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Zebrafish as a model organism in One Health Toxicology: Impact of solvents and exposure routes on the toxicity of platinum anticancer drugs

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ABSTRACT

Zebrafish are widely used not only as a model in ecotoxicology but also to study the potential impact of chemicals on human health. Typically, zebrafish are exposed to chemicals dissolved in egg water or other defined media, which is the standard routine for ecotoxicology testing. This straightforward exposure method is usually also employed to monitor adverse effects in zebrafish to predict potential hazards and modes of action in humans. Here, we compared different exposure media and studied the impact of salinity and solvents relevant to ecotoxicity testing. For comparison, toxicants also were directly injected into the bloodstream of zebrafish embryos, as this method better simulates the exposure scenario for assessing the adverse effects of drugs administered intravenously to patients. As model compounds we studied platinum-based anticancer drugs, which are known micropollutants, but also lead to severe side effects in humans. Striking differences in sensitivity and phenotypes, i.e. adverse outcomes, were observed dependent on the exposure route and media. The bioavailability of the platinum compounds was significantly altered in the different media and by the commonly used solvent DMSO. These findings highlight the relevance of the exposure route and media as well as of solvents to be considered when interpreting zebrafish studies in the field of ecotoxicology or in cross-species comparisons to predict effects on human health.

1. Introduction

Harmful chemicals in the environment, including air, water, and soil, can negatively impact human health, as well as the health of animals and ecosystems. The One Health concept recognizes the interconnectedness of human, animal, and environmental health. Originally, this concept was introduced as a holistic and transdisciplinary approach to tackle global zoonoses, such as bird flu or viral epidemics (Destoumieux-Garzón et al. 2018). The artificial divide between human and veterinary medicine was seen as an obstacle to efficiently address such multifactorial health hazards, which also requires expertise from other fields such as ecology, evolution and environmental sciences. As another example, the widespread use of antibiotics in agriculture and farming has promoted the development of antibiotic-resistant bacteria, posing a serious problem for human medicine. Thus, the One Health concept is not only relevant in the context of pandemics but also in assessing the detrimental effects of chemicals on global health. The health of and complex interactions between humans, animals (wildlife and domestic), and ecosystems is thus further influenced by the chemosphere. The new term "One Health Toxicology" more concisely describes this emerging field (Dinis-Oliveira 2023).

Model organisms such as rodents, fish, frogs, fruit flies, nematodes, and even yeast have long been used to understand fundamental biological principles relevant to humans. Specifically, the response to chemicals has been shaped by evolution and, therefore, many toxicity pathways are conserved across species (PrecisionTox 2023). Nevertheless, human toxicology primarily relies on testing in rodents, while the field of ecotoxicology employs other standard models including lower vertebrates and invertebrates. Findings in one species can often be relevant for another, and thus studies in human and ecotoxicology might

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be mutually beneficial for understanding chemical toxicity.

As such, zebrafish (*Danio rerio*) were initially used as a model organism in ecotoxicology to investigate adverse effects of environmental pollutants on fish (Laale 1977). However, given the significant conservation of genes and associated functions among zebrafish, mice and humans, zebrafish are increasingly recognized as a valuable model to interrogate the mode of action of chemicals in human toxicology (Yang et al. 2009). Zebrafish offer unique advantages over other vertebrate models, such as rodents, due to their rapid development outside the mother, small size, transparency during early stages of development, and ease of genetic manipulation. Zebrafish have a similar number of genes as humans, with a high overlap of orthologues (70 %), which becomes even more pronounced when only considering human disease genes (82 %). Concordant analysis for mouse and human genes reveals a similarly high conservation (83 %), and there is a comparable overlap between mouse and zebrafish genes (77 %) (Howe et al. 2013). A more recent study on genome phylogeny focusing not only on individual disease genes but entire disease gene families (1597 to be found in humans) indicates an even higher conservation in mice (99 %) and zebrafish (92 %) (Colbourne et al. 2022). Thus, the similarity of physiological and disease pathways anchored at the genome level positions zebrafish as an ideal model for cross-species comparison of adverse outcomes in response to chemicals with relevance for human toxicology.

Another driver to more rigorously assess the utility of zebrafish in this respect is the interest to reduce, refine or replace animal experiments (3R concept)(Bauer et al. 2021). Therefore, alternative non-animal models or new approach methodologies (NAMs) need to be explored and established (Schmeisser et al. 2023). In this context, zebrafish embryos up to 5 days post fertilization (dpf) are considered ethically accepted alternatives that are not subject to regulation in the European Union (Strahle et al. 2012). In the USA, stages between 3 dpf to 7 dpf do not require approval dependent on the local Institutional Animal Care and Use Committees (IACUCs) (Bartlett and Silk 2016; Moulder 2016). For ecotoxicological studies, zebrafish are typically exposed by dissolving the chemicals in egg water (Hansjosten et al. 2022; Wahl et al. 2010; Wahl et al. 2008). This exposure route is also commonly employed to monitor adverse effects in zebrafish to predict potential hazards and modes of action in humans (Hayot et al. 2024). However, direct microinjection of toxicants into the blood stream of zebrafish embryos might provide a more relevant exposure scenario for predicting adverse outcomes of drugs administered intravenously to patients (Hayashi et al. 2020; Hentschel et al. 2005; Mane et al. 2018).

Here, we studied the impact of different exposure routes, i.e. classical incubation in egg water versus microinjection of chemicals, on the viability of zebrafish embryos. As model substances we used three different platinum compounds: cisplatin, carboplatin and oxaliplatin. All are widely used in the clinics to treat various types of cancer and are known to exhibit different side effects (Hartmann and Lipp 2003; Jung and Lippard 2007). The founding member of the group, cisplatin (*cis*-diamminedichloroplatinum(II): CDDP), is a simple square-planar complex in a *cis* configuration, where the central Pt (II) atom interacts with two relatively stable ammonia groups and two chlorines. Due to the relatively high chloride concentration in the plasma of patients (about 100 mM), cisplatin is not hydrolyzed. However, at the much lower chloride concentration inside cells (about 4 mM), the chlorine atoms are exchanged by water molecules to form the so-called aqua-complex. This hydrolyzed form of cisplatin can then bind to DNA in cancer cells and inflict DNA damage, ultimately triggering cell death (Jung and Lippard 2007). However, cisplatin can also damage off-target cells, leading to neurotoxicity, ototoxicity, hepatotoxicity, and, notably, kidney toxicity, which is the rate-limiting factor for treatment (Hartmann and Lipp 2003). Additionally, during pregnancy, especially in the first trimester, cisplatin can cross the placenta and cause developmental toxicity (Benoit et al. 2021; Varella and Partridge 2024). In clinical settings, cisplatin is usually dissolved in saline solution to prevent its activation via replacement of chloride ligands with water before administration (Knox et al. 1986). Due to the low solubility of cisplatin in saline solution, some laboratories use the common solvent dimethyl sulfoxide (DMSO) to achieve higher stock concentrations. Yet, DMSO can directly bind to cisplatin and interfere with its reactivity (Dernell et al. 1998; Hall et al. 2014; Massart et al. 1993). Consistent with this, administering cisplatin along with DMSO to Sprague-Dawley rats resulted in reduced nephrotoxicity, but not in impaired anticancer activity of cisplatin (Jones et al. 1991). Thus, apart from salinity, solvents such as DMSO can drastically change the toxicity of cisplatin. Given the severe side effects induced by cisplatin, the analog compounds carboplatin and oxaliplatin were developed. These compounds exhibit less kidney toxicity and are also used to treat cisplatin-resistant tumours (Hartmann and Lipp 2003).

Increasing concentrations of platinum anticancer drugs are detected in the environment due to their constant release from municipal waste water treatment plants and hospital effluents (Kummerer et al. 1999; Rehman et al. 2015). As micropollutants, the hazard of platinum compounds has been assessed in routine ecotoxicity studies, often employing zebrafish embryos (Di Paola et al. 2022; Hung et al. 2019; Karas et al. 2019; Karas et al. 2020; Kovács et al. 2016; Misík et al. 2019; Osterauer et al. 2011; Osterauer et al. 2009). These studies have shown that platinum compounds can interfere with the hatching of zebrafish embryos, provoke developmental malformations and cause lethality in embryos and hatched larvae. However, when various platinum anticancer drugs were assessed for toxicity, different exposure media with varying chemical composition were used. Specifically, the salt concentration, particularly the sodium chloride content, varied depending on the exact exposure media, with unknown consequences for the efficacy of the tested platinum compounds.

Therefore, we exposed zebrafish "classically" by dissolving the three clinically approved platinum anticancer drugs – cisplatin, carboplatin and oxaliplatin – in defined embryo medium, i.e. E3 medium, to directly compare their adverse effects. Zebrafish embryos were exposed at early stages, starting at 6 hpf, and at later stages of development, i.e. 48 hpf. After fertilization, embryos are immersed in an extraembryonic fluid within the chorion, an acellular barrier composed of polypeptides. The chorion and perivitelline fluid provide physical protection and a stable osmotic milieu to prevent damage inflicted by mechanical forces and dehydration (De la Paz et al. 2020). The chorion also shields the embryo from the environment and hinders exposure to chemicals, specifically large molecules and particles (Chen et al. 2016; Kais et al. 2013; Tran et al. 2021). Around 48 hpf, embryos start hatching, and the post-hatched embryo, also referred to as eleutheroembryo, can further develop nourished by the yolk (Strahle et al. 2012). Simple exposure of embryos at early and late stages to chemicals dissolved in embryo medium is commonly used to monitor adversity in zebrafish, both to predict the ecotoxicity of chemicals and to infer their potential hazard and mode of action in humans. However, as the toxicity of specifically cisplatin is heavily influenced by the relative amount of sodium chloride, as outlined above, we also administered the platinum compounds in physiological saline solution, which has a much higher concentration of sodium chloride (140 mM) compared to E3 medium (5 mM). To even more closely resemble the exposure scenario for drugs injected into patients, we established a procedure for the direct microinjection of toxicants into the bloodstream of zebrafish embryos. For mechanistic studies aimed at unraveling the mode of action of a chemical and identifying critical key events, small molecules are often co-administered in human toxicology studies. Such bioactive compounds, which typically target specific enzymes, receptors, or transporters, are frequently dissolved in DMSO. However, whether DMSO itself impacts the toxicity of the chemical in question, in our case platinum compounds, often remains unknown. In the present study on the toxicity of different platinum compounds, we observed striking differences in sensitivity, bioavailability, and phenotypes (i.e., adverse outcomes) depending on the exposure media, the solvent (DMSO), and the route of administration. In essence, we demonstrate the critical importance of exposure conditions in determining the adverse outcomes in response to

chemicals in zebrafish embryos. This has significant implications for interpreting zebrafish studies in the field of ecotoxicology as well as for cross-species comparisons to predict effects on human health.

2. Methods

2.1. Materials

All chemicals were obtained from Sigma–Aldrich Chemie GmbH (Taufkirchen, Germany) or Carl Roth GmbH (Karlsruhe, Germany), if not stated otherwise. Other materials, such as dishes, flasks, and reaction tubes were bought from Sarstedt (Nuembrecht, Germany).

2.2. Zebrafish husbandry

AB strain wild-type zebrafish (*Danio rerio*) obtained from the European Zebrafish Resource Centre (Karlsruhe, Germany) were raised and maintained at standard conditions (28 ± 0.5 ℃ and 14/10 h light/dark cycle) as previously described.(Mane et al. 2018) Before the day of egg collection, female and male individuals (2:1) were kept separated by a sieve in a breeding cage over night. After mating and spawning on the next day, embryos were collected and washed with facility water. 50 embryos with normal characteristics of development were picked and raised in a 100-mm Petri dish with E3 medium containing 1 ‰ methylene blue solution in an incubator at 28 ± 0.5 ℃. All zebrafish husbandry and experimental procedures were performed in accordance with the German animal protection standards and were approved by the Government of Baden-Württemberg, Regierungspräsidium Karlsruhe, Germany (Aktenzeichen 35–9185.64/BH KIT).

2.3. Drug treatment and assessment of malformations and mortality

Cisplatin, carboplatin and oxaliplatin (Cayman Chemical, USA, ≥ 98 % purity) can be readily dissolved in water with a maximum solubility as follows: cisplatin-1.4 mg/mL, carboplatin-14.28 mg/mL, and oxaliplatin-9 mg/mL (Eslami Moghadam et al. 2023). For exposure experiments all platinum compounds were dissolved in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$)(Nüsslein-Volhard, 2002) or NaCl solution (140 mM). Solutions were prepared freshly for each experiment to maintain the stability of platinum compounds. All solutions were assessed for precipitation to ensure that the compounds were fully solubilized. Embryos at 48 hpf (hour post fertilization) were transferred to a 96-well plate and treated with 200 μM, 400 μM, 600 μM, 800 μM, or 1000 μM cisplatin dissolved in E3 medium or NaCl solution (1 embryo per well incubated in 200 μl exposure volume) for 72 h of exposure. Untreated embryos served as control groups. For comparison, embryos at 48 hpf were also exposed to 200 μM, 600 μM, and 1000 μM carboplatin or oxaliplatin dissolved in E3 medium or NaCl solution for 72 h. While most of the embryos already hatched at 48hpf, unhatched eggs were manually dechorionated using forceps. To evaluate the influence of the widely used solvent DMSO, cisplatin was directly dissolved in DMSO and diluted in E3 medium to the desired concentrations (10, 50, 100, 150, 200, 250 μM) resulting in a final concentration of 0.1 % DMSO in all exposure solutions. In addition, 48 hpf dechorionated embryos were co-exposed to 200 μM cisplatin dissolved in E3 medium and 0 %, 0.005 %, 0.01 %, 0.05 %, 0.1 %, or 0.5 % DMSO, respectively. Embryos incubated in E3 medium only or E3 medium with 0.5 % DMSO were used as controls. Morphological abnormalities and mortality of all embryos were evaluated daily by microscopy (stereomicroscope SMZ645, Nikon, Japan). Embryos without heartbeat and blood circulation were considered as dead. The rate of malformations and mortality were calculated as percentage of affected embryos divided by the total number of exposed embryos. 12 embryos were used in each group in an individual experiment, and three independent experiments were conducted (total number of analyzed embryos: 36).

2.4. Drug treatment and assessment of hatching

To explore the effects of cisplatin, carboplatin and oxaliplatin on hatching, embryos at 6 hpf were incubated with 5, 25, 50, 100, 200, 600, or 1000 μM cisplatin dissolved in E3 medium or NaCl solution for 5 days in a 96-well plate. For comparison, embryos at 6 hpf were also exposed to 200, 600, or 1000 μM carboplatin and oxaliplatin. The hatching rate, survival rate and morphological abnormalities of all embryos were evaluated daily by microscopy. Viability of unhatched embryos is evidenced by a regular heartbeat, blood flow, spontaneous movements and twitching. The hatching rate or survival rate were calculated as the number of hatched or alive embryos divided by the total number or exposed embryos, and were expressed as percentage. 12 embryos were used in each group, and three independent experiments were conducted.

2.5. Analysis of platinum accumulation in embryos

Atomic absorption spectroscopy (AAS, Perkin-Elmer PinAAcle 900 T) was applied for platinum detection in embryos similar to protocols used for human cells as described previously (Cetraz et al. 2017). The treated embryos were washed with E3 medium for 3 times and the liquid supernatant was discarded. Remaining embryos were incubated with 0.5 ml mixture of 70 % nitric acid and 30 % H_2O_2 (v/v = 1/1) for lysis at different temperatures and for different periods: samples were consecutively incubated at 65 °C, 75 °C and 85 °C for 1 h, and finally maintained at 95 °C for 3 h to ensure complete evaporation of moisture. Finally, pellets were redissolved in 0.5 ml of 0.2 % nitric acid for AAS analysis. The concentrations of platinum in each sample were calculated based on linear regression analysis of platinum standards covering the concentration range of 1–200 μg/L ($r = 0.99$).

2.6. Zebrafish injection

Dechorionated embryos at 50–55 hpf were anesthetized in E3 medium supplemented with 0.0168 % (w/v) MS 222 (tricaine methanesulphonate) and immobilized in 1 % w/v low melting agarose for documenting the lateral view as previously described (Mane et al. 2018). Then, 3 mM cisplatin, carboplatin or oxaliplatin stock solution dissolved in NaCl solution (140 mM) blended with 3-kDa dextran (red fluorescent probe to monitor successful injection, Molecular Probes, Eugene, OR) were injected into the cardinal vein with a FemtoJet microinjector (Eppendorf). Thereafter, embryos were released from agarose and returned to E3 medium. Embryos injected with NaCl solution and 3-kDa dextran were used as control. Embryos showing well-distributed red fluorescence were further evaluated for survival rate and morphological alterations daily until 5 dpf. 24 embryos were used in each group, and at least three independent experiments were conducted. The distance between the anus and the posterior end of the notochord was defined as tail length. Quantification of length was performed with ImageJ (version 1.52a, National Institutes of Health, USA, https://imagej.nih.gov/ij/).

2.7. Statistical analysis

Statistical analysis was conducted by the statistical software R (R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/). The four-parameter log-logistic function, drc package (Ritz et al. 2015) was used for fitting dose response curves. Statistical differences were examined by one-way analysis of variance (ANOVA) followed by post-hoc Welch's t-test or the Tukey's honestly significant (HSD) difference test. All data points were used for the analysis and plotted in the figures.

3. Results

3.1. Cisplatin, but not oxaliplatin and carboplatin, induces mortality of zebrafish eleutheroembryos dependent on exposure medium and the solvent DMSO

Adverse effects of cisplatin dissolved in E3 medium or NaCl solution on zebrafish were investigated at different concentrations ranging from 200 up to 1000 μM. Dechorionated embryos were exposed at 48 h post fertilization (hpf) and assessed for malformations and mortality 24, 48 and 72 h later (Fig. 1A). Cisplatin induced lethality in a dose and time dependent manner (Fig. 1B and C). Whereas at non-toxic concentrations (below 400 μM) no malformations, such as edema, could be observed, at lethal doses, e.g. 600 or 1000 μM cisplatin, severe histolysis of eleutheroembryos became apparent (Fig. 1B). Moreover, in E3 medium cisplatin caused a higher mortality compared to its effects in NaCl solution (Fig. 1C and Supplementary Fig. S1). Carboplatin and oxaliplatin treatment at 200, 600 and 1000 μM failed to provoke death of the exposed eleutheroembryos in both exposure media, and no significant morphological changes were observed (Fig. 1C). For mechanistic studies, bioactive compounds such as inhibitors of critical signaling proteins, e.g. kinases or proteases, are often dissolved in DMSO and co-administered with cisplatin. Therefore, we addressed the impact of DMSO in the E3 exposure medium on cisplatin toxicity. Surprisingly, when cisplatin was dissolved in DMSO and diluted to a final concentration of 0.1 % DMSO, eleutheroembryo mortality was increased relative to the effects observed for cisplatin dissolved directly in E3 medium (compare Fig. 1D and Fig. 1C). For example, 150 μM cisplatin in the presence of 0.1 % DMSO already caused almost 100 % death of eleutheroembryos at 48 h of exposure (48–96 hpf, Fig. 1D), whereas in the absence of DMSO even 200 μM at 48 h, but also at 72 h, post incubation was without effect (48–96 and 48–120 hpf, Fig. 1C). To further explore the enhanced toxicity of cisplatin due to addition of DMSO, we performed dose response experiments (Fig. 1E and Supplementary Fig. S2) in which a fixed concentration of cisplatin (200 μM) was mixed with increasing levels of DMSO (0.005 up to 0.5 %). As expected, treatment with either 0.5 % DMSO or 200 μM cisplatin for 24–72 h (i.e. from 48 − 120 hpf) did not trigger mortality nor other adverse effects. In contrast, co-treatment of eleutheroembryos with 0.5 % DMSO and 200 μM cisplatin reduced the motility of eleutheroembryos and caused 8 % death already at 24 h after exposure (48 – 72 hpf). Overall, dependent on increasing concentrations of DMSO, the onset of toxicity appeared earlier. Also, at a given time point, the percentage of lethal eleutheroembryos increased with the relative dose of DMSO. As an extreme example, the combination of only 0.005 % DMSO and 200 μM cisplatin was sufficient to cause death of all eleutheroembryos 72 h post exposure (48–120 hpf).

3.2. Cisplatin interferes with hatching of zebrafish embryos at much lower concentrations than those required for the induction of mortality

To assess the impact of the chorion and the developmental stage of zebrafish on the toxicity of platinum compounds, zebrafish embryos were kept inside the chorion and exposed from 6 hpf until 120 hpf (Fig. 2a). Cisplatin inhibited hatching of embryos at 200 μM and induced lethality accompanied by histolysis at high concentrations of 600 and 1000 μM as evidenced by bright field microscopy (Fig. 2b). Quantitative analysis of the mortality rate of cisplatin treated embryos revealed a dose and time dependent response (Fig. 2c and Supplementary Fig. S3). Again, as shown above for the experiments with zebrafish eleutheroembryos, the effects were more pronounced when cisplatin was dissolved in E3 medium compared to NaCl solution. For example, upon treatment with 600 μM cisplatin dissolved in E3 medium just 14 % of embryos were alive, whereas in case of 600 μM cisplatin dissolved in NaCl solution up to 86 % survived at 96 hpf. Hatching similarly was inhibited dependent on dose and exposure time (Fig. 2d, e and Supplementary Fig. S4). Of note, interference with hatching already occurred at lower concentrations of cisplatin dissolved in E3 medium than those required to trigger lethality, i.e. at 100 μM cisplatin compared to 600 μM cisplatin (compare to Fig. 2c). The highest non-lethal dose of 200 μM cisplatin dissolved in E3 completely inhibited hatching at 72 hpf, and even only 8 % of the embryos hatched at 120 hpf (Fig. 2d). As shown in Fig. 2b, embryos were well developed within the chorion at 120 hpf after treatment with 200 μM cisplatin, indicating that inhibition of hatching per se is not lethal. In contrast, embryos exposed to 600 or 1000 μM cisplatin disintegrated within the chorion.

Interestingly, whereas cisplatin caused a higher mortality rate when dissolved in E3 medium versus NaCl solution, the effects in the two different exposure media on hatching showed the opposite trend. Inhibition of hatching already became obvious at 50 μM cisplatin dissolved in NaCl compared to 100 μM cisplatin when dissolved in E3 medium (Fig. 2d and e). 50 μM cisplatin dissolved in NaCl solution, but not in E3 medium, completely blocked hatching at 72–120 hpf.

3.3. Oxaliplatin and to a lesser extent carboplatin inhibit hatching dependent on the exposure medium

Exposure to oxaliplatin and carboplatin at various concentrations (200, 600 and 1000 μM) had no significant effect on the survival rates of embryos (Fig. 3a and 3c), in keeping with the absence of adverse effects observed in eleutheroembryos (Fig. 1C). However, 600 and 1000 μM oxaliplatin dissolved in NaCl solution, but not in E3 medium, significantly reduced the hatching rate at 72, 96 and 120 hpf (Fig. 3b and Supplementary Fig. S5). Different to oxaliplatin, only the highest concentration of carboplatin (1000 μM dissolved in NaCl solution) caused a slight inhibition of hatching at 96 and 120 hpf (Fig. 3d).

3.4. Accumulation of platinum in eleutheroembryos is impacted by the exposure medium, the solvent DMSO, and the type of platinum compounds

The toxicity of the various platinum compounds was influenced by the time of exposure, the exposure medium and by the solvent DMSO. In order to address the relevance of the delivered dose, which might explain the different sensitivities of the exposed larvae, we measured the absolute platinum levels by atomic absorption spectroscopy (AAS). First, the amount of the platinum compounds was determined and compared in dechorionated embryos treated with 1000 μM cisplatin, carboplatin, or oxaliplatin at 48 hpf for 24 h. At this time point and concentration, lethality is not yet triggered by cisplatin (Fig. 1C). Accumulation of platinum in embryos treated with cisplatin dissolved in E3 medium was about 7.6 times and 4.8 times higher than in embryos treated with oxaliplatin and carboplatin, respectively (Fig. 4a). Furthermore, when cisplatin was dissolved in E3 medium versus NaCl solution, the dose delivered per eleutheroembryo was 2.2-fold higher, which correlates well with the increased toxicity of cisplatin dissolved in E3 medium at later stages. However, in the case of carboplatin and oxaliplatin no difference in the total platinum amount dependent on the exposure medium could be observed. The time dependent accumulation of oxaliplatin and carboplatin was also studied at a later time point, i.e. at 120 hpf. When treated with oxaliplatin and carboplatin, platinum levels further increased by roughly 3-fold when the exposure time was prolonged from 24 to 72 h (Fig. 4 a and b). Again, no significant differences were observed when considering E3 medium and NaCl solution to dissolve the compounds (Fig. 4b). After treatment with 600 μM cisplatin at 48 hpf, accumulation of platinum in eleutheroembryos was steadily and drastically enhanced over time, and again higher when administered in E3 medium versus NaCl solution (Fig. 4c). Finally, we wanted to assess the effect of the solvent DMSO on the total platinum amount. To this end, eleutheroembryos were co-treated with 200 μM cisplatin and 0.005 %, 0.01 % or 0.05 % DMSO similar to the survival experiment described above (Fig. 1E). Indeed, in the presence of 0.005 or 0.01 % DMSO the levels of cisplatin doubled, and at 0.05 % DMSO the relative

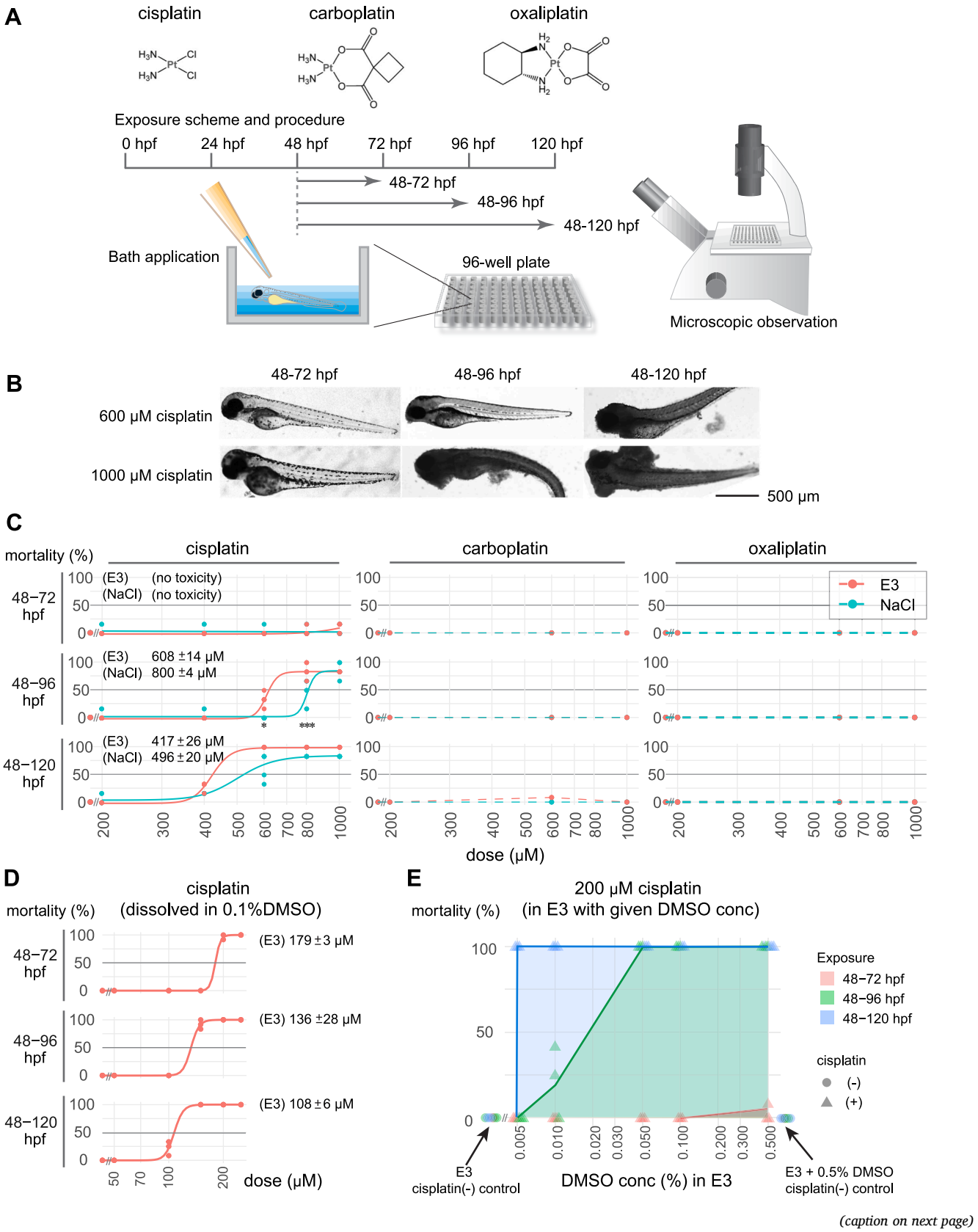

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Fig. 1. Differential mortality of zebrafish eleutheroembryos induced by three platinum compounds, cisplatin, carboplatin and oxaliplatin. A) Structures of the investigated platinum-based anticancer drugs and schematic diagram of the exposure scheme and experimental procedure. Eleutheroembryos were exposed in a 96-well plate to the indicated platinum compounds at 48 hpf and assessed for survival at 72-, 96- and 120 hpf. B) The typical morphology of eleutheroembryos exposed to 600 or 1000 μM cisplatin. Scale bar: 500 μm. C) Mortality of zebrafish eleutheroembryos upon treatment with each platinum compound dissolved either in E3 medium (red line) or NaCl solution (green line). The three different exposure periods (48–72 hpf, 48–96 hpf, or 48–120 hpf) and 5 tested doses (200, 400, 600, 800 and 1000 μM) are indicated. Mortality, normalized to the total number of analyzed embryos, is shown as a function of platinum compound concentration for each exposure period. LD50 values are shown if the four-parameter log-logistic model can be fitted. ANOVA revealed that the mortality induced by cisplatin either dissolved in E3 or NaCl solution is significantly different for the 48–96 hpf period ($p = 0.0004$), with differences seen at 600 μM and 800 μM (Tukey's HSD test, $p = 0.011$ labeled as * and 0.0008 labeled as ***, respectively). D) Enhanced mortality of zebrafish eleutheroembryos exposed to cisplatin dissolved in 0.1 % DMSO. E) Mortality of zebrafish eleutheroembryos exposed to 200 μM cisplatin in E3 medium increases with exposure time (48–72, 48–96 and 48–120 hpf) and the DMSO dose (0.005, 0.01, 0.05, 0.1 and 0.5 %). Depicted are fitted dose–response curves based on all data points of 3 independent experiments (number of exposed larvae: $n = 36$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

difference even further increased to 34-fold compared to cisplatin treatment alone.

3.5. Microinjection of cisplatin, oxaliplatin and carboplatin leads to different phenotypes in zebrafish eleutheroembryos than those observed upon simple addition to exposure media

Zebrafish is not only used as a model organism in ecotoxicology, but also frequently employed to assess the efficacy and toxicity of drugs and toxicants relevant for humans. As in the clinics platinum drugs are normally administered to patients by intravenous injection, we established procedures to directly deliver the different platinum compounds into the bloodstream of zebrafish embryos. Thus, dechorionated embryos at 48 hpf were injected with 3 mM cisplatin, carboplatin, or oxaliplatin, respectively, resembling a clinically relevant concentration as found in solutions for infusion. Mortality and malformations were recorded up to 120 hpf (Fig. 5a). Dependent on the type of platinum compound, different phenotypes were observed (Fig. 5b). Whereas cisplatin provoked edema formation, which is a known consequence of kidney injury, and increased spinal curvature, carboplatin selectively reduced body length. By contrast, oxaliplatin at this concentration was without effect. Quantification of the various adverse effects revealed that about 60 % of the embryos displayed severe pericardial and yolk-sac edema 48 h post injection (hpi) of cisplatin, with a further increase to about 70 % at 72 hpi (Fig. 5c). Likewise, a time-dependent increase in spinal curvature and mortality upon injection of cisplatin could be observed (Fig. 5d and e). Higher concentrations of cisplatin (5 and 10 mM) did not further enhance edema formation nor increase the percentage of eleutheroembryos with curved body axis, but triggered higher mortality (Supplementary Fig. S6a-d). Unlike cisplatin, injection of 3 mM oxaliplatin did not cause any significant effects (Fig. 5b-f). However, at the highest concentration of 10 mM, oxaliplatin provoked a high mortality (Supplementary Fig. S6d). Finally, carboplatin reduced the body length of more than 40 % of injected embryos (Fig. 5f). The tail length of carboplatin-treated larvae was significantly reduced by about 25 % (Fig. 5g). Furthermore, injection of higher concentration of carboplatin (10 and 20 mM) further increased the percentage of affected embryos with reduced body length (Supplementary Fig. S6e). Interestingly, the highest carboplatin concentration of 20 mM led to strong edema formation, yet without concomitant increase in lethality and spinal curvature (Supplemental Fig. S6a-d).

4. Discussion

Numerous studies have addressed the toxicity of different platinum drugs, including cisplatin, carboplatin, and oxaliplatin, in zebrafish. However, the impact of the exposure media, the solvent DMSO and the route of administration on adverse outcomes have not yet been critically evaluated in a systematic and comparative analysis. In the present study, we exposed zebrafish embryos at early developmental stages (thus still within the chorion), and hatched (eleuthero-)embryos at later stages, to three clinically and environmentally relevant platinum compounds. Zebrafish were exposed in E3 medium, which is widely used in many laboratories, to assess lethality and interference with hatching as typical readouts for ecotoxicity studies. Since zebrafish are also used as a model to predict human toxicity, we additionally applied the drugs in physiological saline solution. To more closely resemble the exposure scenario encountered in clinical settings, we also directly injected the compounds dissolved in saline solution into the bloodstream of embryos. First, we found that the survival of eleutheroembryos, dependent on dose and time, was more strongly reduced in E3 medium compared to saline solution. This difference in toxicity might be attributed to the lower concentration of Cl^- ions in E3 medium (5 mM) relative to saline (140 mM). At low Cl^- concentrations, the formation of the diaquo complex of cisplatin is enhanced, which has been shown to be much more toxic when injected into mice (Rosenberg 1978). Furthermore, it has also been suggested that the diaquo complex reacts with the brush border in the proximal tubules, thereby promoting renal toxicity. Co-administration of DMSO suppresses the renal toxicity of cisplatin in rats, but not its anticancer activity in a leukemia or sarcoma rat model. In the extracellular space, DMSO may specifically bind to and inactivate the hydrolyzed cisplatin species but not to the neutral form of cisplatin, which is taken up by the tumor cells (Jones et al. 1991). At low intracellular Cl^- levels, cisplatin is activated by hydration to ultimately kill the tumor cells (Jung and Lippard 2007). Thus, the extracellular action of the diaquo complex of cisplatin may explain not only the high kidney toxicity, but also the increased toxicity of cisplatin to zebrafish eleutheroembryos when exposed in E3 medium.

In addition to considering phenotypic endpoints, we also measured the actual dose of platinum delivered to zebrafish eleutheroembryos under the different conditions. Interestingly, the amount of cisplatin detected in eleutheroembryos exposed in E3 medium was always higher compared to those exposed in saline solution. Thus, not only is the reactivity of cisplatin increased at low chlorine concentrations, but also its relative internal dose was higher, contributing to the higher toxicity observed. However, as we measured only the total amount of cisplatin in the whole embryo, we cannot infer the relative distribution of cisplatin in the embryo. In fact, decreased renal clearance due to enhanced protein binding at low Cl^- concentrations could contribute to the overall increase in cisplatin levels. The extent to which cisplatin delivery into cells is affected by the different media remains unknown. Future experiments are warranted to quantify the relative amount of cisplatin in total lysates of different organs, such as the kidney, in the eleutheroembryo. To monitor intracellular delivery, direct binding to DNA can also be analyzed by AAS (Cetraz et al. 2017). For ecotoxicity studies, low chlorine concentrations in the exposure media are more relevant, as chlorine levels in freshwater are also low. The salinity of waterbodies varies substantially, ranging from less than 0.05 % in freshwater to 0.05–2 % in brackish water and 3–5 % in sea water. As NaCl is not the only salt contributing to salinity, the relative amount of NaCl in the wild ranges from 8 mM over 30 mM up to 500 mM in fresh, brackish and saline water, respectively (Nthunya et al. 2018). Here, we used E3 medium with 5 mM NaCl, which is often used in laboratories for experiments with zebrafish. However, other media containing different levels of NaCl are also quite common, such as diluted "Instant Ocean" sea salts (0.8 mM), embryo medium (14 mM), or Holtfreter's medium (60 mM)

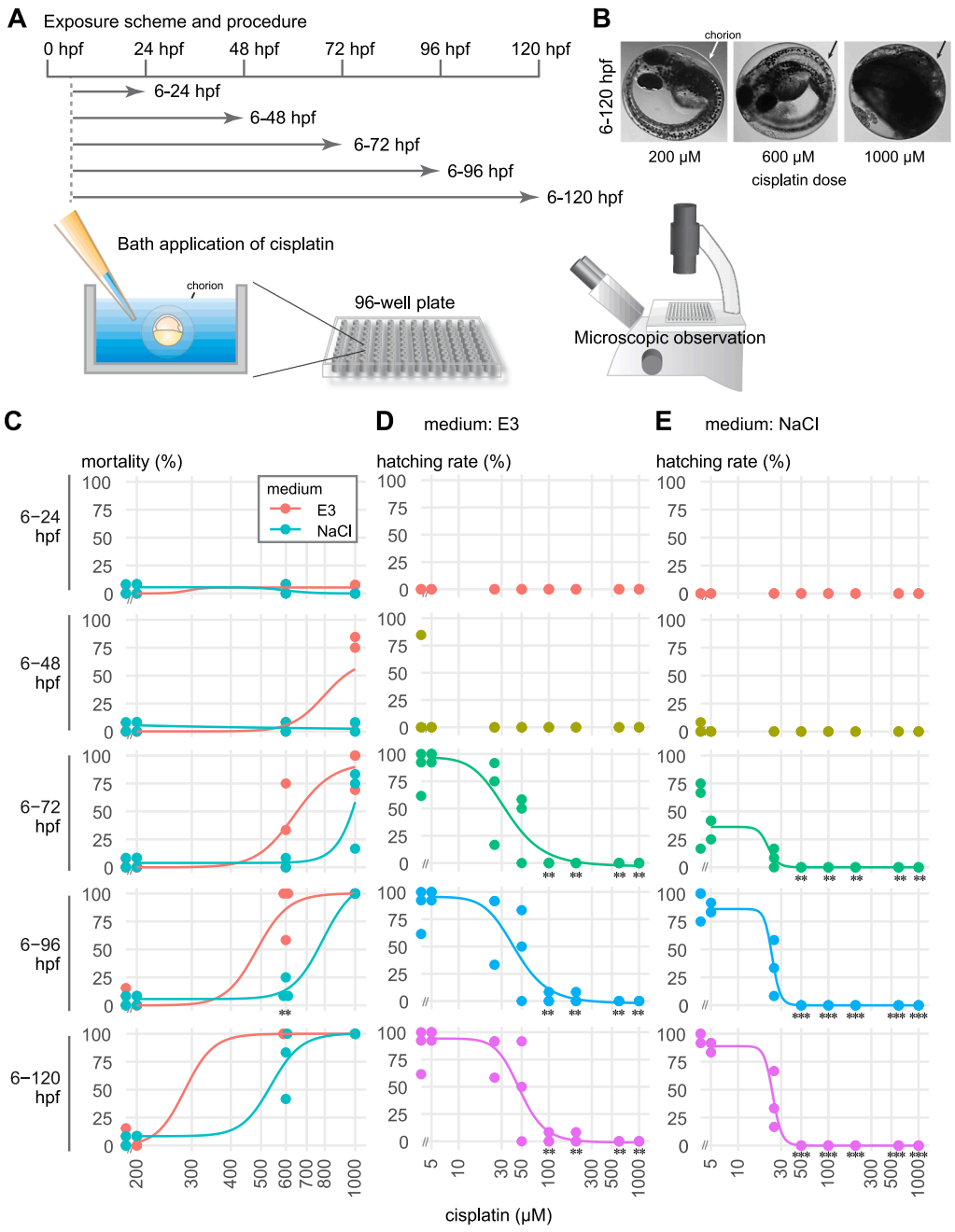

Fig. 2. Effects of cisplatin on the mortality and hatching rate of embryos. A) Schematic diagram of the exposure scheme. Embryos at 6 hpf were exposed in a 96-well plate to 3 different doses of cisplatin (200-, 600-, or 1000 µM) and assessed for the mortality and hatching rate at 24-, 48-, 72-, 96- and 120 hpf. B) Representative images of unhatched embryos at 120 hpf exposed to three different concentrations of cisplatin. The arrows indicate the chorion. C) Mortality of embryos evaluated at indicated stages treated with cisplatin dissolved in E3 medium (red data points) or NaCl solution (green data points). For each exposure period, the difference in the effect of cisplatin between the two media at a given concentration was evaluated using Tukeýs HSD test, following the identification of statistically significant differences by ANOVA ($p < 0.05$). A statistically significant difference was observed at 600 µM cisplatin under the 6–96 hpf exposure condition (**, $p < 0.01$). D-E). Hatching rate of embryos treated with cisplatin dissolved in E3 medium (D) or NaCl solution (E) for each exposure period. Within each exposure period, the effect of cisplatin was compared to a cisplatin-free control using Tukeýs HSD test, following the identification of statistically significant differences by ANOVA ($p < 0.05$). The results are indicated with asterisks as follows: **, $p < 0.01$, and ***, $p < 0.001$. Mortality and hatching rate, normalised to the total number of analysed embryos, are shown as a function of exposure concentration for each exposure period. Depicted are fitted dose–response curves of 3 independent experiments (total number of embryos for each time period and dose: $n = 36$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Christy and Dickman 2002; Hansjosten et al. 2022; Westerfield 1995). Therefore, the NaCl concentration in exposure media employed in ecotoxicity experiments should be considered more carefully. Indeed, the toxicity of metals in crustaceans and fish usually inversely correlates with decreased salinity, likely due to the greater bioavailability of the free metal ions (Hall and Anderson 1995). However, for organic chemicals no consistent trend could be observed with the exception of organophosphate insecticides, for which toxicity increases with higher salinity. Of note, due to climate change, the salinity of freshwater systems is increasing as precipitation decreases and evaporation is enhanced (Jeppesen et al. 2015). These changes in salinity can affect ecosystems and might as well influence the toxicity of micropollutants.

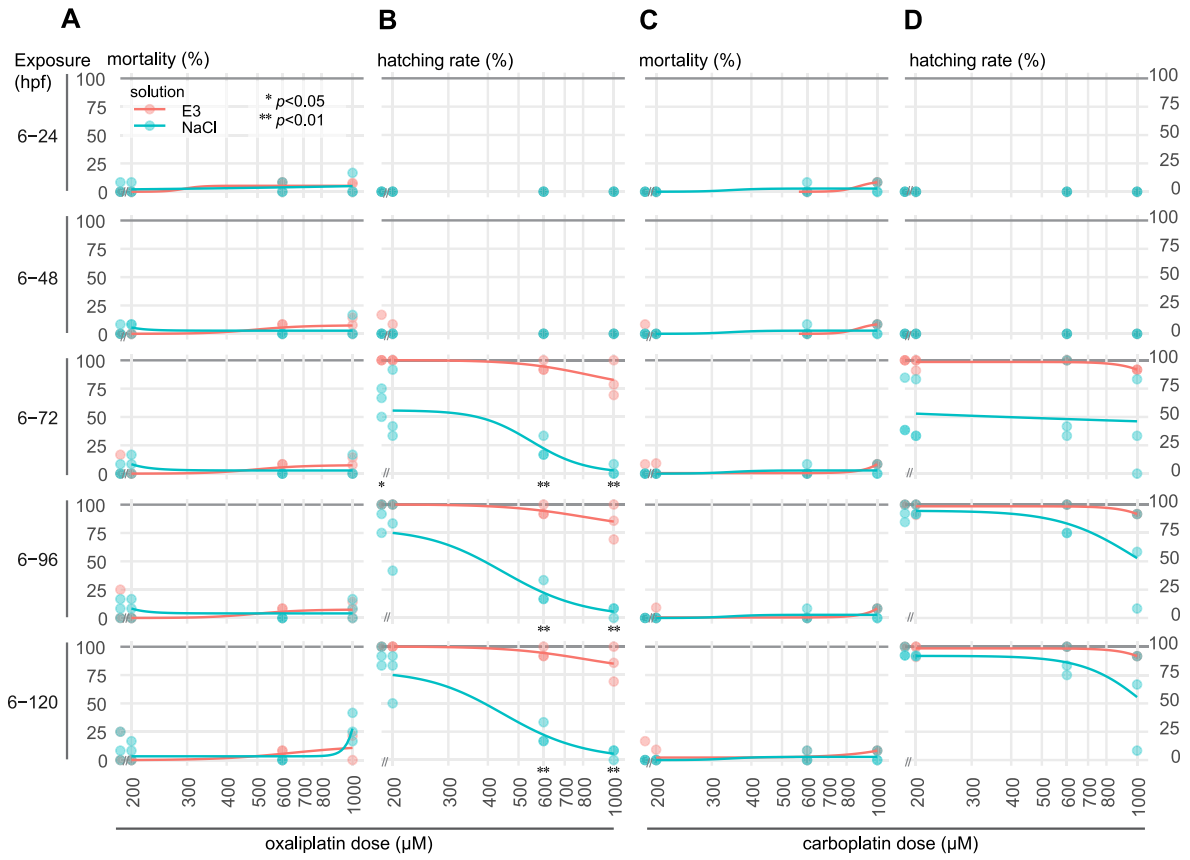

Fig. 3. Effects of oxaliplatin (A, B) and carboplatin (C, D) dissolved in E3 medium or NaCl solution on the mortality (A, C) and hatching rate (B, D) of embryos. Each compound is tested at 200-, 600-, and 1000 μM for five exposure periods of 6–24, 6–48, 6–72, 6–96 and 6–120 hpf. When ANOVA revealed a significant difference between E3 medium and NaCl solution for a given compound and exposure period, a follow-up Welch *t*-test was performed at each dose. Significant differences are indicated by asterisks (*$p < 0.05$ and **$p < 0.01$).

For typical ecotoxicological endpoints, such as lethality and inhibition of hatching, mostly low NaCl concentrations in the exposure media are used. However, zebrafish are also employed as a model organism to study other readouts relevant to human oto-, neuro- and nephrotoxicity. Specifically, hair cell death in lateral neuromasts of embryos serves as a proxy for the toxicity in sensory cells of the human inner ear (Choi et al. 2013; Ou et al. 2007). By screening compounds that improve hair cell survival in combination with cisplatin, potential drug candidates can be identified to reduce ototoxicity in patients. However, these experiments are often performed at very low NaCl concentrations (e.g., 0.015 mM), while levels up to 140 mM, as found in human plasma, might provide more physiologically realistic exposure conditions relevant for human toxicity evaluation. Therefore, cisplatin has also been injected into adult zebrafish in saline (0.9 % / 154 mM NaCl) to monitor kidney toxicity, yielding results similar to those observed in rodent models (Kim et al. 2020). Moreover, transparent Casper zebrafish embryos were exposed to cisplatin dissolved in saline (f.c. 2–4 mM NaCl) at 35 °C to more closely mimic human conditions. Administration of cisplatin reduced the survival of hair cells and diminished the glomerular filtration rate (GFR) (Wertman et al. 2020). Although GFR was impaired, no signs of acute kidney damage, such as edema or lesions in histological sections, were observed, in contrast to earlier studies where cisplatin was directly injected into the bloodstream of embryos (Hentschel et al. 2005). By comparing the adverse phenotypes in eleutheroembryos either exposed to cisplatin via E3 medium or by direct injection, we demonstrate that edema formation is only observed in the latter case. Hence, we propose using a microinjection approach in zebrafish eleutheroembryos to assess the effects of drugs usually administered intravenously to patients. However, for ecotoxicity studies, exposure to chemicals via media with low chlorine concentrations is the appropriate procedure.

Another important finding from our investigations is the strong impact of the solvent DMSO on the toxicity of cisplatin. DMSO is frequently used to dissolve hydrophobic compounds and to apply small molecules intended to ameliorate the toxicity of cisplatin in different models. For example, ototoxicity in rats or toxicity to neuronal cells in vitro can be decreased by cotreatment with certain drugs or carotenoids dissolved in DMSO (Dos Santos et al. 2012; Fang and Xiao 2014). In experiments with zebrafish embryos and eleutheroembryos, chemical libraries of bioactive compounds and drugs are usually dissolved in DMSO and often screened to identify modifiers of various biological processes, such as the suppression of hair cell death induced by cisplatin (Domarecka et al. 2020; Thomas et al. 2015). Yet, the inactivation of cisplatin by DMSO needs to be carefully considered in such studies. Previous reports have shown that the strong nucleophilic sulfur present in DMSO can directly bond with Pt(II), displacing ligands and reducing cisplatin-induced toxicity and anticancer activity (Casolaro et al. 2009; Dernell et al. 1998; Kerrison and Sadler 1977). Contrary to the general suppression of cisplatin activity by DMSO, hair cell death in zebrafish eleutheroembryos provoked by cisplatin was found to be enhanced in the presence of DMSO. Interestingly, this effect was specific to cisplatin and could not be replicated with other organic solvents or with another ototoxic agent, aminoglycoside. A moderate increase (approximately 1.3–2-fold) in the uptake of fluorescently-tagged cisplatin into hair cells was visualized over 48 min, suggesting enhanced bioavailability of cisplatin in the presence of 0.1 % DMSO (Uribe et al. 2013). Similarly, but much more pronounced, we found a 15-fold increase in the total amount of cisplatin in treated eleutheroembryos when co-incubated with 0.05 % DMSO over 24 h. Possibly, the DMSO-cisplatin complex

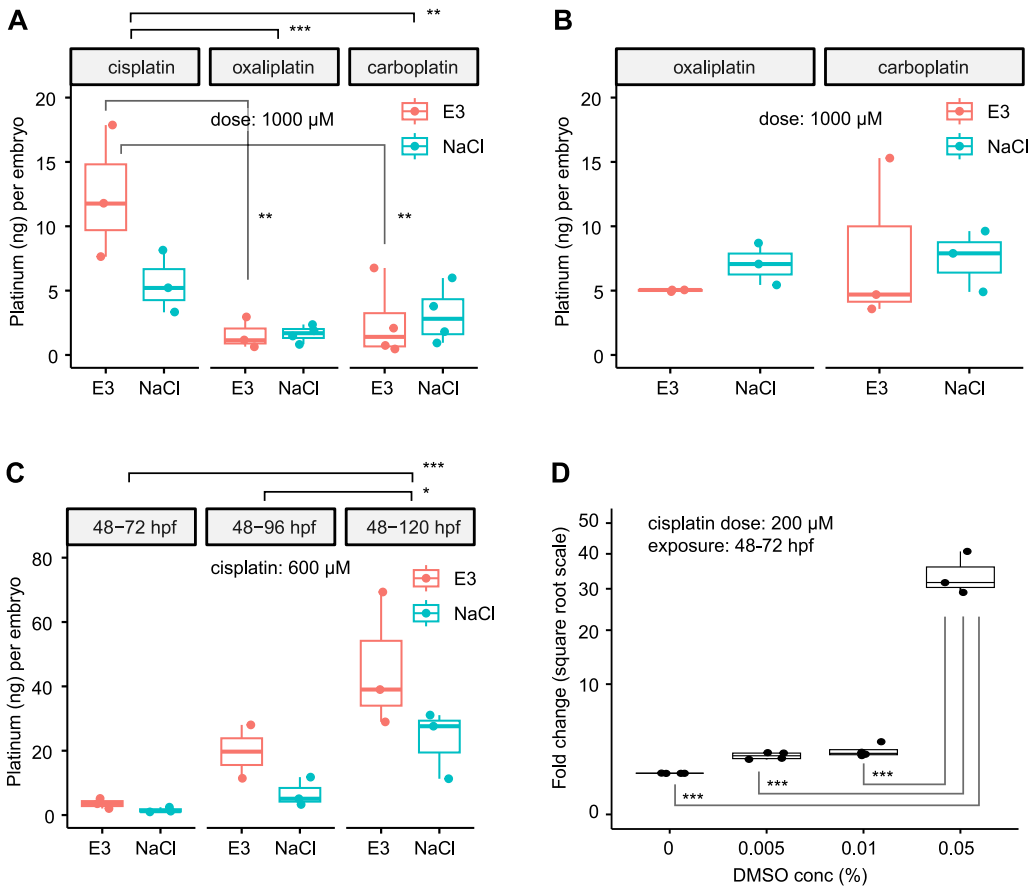

Fig. 4. Accumulation of platinum in the eleutheroembryo depends on the exposure medium, exposure period and platinum compounds. A) Averaged platinum amount (ng) detected per embryo at 72 hpf after 48–72 hpf exposure to 1000 µM of cisplatin, oxaliplatin and carboplatin in E3 medium (red) or NaCl solution (green). Detected platinum levels per embryo differ between platinum compounds ($p = 0.0005$***, ANOVA), with cisplatin showing the highest accumulation compared to oxaliplatin ($p = 0.0006$***, Tukey's HSD test) and carboplatin ($p = 0.002$**, Tukey's HSDtest). In E3 medium, but not in NaCl solution, cisplatin showed higher levels of platinum in comparison to oxaliplatin ($p = 0.002$**, Tukey's HSD test) and carboplatin ($p = 0.003$**, Tukey's HSD test). B) Platinum amounts (ng) in the embryo at 120 hpf after 48–120 hpf exposure to 1000 µM carboplatin or oxaliplatin do not differ between compounds ($p = 0.4$, ANOVA) or exposure groups (E3 medium and NaCl solutions, $p = 0.7$, ANOVA). C) Platinum content (ng) per embryo after cisplatin-exposure depends on the exposure period ($p = 0.001$**, ANOVA) and E3 media/NaCl solution; $p = 0.03$*, ANOVA). Exposure during 48–120 hpf showed significantly higher platinum accumulation in comparison to shorter exposures (48–72 hpf, $p = 0.0008$***, Tukey's HSD test; 48–96 hpf, $p = 0.02$*, Tukey's HSD test). D) Zebrafish embryos were exposed to 200 µM cisplatin during 48–72 hpf in E3 medium containing different DMSO concentrations (0.005, 0.01, or 0.05 %). DMSO concentration affected the platinum accumulation in the treated embryo ($p = 1x10^{-8}$***, ANOVA) with significant increase at 0.05 % DMSO in comparison to lower doses ($p < 0.00001$***, Tukey's HSD test). The platinum content per embryo is shown as a fold change relative to a group exposed only to E3 medium. For each experiment, embryos ($n = 12$) were exposed in a 96 well plate to the indicated platinum compounds and platinum levels were determined. Depicted are the results of 3 independent experiments (number of exposed embryos: $n = 36$). Boxplots show the median (bold line), first and third quartile (box), and 1.5-fold interquartile range (IQR) (whiskers). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

can cross cell membranes more easily compared to cisplatin alone. In addition, it is well known that DMSO affects the permeability of the skin and thereby enhances uptake of administered compounds (Notman et al. 2007). Thus, although the intrinsic reactivity of cisplatin is reduced by complexation with DMSO, the enhanced available internal dose might compensate and even override this effect, resulting in a net increase in toxicity.

In comparison to cisplatin, for both carboplatin and oxaliplatin no significant toxic effect was observed after exposure via E3 medium or saline, even at concentrations as high as 1 mM. Similar results indicating reduced toxicity of carboplatin and oxaliplatin have been reported in preclinical studies in cell culture and rodents as well as in the clinics (Adams et al. 1989; Hartmann and Lipp 2003). Furthermore, the relative levels of carboplatin and oxaliplatin in treated eleutheroembryos were lower compared to the accumulated amount of cisplatin, which might also partially explain the absence of toxicity for both platinum compounds. However, when directly injecting all platinum drugs into the bloodstream of eleutheroembryos, thus delivering the same dose for all, the toxicity of carboplatin and oxaliplatin was still lower compared to that of cisplatin. This is in line with the notion that the intrinsic reactivity of cisplatin is the highest among the three platinum drugs (Hartmann and Lipp 2003; Jung and Lippard 2007). Whereas cisplatin triggered signs of nephrotoxicity, evidenced by increased edema formation and enhanced spinal curvature, carboplatin and oxaliplatin were ineffective at the same injected dose. Thus, the clinical adverse outcome of kidney injury, which is more pronounced for cisplatin than for carboplatin and oxaliplatin, can be recapitulated in zebrafish. At roughly threefold higher doses, carboplatin injection also led to edema formation, indicating a similar mode of action as cisplatin, which occurs simply at higher doses (English et al. 1999). Nevertheless, in contrast to cisplatin, carboplatin reduced the body length of exposed eleutheroembryos by a so far unknown mechanism. A number of mutations leading to short axis and notochord phenotypes have been identified in large-scale screens in zebrafish, which might provide candidate genes and pathways affected by carboplatin (Stemple et al. 1996). Oxaliplatin did not induce edema at 3 or 10 mM, but did provoke strong lethality at

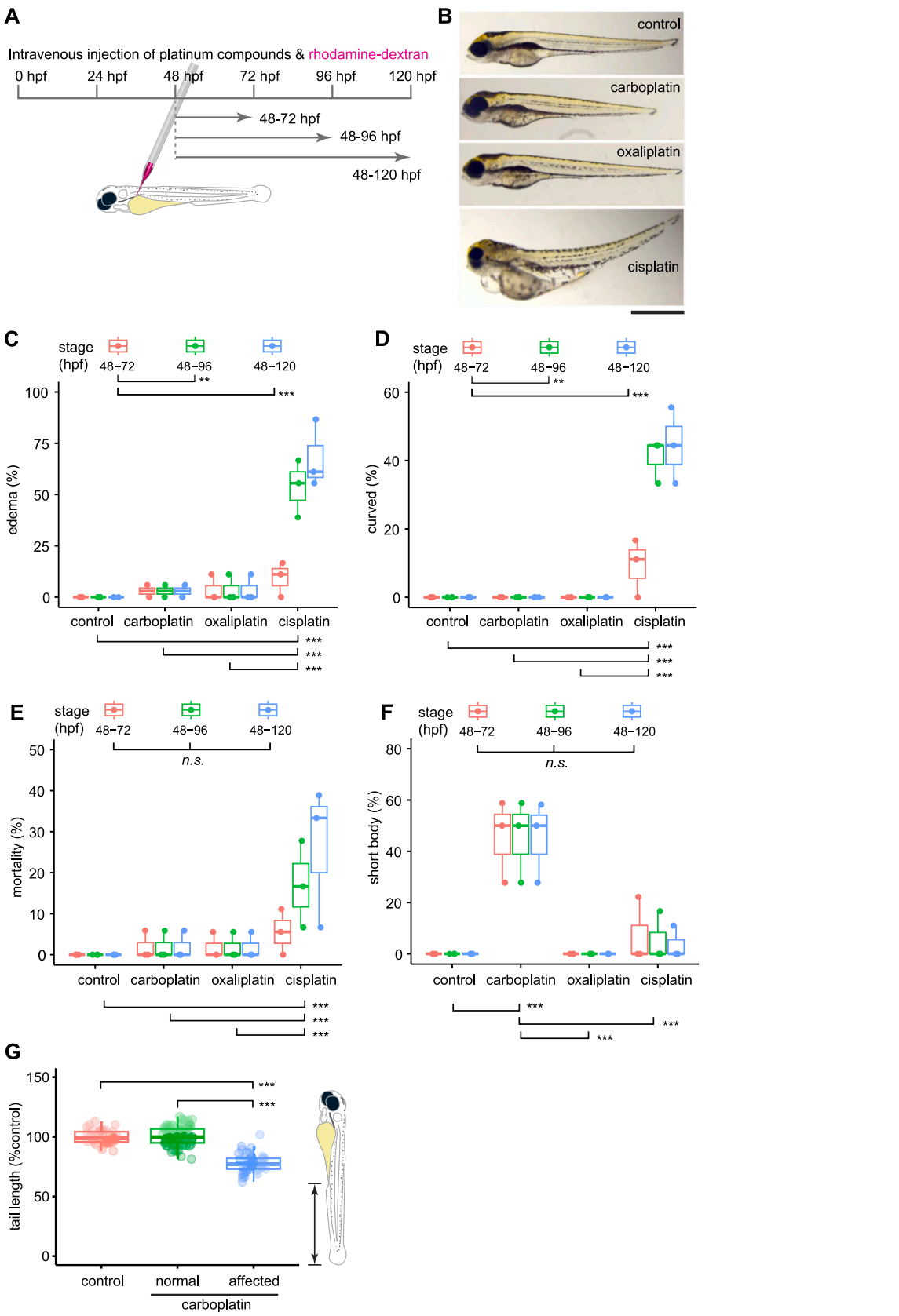

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Fig. 5. Adverse effects on embryos after intravenous injection of cisplatin, carboplatin and oxaliplatin. A) Schematic diagram of the experimental procedure. Platinum compounds (3 mM, spiked with rhodamine-dextran to monitor successful administration) were injected into the cardinal vein of embryos ($n = 24$) at 48 hpf and assessed at 72-, 96- and 120 hpf for edema formation (C), abnormal body axis curvature (D), mortality (E) and abnormal short body (F). (B) Representative images of eleutheroembryos at 120 hpf injected with either cisplatin, carboplatin or oxaliplatin, together with the platinum-free control injected solely with rhodamine-dextran. (C) Edema formation depends on the type of injected compound ($p = 1x10^{-8}$***, ANOVA) and is significant for cisplatin ($p < 0.001$***, Tukey's test). Edema formation was observed at particular stages ($p = 0.0008$***, ANOVA) and evident at 96 hpf ($p < 0.01$**, Tukey's test) and 120 hpf ($p < 0.001$***, Tukey's test). (D) Abnormal body axis curvature is dependent on the injected compound ($p = 1x10^{-14}$***, ANOVA) and significant for cisplatin ($p < 0.001$***, Tukey's test). The induction of axis abnormality was observed at particular stages ($p = 1x10^{-5}$***, ANOVA) and evident at 96 hpf ($p < 0.01$**, Tukey's test) and 120 hpf ($p < 0.001$***, Tukey's test). (E) The observed mortality solely depends on the injected compound ($p = 5x10^{-5}$***, ANOVA) and is significant for cisplatin ($p < 0.001$***, Tukey's test). (F) The abnormal short body phenotype was induced in a compound dependent manner ($p = 2x10^{-10}$***, ANOVA) regardless of the evaluation stage, identifying carboplatin as the sole responsible compound ($p < 0.001$***, Tukey's test). Depicted are the results of 3 independent experiments (number of exposed embryos: n = 72). (G) Injection of 3 mM carboplatin affected the tail length (distance between the cloaca and the caudal end; $p < 2x10^{-16}$***, ANOVA). A subpopulation of 3 mM carboplatin-injected embryos (37 % out of 117 embryos) showed significantly shorter tail length in comparison to its unaffected siblings (Tukey's test, $p < 1 \times 10^{-7}$***) and control (Tukey's test, $p < 1 \times 10^{-7}$***). Boxplots show the median (bold line), first and third quartile (box) and 1.5-fold IQR (whiskers).

the higher dose. The difference in toxicity phenotypes in response to oxaliplatin treatment is also seen in rodent models and patients, where oxaliplatin is not linked to acute kidney injury but rather initiates peripheral neuropathy (Rabik and Dolan 2007).

Higher concentrations of cisplatin were required to trigger lethality when embryos within the chorion were exposed compared to experiments with dechorionated embryos. This suggests that either the chorion prevents the accumulation of cisplatin in the embryo and/or that embryos at earlier stages of development appear to be less sensitive to the deleterious actions of cisplatin. Previously, ICP-MS studies showed a strong accumulation of cisplatin in the chorion, supporting the barrier function of the chorion reducing the actual delivered dose by up to 90 % (Karas et al. 2019; Kovács et al. 2016). Toxicity was again more pronounced in E3 medium compared to saline, showing that the impact of salinity is independent of the stages of development at which embryos are exposed. Interestingly, cisplatin interfered with hatching already at much lower concentrations than those resulting in lethality. When exposed in E3 medium, around 100 μM completely suppressed hatching, whereas at 200 μM all embryos survived, and maximal lethality was only observed starting at 600 μM. Surprisingly, inhibition of hatching by cisplatin treatment was more pronounced in NaCl solution, as none of the embryos hatched at a concentration as low as 50 μM. Although carboplatin and oxaliplatin were not toxic up to a concentration of 1000 μM, both interfered with hatching as well, but only when dissolved in saline. Oxaliplatin started to be effective at 600 μM, whereas carboplatin only showed partial hatching inhibition at 1000 μM. Thus, inhibition of hatching turns out to be the most sensitive endpoint after exposure to the different platinum compounds. Digestion of the chorion before hatching is mediated by the hatching enzyme ZHE1, a metalloprotease in which Zn^{2+} is bound in the active center, mediated by three critical histidine residues (Okada et al. 2010). A number of metal ions, such as Cu^{2+}, Ni^{2+}, and Cr^{3+} can bind to the metal-sensitive sites, replacing the active Zn^{2+} in the ZHE1 (Uribe et al. 2013). Notably, also $PtCl_2$ has been shown to interfere with hatching at a concentration as low as 0.2 μM (Osterauer et al. 2011). Thus, we hypothesize that the bivalent Pt atom in the different platinum complexes (cisplatin, carboplatin and oxaliplatin) might also interfere with Zn^{2+} binding and inhibit ZHE1 activity. The different structural properties of cisplatin, carboplatin and oxaliplatin could possibly explain the varying degrees of hatching inhibition due to altered binding to ZHE1, which warrants further investigations. The fact that hatching inhibition was much stronger in the presence of saline suggests an impact of sodium and chlorine ions on the interaction of platinum compounds with ZHE1. Another proposed mechanism by which cisplatin can interfere with hatching involves crosslinking of proteins in the chorion, thereby hindering its breakdown (Karas et al. 2020). Clearly, hatching is a complex process that is controlled not only by the enzymatic activity of ZHE1, but also by other physiological pathways such as the circadian clock or the dopaminergic system. The somatosensory system, which innervates and interacts with the hatching gland, may also be a potential target for platinum compounds. Physical forces due to embryo motility and spontaneous contractions also contribute to the rupture of the chorion. Further studies are needed to elucidate which of these regulatory factors are affected by the synergistic action of NaCl and platinum compounds (Cowan et al. 2024; Schoots et al. 1983; Villamizar et al. 2014). Regardless of the detailed mechanisms by which platinum compounds suppress hatching, the relatively low concentration needed (at least for cisplatin) to affect this adverse outcome are still higher than what has been maximally detected in real aquatic environments or polluted hospital wastewater (up to 250 μg/L cisplatin, carboplatin and oxaliplatin)(Misík et al. 2019; Queiros et al. 2021). Nevertheless, dependent on the potential bioaccumulation of cisplatin, inhibition of hatching seems to be a relevant acute endpoint to consider for ecotoxicological studies.

5. Conclusions

In this study, several factors were identified that critically influence the outcome of toxicity studies employing zebrafish. First, the impact of the exposure media, i.e. E3 medium versus saline, on the adverse outcome in response to different platinum anticancer drugs was demonstrated. Therefore, in future investigations, the composition of various media utilized to expose zebrafish to chemicals should be considered more carefully. Second, the mode of administration is key to the observed phenotypes. Whereas for ecotoxicity studies the compounds should be dissolved in aqueous embryo media, prediction of human toxicity needs to take into account the physiologically relevant exposure route. In case of drugs administered intravenously, direct microinjection of chemicals into the bloodstream of zebrafish embryos is recommended. As demonstrated here for platinum anticancer drugs, the conventional exposure protocol results in strikingly different phenotypes compared to the more demanding microinjection approach. Finally, the commonly used solvent DMSO not only affects the relative sensitivity towards cisplatin but also profoundly the delivered dose. Thus, the interaction of DMSO with chemicals to be tested in the zebrafish model must be assessed more rigorously. Our findings therefore should further improve the utility of zebrafish as a model organism to address the impact of chemicals on environmental as well as human health.

CRediT authorship contribution statement

Jin Yan: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Masanari Takamiya:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ding Zhang:** Methodology, Investigation, Formal analysis. **Giuseppina Pace:** Supervision, Methodology, Investigation. **Sepand Rastegar:** Writing – review & editing, Resources, Methodology. **Huili Wang:** Writing – review & editing. **Sarah Schoch:** Methodology, Investigation, Formal

analysis. **Beate Köberle:** Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization. **Andrea Hartwig:** Writing – review & editing, Supervision, Formal analysis. **Thomas Dickmeis:** Writing – review & editing, Supervision, Resources, Formal analysis, Conceptualization. **Carsten Weiss:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2025.109349.

Data availability

Data will be made available on request.

All data generated or analysed during this study are included in this published article (and its Supplementary Information files/source data files).

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