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Embryonic developmental toxicity of selenite in zebrafish (*Danio rerio*) and prevention with folic acid

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ABSTRACT

Selenium (Se) is an essential micronutrient, but also a potential toxin, which may be absorbed in excess. Relatively little is known about selenium embryotoxicity in zebrafish. In this study, we evaluated the effect of selenite exposure in zebrafish embryos. Selenite treatment decreased survival and resulted in abnormal development in a dose- and time-dependent manner. We observed irregular growth of neurons in selenite treated embryos, characterized by the absence of neurons in the brain, trunk and tail. Selenite exposure also induced defects in heart function, such as bradycardia and cardiac dysplasia with irregular and smaller chamber shape. In addition, selenite exposure caused ectopic cell proliferation, apoptosis, and a change in the pattern of DNA methylation. Our results suggested that supplementation with folic acid (FA) ameliorated the cardiac and neural defects in selenite-treated embryos. In conclusion, we demonstrated that selenite exposure caused cardiac and neural defects in zebrafish embryos and that folic acid protected against this embryotoxicity. It will give insight into the risk assessment and prevention of Se-mediated embryotoxicity.

1. Introduction

Selenium is an essential dietary element for health, but is also toxic in excess. Activity from agricultural and industrial, including agricultural drainage waters (Baesman et al., 2007), oil refining wastewaters (Viamajala et al., 2006) and coal combustion residues (Rowe et al., 2002) has accelerated the release of selenium from geologic sources, which could be absorbed by animals from contaminated food and water. It can be supplied at different steps of metabolism in two forms, organic and inorganic. Inorganic selenium (e.g., selenite) is more likely to cause toxicity than organic forms (e.g., selenomethionine) (Shenkin, 2009). Low concentrations are required for normal growth and development, moderate concentrations can be stored and maintain homeostatic functions, and elevated concentrations can result in toxic effects. The threat that the toxic forms of selenium to wildlife can be increased by bioaccumulation and biomagnifications (Lenz and Lens, 2009). Chronic over-dose exposure to Se led to dermal and neurological problems (Letavayova et al., 2006). Intake of high doses of Se can also cause acute poisoning with immune and reproductive system damage, cardiovascular diseases and carcinogenesis. It may affect embryonic development by active placental transferring and increase the incidence of malformations, including those of the neural system in mice embryos (Danielsson et al., 1990; Dodig and Cepelak, 2004; Usami et al., 2008; Bansal and Kaur, 2005; Lenz and Lens, 2009). Several different forms of Se, including selenite, selenate, selenomethionine, and selenocysteine, caused malformations in cultured rat embryos, such as deformation of the optic vesicle and swelling of the rhombencephalon (Usami and Ohno, 1996). The toxicity of Se varies among teleost species during different life stages. Recent studies suggested that exposure of freshwater fish to Se caused growth depression, abnormal swimming patterns, reproductive impairment, morphological deformities, and histopathological changes in the liver, gills and kidneys (Kennedy et al., 2000; Lemly, 2002; Hamilton, 2004; Teh et al., 2004; Holm et al., 2005; Muscatello et al., 2006). The mechanism of selenium toxicity has most often been ascribed to its sulfur-like chemical characteristics and its tendency to substitute for this element in amino acids during proteins assembly (Maier and Knight, 1994). More recently, it was suggested that selenium toxicity may be a result of oxidative damage (Misra and Niyogi, 2009). Furthermore, quantitative changes of several proteins, including

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the actin-binding proteins and antioxidant proteins, were thought to be partially responsible for Se embryotoxicity (Usami et al., 2008).

Zebrafish is an ideal vertebrate model for studying the effects of environmental contaminants on developmental processes. The transparency of the zebrafish embryos allows continuous observation during organogenesis and simplifies the process of embryologic manipulation (Lohr and Yost, 2000). The mortality of organic and inorganic compounds of selenium to newly hatched zebrafish has been evaluated previously (Niimi and LaHam, 1976). However, the effects of Se on cardiogenesis and neural development in zebrafish have not yet been evaluated.

Folic acid (FA) is an essential methyl donor and is used for the generation of endogenous methionine (Henning et al., 1997). It cannot be synthesized *in vivo* but is instead absorbed from green leafy vegetables and citrus fruits. It is well known that dietary FA supplementation reduces neonatal mortality from neural tube disorders. Meanwhile, several studies have shown that periconceptional multivitamins containing FA may reduce the risk of congenital heart defects. In addition, dietary supplementation with FA prevented ethanol-induced cardiac birth defects in mice (Serrano et al., 2010). Han et al. reported that FA was involved in the canonical Wnt pathway to rescue lithium-induced vertebrate cardiac anomalies (Han et al., 2009).

In this study, we evaluated the effect of selenite on embryonic development in zebrafish and found that selenite could affect the development of the cardiovascular and nervous systems during embryonic period, accompanied with disturbance of proliferation and apoptosis status of the whole body. This study suggested that folic acid could be an effective protector for Se toxicity.

2. Materials and methods

2.1. Zebrafish maintenance and embryo collection

Individuals belonging to the AB strain of zebrafish (*Danio rerio*) were cultured in a breeding colony according to standard zebrafish breeding protocols (Whitlock and Westerfield, 2000). The water supplied to the system was treated by UV irradiation, and Instant Ocean® salt was added to the water to increase conductivity to approximately 1500 μS/cm. Adult zebrafish were maintained in groups at 28 °C under a 14:10 light:dark (L:D) cycle in a closed flow-through system. Group mating occurred during the first 30 min of the light period. Embryos were collected during the first hour of the light period of the L:D cycle and incubated in egg water (distilled deionized water containing 60 μg/ml Instant Ocean) at 28.3 °C. According to the stages of embryonic development as described before (Kimmel et al., 1995), normally developing embryos were selected under a stereomicroscope at the blastula stage (4 h post-fertilization, hpf) and subjected to a range of treatments to investigate the developmental toxicity of selenite.

2.2. Selenium exposure

Sodium selenite (purum p.a., ⩾99.0%) was purchased from Sigma–Aldrich (St. Louis, MO, USA). A 10 mM stock solution of sodium selenite was prepared in egg water and aliquots were dispensed into Eppendorf tubes for storage at −20 °C. Before each bioassay, stock solutions were warmed to room temperature (RT, 25 °C) and used to prepare the final test concentrations in sterilized water. One control group was designated for each exposure test.

The above zebrafish embryos were assigned to experimental treatment groups at 4 hpf (n = 30 individuals/group) and were either exposed to sodium selenite (1, 10, 20, 50, 100, or 200 μM) or subjected to control treatments (egg water only) until 120 hpf. Thirty embryos were maintained in 20 ml of the control solution or sodium selenite solution in 90 mm Petri dishes. Each treatment was replicated three times on different days/spawns. To suppress pigment expression, 0.003% (w/v) 1-phenyl-2-thiourea (PTU) (Sigma–Aldrich, St. Louis, MO, USA) was added to perform immunostaining experiments, while it was not necessary to add PTU when we observed the effect of selenite exposure on zebrafish embryo development via the bright-field stereomicroscope (Karlsson et al., 2001). The selenite egg water was replaced daily with fresh selenite solution in each dish. The percentages of surviving and affected fish were calculated and plotted against the hour post-fertilization (hpf).

2.3. Effects of selenite on hatching, survival, and morphological development of zebrafish embryos

The time points at which specific embryonic structures were formed were compared with those described in an atlas for normal zebrafish development (Kimmel et al., 1995; Whitlock and Westerfield, 2000). For all experiments, mortality data were collected at 24, 48, 72, 96, and 120 hpf (n = 30, 3 replicates per concentration). Live embryos/larvae were observed using a stereomicroscope to assess developmental progression (i.e., completion of gastrulation, formation of somites, regular heartbeat and spontaneous movement) (Kimmel et al., 1995), as well as alterations in morphology and signs of toxicity (i.e., alteration of body axis, malformation of the eye, jaw, heart and fins, failure to inflate the swim bladder, yolk sac deformity, growth retardation, and edema) (Xiang et al., 2008). Subsequently, the number of hatched embryos was recorded at 48, 60, and 72 hpf. At 72 hpf, 10 embryos were randomly selected to obtain the mean body length for each treatment. Body length was measured along the body axis, from the rostrum to the base of the tail, using a digital camera attached to a microscope (Nikon TE2000-U).

2.4. Morphological observation and functional evaluation of the heart

The bend of the heart that marks the division between the atrium and ventricle becomes prominent at 48 hpf. The heart then begins to beat with the atrial beat preceding the ventricular beat. Abnormal phenotypes of the heart were observed under the microscope after labeling with chamber-specific antibodies MF20 and S46 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa, IA, USA) for the ventricle and atrium, respectively. AlexaFluor568-conjugated goat anti-mouse IgG2b (MF20) and AlexaFluor488-conjugated goat anti-mouse IgG1 (S46) (Molecular Probes Inc., Eugene, OR) were used as secondary antibodies (Yelon et al., 1999; Lin et al., 2007).

An index of the effect of selenite exposure on cardiac function in the zebrafish was obtained by measuring heart rates at RT for 15s (4 replicates) under a stereomicroscope (SteREO Lumar.V12, Carl Zeiss GmbH, Germany). The sample size was 10 fish for each treatment (n = 3) and developmental stage (Ton et al., 2006; Li et al., 2009).

2.5. Proliferation status of the selenite-treated embryos

Expression of the proliferating cell nuclear antigen (PCNA) was detected with anti-mouse PCNA primary antibody (Sigma–Aldrich, St. Louis, MO, USA), followed by horseradish peroxidase-labeled goat anti-mouse secondary antibody (Zhongshan Biological Company, Beijing, China) for development of the signals at 24 and 48 hpf.

2.6. TUNEL assay

To detect apoptosis in selenite-treated embryos, the TUNEL assay (Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling, Promega, San Luis Obispo, CA, USA) was carried out according to the manufacturer's instructions. Briefly, embryos were fixed in 1.5 ml Eppendorf tubes with 4% paraformaldehyde (PFA) for 1 h at RT, then incubated in phosphate buffered saline (PBS) with 0.2% TritonX-100 overnight. The embryos were then washed in PBS for 10 min and, after addition of 100 μL equilibration buffer, incubated for 15 min at RT. The solution was then removed and the embryos were incubated with 50 μL TdT working solution for 1 h at 37 °C. The reaction was stopped by removing the TdT working solution and incubating the embryos in 2× standard saline citrate (SSC) buffer for 15 min at RT. The whole embryos were mounted on slides and observed under a stereomicroscope finally (SteREO Lumar.V12, Carl Zeiss GmbH, Germany).

2.7. Effects of selenite on genomic DNA methylation

The global methylation status of zebrafish embryos was assessed at 24 and 48 hpf with 5-methylcytidine-specific antibody (Santa Cruz, CA, USA), followed by goat anti-mouse IgG-FITC secondary antibody (Zhongshan Biological Company, Beijing, China).

2.8. Whole embryo immunostaining

Embryos were dechlorinated with forceps and fixed in 4% PFA dissolved in PBS containing 0.1% Tween-20 (PBST) at RT for 1 h. The embryos were then washed and incubated in PBST containing 10% normal goat serum (Zhongshan Biological Company, Beijing, China) and 2 mg/ml bovine serum albumin (Sigma–Aldrich, St. Louis, MO, USA) for 2 h at RT. The goat serum was then removed and the embryos were incubated with primary antibody at 4 °C overnight. They were then washed and incubated with a specific secondary antibody for 2 h at RT. Positive signals were developed with fluorescent labels or a DAB kit (Zhongshan Biological Company, Beijing, China). Micrographs were taken using a fluorescence stereomicroscope (SteREO Lumar.V12, Carl Zeiss GmbH, Germany).