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Assessment of the in vitro developmental toxicity of diethylstilbestrol and estradiol in the zebrafish embryotoxicity test

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ABSTRACT

The present study investigated the developmental toxicity of diethylstilbestrol (DES) in the zebrafish embryotoxicity test (ZET). This was done to investigate whether the ZET would better capture the developmental toxicity of DES than the embryonic stem cells test (EST) that was previously shown to underpredict the DES-induced developmental toxicity as compared to in vivo data, potentially because the EST does not capture late events in the developmental process. The ZET results showed DES-induced growth retardation, cumulative mortality and dysmorphisms (i.e. induction of pericardial edema) in zebrafish embryos while the endogenous ERα agonist 17β-estradiol (E2) showed only growth retardation and cumulative mortality with lower potency compared to DES. Furthermore, the DES-induced pericardial edema formation in zebrafish embryos could be counteracted by co-exposure with ERα antagonist fulvestrant, indicating that the ZET captures the role of ERα in the mode of action underlying the developmental toxicity of DES. Altogether, it is concluded that the ZET differentiates DES from E2 with respect to their developmental toxicity effects, while confirming the role of ERα in mediating the developmental toxicity of DES. Furthermore, comparison to in vivo data revealed that, like the EST, in a quantitative way also the ZET did not capture the relatively high in vivo potency of DES as a developmental toxicant.

1. Introduction

Diethylstilbestrol (DES) is a synthetic hormone that was first prescribed in the period of 1938 to 1971 for pregnant women to prevent miscarriage and premature delivery, for menstrual problems and for cancer treatment (Herbst and Anderson 2015; Reed and Fenton 2013; von Schilling, 1980). However, while these claimed beneficial effects were not observed, adverse effects were reported including spontaneous abortion, second trimester pregnancy loss, preterm delivery, neonatal death, sub−/infertility and cancer of reproductive tissues in neonates, all pointing at developmental toxicity (IARC 2012; Newbold 2004; Reed and Fenton 2013). Subsequent in vivo animal studies confirmed the developmental toxicity of DES including the induction of embryonic death, resorptions and morphological changes in mice and rats (Cornwall et al. 1984; Nagao et al. 2013; Nagao and Yoshimura 2009; Odum et al. 2002).

It was also shown that the estrogen receptor alpha (ERα; ESR1) is involved in the mode-of-action underlying DES-induced malformations and phenotypic changes in the reproductive tract in male and female mice neonates, while this effect was not observed in ERα knockout mice (Couse et al., 2004; Couse and Korach 2004; Prins et al. 2001). The role of ERα (ESR1) in DES-mediated developmental toxicity was also confirmed in our recent in vitro developmental toxicity study in which DES tested positive in the ES-D3 cell differentiation assay of the embryonic stem cell test (EST) and this DES-induced inhibition of ES-D3 cell differentiation into beating cardiomyocytes could be counteracted by the ERα antagonist fulvestrant (Adam et al. 2019). However, when correlating EST data to in vivo developmental toxicity data for DES and other developmental toxicants, the EST appeared to largely underpredict the developmental toxicity of DES (Adam et al. 2019). Similarly, Zurlinden et al. (2020) found human embryonic stem cells (hESC) to underpredicted DES teratogenicity, an observation that is consistent with our findings in the EST. Thus, it was concluded that although the EST did capture ERα-mediated adverse developmental effects of DES in vitro, it apparently did not adequately capture all processes underlying DES-induced developmental toxicity. A possible explanation for this

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might be that the ES-D3 cell differentiation assay of the EST lacks the complex biological system and the metabolic capacity of an intact organism (Spielmann et al. 2006), and may only be suitable for detection of adverse effects in the early developmental stages (Pera and Trounson 2004).

Such late developmental effects may in theory be better reflected by the zebrafish embryotoxicity test (ZET). The ZET is one of the most recent in vitro alternative assays for developmental toxicity testing and is often used in addition to the validated embryonic stem cell test (EST), the rat limb bud micromass test (MM) and the rat post implantation whole embryo culture (WEC) (Busquet et al. 2014: Genschow et al. 2004; Hill et al. 2005; Kamelia et al., 2019; Lee et al. 2012; Piersma et al. 2004; Spielmann et al. 2004). The use of zebrafish embryos as developmental toxicity in vitro model is facilitated by the transparency of the organism throughout embryonic development and a rapid embryonic growth (Panzica-Kelly et al. 2012). Furthermore, development of the zebrafish embryos is considered to be very similar to the embryogenesis in vertebrates including humans (Sipes et al. 2011).

The aim of the present study was to assess the developmental toxicity of DES in the ZET, and to compare the effects obtained for this synthetic ERα agonist with the effects of the endogenous ERα agonist 17β-estradiol (E2). In addition, it was investigated whether the role of ERα, observed in vivo and in the EST, could also be demonstrated for the in vitro developmental toxicity of DES in the ZET. To this end, the in vitro embryotoxicity of DES was quantified in the ZET in the absence and presence of the ERα antagonist fulvestrant.

2. Materials and methods

2.1. Materials

Diethylstilbestrol (DES; CAS no. 56–53-1), estradiol (E2; CAS no. 50–28-2), fulvestrant (CAS no. 129453–61-8) and 3,4-dichloroaniline (CAS no. 95–76-1) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Dimethyl sulfoxide (DMSO) was purchased from Acros Organics (Geel, Belgium).

2.2. Zebrafish maintenance and embryo collections

The wild-type adult zebrafish (*Danio rerio*) AB line was obtained from the research facility Carus, Wageningen University and Research (Wageningen, The Netherlands) and maintained in a flow-through aquarium system at 27 °C with 14 h light/10 h dark cycle. The zebrafish embryos were obtained via spawning groups by placing adult males and females with a ratio of 1:2 in spawning tanks 4–5 h before the beginning of the dark cycle on the day before the test. Spawned eggs were collected, rinsed with egg water (prepared by mixing 1.5 ml salt stock solution in 1 l distilled water), pH was adjusted to 7–8 and incubated at 26 °C until further steps. The salt stock solution was prepared by dissolving 40 g "Instant Ocean" sea salt (Blacksburg, Virginia, USA) in 1 l distilled water. The egg water was also used as the assay medium for the zebrafish embryotoxicity test. Collected eggs were examined under a stereomicroscope, and embryos that developed normally and reached the blastula stage were selected for subsequent experiments while embryos that showed anomalies were discarded. These maintenance and selection criteria are based on the OECD guideline 236 for fish embryo acute toxicity (OECD 2013).

2.3. Zebrafish embryotoxicity test (ZET)

The ZET was initiated at 4–5 h post fertilization (hpf) at the gastrulation period and ended at 96 hpf, as this covers the entire organogenesis in a zebrafish embryo (Beekhuijzen et al. 2015; OECD 2013). The chorion of zebrafish embryos remains intact when used for exposure at 4–5 hpf. The zebrafish embryo exposure was performed in 24-well plates (Greiner Bio-one, Frickenhausen, Germany), sealed with a self-adhesive film cover (Sigma-Aldrich, Zwijndrecht, The Netherlands). Twenty wells of the 24-well plate were used for exposure to one concentration of test compound and the other four wells were used for the internal plate control. Exposure medium was prepared by mixing 400 times concentrated stock solutions of the test compounds (dissolved in DMSO) with egg water. The exposure medium was then transferred into 20 wells of the 24-well plate, at 2 ml exposure medium/well, and for the internal plate control, 2 ml egg water was added into each of the four remaining wells. The zebrafish embryos (1 embryo per well) were added after filling the wells with either exposure medium or egg water. The plate was then sealed with self-adhesive film cover to prevent evaporation of test compound throughout the exposure period (up to 96 hpf). E2 was tested at a range of concentrations up to 30 μM in egg water, while DES was tested at a range of concentrations up to 10 μM. Solvent controls (0.25% v/v DMSO), positive controls (4 μg/ml 3,4-dichloroaniline) and negative controls (egg water only) were included in each independent experiment. Plates were incubated at 26 °C with a photo period of 14 h light:10 h dark. Embryos were scored every 24 h ($t = 0$ is 0 hpf) for developmental abnormalities and cumulative mortality using an inverted microscope until 96 hpf, based on the extended general morphological scoring (GMS) system described by Beekhuijzen et al. (2015). Deviation from normal developmental stages, for example incomplete detachment of tail, incomplete development of eyes, fin, and mouth, unhatched embryos, will result in a lower total GMS value corresponding to a certain extent of developmental retardation. The GMS used for the exposure time window of 0–96 hpf is based on the 96 hpf endpoints, as described in detail by Beekhuijzen et al. (2015). The ZET was considered valid if the following was observed: $\leq$ 1 dead embryo (out of 4) in the internal plate control of every exposed-plate; $\leq$ 3 dead embryos (out of 24) in the negative control plate (at least 87.5% survival rate); $\leq$ 2 dead embryos (out of 20) in the solvent control plate (0.25% v/v DMSO); $\leq$ 14 live embryos (out of 20) in the positive control plate (4 μg/ml 3,4-dichloroaniline; exposure to positive control should result in a minimum of 30% mortality by 96 hpf). An embryo was considered dead when it was coagulated (dead milky white embryo appearing dark under the microscope) after 24 h. If the somites did not form after 48 h, the embryos were also considered dead, and when the heartbeat was absent after 48, 72 and 96 h the embryo was considered dead as well. In addition to the GMS, embryos were also assessed for six dysmorphogenic endpoints that include yolk-sac edema, pericardial edema (heart malformation), deformed body shape, deformed tail (i.e. kinked tail or malformed tail fin), malformation of the jaw and head, and malformation of sacculi/otoliths, which are all considered teratogenic endpoints in the harmonized zebrafish embryotoxicity assay guideline (Beekhuijzen et al. 2015). At least four independent experiments were performed for each test compound.

Previous published studies (Beekhuijzen et al. 2015; Hermsen et al. 2011; Selderslaghs et al. 2012) used the so-called teratogenicity index (TI) for classifying teratogenic compounds using the ZET. To that purpose, the TI for both DES or E2 were also defined in the present study. Within the ZET, the TI is defined as the ratio between the 50% lethal concentration (LC50) and the 50% effect concentration (EC50) based on the above-mentioned dysmorphogenic endpoints described by Beekhuijzen et al. (2015) and Selderslaghs et al. (2012). The list of these endpoints described by Beekhuijzen et al. (2015) and Selderslaghs et al. (2012) is presented in the Supplementary materials.

To assess whether the observed developmental toxicity in the ZET was mediated via the ERα, studies in which the effects of DES on zebrafish embryo development were assessed, were performed in the absence or presence of the ERα antagonist fulvestrant. To that end, first a concentration of fulvestrant that did not affect development of zebrafish embryo by itself was determined, which was then applied in the co-exposure studies. Final solvent concentrations in these studies was also 0.25% v/v DMSO.

2.4. Data analysis

Figures of concentration-response curves for the effect of test compounds in the ZET were made using GraphPad Prism 5.0 (California, US). Data were fitted to a sigmoid concentration-response curve with three parameters. For this analysis, results obtained in the ZET were expressed as fraction of the GMS score at 96 hpf compared to the GMS score of the solvent control (0.25% v/v DMSO, also at 96 hpf), and are presented as mean ± standard error of the mean (SEM) of 4 experiments. In vitro concentration-response curves from the ZET data were also analysed using the proast web-tool for BMD analysis, based on the PROAST software version 67.0 developed by the Dutch Institute for Public Health and the Environment (RIVM, The Netherlands) (Slob 2019), in which the benchmark concentration (BMC) at a predefined benchmark response (BMR) was calculated using a fitted dose–response curve. For these analyses, concentration-response data for the GMS were used. For both DES and E2, a decrease of 5% in GMS and a decrease of 5% in the survival were defined as the BMR (BMR05) for calculating the corresponding BMC (BMC05). The model with the lowest BMC outcome was selected to calculate the BMC05 (Supplementary materials). For TI calculation, both LD50 and EC50 values were determined from the concentration response curves obtained in the ZET using the same PROAST web-tool for BMD analysis. To this purpose, the BMR was set to 50%, representing the concentration causing either 50% cumulative mortality or lethality (LD50) or 50% dysmorphogenic-related effects (EC50), such as pericardial edema formation.

To assess for statistical differences of treatment effects, multiple paired *t*-tests between the treatments and the solvent control were performed using GraphPad Prism 5.0.

3. Results

3.1. In vitro developmental toxicity of E2 and DES in the ZET

Fig. 1 shows the effects of DES and E2 in the ZET. As shown in Fig. 1, exposure to both E2 or DES induced concentration-dependent effects on the GMS score and the embryo survival in the ZET (scored at 96 hpf). BMD analysis for GMS indicated a 2.8-fold higher potency for DES compared to E2 as reflected by the BMC05 values of 0.63 and 1.77 μM, respectively. The concentration-dependent effects on the survival at 96 hpf are also shown in Fig. 1 and related results of the BMD analysis provide BMC05 values of 1.5 and 4.7 μM for DES and E2, respectively, indicating that based on this endpoint DES is, 3.1-fold more potent than E2, in the ZET. Results of the BMD analysis are presented in the Supplementary materials.

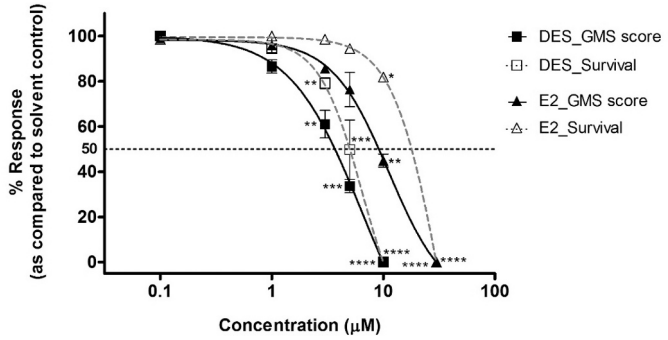

Fig. 1. Concentration-dependent effects of E2 and DES in the ZET on GMS score (black line with filled symbols) and survival (grey dotted line with unfilled symbols). The horizontal dotted line indicates the 50% response in either GMS score or survival. The statistical analysis that indicates a significant difference of results between the compound tested and solvent control at **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ is also presented.

3.2. Malformations and dysmorphogenicity in zebrafish embryos and larvae

In addition to the GMS, the dysmorphogenicity of E2 and DES was evaluated for some specific dysmorphogenic endpoints with emphasis on those endpoints that have been shown to be specifically affected upon exposure to known developmental toxicants, as described by Beekhuijzen et al. (2015). The endpoints included are yolk-sac edema, pericardial edema (heart malformation), deformed body shape, deformed tail (i.e. kinked tail or malformed tail fin), malformation of the jaw and head, and malformation of sacculi/otoliths. The outcome for these individual endpoints at 96 hpf, including induction of pericardial and yolk sac edema are presented in Figs. 2 and 3. Fig. 2 shows that no substantial induction of pericardial edema was observed in embryos exposed to E2. Interestingly, and in contrast to E2, DES induced pericardial edema in a concentration-dependent manner (Fig. 3) with significant differences compared to the solvent control already at 3 and 5 μM (Fig. 4). Furthermore, DES induced several other morphological effects not observed for E2, including short tail, curved tail and yolk sac edema (Fig. 5).

Regarding the teratogenicity or dysmorphogenicity classification (i.e. TI), 3 dysmorphogenic endpoints were observed mainly in zebrafish embryos exposed to DES including malformation of the heart (pericardial edema), malformation of the tail (short and curved tail) and yolk deformation (yolk sac edema), while none of these endpoints were observed to a significant extent upon exposure to E2, as upon exposure to E2 no concentration-dependent adverse effects, nor a statistically significantly effect different from the solvent control were observed. Furthermore, it is worth mentioning that a BMC50 could only be defined for the DES-induced pericardial edema scoring, but not for the other two aforementioned endpoints due to the presence of unhatched embryos at 96 hpf. In other words, it is hard to evaluate the malformation of the tail and body for the unhatched zebrafish embryo. Thus, based on the pericardial edema as a sole dysmorphogenic endpoint, a TI of 3.5 was obtained for DES by dividing the LC50 (BMC50) of the survival (cumulative mortality) (8.8 μM) by the BMC50 of the pericardial edema formation (2.5 μM). Given that E2 did not induce this adverse effect, a TI for E2 could not be established.

3.3. Effect of the ERα antagonist fulvestrant on DES-induced developmental toxicity in zebrafish embryos

To first establish a non-toxic concentration of the ERα antagonist fulvestrant in the ZET, the developmental effects and the in vitro embryotoxicity potential of fulvestrant in the ZET were evaluated. The results obtained (Fig. 6A) indicate that fulvestrant does not affect the GMS score up to 3 μM, inducing no malformations or deformations. At 10 μM the GMS score decreased without mortality being observed, while the highest concentration of 30 μM resulted in cumulative mortality (at 96 hpf) for all exposed zebrafish embryos. Based on these results, a concentration of 3 μM fulvestrant was selected to investigate the effect of the ERα antagonist on DES-induced developmental toxicity in the ZET, because at this concentration fulvestrant did not interfere with the zebrafish development. Furthermore, 3 μM is considerably (> 3000 times) higher than the IC50 for antagonist activity of fulvestrant to the ERα, which amounts to 0.8 to 0.9 nM as reported in the literature (Wakeling et al. 1991; Weir et al. 2016). Fig. 6B presents the results obtained when DES was tested in the ZET in the absence or presence of 3 μM fulvestrant. These results reveal that DES-induced pericardial and yolk sac edema formation in zebrafish embryos is significantly reduced in the presence of the ERα-antagonist fulvestrant at 3 μM, although at higher concentrations of DES (3 and 5 μM) inhibition was not complete.

4. Discussion

DES has been reported to induce developmental toxicity in vivo

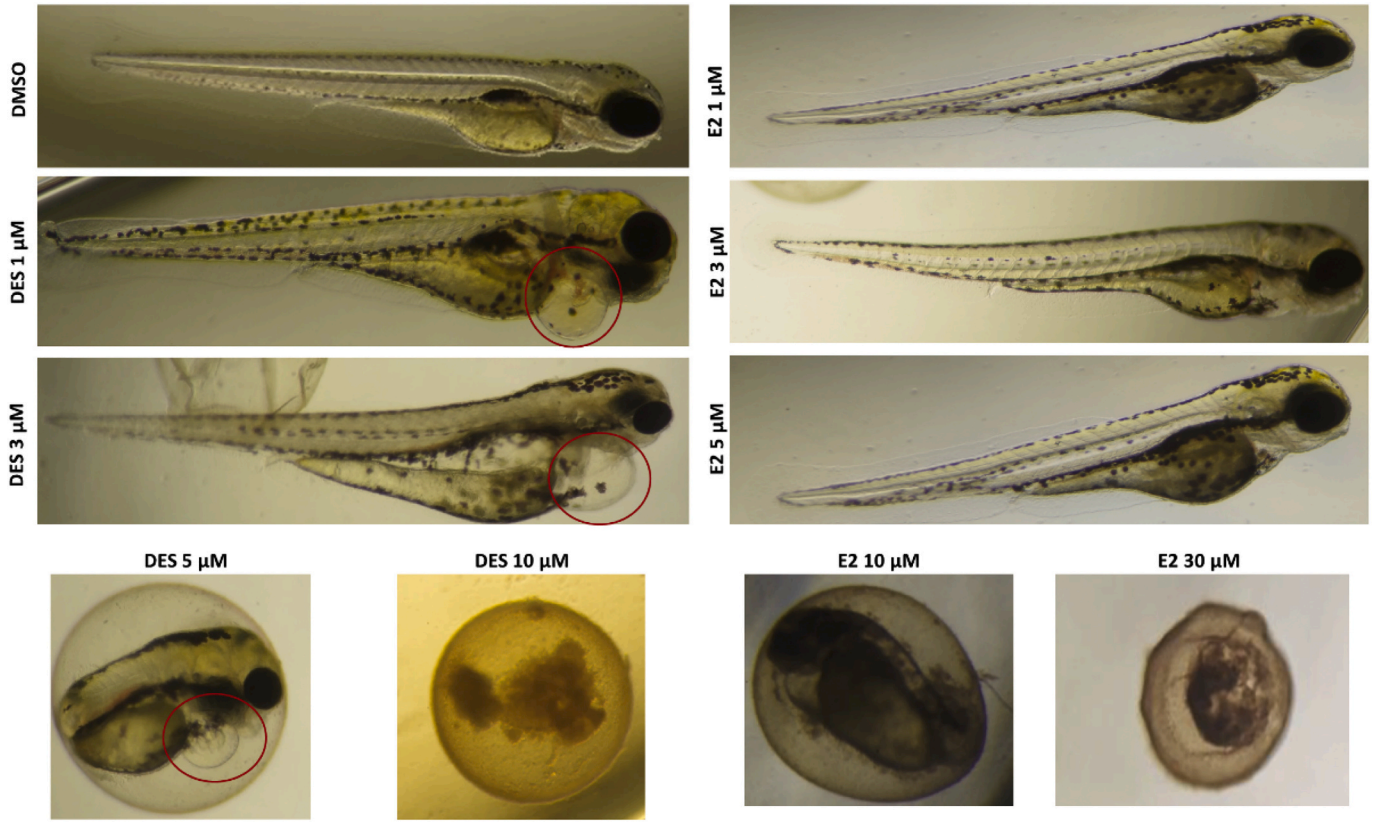

Fig. 2. Representative images of morphological analysis of zebrafish embryos exposed to different concentrations of E2 and DES showing DES-induced pericardial edema (red circles), which is not observed upon exposure to E2 at concentrations <10 µM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

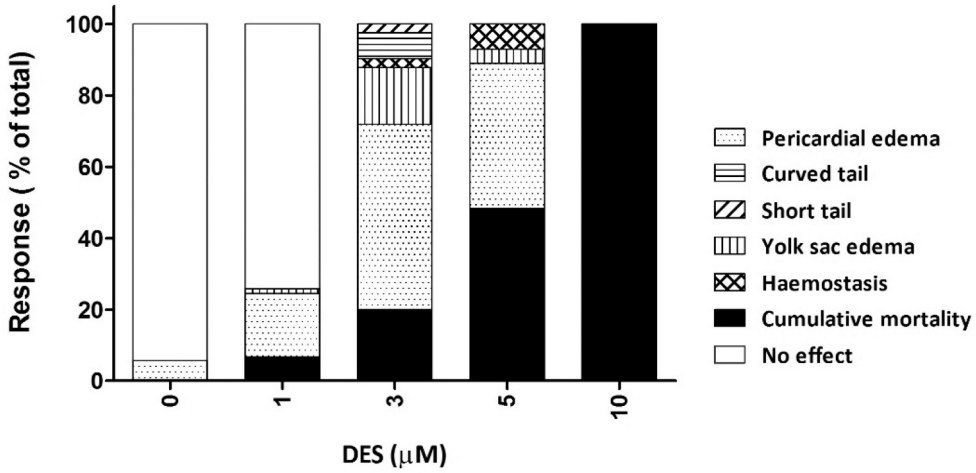

Fig. 3. Concentration-dependent developmental effects of DES in the ZET. Patterns within the bars illustrating different dysmorphogenic endpoints scored including pericardial edema, malformation in tail (short and curved tail), yolk sac edema, haemostasis and cumulative mortality.

including embryo mortality and malformations, with an important role for its ERα agonist activity in the underlying mode of action (Wardell et al. 1982; Cornwall et al. 1984; Nagao et al. 2013; Nagao and Yoshimura 2009; Odum et al. 2002). However, no in vivo developmental toxicity data have been reported for the endogenous ERα agonist E2. In our previous study, also in vitro data from the EST appeared to capture the differential effect of DES and E2 showing DES to be active in inducing in vitro developmental toxicity, while E2 was not, since it appeared to be active only at cytotoxic concentrations (Adam et al. 2019). The EST data also revealed that the DES-induced in vitro developmental toxicity was counteracted by the ERα antagonist fulvestrant thus demonstrating that the EST captured the role of ERα in the mode of action of DES. However, compared to other developmental toxicants tested in the EST, the assay seemed to underpredict the developmental toxicity potency of DES, possibly because the EST does not capture late events in the developmental process. Therefore, given that the ZET may be better able to detect late developmental effects, the aims of the present study were (1) to study the developmental toxicity of DES and E2 in the ZET and assess whether the ZET better predicts the in vivo DES-induced developmental toxicity, and (2) to determine the

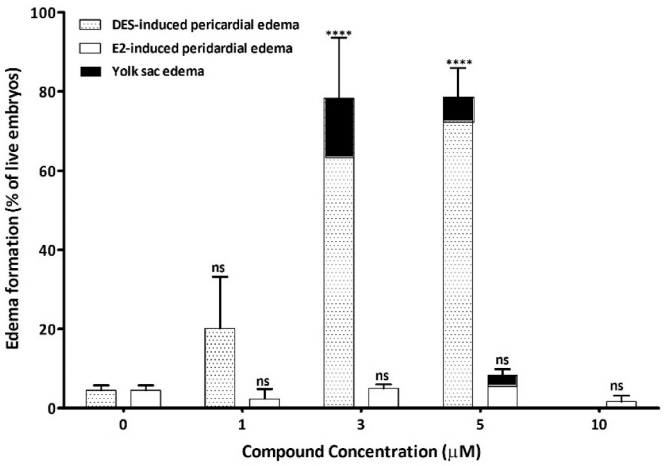

Fig. 4. Concentration-dependent effect of DES and E2 on edema (pericardial and yolk sac (black bars)) formation in zebrafish embryos. For the statistical analysis, **** $p < 0.0001$, ns is non-significant.

applicability of the ZET to capture the role of ERα in the developmental toxicity of DES, in order to investigate the potential of the ZET as a tool to study the role of ERα in developmental toxicity of estrogenic compounds.

Notable adverse developmental effects, including edema (pericardial and yolk sac), and dorsal curvature were observed in the DES-exposed zebrafish embryos, in addition to a concentration-dependent response for cumulative mortality and GMS. E2 appeared to be less active in the ZET, showing an induction of growth retardation and embryo mortality only starting at 10 μM. These findings are partially in line with a previous study reporting E2-induced malformation and mortality in zebrafish embryos at 10 μM (Kishida et al., 2001; Ren et al. 2012). In contrast to E2, DES induced in vitro developmental toxicity, as measured in the ZET, reflected by cumulative mortality, growth retardation and pericardial edema formation (scored at 96 hpf) occurring in a concentration-dependent manner, starting at 1 μM. Other dysmorphogeniceffects than edema formation, including deformed head, deformed tail (i.e. short and curved-tail), haemostasis and yolk sac edema were also recorded in zebrafish embryos exposed to DES (Figs. 3 and 5). These dysmorphogenic effects of DES corroborate previously reported findings that DES altered heart development and function of zebrafish embryos (Campinho and Power 2013). It has been indicated that pericardial edema may also reflect a non-specific stress-related outcome instead of compound-induced dysmorphisms (Bugel et al., 2015; Truong et al. 2011, 2020). However, given that pericardial dysmorphism is considered one of the parameters representative of heart malformation (Beekhuijzen et al. 2015; Li et al., 2019, Sarmah and Marrs 2016; Tournaire et al. 2016), and because the TI could not be defined based on other dysmorphisms observed in the DES-exposed zebrafish embryos, and pericardial edema was observed in a concentration-dependent way, a TI was calculated based on this sole endpoint. As a result, in the present study, DES classified to be a teratogenic compound based on the calculated TI of 3.5, while E2 could not be classified as teratogenic, based on the fact that E2 did not induce malformations in the ZET at the tested concentrations.

Additional experiments of the present study investigated the role of ERα in the developmental toxicity induced by DES in the ZET. Activation and disruption of the ERα pathway might contribute to disruption of embryonic development (Bondesson et al. 2015; Greco et al. 1993). Strong relationships between ERα activation and the adverse developmental effects of DES have been reported before. It has been reported, for example, that ERα is essential for DES to induce phenotypic changes in the reproductive tract (malformed reproductive tract) and alterations of several genes that are involved in regulation of embryonic

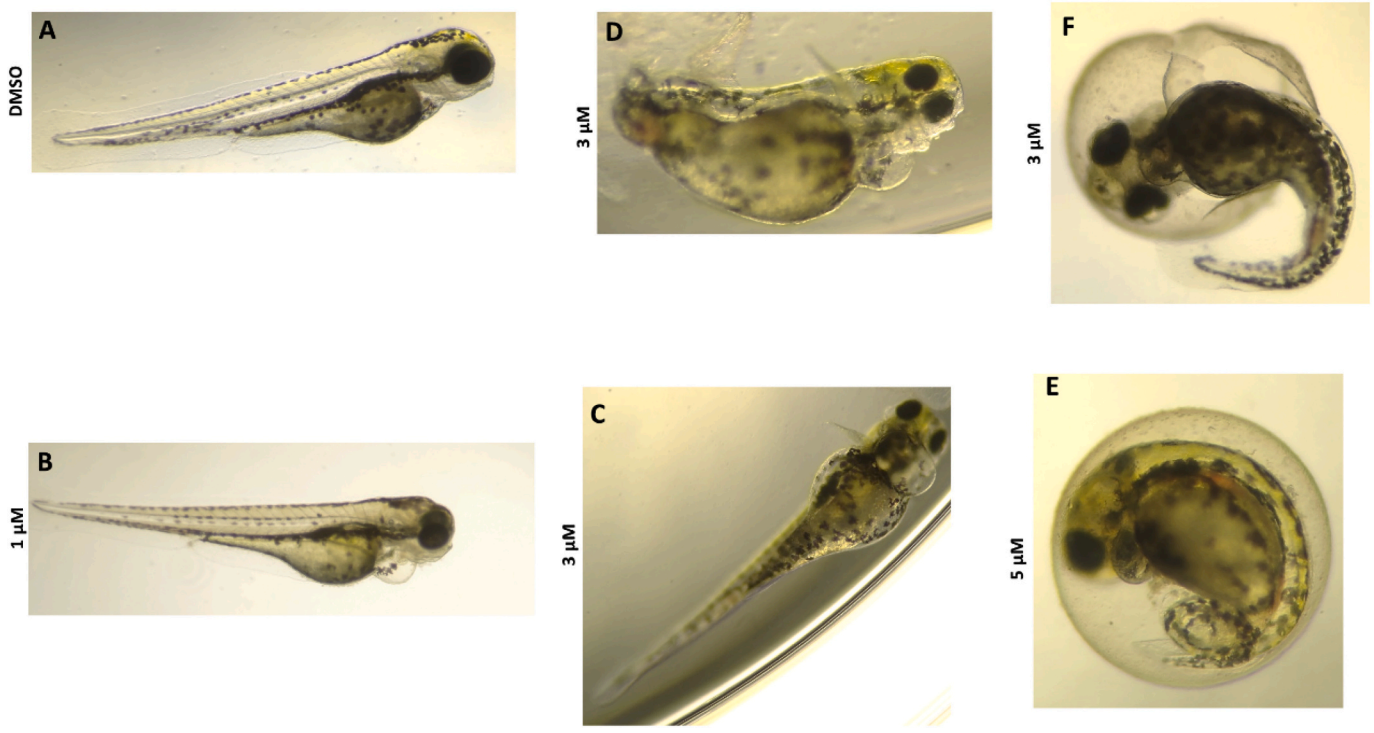

Fig. 5. Presentation of (A) normal embryos at 96 hpf (without phenotypic changes) and of adverse developmental effects observed mostly for DES- but not E2-exposed zebrafish embryos in the ZET, including (B) pericardial edema formation, (C) deformed head with small eyes, and a deformed body shape with yolk sac edema, (D) the "Short Tail" phenotype with a tail shorter than normal with haemostasis in the tail and yolk sac edema, (E) unhatched phenotype representing embryos that are still located in their chorion with yolk sac edema and haemostasis, and (F) down curved tail, where the tail is oriented downward compared to the horizontal orientation/deformed body shape.

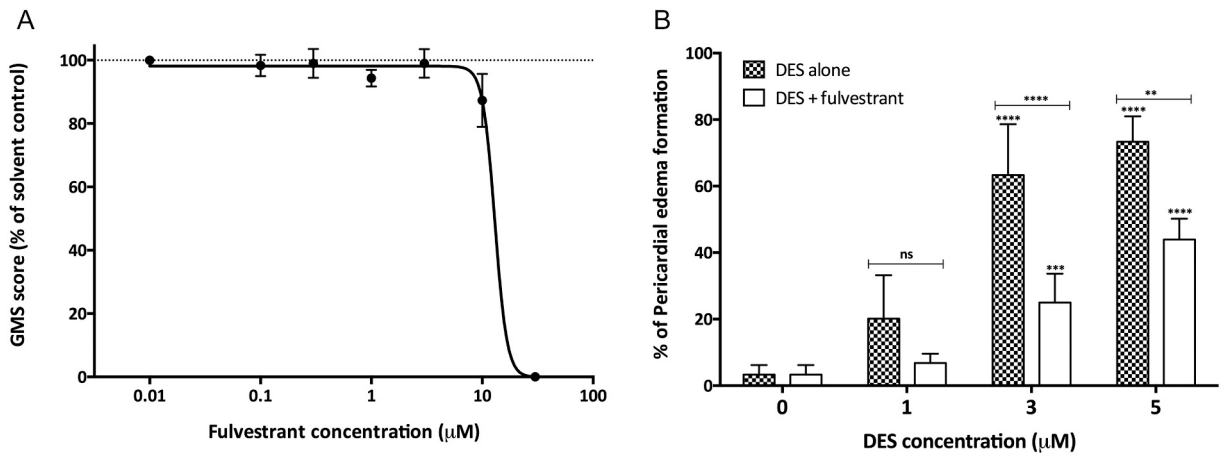

Fig. 6. (A) Concentration-dependent effect of fulvestrant (ERα antagonist) in the ZET (scored at 96 hpf) based on the decrease in GMS score (GMS solvent control set at 100%) and (B) fulvestrant-mediated inhibition of the DES-induced pericardial edema formation tested in the absence or presence of 3 μM fulvestrant. Edema formation is presented as percentage of surviving embryos. For the statistical analysis, **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$.

development (Block et al. 2000; Couse et al. 2001; Couse and Korach 2004; Ma et al. 1998; Miller et al., 1998). Also in the EST, a role for ERα in the developmental toxicity of DES has been elucidated (Adam et al. 2019). With respect to the ZET, the results of the present study corroborate a role for ERα in the DES-induced developmental toxicity, as demonstrated by the fact that the ERα antagonist fulvestrant significantly counteracted the DES-induced pericardial edema formation. Fulvestrant at 3 μM fully prevented edema formation induced by 1 μM DES, while the effect was only partially prevented at 3 and 5 μM DES. This partial effect of fulvestrant might be due to the relative ERα affinity of the two ligands, DES and fulvestrant, and their internal concentration at the relevant target organ. Comparison of the relative ERα binding affinities of DES and fulvestrant reveal the EC50 values for binding of DES and fulvestrant to be similar (Blair et al. 2000; Wakeling et al. 1991; Weir et al. 2016), so the competition may no longer be effective at equimolar concentrations (as now observed). Interestingly, when ERα activity was blocked in the EST by addition of fulvestrant, 0.15 μM fulvestrant was able to fully block the DES-induced inhibition of ES-D3 cell differentiation (Adam et al. 2019). The differences between the fulvestrant-mediated inhibition of DES-induced developmental toxicity in the EST and ZET assays could be due to differences in kinetics of fulvestrant and/or DES in the two model systems resulting in different concentrations at the cellular location of relevance, and/or to the fact that the EST detects other developmental stages than the ZET (Pera and Trounson 2004).

The partial, but not full, inhibition of DES-induced edema formation in the ZET, in the presence of the ERα antagonist fulvestrant, may also be due to the fact that in addition to ERα-mediated effects also other modes of action contribute to the DES-induced developmental toxicity. For instance, the retinoic acid receptor (RAR) pathway has been shown to play an important role in embryo development by supporting normal growth and differentiation (Kam et al. 2012; Mark et al. 2009; Rhinn and Dolle 2012), and results reported in our previous study in T47D cells in vitro (Adam et al., 2020) pointed at DES interference with retinoic acid receptor (RAR) pathways via upregulation of CYP26 genes. Given these results and the fact that disturbance of retinoic acid signaling has been frequently implicated in the toxicological mode of action of known developmental toxicants (Collins and Mao 1999; Dimopoulou et al. 2016; Loudig et al., 2000; Luijten et al., 2010; Thatcher and Isoherranen, 2009; Tembe et al. 1996; Turton et al. 1992), it is tempting to speculate that disturbance of RAR pathways may be involved in the possible embryotoxicity of DES. Thus, future research is needed to clarify also the role of RAR, in addition to the ERα, in mediating DES-induced developmental toxicity.

The present study shows that effect concentrations of DES in the ZET are in the low micromolar range. Since limited data are available to perform a correlation analysis between in vitro effect concentrations in the ZET and in vivo effect doses in rodents, we could not directly assess whether the ZET better predicts the in vivo developmental toxicity in a quantitative way than the (ES-D3 cell differentiation assay of the EST (Adam et al. 2019). However, given that effect concentrations of DES in the EST are also in the low micromolar range, and given that also for other chemicals (for example azole fungicides (de Jong et al. 2011), glycol ether metabolites (Hermsen et al. 2011)) effect concentrations are quite similar in the ZET as in the EST, it is concluded that the ZET is not more sensitive than the EST to detect developmental toxicity of DES. This may be related to the fact that these two in vitro assays do not reflect repeated dose toxicity and/or epigenetic effects that may play a role in the mode of action of DES-induced developmental toxicity in vivo (Titus-Ernstof et al. 2010; Bromer et al. 2009).

Altogether, it is concluded that the ZET can detect the in vitro developmental toxicity of DES and reveal differences between DES and E2. Furthermore, the present study also confirms the role of ERα in mediating the developmental toxicity of DES in the ZET, while the ZET does not outperform the EST in terms of the assay sensitivity.

Declaration of Competing Interest

The authors declare that there are no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tiv.2021.105088.

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