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Copper impairs zebrafish swimbladder development by down-regulating Wnt signaling

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ABSTRACT

Copper nanoparticles (CuNPs) are used widely in different fields due to their attractive and effective abilities in inhibiting bacteria and fungi, but little information is available about their biological effects and potential molecular mechanisms on fish development. Here, CuNPs and copper (II) ions (Cu^{2+}) were revealed to inhibit the specification and formation of three layers of zebrafish embryonic posterior swimbladder and impair its inflation in a stage-specific manner. CuNPs and Cu^{2+} were also revealed to down-regulate Wnt signaling in embryos. Furthermore, Wnt agonist 6-Bromoindirubin-3′-oxime (BIO) was found to neutralize the inhibiting effects of CuNPs or Cu^{2+} or both on zebrafish swimbladder development. The integrated data here provide the first evidence that both CuNPs and Cu^{2+} act on the specification and growth of the three layers of swimbladder and inhibit its inflation by down-regulating Wnt signaling in a stage-specific manner during embryogenesis.

1. Introduction

Heavy metals in water, such as Cadmium (Cd), Copper (Cu), Plumbum (Pb), Zinc (Zn), and so on, have been revealed to be toxic to aquatic ecosystem and affect the development of fish. Among them, Cu has been found to be the most harmful to fish development (Hou et al., 2013; Li et al., 2012; Wang et al., 2013). Copper nanoparticles, another form of copper, are widely used in industrial, medical and electronic devices, livestock and poultry feed, and other consumer products for their antibacterial and antifungal activities (Adeleye et al., 2016; Cushen et al., 2014; Dankovich and Smith, 2014; Ganesh et al., 2010; Kahru and Savolainen, 2010; Lee et al., 2016; Sotiriou and Pratsinis, 2010). The increased consumption of products containing CuNPs could increase their amount in the environment, thus posing risks to live organisms including humans. Several studies have predicted the risks of CuNPs to aquatic organisms in major Taiwanese rivers (Chio et al., 2012). However, few studies have been performed to reveal the biological effects of CuNPs on vertebrate embryogenesis, and little is known about the molecular mechanisms underlying CuNPs induced developmental defects.

It is reported that CuNPs can cause cumulative mortalities in zebrafish (Kovriznych et al., 2013), negatively affect body length, induce irregular structure of epidermis and aplasia of lamellae, and overfill the blood vessels in both larvae and adult fish (Griffitt et al., 2009, 2008; Ostaszewska et al., 2016). However, literatures are still limited relating to the molecular roles of CuNPs during fish embryogenesis and the potential mechanisms, although the transcriptional responses of zebrafish embryos to CuNPs' toxicology have been studied based on general RNA-Seq data by our lab recently (manuscript under review by another journal). In addition, almost no inflated swimbladder is observed in embryos treated with CuNPs (lab data), however, the molecular characteristics for swimbladder development in CuNPs-treated embryos and the potential mechanisms are still limited.

During the past decade, many researchers have investigated the roles of Cu ions, especially Cu^{2+}, in vertebrate embryogenesis, metabolism, and diseases like cancer (Carreau and Pyle, 2005; Hernandez et al., 2011; Hordyjewska et al., 2014). During fish embryogenesis, Cu^{2+} has been unveiled to delay hatching (Johnson et al., 2007; Tilton et al., 2011), and to induce dysfunctional locomotor behavior in fish (Zhang et al., 2015a). However, few researchers have discussed its effects on fish swimbladder development.

Fish swimbladder is formed with three layers (epithelium, mesenchyme, and mesothelium) (Winata et al., 2009; Yin et al., 2011). Its development consists of three phases: (i) epithelial budding between 36 and 48 hpf, (ii) growth with the formation of two additional mesodermal layers up to 4.5 dpf, and (iii) inflation of posterior and anterior

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chambers at 4.5 and 21 dpf respectively (Winata et al., 2009; Yin et al., 2011). As an earliest marker for swimbladder epithelium progenitors, expression of *sox2* (sex determining region Y) is initiated from 24 hpf in the endoderm (Yin et al., 2011). However, before its expression in endoderm, it is firstly expressed in the neural primordium in early embryos (Streit et al., 1997; Wood and Episkopou, 1999), functioning in the maintenance of neural progenitor identity (Graham et al., 2003; Pevny and Placzek, 2005). *Pbx1* (pre-B-cell leukemia homeobox) is the well-known early marker for the initiation of the swimbladder, which can be detected as early as 28 hpf in the swimbladder anlage (Teoh et al., 2010), and *pbx1* may be required during the late stage of swimbladder development (Teoh et al., 2010). *Hb9*, now named *mnx* (motor neuron and pancreas homeobox 1) in ZFIN (http://zfin.org/ZDB-GENE-040409-1), is initially expressed in the swimbladder bud from 36 hpf (Wendik et al., 2004) and serves as the epithelial marker for the swimbladder. In addition, *fgf10a* (fibroblast growth factor 10a) and *acta2* (actin, alpha 2, smooth muscle, aorta) genes are used as bladder mesenchymal markers, which are detected in swimbladder from 48 hpf and 65 hpf respectively. The expression of *anxa5* (annexin A5a), compared to *hprt1l* (hypoxanthine phosphoribosyltransferase 1) and *elovl1a* (fatty acid elongase 1a), which starts from 48 hpf in the mesothelium layer of swimbladder, starts much later (after 60 hpf) in this layer (Winata et al., 2009; Yin et al., 2011).

Development of fish swimbladder and its inflation can be influenced by nutritional and environmental factors. Perfluorooctane sulphonate (PFOS) has been reported to affect posterior swimbladder chamber inflation and swimming performance of zebrafish larvae (Hagenaars et al., 2014). Another study, 50% of larvae fish at 14 days post-fertilization (dpf) failed to have an inflatable anterior swimbladder after exposure to the environmental pollutant 2-mercaptobenzothiazole (MBT), and the ratio of the anterior/posterior chamber length was significantly reduced compared to that in the control group (Stinckens et al., 2016).

Environmental factors might regulate development and inflation of swimbladder *via* innate genes and signals in organisms. The canonical Wnt pathway is required for bud formation and to the development of posterior chamber of swimbladder (Yin et al., 2011), and the early canonical Wnt signals from mesenchyme promote specification of epithelial bud between 12–14 hpf. Then, the canonical Wnt signals from mesenchyme and mesothelium between 14–36 hpf coordinate the formation of epithelium, mesenchyme and mesothelium layers. Later, between 36–48 hpf, sufficient canonical Wnt signals from each of the three layers guide proper morphogenesis of the swim bladder layers, and signals in swimbladder layers also act in a feedback loop to inhibit Wnt signaling (Yin et al., 2011). Extracellular Wnt activity is transmitted to intracellular cell *via frizzled* class receptors such as *fzd3* and *fzd7b* (Nikaido et al., 2013), to stimulate the disintegration of destruction complex which formed by proteins Gsk3, APC, and Axin2 for phosphorylating and degradation of β-catenin (*ctnnb*) (Liu et al., 2013). Then, the stabilized β-catenin protein translocates to nucleus and activates the transcriptions of the Wnt target genes. *Axin2*, a direct target of Wnt signaling, is usually used as an indicator of Wnt signaling activity in organisms (Liu et al., 2013). Additionally, it is reported that 6-Bromoindirubin-3′-oxime (BIO) is an effective agonist for activating Wnt signaling in cells (Sato et al., 2004; Zhang et al., 2015b). Besides Wnt signaling, Hedgehog signals are reported to be essential in specification and organization of the three layers of swimbladder (Winata et al., 2009).

Zebrafish has been used as a prominent model species in different research fields (Driever et al., 1994; Roush, 1996), and has been used to monitor the effect of environmental contaminants on fish organogenesis due to the sensitivity of the development of its embryos and larvae to environmental factors and the transparency of its eggs which allows to easily follow development (Mena, 2014; Quesada-García et al., 2013; Zhang et al., 2015a,b). In order to unveil the biological effects and the potential mechanisms of copper on fish swimbladder development, zebrafish embryos were exposed to CuNPs and Cu^{2+} respectively, and the initiation and specification of swimbladder during embryogenesis were tested in the treated embryos in the present study. CuNPs and Cu^{2+} were revealed to inhibit the inflation of zebrafish swimbladders and reduce their size significantly. Down-regulated expressions of bladder marker genes were observed in copper treated embryos at 36, 60, and 120 hpf in a stage-specific manner. Additionally, it was revealed that Wnt signals were down-regulated and the Wnt signaling agonist BIO could recover inflation and expressions of bladder layer markers in copper treated embryos.

2. Materials and methods

2.1. Maintenance of fish stocks and embryos collection

According to standard procedures, adult wild-type zebrafish (AB) strain specimens were maintained in a recirculation filtration system (28 ± 0.5 °C, 14:10 h light: dark). Male and female zebrafish specimens were kept separately until mating and spawning. After natural spawning, fertilized embryos were selected and staged by morphological features as reported previously (Zhang et al., 2015a), and were then used for different bioassays as described below.

2.2. Copper exposure and chemical treatment

Copper exposures were initiated before sphere stage (4 hpf), and exposures (50 embryos/60-mm-diameter plastic dish containing 10 ml water) were conducted in a 28 ± 0.5 °C incubator as previously reported (Zhou et al., 2016). Embryos from the same batch were used for the exposures of CuNPs (40–60 nm particle size, Cat # 774111, Sigma-Aldrich), Cu^{2+} ($CuSO_4{\cdot}5H2O$), and the solvent controls in each experiment. CuNPs were dissolved in 0.01% sodium citrate at 0.1 mg/mL as stock solution. Briefly, CuNPs in 0.01% sodium citrate were dispersed and sonicated by an Ultrasonic processor JY92-IID 900W (Scientz, China) (frequency 25 kHz, tip diameter 2 mm) for 20 min in an ice-water bath. Characteristics of CuNPs in egg water (Cui et al., 2016) had been tested by TEM (HITACHI-7650, Janpan) and Zetasizer (Malvern ZETASIZER Nano-ZS). High percentage of defective embryos was observed in embryos exposed to CuNPs at 0.25 mg/L (3.9 μM). Thus, 0.25 mg/L CuNPs (3.9 μM) was used for all treatments in the present study, and the 0.01% sodium citrate was diluted accordingly as solvent controls.

Cu^{2+} at 0.25 mg/L (3.9 μM) induces dysfunctional locomotor behaviors and elevates hemoglobin in embryos (Zhang et al., 2015a; Zhou et al., 2016). 0.25 mg/L Cu^{2+} (3.9 μM) was used in this study to test its effect on swimbladder development. All stock solutions were diluted with autoclaved ultrapure water, but not with E3 water (5.0 mM NaCl; 0.17 mM KCl; 0.33 mM CaCl; 0.33 mM MgSO4; 0.05% methylene blue, pH ~ 7.4) (Teoh et al., 2010) which was used usually for zebrafish embryos culture to eliminate the influence of other ions in E3. Each exposure group was replicated 3 times. The exposed and the control embryos were collected at 24 hpf, 36 hpf, 60 hpf, 96 hpf, 120 hpf respectively for different analysis including qRT-PCR, WISH, and others.

Wnt agonist BIO (Sigma-Aldrich, USA) was dissolved in Dimethyl Sulphoxide (DMSO) (Biosharp, China) at 5 mM for stock solutions (Zhang et al., 2015b). As reported in our previous study (Zhang et al., 2015b), BIO was used in this study to detect its neutralization effects on Cu^{2+} or CuNPs toxicity on swimbladder development and inflation. As Scheme 1 shown, embryos were exposed to the indicated concentrations of CuNPs or Cu^{2+} before 4 hpf, then 0.05 μM BIO was added separately to the copper and the control solvent treated groups. Each 2–3 parallel groups, respectively for the controls, CuNPs-exposed, and Cu^{2+}-exposed, were kept as negative or positive controls to detect the neutralizing effects of BIO on copper induced swimbladder developmental defects. All the treatments were performed in triplicate. BIO was added to the medium when embryos were developed to 6 hpf for three