Increased apoptosis, reduced Wnt/β-catenin signaling, and altered tail development in zebrafish embryos exposed to a human-relevant chemical mixture

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HIGHLIGHTS

- A human-relevant chemical mixture induced apoptosis in zebrafish embryos.
- The mixture reduced Wnt/β-catenin signaling in the embryonic caudal fin.
- The shape of the caudal fin was altered in embryos exposed to the mixture.
- The results indicate that the mixture interferes with basic developmental processes.

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ABSTRACT

A wide variety of anthropogenic chemicals is detected in humans and wildlife and the health effects of various chemical exposures are not well understood. Early life stages are generally the most susceptible to chemical disruption and developmental exposure can cause disease in adulthood, but the mechanistic understanding of such effects is poor. Within the EU project EDC-MixRisk, a chemical mixture (Mixture G) was identified in the Swedish pregnancy cohort SELMA by the inverse association between levels in women at around gestational week ten with birth weight of their children. This mixture was composed of mono-ethyl phthalate, mono-butyl phthalate, mono-benzyl phthalate, mono-ethylhexyl phthalate, mono-isononyl phthalate, triclosan, perfluorohexane sulfonate, perfluorooctanoic acid, and perfluorooctane sulfonate. In a series of experimental studies, we characterized effects of Mixture G on early development in zebrafish models. Here, we studied apoptosis and Wnt/β-catenin signaling which are two evolutionarily conserved signaling pathways of crucial importance during development. We determined effects on apoptosis by measuring TUNEL staining, caspase-3 activity, and acridine orange staining in wildtype zebrafish embryos, while Wnt/β-catenin signaling was assayed using a transgenic line expressing an EGFP reporter at β-catenin-regulated promoters. We found that Mixture G increased apoptosis, suppressed Wnt/β-catenin signaling in the caudal fin, and altered the shape of the caudal fin at water concentrations only 20–100 times higher than the geometric mean serum concentration in the human cohort. These findings call for awareness that pollutant mixtures like mixture G may interfere with a variety of developmental processes, possibly resulting in adverse health effects.

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1. Introduction

Humans and animals are exposed to mixtures of chemicals derived from a variety of sources as reflected by the large number of exogenous compounds found in blood and tissue samples (Bergman et al., 2013). Long-term effects of such exposures are not well understood, but developing individuals tend to be more sensitive than adults. Epidemiological and experimental data suggest that developmental exposure to endocrine disrupting compounds (EDCs) increases the risk for disease later in life (Gore et al., 2015). Most studies have focused on effects by individual compounds although combined exposure to different chemicals probably is more important for health effects in the general population.

Birth weight is an important marker and predictor for the present and future health of a newborn. Low birth weight, defined by the World Health Organization (WHO) as a weight at birth less than 2500 g, is associated with an increased risk for disease and premature death (Rises et al., 2011; Barker, 2012). A recent study reports that on average 15% of all children born in 2015 worldwide exhibited low birth weight with a higher incidence in low- and middle-income countries than in high-income countries (Blencowe et al., 2019). There are many causes for low birth weight, including malnutrition, drug use, infection, and stress of the mother, genetic/chromosomal abnormalities of the fetus, and impaired placental function (Nardozza et al., 2017). In terms of environmental pollutants, maternal exposure to e.g., perfluorooctanoic acid (PFOA), cadmium, dioxin-like compounds, and ambient air pollution has shown negative associations with fetal growth or birth weight (Konishi et al., 2009; Pedersen et al., 2013; Knutsen et al., 2018; Khoshhali et al., 2019).

Pollutants can interfere with basic cellular functions, such as apoptosis or proliferation, resulting in various health outcomes, depending on cell type, tissue, and developmental stage at exposure. The extrinsic and intrinsic pathways of apoptosis are evolutionarily conserved and tightly regulated suicide programs that function to remove superfluous and irreversibly damaged or harmful cells (Galluzzi et al., 2018). Apoptosis is essential in morphogenesis and organogenesis, processes that often involve an over-production of cells and subsequent removal of groups of certain cells for proper development of a tissue or organ (Meier et al., 2000). The apoptotic pathways involve activation of caspase cascades leading to degradation of DNA and proteins, and ultimately cell death. The activated caspases also play an important role in maintenance of tissue homeostasis following stress-induced cell death by inducing compensatory cell proliferation (Fogarty and Bergmann, 2017). The extrinsic pathway is activated by extracellular factors detected by “death” receptors at the cellular membrane, while the intrinsic pathway is induced by intracellular triggers, including DNA damage, mitotic errors, oxidative stress, and growth factor withdrawal (Galluzzi et al., 2018). Various environmental pollutants have been found to induce apoptosis (e.g., Robertson and Orrenius, 2000).

Wnt signaling represents an evolutionarily conserved signal transduction network that organizes proliferation, differentiation, migration, polarization, and patterning of cells, thus playing central roles in development and growth of multicellular animals (Loh et al., 2016). Increasing evidence also shows its important roles in cellular metabolism and metabolic disease (Sethi and Vidal-Puig, 2010; Ackers and Malgor, 2018). The network is controlled by a multitude of autocrine- or paracrine-acting glycoproteins – Wnts — that activate different parts of the network via cell surface receptors (Niehrs, 2012). The canonical Wnt signaling pathway involves β-catenin, a protein that binds to the T-cell factor/lymphoid enhancer factor (Tcf/Lef) family of transcription factors, thus regulating transcription of genes implicated in cell proliferation, differentiation, and other processes (Hurlstone and Clevers, 2002; Herbst et al., 2014). The activity of β-catenin is controlled by positive and negative regulators, which in addition to Wnts include physiological inhibitors and enhancers, various transcription factors (cross-talk), and kinases that mediate its proteasomal degradation (Mulholland et al., 2005; Kikuchi et al., 2011). Pollutants reported to interfere with β-catenin function include dioxins, benzo(a)pyrene, and bisphenol A (Ustundag et al., 2017; Yi et al., 2019; Yoshioka and Tohyama, 2019).

The zebrafish (Danio rerio) is commonly used as a model to study developmental toxicity. It has been used to explore molecular mechanisms of apoptosis and Wnt/β-catenin signaling and the effects of pollutants on these processes (Yamashita, 2003; Holley, 2006; Mathew et al., 2008; Shi et al., 2008; Eimon and Ashkenazi, 2010; Yoshioka et al., 2011).

The current paper is part of the EU project EDC-MixRisk where data from the Swedish environmental longitudinal, mother and child, asthma and allergy (SELM) cohort (Bornehag, 2012) were used to identify chemical mixtures of concern. Within EDC-MixRisk, effects of these mixtures were studied in various experimental in vivo and in vitro models, with the goals to learn more about biological mechanisms and to establish dose response data for developing better risk assessment tools. The particular mixture studied here, Mixture G, was designed based on the inverse association between levels measured in the pregnant women and birth weight of their newborns (Birgersson et al., 2017). Mixture G is composed of five phthalate-monoesters, triclosan, and three perfluoralkyl acids (PFAAs) (see Table S1 and subsection 2.2.1). Phthalates, PFAAs, and triclosan are widespread environmental pollutants and thus mixtures of these compounds are relevant for both human and wildlife. We postulated that Mixture G could interfere with various developmental processes and used zebrafish models to gain mechanistic insights into its actions. In a previous study, we examined the effects of Mixture G on metabolic endpoints in developing zebrafish and found that it stimulates adipogenesis and alters metabolic rate (Mentor et al., 2019). In this study, we hypothesized that mixture G can disrupt early development by interfering with apoptosis and/or Wnt/β-catenin signaling. Thus, our aim was to investigate effects of Mixture G on these two processes in zebrafish embryos.

2. Materials and methods

2.1. Animals

A wildtype zebrafish line (AB) was used to assess the effect of Mixture G on apoptosis. The transgenic zebrafish line, Tg(6xTcf/Lef)Bsh-miNiP:d2EGFP)6918 (AB; homozygous), developed by Shimizu et al. (2012), was used to investigate effects on Wnt/β-catenin signaling. To acquire breeding stocks of wildtype fish, embryos were obtained from SciLifeLab zebrafish facility (Evolutionary Biology Centre, Uppsala University; https://www.scilifelab.se/facilities/genomeengineeringzebrafish/). Embryos of the transgenic line were obtained from the original stock which is
maintained by the National BioResource Project, Zebrafish Core Institution, the Institute for Physical and Chemical Research (RIKEN) in Japan. Breeding stocks from both lines were kept at the SciLifeLab facility at 27–28 °C and under a 14 h light/10 h dark diurnal photoperiod. Eggs for use in the experiments were generated through group breeding of three female and three male fish. Fish husbandry and experimental procedures were in accordance with the EU legislation and the protocols were approved by the Uppsala Animal Experiments Ethics Board (Uppsala District Court), reference numbers C96/15 and C109615/16.

2.2. Chemicals

All chemicals used in the exposures were purchased from Sigma-Aldrich (Saint Louis, MO, USA) or Toronto Research Chemicals (North York, ON, Canada).

2.2.1. Mixture G. The mixture was composed within EDC-MixRisk using data from the Swedish mother-child cohort SELMA (Bornehag, 2012; Birgersson et al., 2017, preprint). Urine and blood for chemical analyses were sampled from the mothers around the 10th week of gestation. Levels of bisphenol A, triclosan, and ten metabolites of bisphenol A, triclosan, and ten metabolites of in total five phthalate diesters were analyzed in serum. A mixture of nine compounds of concern was designed in a three-step procedure. Firstly, negative associations with birth weight were identified in data from 1874 mother-child pairs by the use of weighted quantile sum (WQS) regression analysis (Carrió et al., 2015). Next, serum concentrations of six compounds measured in urine (i.e., five phthalates and triclosan) were calculated based on estimations of the corresponding daily intakes (DI). Finally, we used the geometric mean serum concentrations of the nine chemicals from all SELMA mothers (>2300) to establish mixing proportions for the mixture on a molar basis. The Mixture G components and their estimated/measured geometric mean serum concentrations in the mothers were: Mono-ethyl phthalate (MEP; 28 nM), mono- butyl phthalate (MBP; 23 nM), mono-benzyl phthalate (MBzP; 11 nM), mono-ethylhexyl phthalate (MEHP; 15 nM), mono-isonyl phthalate (MINP; 21 nM), triclosan (3.1 nM), perfluorohexane sulfonate (PFHxS; 3.3 nM), perfluorooctanoic acid (PFOA; 3.4 nM), and perfluorooctane sulfonate (PFOS; 10 nM). More information about mixture G is given in Tables S1 and S2, and the concept and mathematical procedure is further described in Bornehag et al. (2019) and Birgersson et al. (2017, preprint).

A stock solution of Mixture G (in DMSO) to be used for experimental studies within EDC-MixRisk was prepared by the partners at Lund University. In this paper, Mixture G concentrations are expressed as “x hsc,” where 1x hsc represents the above-mentioned geometric mean serum concentrations.

2.2.2. Positive controls. XAV939 is a small molecule which selectively inhibits the Wnt/β-catenin pathway (Huang et al., 2009). PFOS is one of the components of Mixture G and known to induce apoptosis in zebrafish (Shi et al., 2008; Sant et al., 2018). Stock solutions of XAV939 and PFOS were prepared in DMSO.

2.3. Exposure

We chose to expose the embryos via ambient water since it is an efficient and non-invasive way of administration. Exposure solutions were prepared by mixing vehicle (DMSO) or stock solutions of Mixture G, XAV939, or PFOS into carbon-filtered Uppsala municipality tap water to yield the final nominal water concentrations of 0.01% of DMSO, 1, 20 or 100x hsc of Mixture G, 10 nM of XAV939, and 0.2, 2, 5, or 10 μM of PFOS. The Mixture G concentrations were selected based on results from our previous study in which 20x hsc was found effective in stimulating adipogenesis and concentrations higher than 100x hsc were toxic (Mentor et al., 2019). The concentration of PFOS in 20x hsc of Mixture G is 0.2 μM, but to ascertain an effect of PFOS (which was used as positive control), we added two higher concentrations, i.e., 2 and 10 μM (or 5 μM). In some experiments, the lowest concentrations of Mixture G and PFOS were not included for practical reasons.

Before exposure started, the exposure vials (glass petri dishes or 12- or 24-well polystyrene plates) were pretreated with exposure solutions overnight. At 3–4 h post-fertilization (hpf), the solutions were renewed and exposure started by the transfer of embryo groups to glass petri dishes or individual embryos to wells of the polystyrene plates (density: 1 egg mL−1). Glass petri dishes were used in early experiments to avoid the risk of leakage of interfering substances from plastics. After testing and finding no difference in effect in embryos exposed in glass versus polystyrene vials, we switched to using polystyrene plates to be able increase the number of biological replicates in each experiment. The embryos were incubated at 28.5 °C with a 12-h light/12-h dark diurnal photoperiod and with daily renewal of exposure solutions. Apoptosis, Wnt/β-catenin signaling, caudal fin (CF) morphology, standard length, and relative mRNA levels were determined according to the schedule shown in Fig. 1. An experiment to compare hatching rates over time was performed in wildtype and transgenic embryos exposed to Mixture G in the period 48 to 72 hpf. More details on the exposures are given in the figure legends.

2.4. Wnt/β-catenin signaling

Wnt/β-catenin signaling was studied using zebrafish of the transgenic line, which carry a Tcf/Lef-linked reporter that indicates β-catenin-dependent gene expression by enhanced green fluorescent protein (EGFP). Initially, a pilot study was performed where EGFP fluorescence was localized in unexposed embryos at 2 and 3 days post-fertilization (dpf). Images were obtained using a Nikon SMZ1500 microscope with GFP HQ filter (Ex 470/40, DM495, BA 525/50). Based on the results from this study, we settled on analyzing Wnt/β-catenin signaling in the caudal fin in 2-day-old hatched embryos.

After exposure and hatching, embryos (54–60 hpf) were anesthetized in tricaine (0.2 mg mL−1) and photographed under microscope. A light microscopy image was taken of the whole embryo using the 4× objective. Switching to the 8× objective, the caudal fin was photographed with both light and fluorescence microscopy. By means of a script in CellProfiler (Lamprécht et al., 2007), the fin was localized and encircled in the bright-field images; the total fluorescence within the circle was analyzed in the respective overlapping dark-field images (Fig. 2B). The CellProfiler pipeline is described in the first section in supplementary
Each data point represents one individual embryos exposed in 24-well plates to vehicle (0.01% DMSO, denoted 54-60 hpf, the caudal signaling. B) The total fluorescence within a circular area centered on the fin was quantified by automated imaging analysis using CellProfiler. C-D) The graphs show results from individual embryos exposed in 24-well plates to vehicle (0.01% DMSO, denoted “0”) or XAV939 (10 nM), or vehicle or Mixture G (20 or 100x hsc). Exposure started at 3-4 hpf and at 54-60 hpf, the caudal fins were photographed by light- and fluorescence microscopy. The results are expressed as percentages of the respective DMSO group mean (hatched line). Each data point represents one fish and the solid lines group means. Statistically significant differences between control and exposed groups were determined using t test with Welch’s correction (n=18-19) and D one-way ANOVA followed by Dunnett’s test (n=23-24) and are indicated by * p<0.05 and *** p<0.001.

The above method is very robust and fully automated. However, it gives only a rough definition of the fin and the analyzed area includes background. In order to obtain more precise data we also measured fluorescence in a specified area of the caudal fin (denoted “fin area”, or “A”; Fig. 3A) using ImageJ (Schneider et al., 2012). Manual image analysis was performed without knowledge of the exposure group. For each fish, part of the caudal fin was outlined in the 8x-light microscopy images using the selection tool, with the anterior border (line c in Fig. 4A) positioned 300 pixels anterior to the notochord tip. These selections (representing A) included most of the caudal fin fluorescence when applied to the corresponding fluorescence images. The following parameters were analyzed: The fin area (A) measured in pixels; the mean fluorescence in A, defined as the mean pixel intensity (MPI) within A subtracted by the background (MPI in the area outside the fish); and the total fluorescence in A (A × MPI).

2.5. Caudal fin morphometry

Morphometric analyses of the caudal fin were performed in ImageJ without knowledge of the exposure groups. Tail and fin lengths and heights were measured in the 8x-light microscopy images as indicated in Fig. 4A. The length of the fin tip (length a) was measured from the posterior notochord tip to the posterior end of the fin. The total height of the tail, including the fin, was measured at the level of the notochord tip (height b) and at a level 300 pixels anterior to the notochord tip (height c). At the same level as tail height c, height of the tail excluding the fin was also measured (height d). Embryo standard length (from crown of the head to the posterior notochord tip) was determined in the 4x-images.

2.6. Apoptosis

Apoptosis was determined by three methods; acridine orange staining, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and an assay for caspase 3-like activity. The acridine orange and caspase 3-like activity assays were used to assess apoptosis in the whole body whereas the TUNEL assay was used for localization of apoptotic cells in the tail fin. For the illustrations in Fig. 5, fluorescence images were taken of acridine orange-stained embryos at 36 and 60 hpf (about 20 embryos per exposure and day) using the Nikon SMZ1500 microscope with the GFP HQ filter (Ex 470/40, DM495, BA 525/50) and 4x objective.

2.6.1. Acridine orange staining. Acridine orange is a fluorescent, DNA intercalating dye that is used to study apoptosis in zebrafish and other models (Parng et al., 2004). Based on methods described by Tilton et al. (2008) and Sant et al. (2018) we established two slightly different assays where acridine orange fluorescence was measured in intact stained embryos and in supernatants from homogenized stained embryos, respectively.

The first assay was performed with 1-day-old embryos that were exposed in groups in glass petri dishes. Before processing, embryos from all dishes with the same exposure were pooled together. Dechorionation was performed as described by Westerfield (2000), but with a lower pronase concentration
The embryos were stained by incubation in aqueous acridine orange solution (5 mg L⁻¹) in the dark at room temperature (RT). After 60 min, the embryos were thoroughly washed in water and then placed individually in wells of a 96-well plate containing a euthanizing dose of tricaine (0.5 mg mL⁻¹ water) for immediate measurement in a plate reader, as described below. This experiment was repeated three times.

The second assay was run on three consecutive days with embryos exposed in groups of four in 12-well plates. At 28, 52, and 76 hpf, one embryo from each replicate well was transferred to a dish to be processed together with the other of the same exposure. Embryos processed at 28 hpf were first dechorionated by pronase (0.08 mg mL⁻¹ water). Each day one batch of acridine orange solution was prepared fresh to be split among all exposure groups. The staining procedure was identical to that of the first assay, except that lower acridine orange concentration (1 mg mL⁻¹) and shorter incubation time (30 min) were used in order to reduce background staining. After staining and thorough rinsing in water, the embryos were placed individually in Eppendorf tubes containing 50 µL of ice-cold water; then 125 µL of 70% ethanol (v/v) was added to each tube and the tubes were put on ice in the dark. The embryos were homogenized with an ultra sound probe and subsequently the homogenates were centrifuged at 11000 × g for 15 min (4 °C). An aliquot (50 µL) of each supernatant were pipetted into each of two 96-well plates. This experiment was performed once.

In both assays, the fluorescence was measured at 485 nm (ex) and 535 nm (em) with a plate reader (Victor 3, PerkinElmer, Boston, MA, USA). Unstained embryos, processed in the same way as...
stained, were included as background controls. A dilution series of acridine orange in 50% ethanol showed that the data were within the linear range of the acridine orange fluorescence/concentration curve.

2.6.2. TUNEL. Localization of apoptotic cells was studied by TUNEL technique using the in situ cell death detection kit, POD (Sigma-Aldrich), according to the manufacturer’s instructions. At 54 hpf (or 54, 78, and 102 hpf), hatched embryos were euthanized in tricaine (ca. 0.5 mg mL⁻¹ water) and then fixed in 4% formaldehyde in phosphate buffer overnight (4 °C). Fixed specimens were rinsed in phosphate-buffered saline (PBS), treated with 3% H₂O₂ in methanol at RT (15 min), rinsed with PBS, and then treated with 0.1% of Triton X-100 in ice-cold 0.1% sodium citrate (2 min). After thorough rinsing with PBS and removal of excess buffer, the specimens were incubated (37 °C) with TUNEL reaction mixture (60 min), rinsed with PBS, and incubated (37 °C) with converter POD solution (30 min). Finally, they were rinsed in PBS and then treated with peroxidase substrate solution (0.05% DAB and 0.015% H₂O₂ in 0.01 M PBS, pH 7.2; 10 min, RT). Labeled embryos were photographed under light microscope and stained spots were counted (Fig. 6A).

2.6.3. Caspase 3 assay. Caspase 3-like activity was assayed at 78 hpf using Caspase 3 Assay Kit (Colorimetric, Sigma Aldrich). The embryos were anesthetized with ice and washed with ice-cold PBS. Samples of 40 pooled embryos were placed in micro tubes, snap-frozen in liquid N₂, and stored at −80 °C awaiting analysis. For caspase 3 assay, defrosted samples were mixed with 100 μL of lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS, 5 mM DTT), and homogenized. The homogenate was centrifuged at 13000 × g (4 °C) for 17 min and the supernatant was collected. From each sample, duplicate 20-μL aliquots of the supernatants and 160 μL of assay buffer containing 2 mM caspase 3 substrate (Acetyl-Asp-Glu-Val-Asp-p-nitroanilide), 20 mM HEPES, 2 mM EDTA, 0.1% CHAPS and 5 mM DTT (pH 7.4) were placed in wells of a 96-well plate. The plate was incubated at 37 °C. The release of product (p-nitroaniline, pNA) was measured after 0.5 and 2 h and then repeatedly every 2 h for up to 24 h by absorbance at 405 nm using a plate reader (Victor 3 from PerkinElmer or Bioscreen C Growth Curve Analysis System from Oy...
Growth Curves AB Ltd, Helsinki, Finland). Total protein concentration was determined with the Pierce Coomassie Plus Assay Reagent (ThermoFisher, Waltham, MA, USA) according to the manufacturer’s instructions. The pNA absorbance values were adjusted for protein content (μmoles/μg) of each sample and normalized to the mean values of the control (DMSO). For each replicate, the 30-min data were subtracted from the data at all other time points.

### 2.7. Quantitative real-time RT PCR

Zebrafish embryos were exposed in glass petri dishes to vehicle (0.01%), Mixture G (1x, 20x, or 100x hsc), or PFOS (0.2 or 2 μM). At 54 and 78 hpf, groups of 15–20 embryos were anesthetized with ice-cold water, pooled in micro tubes, flash frozen in liquid nitrogen, and stored at −80 °C awaiting analysis. Preparation for

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**Fig. 5. Effects of Mixture G and PFOS on apoptosis analyzed by acridine orange staining.** A) The images show localization of acridine orange staining (representing apoptosis) at ca 36 and 60 hpf with arrows indicating examples of stained cells. B) The graphs show the relative quantity of acridine orange fluorescence as measured in supernatants of homogenized embryos at 36, 60, and 84 hpf. The embryos were exposed in groups of four in 12-well plates to vehicle (0.01% DMSO; “0”), Mixture G (20 or 100x hsc), or PFOS (10 μM). Exposure started at 3 hpf. The upper panel in A was made from two images of the same individual taken at different focal planes and merged at the dotted line. The Mixture-G-exposed embryo shown in the lower panel in A was among those having the largest number of visible stained cells. Data shown in the graphs are expressed as percentages of the respective DMSO control medians (hatched line). Each data point represents one fish and the solid lines indicate group medians. Statistically significant differences between the DMSO control and exposed groups were determined using Kruskal-Wallis test followed by Dunn’s test (n = 15-24). * p<0.05, ** p<0.01, and *** p<0.001.
quantitative PCR (qPCR) was performed as follows: total RNA was isolated and DNase-treated using the Aurum™ total RNA fatty and fibrous tissue kit (Bio-Rad, Hercules, CA, USA) according to Bio-Rad’s instructions. Integrity of RNA was verified with agarose gel electrophoresis and purity and quantity of RNA were determined spectrophotometrically using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). Samples with 260/280 and 260/230 nm ratios >1.9 were included in subsequent procedures. Complementary DNA was synthesized using the iScript cDNA Synthesis kit (Bio-Rad). Sequences of gene-specific primers were found in the literature (Goldstone et al., 2010; Rhodes et al., 2010; Sarkar et al., 2014; Du et al., 2017; Sant et al., 2018) or designed by us and then synthesized by Sigma-Aldrich (Table S3). Samples of 4–6 biological replicates were analyzed using a Rotor Gene 6000 real-time PCR machine (software version 1.7; Qiagen, Hilden, Germany). The reaction mixtures (20 µl) consisted of iQ SYBR Green Supermix (Bio-Rad), 5 pmol of each of forward and reverse primers, and cDNA derived from 25 ng of total RNA. All samples were analyzed in duplicate with the following protocol: 95 °C for 4 min followed by 30–40 cycles (cycle numbers varying with transcript levels) of 95 °C for 15 s and 62 °C for 45 s. At the end of each qPCR run, a melt curve analysis was performed in the range from 55 °C to 95 °C to confirm that single PCR products were formed. The reference genes used were arnt2 and ef1a.

2.8. Statistical analysis

For the statistical analyses, GraphPad Prism 5 from GraphPad Software (San Diego, CA, USA) was used. Statistically significant differences between the control and multiple exposure groups were determined by one-way ANOVA followed by Dunnett’s post-hoc test. Kruskal-Wallis test followed by Dunn’s test with selected pairs was used if the residuals were not normally distributed or if the variance differed among exposure groups even after logarithmic transformation. For comparison of two groups, students t-test, t-test with Welch’s correction (when variances differed), or Mann-Whitney’s test (when the assumption of normality was not met) was used. Data from replicate dishes or different experiments were combined if no statistical difference (p < 0.05) was detected by student’s unpaired t-test or ANOVA followed by Dunnett’s test. The figures were made using GraphPad Prism 5 and Adobe Photoshop CS5 (v12.0) from Adobe (San Jose, CA, USA).

3. Results

3.1. Mixture G

The geometric means and 95th percentiles of the levels of mixture components measured in urine or serum in pregnant women in the SELMA cohort are shown in Table S2. Relative to the geometric means, the 95th percentile values of the PFASs and phthalate metabolites were 2–3 and 3–5 times higher, respectively. The corresponding value for triclosan was 280.

3.2. Wnt/β-catenin signaling

3.2.1. Localization of EGFP fluorescence. The transgenic fish exhibited a relatively strong fluorescence in various tissues at 2 dpf while the fluorescence was weaker one day later. At 2 dpf (48–60 hpf), the most prominent locations of fluorescence were brain, otic vesicles, jaw, lateral line primordia, and pectoral and caudal fins (Fig. 2A). Two difficulties with measuring fluorescence in whole...
zebrafish embryos are that the fluorescence cannot be quantified with precision in three-dimensional structures and that pigmentation may block the fluorescence (e.g., see the brain in Fig. 2A). However, the caudal fin is flat and only a few cell layers thick, making it suitable for quantitative measurements by image analysis. Furthermore, at 2 dpf the caudal fin showed a high abundance of fluorescent cells and few melanocytes (Fig. 2A).

3.2.2. Effects of XAV939 and Mixture G on Wnt/β-catenin signaling in the caudal fin. The fluorescence in the caudal fin was screened in images from all fish using CellProfiler. The program identified the caudal fin and determined the fluorescence within a circle centered on the fin (Fig. 2B). The total fluorescence in this area was found to be reduced in groups exposed to the positive control XAV939 (10 nM) and in groups exposed to Mixture G at 100x hsc, while no statistically significant effect was seen at 20x hsc (Fig. 2C and D). Exposure to 2 or 5 μM of PFOS had no significant effect on Wnt/β-catenin signaling in the fin (Fig S1).

In order to refine the analysis, an area defined by the shape of the caudal fin (A) was outlined as shown in Figs. 3A and 4A; the area, as well as the mean and total fluorescence (pixel intensities) in this area were determined (Fig. 3B–G). In embryos exposed to XAV939 there was no statistically significant effect on the size of the fin area, but mean and total pixel intensities were reduced (Fig. 3B–D). Embryos exposed to Mixture G at 20x hsc showed no effect on any of these parameters, but those exposed at 100x hsc exhibited a small but statistically significant reduction in fin area and pronounced reductions in the mean and total pixel intensities (Fig. 3E–G). Data in the graphs in Figs. 2 and 3 are from the same images analyzed by the two methods, and both methods indicate that the positive control XAV939 and Mixture G at 100x hsc reduce EGFP fluorescence (representing Wnt/β-catenin signaling) in the fin.

3.3. Fin morphology

To further analyze the effect on the shape of the caudal fin, four defined distances within the fin area A were measured; length of the fin tip (a) and three heights of the tail (b, c, and d; indicated in Fig. 4A). In embryos exposed to XAV939 there was no effect on a, b, or c (Fig. 4B–D), while height d (tail height c excluding the fins) was slightly shorter than in controls (Fig. 4E). In embryos exposed to Mixture G at 20x hsc there was no effect on a, c, or d, while height b (the highest part of the caudal fin) was somewhat shorter than in controls (Fig. 4F–I). Mixture G at 100x hsc had no effect on length a, but the heights b, c, and d were shorter than those in the DMSO control group.

The standard length of the embryos shown in Figs. 2–4 was not affected by exposure to XAV939 or Mixture G. In the experiment with XAV939, the standard lengths (mean ± SD) were 1.50 ± 0.07 mm in controls and 1.50 ± 0.04 mm in XAV939-exposed fish. In the Mixture G experiment, the lengths were 1.49 ± 0.05 mm (controls), 1.49 ± 0.05 mm (20x hsc) and 1.49 ± 0.04 mm (100x hsc).

3.4. Apoptosis

3.4.1. Acridine orange staining. Fig. 5A shows the distribution of acridine orange fluorescence in zebrafish embryos (ca 36 and 60 hpf) exposed to DMSO and 100x hsc of Mixture G. Scattered fluorescent cells were observed in several parts of the fish at both stages but the number of fluorescent cells was visibly larger in the older embryos. At 36 hpf, strong staining was present in the hatching gland which is known to have apoptotic cells at this stage. However, no marked difference in number of stained cells was discerned between DMSO- and Mixture G-exposed fish by visual inspection. At 60 hpf, staining was present in the brain, fin, and eyes, structures that have a high incidence of apoptosis in embryonic zebrafish. At this stage, many Mixture G-exposed embryos appeared to have an increased number of stained cells compared with DMSO controls, although the amount of stained cells varied largely within the Mixture G-exposed group. Diffuse staining, interpreted as unspecified, was present in the yolk.

Acridine orange staining (representing apoptosis) was quantified using two assays, one based on supernatant of homogenate from stained embryos (Fig. 5B) and one based on intact stained embryos (Fig S2). With the assay using homogenized embryos, effects on apoptosis were determined at 36, 60, and 84 hpf (Fig. 5B). Exposure to 20x hsc of Mixture G had no effect on acridine orange staining at any time point studied, while exposure to 100x hsc led to statistically significant increases in acridine orange staining at 36 and 60 hpf. Embryos exposed to PFOS (10 μM) showed stronger acridine orange staining than the controls at 36 hpf and weaker staining than the controls at 60 hpf. At 84 hpf, none of the exposed groups showed a statistically significant difference versus the control.

With the assay based on intact embryos three experiments were performed at 36 hpf, and since the results differed slightly among the experiments each experiment is presented separately (Fig S2). Mixture G at 20x hsc induced apoptosis in all three experiments, while exposure to 20x hsc of Mixture G or to PFOS (10 μM) resulted in statistically significant induction in two out of the three experiments (Fig S2).

3.4.2. TUNEL. The effect of Mixture G on apoptosis was also determined using TUNEL technique, in which spots of labeled cells were counted in the fins surrounding the notochord of the embryo (tail fin). The DMSO control fish showed a few labeled spots, generally localized at the edge of the caudal fin and in the area near the cloaca while increased labeling was observed around the edges of the whole fin after exposure to Mixture G. Examples of TUNEL staining in the tail fins of DMSO- and Mixture G-exposed embryos are shown in Fig. 6A in which four and ninety spots were recorded, respectively. Fig. 6B shows the results from two combined experiments where embryos were exposed to Mixture G or PFOS (positive control) and analyzed at 54 hpf. The relative amount of labeling increased compared with the DMSO group in embryos exposed to Mixture G at 20 and 100x hsc and to PFOS at 2 and 10 μM (Fig. 6B). In a time course study, the effect of Mixture G (20x hsc) on TUNEL labeling was analyzed at 54, 78, and 102 hpf; only at 54 hpf, the amount of labeling was higher than in the DMSO control group (Fig. 6C).

3.4.3. Caspase activity. Caspase 3-like activity was assayed in embryos exposed to Mixture G or PFOS by repeated measurements for up to 24 h starting at 78 hpf. During the first hours of the assay, the fluorescence values were low and showed a large variation (Fig S3). In the period of 4–8 h, stabilization was observed; i.e., the fold-DMSO control values were similar at 4, 6, and 8 h (Fig S3). At 4 h there was no effect of Mixture G or PFOS at the lowest concentrations examined (1x hsc and 0.2 μM, respectively), while exposure to higher concentrations, 20x and 100x hsc of Mixture G and 2 and 10 μM of PFOS, led to an increased caspase 3-like activity (Fig. 7).

3.5. mRNA expression

Effects on mRNA expression of genes involved in apoptosis and oxidative stress were assessed by qPCR at 54 and 78 hpf. The genes involved in apoptosis were tumor protein p53 (p53), BCL2-associated X, apoptosis regulator (bax), and MDM2 proto-oncogene (mdm2) and those involved in oxidative stress were catalase (cat), glutathione peroxidase 1a (gpx1a), and nuclear factor E2-related factor 2a (nrf2a). We found no effect by PFOS or Mixture G on the mRNA expression of any of the genes analyzed (Fig S4).
Statistically significant differences among groups with the same exposures from different experiments, as assessed by one-way ANOVA followed by Dunnett's test. Statistically significant differences between the DMSO control and exposed groups were determined using Kruskal-Wallis test followed by Dunn's test (**p ≤ 0.01, and ***p ≤ 0.001).

3.6. Hatching rate

Hatching rate was not significantly affected by exposure to Mixture G or by embryo origin (wildtype or transgenic; Fig S5). At 48 hpf, one wildtype embryo (2%) and no transgenic embryo had hatched, while at 72 hpf, 97% of the embryos of both types had hatched. At 60 hpf, the hatching rates were 90% in the wildtype embryos (n = 89) and 92% in the transgenic embryos (n = 89).

4. Discussion

In this study we show that exposure of embryonic zebrafish to a chemical mixture that was inversely associated with birth weight in humans led to increased incidence of apoptosis, suppressed Wnt/β-catenin signaling, and altered caudal fin shape.

4.1. Apoptosis

The pro-apoptotic effect of Mixture G was demonstrated with three different methods (acridine orange staining, TUNEL, and caspase-3 activity). The three methods also indicated increased apoptosis by PFOS, a compound previously shown to have this effect in zebrafish embryos (Shi et al., 2008; Sant et al., 2018).

Mixture G induced apoptosis at 20x hsc and appeared approximately as potent as 2 μM PFOS. The concentration of PFOS in 20x hsc of the mixture is 0.2 μM, but this concentration of PFOS had no effect by its own. This suggests that the pro-apoptotic effect of Mixture G involves other compounds in addition to PFOS. Indeed, the other perfluoralkyl acids (PFAAs) present in the mixture, PFOA and PFFtks, have also been shown to induce apoptosis in rodents (Fang et al., 2010; Lee et al., 2014; Lopez-Arellano et al., 2019). In a recent epidemiological study, levels of several PFAAs were associated with an increased response of biomarkers for hepatocyte apoptosis (Bassler et al., 2019). Furthermore, the diethylhexyl phthalate metabolite MEHP is known to trigger apoptosis in germ cells of mouse and rat testes (Richburg and Boekelheide, 1996; Lee et al., 1997). The antimicrobial compound triclosan was shown to affect energy expenditure in rat liver mitochondria, and was proposed to be pro-apoptotic (Newton et al., 2005). In support of this, a recent study showed that triclosan increased the formation of reactive oxygen species and induced apoptosis in primary cultures of mouse neocortical neurons (Szychowski et al., 2019). Thus, a number of studies on Mixture G components support our finding that the mixture was pro-apoptotic.

4.2. Wnt/β-catenin signaling

We also found that Mixture G suppressed Wnt/β-catenin signaling. The transgenic zebrafish line used, Tg(6xTcf/LefBS-mini-P:d2EGFP)H04, was developed by Shimizu et al. (2012). Shimizu et al. (2012) investigated the localization of fluorescence in these fish during embryogenesis, organ development, and fin regeneration and found a strong signal at sites known to have active Wnt/β-catenin signaling, such as the brain, jaw and fins. They also found that the EGFP fluorescence increased and decreased in embryos by exposure to an activator (BIO) and a repressor (XAV939) of the pathway, supporting the relevance of the model for studies on the Wnt/β-catenin pathway (Shimizu et al., 2012). Similar to Shimizu et al. (2012), we found strong fluorescence in mesenchymal cells in the caudal fin at 2 dpf, and exposure to XAV939 reduced the fluorescent signal in the fin at this stage. Mixture G had an effect very similar to that of XAV939, reducing the mean fluorescence in the fin, indicating that Mixture G downregulated Wnt/β-catenin signaling in the fin cells. There was also a reduced fin area in embryos exposed to Mixture G. Notably, height but not length of the fin was affected and at present it is unclear whether this effect on the fin involved aberrant apoptosis, and/or Wnt/β-catenin signaling.

There are relatively few studies on effects of Mixture G components on Wnt/β-catenin signaling. In mice, exposure to MEHP led to an increased level of phosphorylated β-catenin in bone marrow stromal cells (suggesting increased β-catenin degradation); this was associated with reduced osteogenesis and increased adipogenesis (Chiu et al., 2018). The parent compound of MBP, DBP, was found to down-regulate Wnt/β-catenin signaling in genital tubercle cells in male fetal rats (Zhang et al., 2011). On the other hand, a high concentration of DBP (20 μM) was reported to stimulate Wnt/β-catenin signaling leading to disrupted dorsal-ventral patterning in embryonic zebrafish (Fairbairn et al., 2012). In the present study, no effect of 2 or 5 μM PFOS on Wnt/β-catenin signaling was observed in the caudal fin of the zebrafish embryo, whereas in vitro studies found that a lower concentration of PFOS (0.2 μM) downregulated the Wnt/β-catenin signaling pathway in neural and mesenchymal stem cells (Dong et al., 2016; Liu et al., 2019). β-Catenin interacts with a wide variety of transcription factors, including steroid hormone receptors and nuclear receptors (Mulholland et al., 2005). It is thus likely that the effect of chemicals on this pathway can vary with cell type. Although mixture components may both upregulate and repress the Wnt/β-catenin pathway, we found that the total effect of the mixture in the embryos was a suppressed Wnt/β-catenin signaling.

4.3. Mixture G in humans versus zebrafish

The composition of Mixture G was based on measured and estimated serum levels of selected compounds in pregnant women in the SELMA study and the negative associations of these compounds with birth weight in their newborns (Birgersson et al., 2017, preprint). We found that the mixture affected apoptosis and Wnt/β-
catenin signaling in zebrafish embryos at water concentrations only 20–100 times higher than the geometric mean levels in maternal serum in the cohort. In the cohort, the 95th percentiles for the different components varied generally between 2- and 10-fold the geometric means depending on compound (Table S2). Thus, several women in the cohort exhibited serum levels of mixture G components of similar magnitude as the water levels that induced apoptosis and suppressed Wnt/β-catenin signaling in zebrafish (20 and 100x hsc). However, the experimental exposure of zebrafish embryos and the exposure to Mixture G compounds in the human embryo/fetus are expected to differ because of differences in distribution of chemicals in water versus blood, and in uptake, distribution, metabolism, and clearance from the body. Since the zebrafish embryo is small (ca 1.5 mm in length at 2.5 dpf) and does not eat or have gills at this stage, the only way compounds in the ambient water can enter the embryo is via diffusion over the body surface. In the human, compounds in maternal blood need to pass the placenta to enter the embryo/fetus. Transfer to the fetus will differ for the different compounds (Griffiths and Campbell, 2015) and thus the levels and proportions of Mixture G components (e.g., PFAAs and phthalates) in fetal blood will diverge from those in maternal blood.

In addition to entering the embryonic/fetal circulation and potentially causing direct effects on the developing organism, the Mixture G components may impact the placenta. The syncytiotrophoblast in the placental villi is a cell layer that forms a large contact area between maternal and fetal blood for transfer of oxygen and nutrients to the fetus. In studies of pregnancies complicated by fetal growth restriction, the placenta showed increased apoptosis, decreased cell proliferation, and a reduced syncytiotrophoblast area (Sharp et al., 2010; Heazzl et al., 2011). Increased apoptosis was observed in a human first-trimester placental cell line exposed to MEHP and in human placentatic syncytiotrophoblasts exposed to triclosan (Benachour and Aris, 2009; Zhang et al., 2015; Meruvu et al., 2016). Differentiation and expansion of trophoblast cells in the placenta are controlled by β-catenin (Knöfler and Pollheimer, 2013). Disrupted Wnt/β-catenin signaling and increased apoptosis have been observed in these cells in placentas from experimental animals exposed to cadmium, 1-nitropyrene, or reactive oxygen species (ROS) (Chapple et al., 2015; Erboga and Kanter, 2016; Li et al., 2018). Thus, it is plausible that Mixture G could induce apoptosis and inhibit Wnt/β-catenin signaling in placental cells with impaired placental function and fetal growth restriction as a result.

4.4. Mixture G components in the environment

Perfluoroalkyl acids are highly persistent pollutants that are found worldwide in the aquatic environment, even in more remote areas, and they biomagnify in aquatic and terrestrial food webs (OECD, 2013). Phthalates are additives to plastic and when present in plastic debris they can leach to the environment; consequently they are found in samples of dust, soil, water, sediments, and fish (Gunaalan et al., 2020; Panio et al., 2020). In the Mediterranean Sea, DEHP and MEHP were found in plankton and blubber from filter feeding whales (Fossi et al., 2012). The antimicrobial agent triclosan has been widely used in personal care products and it can be found in urban waters near wastewater treatment plants as well as in rural areas where biosolids are used as fertilizers (Goldsmith et al., 2020). Therefore, although the proportions are likely to vary from those in mixture G, mixtures of phthalates, PFAAs, and triclosan are present in the aquatic environment at locations influenced by human activities.

4.5. Conclusion

Our results show that exposure to a chemical mixture that has been inversely associated with birth weight in human increases apoptosis and suppresses Wnt/β-catenin signaling in embryonic zebrafish. These effects are accompanied by an altered shape of the caudal fin of the embryo, showing that mixture components affect morphogenesis. Thus, the mixture interferes with developmental processes in a way that may result in malformations and/or mal-function of tissues and organs and consequently adverse health effects.

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 data

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