Aquatic Toxicology 152 (2014) 152–163

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Embryonic exposure to sodium arsenite perturbs vascular development in zebrafish

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ARTICLE INFO

Article history:
Received 7 February 2014
Received in revised form 2 April 2014
Accepted 5 April 2014
Available online 16 April 2014

Keywords:
Zebrafish
Arsenic
Arsenite
Angiogenesis
Vascular development

ABSTRACT

Exposure to arsenic in its inorganic form, arsenite, causes adverse effects to many different organs and tissues. Here, we have investigated arsenite-induced adverse effects on vascular tissues in the model organism zebrafish, *Danio rerio*. Zebrafish embryos were exposed to arsenite at different exposure windows and the susceptibility to vascular tissue damage was recorded at 72 hours post fertilization (hpf). Intersegmental vessel sprouting and growth was most perturbed by exposure to arsenite during the 24–48 hpf window, while disruption in the condensation of the caudal vein plexus was more often observed at the 48–72 hpf exposure window, reflecting when these structures develop during normal embryogenesis. The vascular growth rate was decreased by arsenite exposure, and deviated from that of control embryos at around 24–26.5 hpf. We further mapped changes in expression of key regulators of angiogenesis and vasculogenesis. Downregulation of *vascular endothelial growth factor receptor 1/fms-related tyrosine kinase 1 (vegfr1/flt1)* expression was evident already at 24 hpf, coinciding with the decreased vascular growth rate. At later time points, *matrix metalloproteinase 9 (mmp9)* expression was upregulated, suggesting that arsenite affects the composition of the extracellular matrix. In total, the expression of eight key factors involved in different aspects of vascularization was significantly altered by arsenic exposure. In conclusion, our results show that arsenite is a potent vascular disruptor in the developing zebrafish embryo, a finding that calls for an evaluation of arsenite as a developmental vascular toxicant in mammalian model systems.

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1. Introduction

Arsenic has been ranked number one on the hazardous substances list prioritized by the Agency for Toxic Substances and Disease Registry (ATSDR) and U.S. Environmental Protection Agency (EPA) since 1997. Humans are exposed to arsenic primarily through drinking water and food (Huq et al., 2006; Sambu and Wilson, 2008; Bundschuh et al., 2012), as well as industrial, tobacco and cosmetic products (Garrod et al., 1999; Sainio et al., 2000; Fresquez et al., 2013). Several health problems have been closely linked to arsenic exposure, such as cancers of the liver, kidney, bladder and lung, skin lesions and cardiovascular diseases (Kapaj et al., 2006; Heck et al., 2009; Otles and Cagindi, 2010; Moon et al., 2013). As of 2002, EPA ruled that the maximum standard for arsenic in drinking water in the U.S. was at 10 parts per billion (ppb), or 0.01 mg/L (EPA 816-F-01-004). Interestingly, arsenic exposure at much lower levels has been reported to strongly correlate with many adverse health effects in humans (Engel and Smith, 1994; Kurttio et al., 1999). Additionally, arsenic levels higher than 0.01 mg/L have been measured in drinking water (Lewis et al., 1999; Steinmaus et al., 2003) in several regions in the U.S., as well as groundwater in other countries, such as Bangladesh and Taiwan (Maity et al., 2011; Islam et al., 2012). Surprisingly, even the reference dose of arsenic at 0.3 μg/kg/day, recommended by the EPA as its permissible limit of daily exposure in humans with no likelihood of adverse effect, was able to induce genotoxicity in the form of micronuclei in erythrocytes of mice after 15 days of exposure (Khan et al., 2013). Another study reported altered antioxidant responses in the gill cells of adult zebrafish after 48 hours

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http://dx.doi.org/10.1016/j.aquatox.2014.04.006
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of exposure to 0.01 mg/L of arsenic in aquatic solution, suggesting bioaccumulation of arsenic in the animals (Ventura-Lima et al., 2009). Dose-dependent lethality was also evident from embryonic exposure to arsenic at low levels in golden hamsters (Unis et al., 2009).

Arsenic exposure has been described to affect both vasculogenesis and angiogenesis. Vasculogenesis refers to the process by which endothelial precursors, or angioblasts, proliferate, migrate and differentiate to form *de novo* blood vessels, while angiogenesis defines the process by which new blood vessels sprout from pre-existing ones. Both processes are regulated by several key factors such as vascular endothelial growth factor (VEGF) and extracellular matrix proteins (Flamme et al., 1995; Liang et al., 2001; Habeck et al., 2002; Oh et al., 2002; Ferrara et al., 2003; Davis and Senger, 2005; Stringer, 2006; Bahary et al., 2007; Chappell et al., 2009; Salvucci and Tosato, 2012). VEGF, widely accepted as a master angiogenic regulator, has been well-studied for its angiogenic properties *in vitro* and *in vivo* (Millauer et al., 1993; Hoeben et al., 2004; Gerhardt, 2008). Prenatal exposure to arsenic has a negative impact on pregnancy in humans and mice by causing abnormal placental vasculogenesis and other placental deficiencies, ultimately leading to stillbirths and spontaneous abortions (He et al., 2007; Sen and Chaudhuri, 2008). Other studies have shown that angiogenesis can be induced by low concentrations of arsenic exposure, while blocked by high concentrations of arsenic exposure in *in vitro* assays using HUVECs (Kao et al., 2003). A similar phenotype has been observed in some *in vivo* studies (Soucy et al., 2003, 2005; Straub et al., 2007). However, there are conflicting reports in other *in vivo* studies when examining high arsenic exposure, in which angiogenesis was inhibited in a chicken chorioallantoic-membrane assay, while induced in a matrigel plug study in mice (Soucy et al., 2003). Additionally, *in vivo* chronic exposure to arsenic has been shown to increase vascularization at low concentrations and decrease vascularization at high concentrations (Soucy et al., 2005). In humans, chronic exposure to high levels of arsenic has been linked to peripheral vascular disease, more commonly known as blackfoot disease (Tseng et al., 1996), and other cardiovascular mortalities (Tseng et al., 2003; Chen et al., 2011).

Zebrafish, *Danio rerio*, has been largely utilized in studying developmental biology and molecular genetics due to advantages such as transparent embryos, rapid external development and high gene homology with humans and other mammalian models. Because of these advantages, many genetic tools have been developed in the field, and advances in understanding basic embryonic development have been made. For instance, essential progress toward understanding vascular development has been credited to zebrafish as a model organism (Cannon et al., 2010; Wiens et al., 2010; Quaife et al., 2012). The anatomy and formation of zebrafish vasculature are vastly similar to that in humans and other mammals. In zebrafish, vasculogenesis begins with the specification and migration of endothelial precursor cells, or angioblasts, from the ventral-lateral mesoderm to form the axial vessel primordia, which give rise to the dorsal aorta (DA) and posterior cardinal vein (PCV) (Brown et al., 2000). This is followed by angiogenesis, during which endothelial cells (ECs) sprout, migrate and proliferate to form a more elaborate vascular network. The zebrafish primary intersegmental vessels (ISVs), patterned by the latter process, begin sprouting at approximately 20 hours post fertilization (hpf; 22-somite stage) from DA and grow dorsally with filopodial extension and retraction from the sprout tips, which act to read guidance cues from the surrounding tissue (Fouquet et al., 1997; Childs et al., 2002; Isogai et al., 2003). As the leading tips of the ISVs reach the dorsolateral roof of the neural tube, they split bilaterally and fuse with neighboring vessels to form the dorsal longitudinal anastomotic vessel (DLAV). By 36 hpf, the zebrafish primary ISV network is established.

To date, a few arsenic toxicity studies have been performed in zebrafish embryos and larvae to examine its effects on general morphology, immune system, cardiac development, ion transporters and bioaccumulation (Nayak et al., 2007; Li et al., 2009; Mattingly et al., 2009; Long et al., 2011; Lopez-Serrano Oliver et al., 2011; Li et al., 2012). Additionally, it has been shown that daily renewal of arsenic at concentrations lower than 65 mg/L (0.5 mM) had no effect on zebrafish embryonic development or survival (Li et al., 2009). We previously developed an image analysis method of quantifying angiogenic sprouts and used arsenic as a test compound for disrupted angiogenic growth (Shirinifard et al., 2013). Here, we describe in detail the effects of arsenic on vascular development and other malformations during specific windows of zebrafish embryonic development. We also show that arsenic exposure retards the growth rate of ISVs from images collected using time-lapse confocal microscopy. Moreover, we demonstrate that arsenic at a range of concentrations, even as low as 10 mg/L, affects the expression of key genes involved in vascular development.

2. Materials and methods

2.1. Fish husbandry

Zebrafish (*Danio rerio*) were reared and maintained at 28.5 °C as previously described (Westerfield, 2007), and in accordance to the standard operating protocols approved by the Institutional Animal Care and Use Committee at University of Houston (protocol nos. 12-042 and 13-028). A stable line of *Tg(kdrl:EGFP)mitfa*^{b692} was generated by crossing *Tg(kdrl:EGFP)*$^{S843/+}$ with *mitfa*$^{b692/b692}$ (Zebrafish International Resource Center, Eugene, OR) to facilitate GFP visualization without obstruction from melanophores. Embryos were collected from natural mating and staged according to Kimmel et al. (1995).

2.2. Chemical treatment

Sodium (meta)arsenite ($NaAsO_2$) was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in ultrapure deionized water (vehicle). *Tg(kdrl:EGFP)mitfa*^{b692} embryos were harvested in a petri dish after mating. Then, they were sorted and placed in 6-well plates (N = 10–30) in 3 mL of embryo medium, E3 (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$), followed by arsenite treatment at 400 mg/L (3.08 mM) without renewal. The embryos were incubated at 28.5 °C until 72 hpf, at which they were manually dechorionated, if necessary, and assessed for vascular perturbation and other developmental malformations. For determining the window of effect, embryos were treated with arsenite at 400 mg/L at 0–24 hpf, 24–48 hpf, or 48–72 hpf. After exposure time was complete, embryos were washed multiple times and allowed to continue to develop in E3 at 28.5 °C until assessment at 72 hpf. For RT-qPCR, 30 embryos were pooled as one biological sample in 3 mL of E3 and treated with arsenite at 10 mg/L, 50 mg/L, 100 mg/L, 200 mg/L, or 400 mg/L up to 72 hpf. To examine RNA levels at different time points *via* RT-qPCR, embryos were treated with arsenite at 400 mg/L up to 18 hpf, 20 hpf, 24 hpf, 28 hpf, or 48 hpf. Control embryos were treated with vehicle.

2.3. Whole embryo globin staining

Globin expression in zebrafish embryos was visualized with *o*-dianisidine staining. *Tg(kdrl:EGFP)mitfa*^{b692} embryos were exposed to arsenite at 400 mg/L, as described above, and fixed at 72 hpf in 4% paraformaldehyde dissolved in phosphate buffered saline solution (PFA-PBS) overnight at 4 °C. Fixed embryos were then washed several times in 1× PBS–0.1% Tween20 (PBT). *o*-Dianisidine staining was carried out as previously described (Detrich et al., 1995) with

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