Biochemical and Biophysical Research Communications xxx (2018) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Sodium benzoate induced developmental defects, oxidative stress and anxiety-like behaviour in zebrafish larva

Himanshu Gaur [a, 1], Srinithi Purushothaman [a, 1], Narasimha Pullaguri [a], Yogesh Bhargava [b], Anamika Bhargava [a, *]

[a] Ion Channel Biology Lab, Department of Biotechnology, Indian Institute of Technology Hyderabad (IITH), Kandi, Telangana, 502285, India
[b] Molecular Engineering and Imaging Lab, School of Biological Sciences, Dr Harisingh Gour Central University, Sagar, MP, 470003, India

ARTICLE INFO

Article history:
Received 14 May 2018
Accepted 25 May 2018
Available online xxx

Keywords:
Sodium benzoate
Zebrafish
Mortality
Hatching
Anxiety
Gsr

ABSTRACT

Sodium benzoate (SB) is a common food preservative. Its FDA described safety limit is 1000 ppm. Lately, increased use of SB has prompted investigations regarding its effects on biological systems. Data regarding toxicity of SB is divergent and controversial with studies reporting both harmful and beneficial effects. Therefore, we did a systematic dose dependent toxicity study of SB using zebrafish vertebrate animal model. We also investigated oxidative stress and anxiety-like behaviour in zebrafish larva treated with SB. Our results indicate that SB induced developmental (delayed hatching), morphological (pericardial edema, yolk sac edema and tail bending), biochemical (oxidative stress) and behavioural (anxiety-like behaviour) abnormalities in developing zebrafish larva. LC_{50} of SB induced toxicity was approximately 400 ppm after 48 h of SB exposure. Our study strongly supports its harmful effects on vertebrates at increasing doses. Thus, we suggest caution in the excessive use of this preservative in processed and convenience foods.

1. Introduction

Sodium benzoate (SB) is a common food preservative with bacteriostatic and fungistatic properties. It is widely used in various food preparations such as jams, jellies, pickles, carbonated drinks etc. throughout the world and is considered generally recognized as safe (GRAS) by FDA [1]. The increased consumption of processed and convenience foods has led to overuse of preservatives such as SB. The allowed limit of sodium benzoate in the food products is 0.1% (1000 ppm) by FDA [2]. However, recent survey suggests that it is being used at very high levels (2119 mg/kg) in the food articles exposing the population to health risks [3].

Several studies in the past have investigated the biological effects of SB using cells and animal models. SB has been linked to urticaria [4], angioedema [5], asthma [6], childhood hyperactivity [7] and other behavioural disorders [8,9]. SB was reported to suppress cellular immune responses at non-toxic concentrations however the comprehensive mechanism underlying the immunomodulatory effects of SB were not investigated [10]. In lymphocytes SB is reported to cause cytostaticity [11], and genotoxicity [12].

In mouse models, dietary SB resulted in death or reduced growth [13]. Intake of SB also increased liver weight and changed serum clinical parameters showing hepatotoxicity [14]. SB significantly impaired memory and induced oxidative stress in mice with decreased glutathione and increased malondialdehyde levels in the brain [15]. In a behavioural study, SB-treated rats showed anxiety-like behaviour and motor impairment [9]. Interestingly, a study by Hovatta et al. showed a relation between oxidative stress and anxiety by the upregulation glyoxalase 1 (glo1) and glutathione reductase (gsr) genes [16], however this link remains to be elucidated in the context of SB. SB has been shown to induce neurotoxicity, nephrotoxicity, and teratogenicity during early embryogenesis in zebrafish larvae but oxidative stress and behaviour due to SB exposure remain poorly understood [17,18].

Studies reporting toxicity of SB are contradicted by the studies that claim the potential beneficial effects of SB. In addition to being used as a therapeutic drug [19], it is also reported to be neuroprotective via over-expression of neurotrophic factors and protein deglycase DJ-1 [20–22]. In PC-12 cell line, low concentrations of SB caused an increase in the cell survival, but cell viability was reduced

* Corresponding author.
E-mail address: abhargava@iith.ac.in (A. Bhargava).
[1] Authors contributed equally.

https://doi.org/10.1016/j.bbrc.2018.05.171
0006-291X/

in high concentrations. In this study SB significantly increased the catalase enzyme activity but could not protect the cells to aluminium-induced free radical toxicity. Therefore, it was suggested that possibly SB improves the symptoms of neurodegenerative disease by other mechanisms [23].

Effects of SB, especially related to its antioxidant effects and effects on the memory and nervous system, are quite controversial as stated above. Further investigations are required to find the dual protective–toxic roles of SB.

In this study we used zebrafish, a vertebrate animal model to investigate potential effects of SB on the development, behaviour and oxidative stress. In the light of controversial data regarding oxidative stress and neuroprotective effects, our study provides important data about its potential toxic effects at low ppm level.

2. Materials and methods

2.1. Zebrafish maintenance and breeding

Adult zebrafish were purchased from local commercial supplier and were housed in static water tanks as described before [24]. Fertilized eggs were produced and collected as described earlier [24]. Eggs were stored in E3 medium (1X sterile E3 medium (in M) as 0.0595 NaCl, 0.021 KCl, 0.039 $CaCl_2.2H_2O$ and 0.048 $MgCl_2.6H_2O$: pH 7.2). The larvae were kept in the incubator at 28 °C for 4–5 h before drug incubation. All animal handling and experiments were performed in accordance with the approved protocols and guidelines prescribed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

2.2. Drug treatment

Effect of SB (SR Laboratories, India) on the hatching rate and mortality of zebrafish embryo was assessed as per OECD guidelines [25]. Briefly, zebrafish embryos 5 h post fertilization (hpf) were treated with different concentrations of SB (100, 200, 500, 1000 and 2000 ppm) prepared in 1X E3 medium. 1X E3 medium without SB was taken as control. All treatments were done as semi-static treatment in 24-well plate till 96 h. Each treatment contained 10–12 developing embryo in their respective wells. The dead embryos were removed from the wells during each day of the experiment period. Mortality rate was calculated using the following formula:

$$\%\ Mortality = (No.\ of\ dead\ embryos / Total\ number\ of\ embryos) * 100.$$

Hatching rate was also recorded at various time points: 43 hpf, 48 hpf and 72 hpf and was calculated using the following formula:

$$\%\ Hatching = (No.\ of\ embryos\ hatched / Total\ number\ of\ live\ embryos) * 100$$

LC_{50} (lethal concentration, 50%), the dose required to kill 50% of the tested population after a specified test duration was obtained from the mortality curve at 48 h post SB exposure.

2.3. Larval behaviour

The neurobehavioural modulatory potential of SB on zebrafish larva was determined. Briefly, zebrafish embryos were treated with either 1X E3 medium (as control) or 50 ppm SB in a 24-well plate. After 72 h of drug exposure, thigmotaxis of live behaving larvae was determined. Thigmotaxis (preference of edge or wall) activity of the larva was considered as an endpoint for anxiety-like behaviour as described before [26]. We used a protocol that has been adapted from Lundegaard et al. [27]. In our protocol, individual larva for each treatment group was collected using pasture pipette and dropped at the centre of the well of 24-well plate containing 500 μl of fresh 1X E3 medium and video was recorded using hand-held digital video camera for 30 s. Larval orientation at the centre or at the wall, at the end of 30 s was used to determine thigmotaxis and was termed as anxiety-like behaviour. Larva oriented towards the wall was equated as thigmotactic. For quantification, % thigmotaxis was determined as the ratio between the numbers of larvae that showed thigmotaxis over the total number of larvae measured in the behavioural test for that treatment group multiplied by 100. Of note, dead and physically impaired larvae were manually removed and discarded before the start of the experiment in all groups.

2.4. Imaging of the larvae

Inverted bright field microscope, Olympus lX73 series equipped with Procam HS-10 MP camera was used to capture images of larvae that were individually placed on a glass slide with minimal amount of their respective solutions.

2.5. RT-PCR analysis

To determine if SB can modulate oxidative stress in the developing zebrafish larva, we determined the gene expression of gsr and glo1 using semi-quantitative reverse transcriptase PCR (RT-PCR) method. Briefly, zebrafish embryo 5 hpf were treated with either 1X E3 medium (as control) or 50 and 400 ppm sodium benzoate for 48 h. After that, RNA was isolated using TRIzol reagent. cDNA was prepared using QuantiTect Reverse Transcription Kit (Qiagen) following manufacturer's instructions. RT-PCR was performed using the following primers (5' – 3'): gsr: Fwd: TGAAAAGGGCAAAATTGAGTTTA, Rev: TTTCGAGAGGTAATGGCGTAATA; glo1 Fwd: TGAAAAGGGCAAAATTGAGTTTA, Rev: TTTCGAGAGGTAATGGCGTAATA; actb1: Fwd: TGAAAAGGGCAAAATTGAGTTTA, Rev: TTTCGAGAGGTAATGGCGTAATA. Following PCR program was used: Initial denaturation at 94 °C (3 min); Denaturation at 94 °C (45 s); Annealing at 42 °C (45 s); Extension at 72 °C (1 min); Final extension at 72 °C (5 min) for 30 cycles. Actin β1 (actb1) gene was used as a housekeeping gene control. Bands in the RT-PCR gels were quantified using an adapted protocol that uses ImageJ software as described before [28].

2.6. Quantitative PCR (qPCR) analysis

The SYBR green based qPCR was performed to check the quantitative gene expression of gsr and glo1. Zebrafish embryo 5 hpf were treated with either 1X E3 medium (as control) or 400 ppm SB for 48 h. Total RNA was prepared and converted into cDNA using Affinity Script qPCR cDNA synthesis kit (Agilent Technologies, USA) as per manufacturer's protocol. qPCR was performed using the following primers (5' – 3'): gsr: Fwd: CCATTGGCAGAGAACCCAAC, Rev: CATAGACGCCTGGACGAGAG, glo1: Fwd: GGCTCAGAAACGGATGACAG, Rev: CTCTCGGTCAGGATCTTCAT, actb1: Fwd: TGGTATCGTGATGGACTCTG, Rev: CTCTCGGTCAGGATCTTCAT. The expression levels were analysed using SYBR Green chemistry (Brilliant II SYBR Green), qPCR master mix (Agilent Technologies, USA) using 1 μL (40 ng/μl) of cDNA in Stratagene mx3005 P instrument (Agilent Technologies, USA). The cycling conditions were – Initial denaturation for 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s, 60 °C for 30 s. The dissociation curve analysis (continuously collect

Please cite this article in press as: H. Gaur, et al., Sodium benzoate induced developmental defects, oxidative stress and anxiety-like behaviour in zebrafish larva, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.05.171