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

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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

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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

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fluorescence from 55 to 95 °C) was performed after amplification for primer specificity using the conditions as 95 °C for 1min, 55 °C for 30 s and 0.2 °C/s increment up to 95 °C. All qPCR experiments were performed by Genotypic Technology Pvt Ltd, Bangalore, India. Analysis of fold expression was done as described before [29].

2.7. Statistics

The data were statistically tested for the level of significance using Graphpad prism version 5.0. One-way ANOVA followed by Dunn's post-test or Bonferroni's post comparison test was performed for non-parametric and parametric data respectively to compare more than two groups. The dose response curve was fitted using Hill's equation using origin software version 9.4 E.

3. Results

3.1. Sodium benzoate exposure affected the survival of zebrafish embryos

To study the effects of SB in zebrafish, embryo 5 hpf were subjected to different concentrations of SB: 100, 200, 500, 1000 and 2000 ppm as described in methods. The mortality rates in percent were calculated at the end of every 24 h of SB exposure till 96 h. Fig. 1 shows cumulative mortality at 48, 72 and 96 h after SB exposure. A dose and time-dependent mortality was observed with increasing concentration and with increased time of exposure respectively. 100% mortality was observed at the end of 48 h in the concentrations 1000 ppm and above, whereas 100% mortality was observed at 72 h in the concentrations 500 ppm and above (Fig. 1A and B). A significantly higher mortality was observed in concentrations 200 ppm and above as compared to control after 72 h of SB exposure (Fig. 1B). 100% mortality was observed in all concentrations except the control at the end of 96 h of SB exposure (Fig. 1C). We observed dose dependent lethality induced by SB with LC_{50} value as 381 ± 10 ppm in the developing zebrafish larvae (Fig. 1D).

3.2. Sodium benzoate exposure affected the hatching rate of zebrafish embryo

Hatching is an important developmental stage in zebrafish embryo which occurs after 48 hpf and any change in the hatching rate indicates a developmental abnormality. We compared the hatching rate at 43, 48 and 72 h after SB exposure between control and treated groups. Hundred percent hatching was observed in the control group (as expected) whereas a statistically significant dose dependent reduction in the hatching rate was observed after SB exposure. After 43 and 48 h of SB exposure, hatching was significantly delayed in 200 and 500 ppm concentrations (Fig. 2A and B). Significant reduction in hatching was only observed for 200 ppm after 72 h of SB exposure (Fig. 2C). These results indicate that SB can induce developmental abnormalities in zebrafish embryos even at low doses. To the best of our knowledge our study is the first to report delayed hatching in zebrafish embryos upon exposure to low doses of SB.

3.3. Sodium benzoate exposure induced teratological lesions in zebrafish larva

The exposure of SB also led to physical malformations in the zebrafish larva (Fig. 3). The occurrence of yolk sac edema and pericardial edema and tail-bending were common phenotypes that were observed (Fig. 3A). We observed teratological abnormalities already after 48 h even in 100 ppm SB concentration (Fig. 3B). Also, we could not precisely determine the EC_{50} (effective concentration at which 50% larva would show any type of malformation) because SB induced abnormality in more than 50% of the larvae even in the lowest concentration (Fig. 3B). Therefore, we speculate that EC_{50} is below 100 ppm. Fig. 3C shows percent abnormalities 72 h after SB exposure.

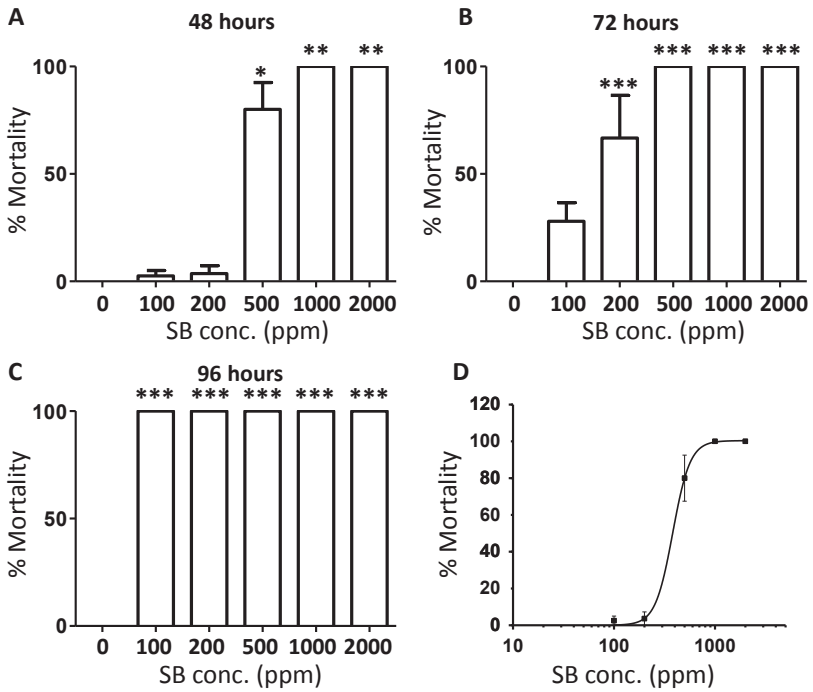

Fig. 1. Sodium benzoate induced mortality in zebrafish larva. Percent mortality at different concentrations is plotted for 48 h (A), 72 h (B) and 96 h (C) of SB exposure. D) Dose response curve is plotted from mortality data at 48 h of SB exposure. LC_{50} was calculated as 381 ± 10.1 ppm. The experiments were performed five times and the data is plotted as mean ± SEM. Number of embryos in all 5 experiments at the start were; control: 46, 100 ppm: 47, 200 ppm: 47, 500 ppm: 47, 1000 ppm: 46 and 2000 ppm: 46. * denotes $P \leq 0.05$; ** denotes $P \leq 0.001$ and *** denotes $P \leq 0.0001$.

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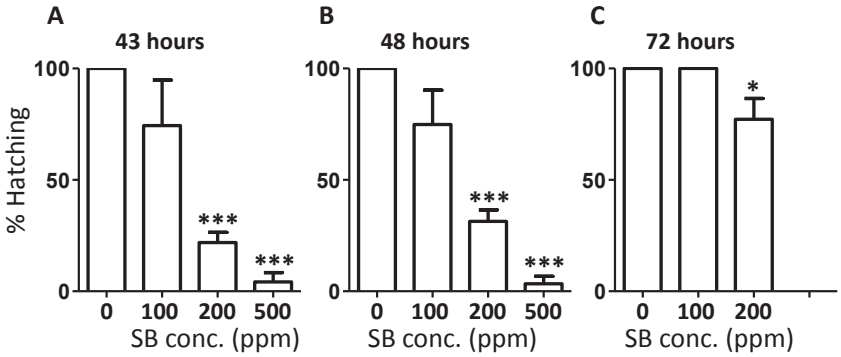

Fig. 2. Sodium benzoate delayed hatching in zebrafish embryos. Percent hatching at different concentrations is plotted for 43 h (A), 48 h (B) and 72 h (C) of SB exposure. The experiments were performed 4–5 times for each concentration and the data is plotted as mean ± SEM. Number of embryos at the start were; control: 46, 100 ppm: 47, 200 ppm: 47, 500 ppm: 47. * denotes $P \leq 0.05$ and *** denotes $P \leq 0.0001$.

3.4. Sodium benzoate induced anxiety-like behaviour in zebrafish larva

To assess anxiety in zebrafish larva we performed behavioural test. We used a protocol as described in methods. Larvae 72 h of SB exposure were chosen for behavioural experiments due to their increased motility as compared to larva after 48 h. We chose 50 ppm SB concentration (half of the lowest concentration of SB tested in our mortality experiments) for behavioural test to reduce physical impairment which may reduce motility and mask anxiety in our experiments. To further dissect anxiety from reduced motility due to physical malformations in our experiments, behavioural experiments were only conducted on larvae that had no visual morphological abnormalities as seen under a bright field inverted microscope. We observed increased thigmotaxis, a measure of anxiety, in larva treated with 50 ppm SB for 72 h (Fig. 4A). Of note 50 ppm SB concentration is very low as compared to the approved 1000 ppm limit by FDA [30].

3.5. Sodium benzoate exposure selectively upregulated gsr without affecting glo1 gene expression in zebrafish larva

Anxiety behaviour in rodent models has been linked to oxidative stress [16]. Therefore, we determined the level of expression of gsr and glo1 genes by both semi-quantitative Reverse Transcription PCR (RT-PCR) and quantitative PCR (qPCR). Our RT-PCR results at two concentrations of SB exposure; 50 ppm (concentration same as behaviour

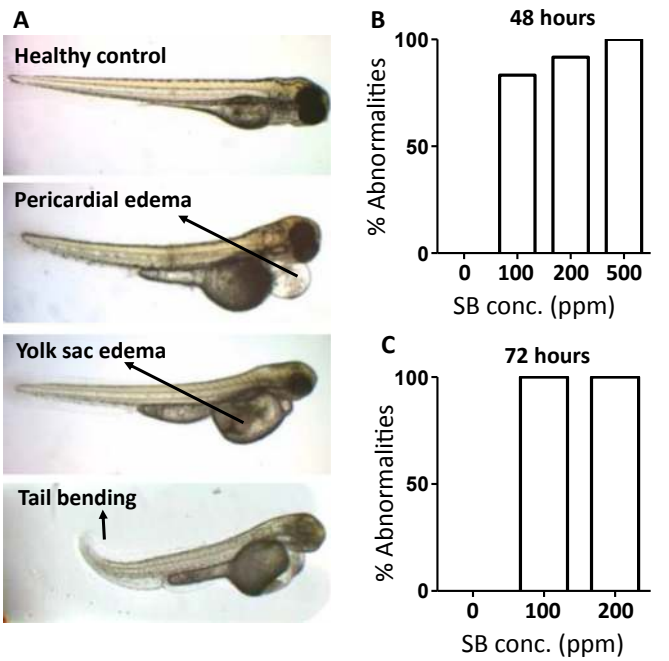

Fig. 3. Sodium benzoate induced physical malformations in zebrafish larva. A) Exemplar images of physical malformations. Total % abnormalities (including any kind of abnormality) that were observed after 48 (B) and 72 (C) hours of SB exposure. Percent abnormalities were calculated from at least 24 images captured for each concentration and time point.

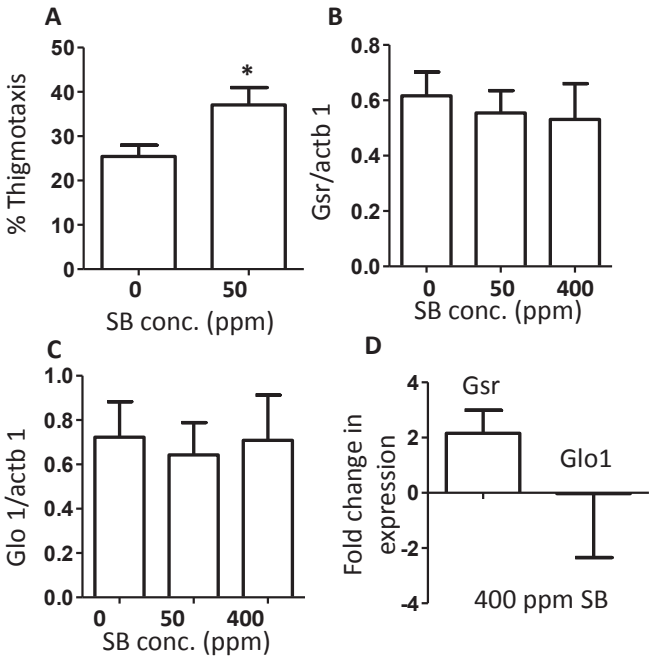

Fig. 4. Sodium benzoate induced anxiety-like behaviour and induced selective upregulation of gsr without affecting the expression of glo1 in zebrafish larva. A) Percent thigmotaxis is plotted for control and treated groups after 0 and 50 ppm SB exposure for 72 h (half of the lowest dose to avoid masking anxiety due to malformations) respectively. * denotes $P \leq 0.05$. RT-PCR band intensities of gsr gene normalized to housekeeping gene actb1 (B) and glo1 gene normalized to housekeeping gene actb1 (C). Data is plotted as mean ± SEM for 3 individual experiments. D) qPCR gene expression data showing fold change as compared to control. The experiments were performed two times (in replicates) and the data is plotted as mean ± SEM. SB concentration used for qPCR experiments was 400 ppm so as to maximize detection of changes in gene expression if any.

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experiments) and 400 ppm (rounded off LC_{50}), did not shown any change in the expression of both gsr and glo1 genes. Fig. 4B and C shows quantification of RT-PCR gel bands normalized to housekeeping gene actb1. However, using qPCR approach (which is more sensitive than RT-PCR), we found that the expression of gsr was found to be 2 fold upregulated in the SB treated group with no change in glo 1 gene expression (Fig. 4D). qPCR experiments were performed at 400 ppm (rounded off LC_{50}) to maximize any changes in the gene expression that may be happening due to treatment with SB.

4. Discussion

Several studies have reported SB induced toxicity or pathology but the mode of action remain inconclusive. It is important to keep all food additives under continuous observation and their effects must be re-evaluated in the light of changing conditions of use and new scientific information (Council directive 89/107/EEC).

Our study has examined SB induced teratology, anxiety-like behaviour and oxidative stress using zebrafish animal model. We used several concentrations of SB lower than the safety limit of 1000 ppm. We observed mortality at even lower concentrations with an LC_{50} of approximately 400 ppm (Fig. 1D). The only study to report LC_{50} of SB on zebrafish larvae is of Tsay H J and colleagues [18]. In their study The LC_{50} was found to be between 1400 and 1500 ppm. This could be attributable to the design of protocol. SB exposure in their study started 48 hpf whereas we have started SB exposure at 5 hpf. Both protocols are described in the OECD guidelines for performing fish toxicity assays. We chose to start early exposure so as to investigate developmental abnormalities like hatching rate. Statistically significant and dose dependent reduction in the hatching rate was observed after SB exposure in our experimental indicating that SB induced developmental abnormalities (Fig. 2). Effect of SB on the hatching rate of zebrafish embryos has not been described before. Teratology observed in our study in agreement with the published study [18] (Fig. 3A).

To investigate whether SB can modulate anxiety in zebrafish we measured thigmotactic behaviour of zebrafish larva. Increased anxiety-like behaviour observed in zebrafish larvae is also in agreement with the published studies on rodents (Fig. 4A) [9].

In the literature, a link between anxiety and oxidative stress is suggested [16]. Gsr and glo1 upregulation can also be considered as a marker for induced oxidative stress [31]. Also, gsr and glo1 expression upon SB exposure has never been investigated in any experimental system. Therefore, we determined the expression of gsr and glo1 genes in SB treated larva. We found a selective upregulation of gsr without any change in glo1 indicating the presence of oxidative stress in the SB treated larvae (Fig. 4D). Whether this upregulation of gsr is also linked to induction of anxiety-like behaviour in SB treated larva, needs further investigation with the help of morpholino based approach.

Overall, in the light of controversial data regarding oxidative stress and neuroprotective effects, our study provides important data about the potential toxic effects of SB and also provides data regarding the dose dependent effects of SB. It is possible that the beneficial effects observed upon SB exposure in diseased state are due to the reduced inflammation [32]. We suggest that SB treatment may be beneficial in certain disease cases where inflammation contributes significantly to the pathology but may be harmful for healthy individuals if taken in excess. We conclude that SB overuse can have potential toxic effects and suggest caution in its extended use as a preservative agent.

Acknowledgements

This work was supported by grants to AB: BioCARe, DBT, India, (BT/BioCARe/01/9701/2013–14), seed grant, IITH (SG/IITH/F145/2016-17/SG-27) and grant to YB: Science and Engineering Research Board (SERB), DST, India, (SB/FT/LS-439/2012). MHRD, India fellowship (HG, SP, NP).

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2018.05.171.

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