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Treatment with sodium benzoate leads to malformation of zebrafish larvae

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Abstract

Sodium benzoate (SB) is a commonly used food preservative and anti-microbial agent in many foods from soup to cereals. However, little is known about the SB-induced toxicity and teratogenicity during early embryonic development. Here, we used zebrafish as a model to test the toxicity and teratogenicity because of their transparent eggs; therefore, the organogenesis of zebrafish embryos is easy to observe. After low dosages of SB (1–1000 ppm) treatment, the zebrafish embryos exhibited a 100% survival rate. As the exposure dosages increased, the survival rates decreased. No embryos survived after treatment with 2000 ppm SB. The 50% lethal dose (LD_{50}) of zebrafish is found to be in the range of 1400–1500 ppm. Gut abnormalities, malformation of pronephros, defective hatching gland and edema in pericardial sac were observed after treatment with SB. Compared to untreated littermates (vehicle-treated control), SB-treated embryos exhibited significantly reduced tactile sensitivity frequencies of touch-induced movement (vehicle-treated control: 27.60±1.98 v.s. 1000 ppm SB: 7.89±5.28; N=30). Subtle changes are easily observed by staining with specific monoclonal antibodies F59, Znp1 and α6F to detect morphology changes in muscle fibers, motor axons and pronephros, respectively. Our data showed that the treatment of SB led to misalignment of muscle fibers, motor neuron innervations, excess acetyl-choline receptor cluster and defective pronephric tubes. On the basis of these observations, we suggest that sodium benzoate is able to induce neurotoxicity and nephrotoxicity of zebrafish larvae.

Keywords: Acetyl-choline receptor; Embryonic development; Motor neuron; Muscle; Pronephros; Sodium benzoate; Toxicity; Zebrafish

1. Introduction

Sodium benzoate (SB) is a sodium salt of benzoic acid and is used in a wide variety of cosmetic products [16]. For food chemistry applications, SB is often used as food preservative because SB can inhibit microbial growth [10,33]. In medical applications, SB can be used as therapeutic agents to treat the acute hyper-ammonaemia in patients who were born with urea cycle disorders [21,28], to block D-dopa induced circling in the hemi-parkinsonian rat [15], to treat dental caries [5], to be an enzyme inhibitor of D-amino acids oxidase (DAO)[20] and to be a free radical scavenger to protect cells from apoptosis induced by reactive oxygen species [18]. These findings indicate that SB has multiple applications on the food chemical and biomedical research. Thus, the bio-safety and the teratogenicity of sodium benzoate is an important issue which should be discussed.

Many biological studies concerning SB-induced toxicities have been reported. Although no genotoxicity was observed using model bacterial cells [22], the protozoan *Tetrahymena pyriformis* as a toxicological model reveals that the treatment with SB caused a statistically significant increase in DNA content suggesting stimulation of the mitotic process [26]. In rats, negative results of carcinogenicity are reported when exposing with diet containing 20,000 ppm SB [25]. In mice, addition of a 20,000 ppm concentration of SB in drinking water daily for life had no apparent carcinogenic effect [27]. In fish, Hatanaka et al. [8] fed adult medaka with 10,000–80,000 ppm of SB for 12–24 weeks, and found that half of the survival fish developed bile duct proliferation. These observations suggested that SB-induced toxicities might differ depending on the species, the route, the dose, and the duration of exposure.

Effects of SB in chronic exposure with mammals were limited to reduced food intake and growth [16], however, no

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significant increase in fetal abnormalities in the pregnant hamsters, mice, rats, and rabbits which were dosed with 20–250 mg/kg SB was observed [16]. In the mammalian system, it is very difficult to observe the very subtle changes during early embryonic development, especially in motor axons growth, kidney formation, muscle progenitors migration, and muscle fibers alignments. In order to further investigate whether the toxicity or teratogenicity are induced by SB exposure during early embryonic stages, development of an alternative animal model is essential.

Recently, zebrafish has become a good model because of their large number of transparent embryos and well characterized developmental stages [17]. To develop an aquatic animal model for studying SB-induced toxicities, we chose zebrafish for this study. We generated a series of time- and dose-dependent SB exposure experiments. Subtle changes in the muscle fibers alignment, kidney formation and motor neurons processing are easily observed by staining with specific monoclonal antibodies. This strategy is excellent for studying SB-induced teratogenicity during early embryonic development.

2. Materials and methods

2.1. Experimental fish

Zebrafish of the AB strain (wild-type, wt) were kept under a 14-h light and 10-h dark photoperiod at approximately 28.5 °C. After fertilization, the eggs were collected and cultured in an aquarium. Embryonic cleavage and somite formation were observed with a light microscope to determine the developmental stage [11].

2.2. Sodium benzoate exposure

Sodium benzoate ($C_7H_5NaO_2$, Sigma-Aldrich) was dissolved in sterile distilled water to the desired concentrations. For dose titration, wt embryos at 48 h post-fertilization (hpf) were collected, randomly divided into several groups of 20 embryos each, and exposed to either water (vehicle-treated control) or water containing sodium benzoate at desired concentrations (1–2000 ppm). All embryos were cultivated in 24-well cell culture plates, and survival rates were counted after 1 day exposure [31]. At the end of exposure, embryos were collected for the antibodies labeling experiment. For time-course exposure experiment, wt embryos were collected at 1-cell stage, randomly divided into four groups of 50 embryos each, and exposed to either water (vehicle-treated control) or water containing 1000 ppm sodium benzoate. All embryos were cultivated in 24-well cell culture plates (1 ml solution for each well), and the survival embryos were counted after exposure to the desired stages.

2.3. Antibodies labeling

Antibody staining was performed as previously described with minor modifications [6,30]. Embryos were fixed in 4% paraformaldehyde in phosphate buffer saline (PBS, pH 7.0)(for F59 and Znp1) or were fixed in Dent's fixative (80% methanol: 20% DMSO)(for α6F) for 4 h at room temperature. Then, embryos were washed in 0.1 M PBS twice for 15 min each, soaked in 100% acetone at −20 °C for at least 10 min, and rehydrated with 0.1% (v/v) Tween 20 in PBS 3 times for 15 min each. After rehydration, the embryos were treated with PBS containing 5% goat serum albumin and subjected to immunofluorescence staining. F59 monoclonal antibodies (Hybridoma Bank; 1:10), Znp1 (Zebrafish Resource Center; 1:200) and α6F (Hybridoma Bank; 1:20) were used as the primary antibodies to detect zebrafish slow muscle fibers, motor neurons and pronephros (pronephric tubes and ducts), respectively, followed by Alexa Fluor 488 rabbit-anti-mouse IgG (Molecular Probes; 1:300) as the secondary antibody.

2.4. Acetylcholine receptor clustering

α-Bungarotoxin (α-BTX) labeling was performed as previously described [19]. Briefly, embryos were fixed in 4% paraformaldehyde in PBS for 4 h at room temperature, and then washed in PBST. Fixed embryos were digested in 0.1% collagenase (Sigma-Aldrich) and washed in PBST. Embryos were incubated for 30 min at room temperature in 10 μg/ml rhodamine-conjugated α-BTX (Molecular Probes) diluted in NCS-PBST (10% normal calf serum and 1% DMSO in PBST), and then washed in PBST and mounted in Vectashield (Vector).

2.5. Microscopy

All embryos were observed at specific stages under a microscope (DM 2500, Leica) equipped with Nomarski differential interference contrast optics and a fluorescent module having a GFP or DsRed filter cube (Kramer Scientific). Photographs of embryos at specific stages were taken with a CoolSNAP CCD (Phtomertics).

2.6. Motility recording

Wild type zebrafish embryos at 48 hpf were exposed in water (0 ppm of sodium benzoate) or in water containing 10, 100, 500 and 1000 ppm of sodium benzoate for 24 h. At the end points of exposure, thirty larvae were picked up in each group (0, 10, 100, 500 and 1000 ppm of sodium benzoate) and each of them was placed in the center of a 5-mm diameter circle generated by marker pens in a 60-mm dish filled with water. Motor function of the animal is measured by quantification the incidences of larvae swam out of the circle after tactile stimulation imposed by an observer at a frequency of 30 stimuli with 5-s intervals. To make sure the strength consistency of tactile stimulation, the identity of each test group was not revealed to the observer.

2.7. Statistical analysis

Motility recording were analyzed statistically with the general linear models procedure (SAS, Inc., Cary, NC. U. S. A). A one-way analysis of variance was used to compare the mean