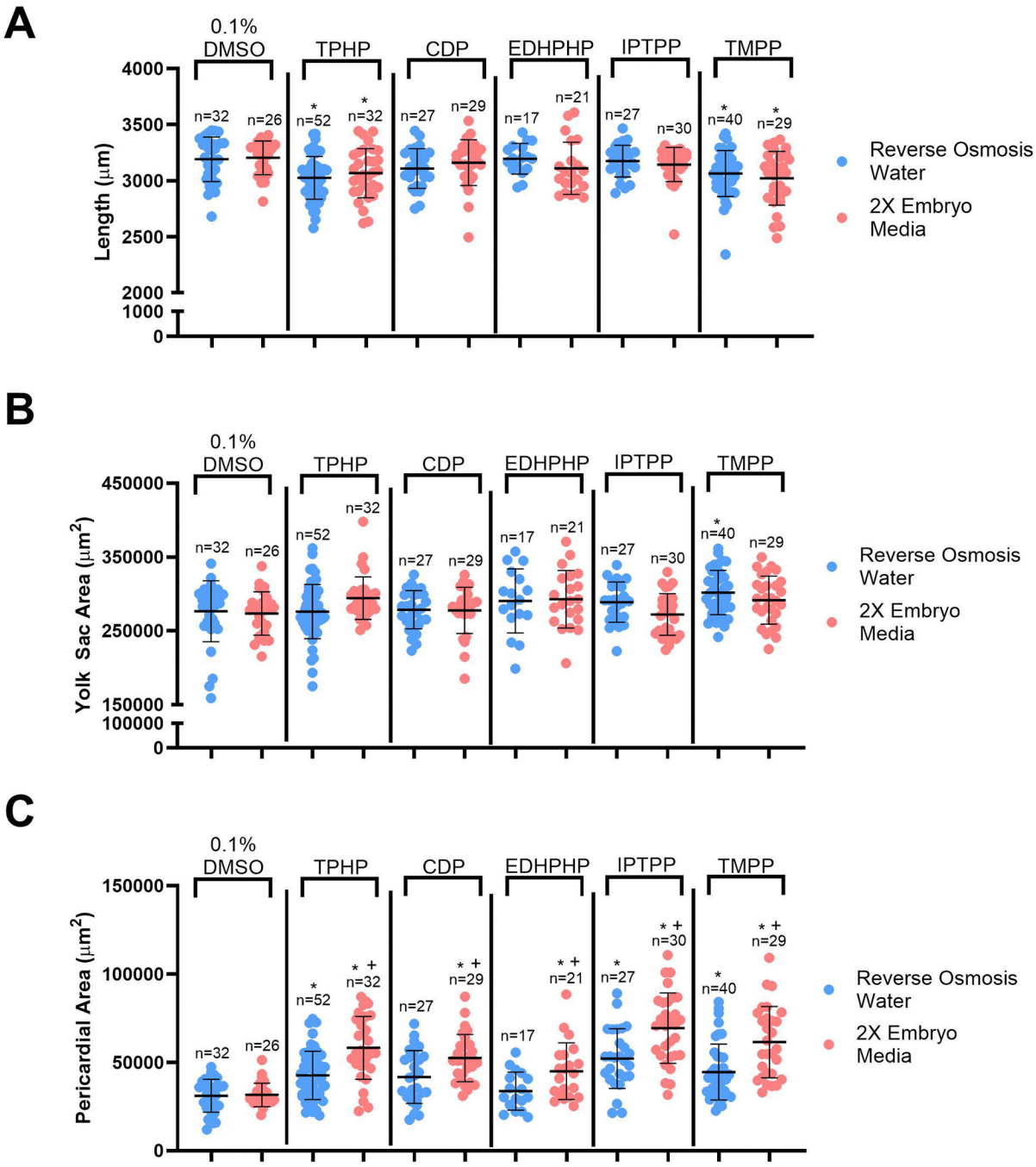

Fig. 5.

Mean (± standard deviation) of length (A), yolk sac area (B) and pericardial area (C) of embryos exposed to vehicle (0.1 % DMSO) or the approximate pericardial area-based EC_{50} of the respective APE (6.25 μM TPHP, 3.125 μM CDP, 100 μM EDHPHP, 3.125 μM IPTPP, or 25 μM TMPP) in Reverse Osmosis (RO) Water or 2X Embryo Media (EM). Plus sign (+) denotes a significant difference ($p < 0.05$) relative to the approximate pericardial area-based EC_{50} in RO water, whereas asterisk (*) denotes a significant difference ($p < 0.05$) relative to

embryos exposed to vehicle (0.1 % DMSO) within the same media group (RO water or 2X EM).