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Exposure to acrylamide induces cardiac developmental toxicity in zebrafish during cardiogenesis☆

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ABSTRACT

Acrylamide (AA), an environmental pollutant, has been linked to neurotoxicity, genotoxicity and carcinogenicity. AA is widely used to synthesize polymers for industrial applications, is widely found in Western-style carbohydrate-rich foods and cigarette smoke, and can also be detected in human umbilical cord blood and breast milk. This is the first study that demonstrated the cardiac developmental toxicity of AA in zebrafish embryos. Post-fertilization exposure to AA caused a clearly deficient cardiovascular system with a shrunken heart and abortive morphogenesis and function. Disordered expression of the cardiac genes, *myl7*, *vmhc*, *myh6*, *bmp4*, *tbx2b* and *notch1b*, as well as reduced number of myocardial cells and endocardial cells, indicated the collapsed development of ventricle and atrium and failed differentiation of atrioventricular canal (AVC). Although cell apoptosis was not affected, the capacity of cardiomyocyte proliferation was significantly reduced by AA exposure after fertilization. Further investigation showed that treatment with AA specifically reduced the expressions of *nkx2.5*, *myl7* and *vmhc* in the anterior lateral plate mesoderm (ALPM) during the early cardiogenesis. In addition, AA exposure disturbed the restricted expressions of *bmp4*, *tbx2b* and *notch1b* during atrioventricular (AV) valve development and cardiac chambers maturation. Our results showed that AA-induced cardiotoxicity was related to decreased cardiac progenitor genes expression, reduced myocardium growth, abnormal cardiac chambers morphogenesis and disordered AVC differentiation. Our study demonstrates that AA exposure during a time point analogous to the first trimester in humans has a detrimental effect on early heart development in zebrafish. A high ingestion rate of AA-containing products may be an underlying risk factor for cardiogenesis in fetuses.

1. Introduction

Acrylamide (AA) has been classified as a “Group 2A carcinogen” and is reported from environmental pollutants and mainstream cigarette smoke from sources as diverse as water samples and tobacco smoke by the International Agency for Research on Cancer (IARC) (Smith et al., 2000). AA is widely used to synthesize polyacrylamide for industrial applications such as soil conditioning, wastewater treatment, research applications, cosmetics, and the paper and textile industries. Residual AA monomers are likely impurities in most polyacrylamide preparations, varying from a low of <1 ppm–600 ppm (van Landingham et al., 2004). Higher levels of residual AA monomers are present in the solid form compared to aqueous solution and inverse emulsions forms, ranging from <0.01% to 0.1% in its polymer (Anderson, 2005). Heightened concerns about AA arose in 2002 when it was first discovered in numerous fried or baked carbohydrate-rich foods, such as French fries, potato chips, breads, and biscuits, as well as coffee (Mottram et al., 2002; Tareke et al., 2002). In 2010, an international evaluation of AA by the WHO/FAO Joint Expert Committee on Food Additives (JECFA) concluded that AA may be “a human health concern” (JECFA, 2011), which was partially based on studies conducted by the US Food and Drug Administration (FDA) National Center for Toxicological Research (NTP, 2012).

Recently, the European Food Safety Authority (EFSA) announced that developmental toxicity has been identified as a probable

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critical endpoint for AA toxicity (EFSA, 2015). AA and its major metabolite, glycidamide (GA) with more genotoxic and carcinogenic properties (Calleman et al., 1990), can be transferred into breast milk and are also able to pass through the placenta in humans during pregnancy (Fohgelberg et al., 2005; Sörgel et al., 2002). AA and GA maintain comparable levels in fetal and maternal blood, indicating that the placenta provides no protection to the fetus against exposure to these compounds if present in maternal blood (Pedersen et al., 2012; Schettgen et al., 2004; von Stedingk et al., 2011). Considering larger ratios of liver-to-body weight and faster bloodstream through the liver in fetus, the metabolism and clearance of toxicants are much more active than those in adults (Greim and Snyder, 2008). In addition, GA is likely to be formed at a high rate through biotransformation *in vivo* in the livers of fetus. Meanwhile, the detoxification pathway through the conjugation of AA and GA with glutathione (GSH) may be less functional due to lower levels of GSH in the livers of fetus (Greim and Snyder, 2008). Previous study has showed that oral administration with both AA and AA-containing fried potato chips induces pre- and post-implantation losses, neonatal mortality, skeletal variations and impaired body weight and nerves in neonatal rats and mice (El-Sayyad et al., 2011a, 2011b; Tyl and Friedman, 2003). Besides, the results from the Norwegian Mother and Child Cohort Study (MoBa), the European prospective mother-child study (NewGeneris) and the recent French Etude des Déterminants pré et post natals du développement et de la santé de l′ Enfant (EDEN) cohort study were all consistent with both experimental and epidemiological studies, which add to the evidence of the effect of AA exposure on the risk at an early gestational age and suggest a toxic effect on fetal growth (Duarte-Salles et al., 2013; Kadawathagedara et al., 2016; Pedersen et al., 2012). Considering the high cell-replication rates, short lifespan erythrocytes and low body weight of fetuses, trans-placental exposure to AA needs to be given more attention (Schettgen et al., 2004).

Given that the heart is the first organ to generate and function during embryonic development, cardiac developmental toxicity induced by AA has been highlighted as a new public health concern. Previous study showed that AA might be an underlying risk factor of congenital heart diseases (CHD) (Huang and Zhang, 2016). Due to various genetic, epigenetic and shared behavioral and environmental risk factors, CHD is a group of serious and common disorders that have a significant impact on morbidity, mortality and healthcare costs in children and adults, and is associated with diminished quality of life (Warnes et al., 2008). Recently, a pioneer study showed that rat cardiomyocytes with prolonged exposure to AA concentrations corresponding to dietary levels appeared with altered morphology, irregular contraction patterns and an increase in the amount of immunoreactive signal for connexin 43 at cell junctions related to some cardiac pathologies (Walters et al., 2014).

Over the past 20 years, zebrafish (*Danio rerio*) has emerged as a powerful vertebrate animal model for investigating cardiac development due to its evolutionary conservation, genetic tractability, external fertilization, optical clarity, rapid development and ability to survive without a functional cardiovascular system during early development (Liu and Stainier, 2012; Stainier, 2001). As a hazardous compound ubiquitously present in our daily diet and around our environment, whether or not AA is associated with CHD and is considered to be a potential risk factor is disclosed in this study.

2. Methods

2.1. Zebrafish maintenance

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Zhejiang University (Hangzhou, Zhejiang, China). The Tuebingen zebrafish strain was wild-type control for all experiments. The transgenic zebrafish lines used in this study are as follows: *Tg(LCR:EGFP)* (Ganis et al., 2012), *Tg(myl7:EGFP)* (Huang et al., 2003), *Tg(myl7:mCherry)* (Palencia-Desai et al., 2011), *Tg(kdrl:mCherry)* (Bertrand et al., 2010), *Tg(myl7:ndsRed)* (Rottbauer et al., 2002) and *Tg(flia:nEGFP)* (Perälä et al., 2010). Zebrafish were raised and maintained according to the standard procedure as described in *The Zebrafish Book* (Westerfield, 1993). The in-tank breeding was used to obtain the embryos. The embryos were collected and raised in the incubator at 28 °C. The larvae were anesthetized with 0.16 mg/mL Tricaine (Sigma-Aldrich, St. Louis, MO, USA) before use.

2.2. Acrylamide exposure

An acute toxicity test of acrylamide (CAS 79-06-1, purity ≥99.9%, Sigma-Aldrich) was performed on zebrafish embryos according to our preliminary experiment mainly based on the OECD guideline No. 236 (FET test) (OECD, 2013). A series of AA concentrations, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 5.0 mM, were chosen in the test according to our preliminary experiments. The AA stock solution was prepared and diluted with egg water for the above levels tested. The egg water was prepared according to *The Zebrafish Book* and used for the vehicle (Westerfield, 1993). Twenty fertilized eggs in a Petri dish were tested at each level and each test was conducted simultaneously in one control and three exposure replicates. The volume of liquid was 20 ml in each Petri dish. The embryos were placed in the Petri dishes within one hour after fertilization at the latest. The tests were terminated after hatching as well as the absorption of the yolk sack in all individuals in the control dish (96–144 h post fertilization (hpf) after placement into the dish). The baths were replaced at 24 h intervals. Early life stage parameters such as egg and embryo mortality, gastrulation, somite formation, movement and tail detachment, pigmentation, heart rate, and hatching success were noted. During the test, the number of dead, deformed, and hatching embryos in individual concentrations was recorded. Overall survival and hatching rate of the control embryos should be ≥ 90% and 80% respectively according to the OECD No. 236, respectively. The bath temperatures were maintained at 28 ± 0.5 °C in test chambers at any time during the test.

The sensibility test of zebrafish exposure to AA at different stages of cardiac development was conducted subsequently based on the acute toxicity test. Briefly, embryos were treated once immediately after fertilization as well as 5.5, 12, 15.5, 19, 24, 36, 48, 72, 96 and 120 hpf by waterborne exposure to either egg water (vehicle) or AA (2.0 mM) in Petri dish. The developing zebrafish in each group were continuously observed for the 72 h following exposure.

2.3. Quantifying cardiac morphology and functions

The assessment of cardiac morphology and functions in zebrafish embryos were conducted according to previously published protocols with some modifications (Glickman and Yelon, 2002; Hoage et al., 2012). Representative images of 72 hpf embryos in the control group illustrating the measurement points are indicated in Fig. S1. To achieve the above measurements, zebrafish were placed on a microscope slide in a thin layer of methylcellulose (Sigma-Aldrich) or egg water. Cardiac morphology changes were measured by the distance between the sinus venous (SV, the attachment region between the atrium and the inflow tract) and bulbus arteriosus (BA, the attachment region between the ventricle and the outflow tract) regions of the heart (mm) as well as the cross-sectional area (CSA) of the ventricle (mm^2) in the state of diastole. The changes of cardiac functions were measured by