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Research Paper

NITRITE (NO_2^-) PERTURBS THE ACTIVITY OF SEROTONERGIC NEURON DURING EARLY DEVELOPMENT OF ZEBRAFISH

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Due to the increasing concern about elevated concentrations of nitrogenous substances in the aquatic environment because of pollution, this study investigates the effect of nitrite (NO_2^-) on the activity of serotonergic neuron in zebrafish embryos and larvae. Fertilized eggs were exposed to nitrite ranging from 0.1-100 mg/L NO_2-N up to 6 days post fertilization (dpf). Nitrite exposure at 1-100 mg/L NO_2-N significantly reduced 5-HT expression in serotonergic neurons at 48 h post fertilization (hpf), whereas nitrate (NO_3^- at 0.1-100 mg/L NO_3^-N showed no effect. Nitrite at 1 mg/L NO_2-N, but not nitrate, significantly reduced heart rate at 48 hpf, while nitrite increased anxiety level at 6 dpf. When the embryos were exposed to nitrite together with an estrogen receptor blocker (ICI 182780), the decreases in 5-HT expression and heart rate caused by nitrite alone were reversed, suggesting the effects of nitrite are mediated through estrogen receptor. The present study indicates that nitrite can be a neurotoxic agent to perturb the activity of serotonergic neuron in early development of zebrafish by disrupting estrogen signaling pathway.

Keywords: Nitrite, Serotonergic neuron, Zebrafish, Early development, Estrogen signaling

INTRODUCTION

Nitrite (NO_2^-) is a chemical compound, which is an intermediate product in bacterial nitrification and denitrification processes in the nitrogen cycle. While nitrite naturally occurs in the environment, excessive nitrite enters freshwater ecosystems from the effluents due to human activities, and its increasing concentrations have been implicated for serious environmental problems (Pitter, 1999). People are exposed to nitrite via drinking water and food. Nitrite is added in food especially in processed meats as preservatives and also to enhance color and flavor (McKnight *et al.,* 1999). Drinking water contaminated with nitrate and nitrite causes methemoglobinemia (blue baby syndrome) in infants by preventing the oxygen carrying capacity of hemoglobin through oxidation (Meybeck *et al*., 1989). In mammals,

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excessive intake of nitrite through drinking water and food has been implicated to cause carcinogenesis in digestive tract (Harte *et al.*, 1991) and renal cell carcinoma (DellaValle *et al.*, 2013). Higher nitrite concentrations in ground and surface water have deleterious effects on fish and invertebrates as well. Nitrite enters fish body quickly from the aquatic environment through gills and can also be generated by reduction of ingested nitrate. The toxicity of nitrite to fish varies depending on number of factors, for example, size, species and age of fish, water quality and individual response (Jensen, 2003; Vosláøová *et al.*, 2006). Although many studies focused on the developmental changes and reproductive and endocrine functions, very few studies to assess the neurotoxic effects of elevated nitrite concentration have been undertaken. Recently, we have shown that nitrate and nitrite act as endocrine disruptors to perturb the activity of dopaminergic neuron during early development of zebrafish (Jannat *et al*., 2014).

Serotonergic neuron is one of the monoamergic neurons, which produces the neurotransmitter, serotonin or 5-hydroxytryptamine (5-HT). Serotonin produced in the central nervous system (CNS) is involved in controlling a wide range of behaviors and physiological processes such as sleep, mood, appetite, temperature regulation, pain perception, etc. (Jessell and Kelly, 1991; Popa *et al.,* 2008). Abnormal serotonin level impairs CNS development, cardiovascular development, neuromuscular coordination, (Chambers *et al.*, 2006; Koren and Boucher, 2009), and perturbation of serotonin signaling during brain development has been implicated to cause adverse effects such as movement disorder, anxiety disorder, depression, autism, etc. (Lisboa *et al.,* 2007; Walsh *et al.*, 2008). Although serotonin alone does not control the development and functions of motor neurons, serotonin is necessary to complete the maturation process of locomotor behavior in vertebrates (Jacobs and Fornal, 1997; Bate, 1999). It has been reported that serotonin has an important role in the organization of locomotor pattern and modulation of motor output during early stage of rats (Nakajima *et al*., 1998; Pearlstein *et al.*, 2005). It has been also demonstrated that the perturbation of serotonin level affects the swimming behavior of zebrafish larvae (Airhart *et al.*, 2007; Gabriel *et al.,* 2009; Airhart *et al.*, 2012).

In recent years, there have been studies reporting that nitrite exposure to zebrafish causes developmental defects in embryos and larvae at concentrations ranging from 10 to 300 mg/L (Simmons *et al*., 2012), high mortality in juveniles at 242 mg/L (Dole•elová *et al.*, 2011) and growth suppression in juveniles and adults from 73 to 130 mg/L (Vosláøová *et al.*, 2008). It has also been reported that excessive nitrite exposure causes developmental defects in heart valve of zebrafish larvae in a dose dependent manner (Li *et al.*, 2014). Additionally, nitrite induces alteration in sex steroids and thyroid hormones in fish, and the possible endocrine disruptive role of nitrite in other vertebrates has been indicated as well (Ciji *et al.,* 2013; Guillette and Edwards, 2005). It has been shown that nitrite activates estrogen receptor in breast cancer cell (Veselik *et al*., 2008), and that the effect of nitrite on dopaminergic neuron is mediated through estrogen receptor in zebrafish (Jannat *et al.*, 2014). On the other hand, estrogen modulates the function of serotonergic neurons (Rubinow *et al.*, 1998), and the interaction between serotonin and estrogen has a significant role in controlling mood and cognition (Amin *et al.*, 2005).

The present study investigates the effect of nitrite exposure on the activity of serotonergic neurons during early development of zebrafish, and whether the effect is mediated through estrogen signaling. We measured the changes in 5-HT expression in serotonergic neurons, and the heart rate and anxiety level as functional parameters of serotonergic neurons.

MATERIALS AND METHODS

Fish Maintenance and Embryo Culture

Adult zebrafish, *Danio rerio*, were purchased from a local pet shop and reared in a 60-L tank containing freshwater with continuous aeration. The water temperature was maintained at 26 – 30 °C. The light regime was 14 h of light starting at 10:00, followed by 10 h of dark. Fish were fed with TetraMin (Tetra Japan Inc.) twice a day. Fertilized eggs were collected and washed in Embryo Medium (EM) (0.004% $CaCl_2$, 0.163% $MgSO_4$, 0.1% NaCl and 0.003% KCl) to remove debris. Embryos were transferred to a 6-well plastic plate (30 embryos in 8 mL of EM per well), and incubated at 28 ± 0.5 °C. The medium was changed daily.

Exposure Experiments

To prepare stock solutions, potassium nitrate (Wako) and potassium nitrite (Sigma) were dissolved in distilled water at 1000 mg/L nitrate-N (NO_2-N) and nitrite-N (NO_2-N). The stock solutions were diluted with EM to make 0.1, 1, 10 and 100 mg/L NO_3-N and NO_2-N for exposure experiments. ICI (182,780) (Tocris) was dissolved in dimethyl sulfoxide (DMSO) to prepare 10 mM stock solution, which was diluted with EM to the final concentration at 10 µM. For the control EM or EM containing 0.1% DMSO were used as necessary. Exposure started at 2 hpf and continued till embryos and larvae were subjected to the assays.

Whole-Mount Immunocytochemistry

Embryos at 48 hpf were fixed in 4% paraformaldehyde in 10 mM phosphate buffered saline (PBS) overnight at 4°C. Fixed embryos were incubated in 3% H_2O_2/0.5% KOH for 20-30 min at room temperature (RT) to remove pigments. Then the embryos were rinsed in PBS and dehydrated in 50% methanol and stored in 100% methanol at –20°C. For immuno-cytochemistry for 5-HT, embryos were first washed in PBS containing 0.1% Tween-20 and 0.5% Triton X-100 (PBSTX). Permealization was achieved by incubation in distilled water for 60 min at RT, followed by acetone for 20 min at –20 °C. Nonspecific binding of the antibody was blocked by incubating in PBSTX (0.1% Tween-20 and 0.3% Triton X-100) containing 10% normal goat serum and 3% bovine serum albumin for 3 h at RT. After washing with PBSTX (1 h×2), embryos were incubated with rabbit polyclonal anti-5HT antibody (ImmunoStar) (1:500, diluted in PBS containing 0.1% Triton X-100 and 10% normal goat serum) for 2 days at 4°C. Then, embryos were washed with PBSTX (1 h×4) and incubated in Alexa Fluor 488 goat anti-rabbit IgG (1:100, diluted in PBS containing 10% normal goat serum) (Invitrogen) for overnight at 4°C. After washing with PBSTX (5 min×3) embryos were mounted on a slide with 1% agarose for observation using the fluorescence stereoscopic microscope (Leica M165 FC). For measurement of positively stained area, a plane with the largest positive area was chosen. Positively stained areas outlined manually were measured and analyzed using ImageJ software. Immuno-cytochemistry was performed using 7-10

embryos per treatment, and the experiments were repeated three times with eggs collected from different spawns.

Heart Rate Measurement

Heart rate was measured at 48 hpf. The heart-beat was counted manually under the microscope. After the habituation of embryos under the microscope for 1 min, the heart-beat was counted for 15 sec. Ten fish were used for each group. Experiments were repeated three times with eggs collected from different spawns.

Thigmotaxis Assay

Thigmotaxis assay was performed according to the protocol described by William and Norton (2012). Zebrafish larvae at 6 dpf were transferred into a clear 6-well tissue culture plate with one fish per well containing 4 mL EM. The bottom of each well was divided into two portions designated as inner and outer zones as shown in Figure 3A (the diameter of inner zone is 2.5 cm and the width of outer zone is 1.0 cm). Larvae were allowed to habituate at 28 °C for 2 h. Then the 6-well plate was placed under the video camera and the larvae were allowed to habituate for 5 min. After habituation swimming activity was recorded for 5 min. For each group, 12 larvae were used. Thigmotaxis was expressed as % of time a fish spent in the outer zone using the following formula.

Thigmotaxis (% time in outer zone) = Time in outer zone (s) /test duration (300 s) × 100

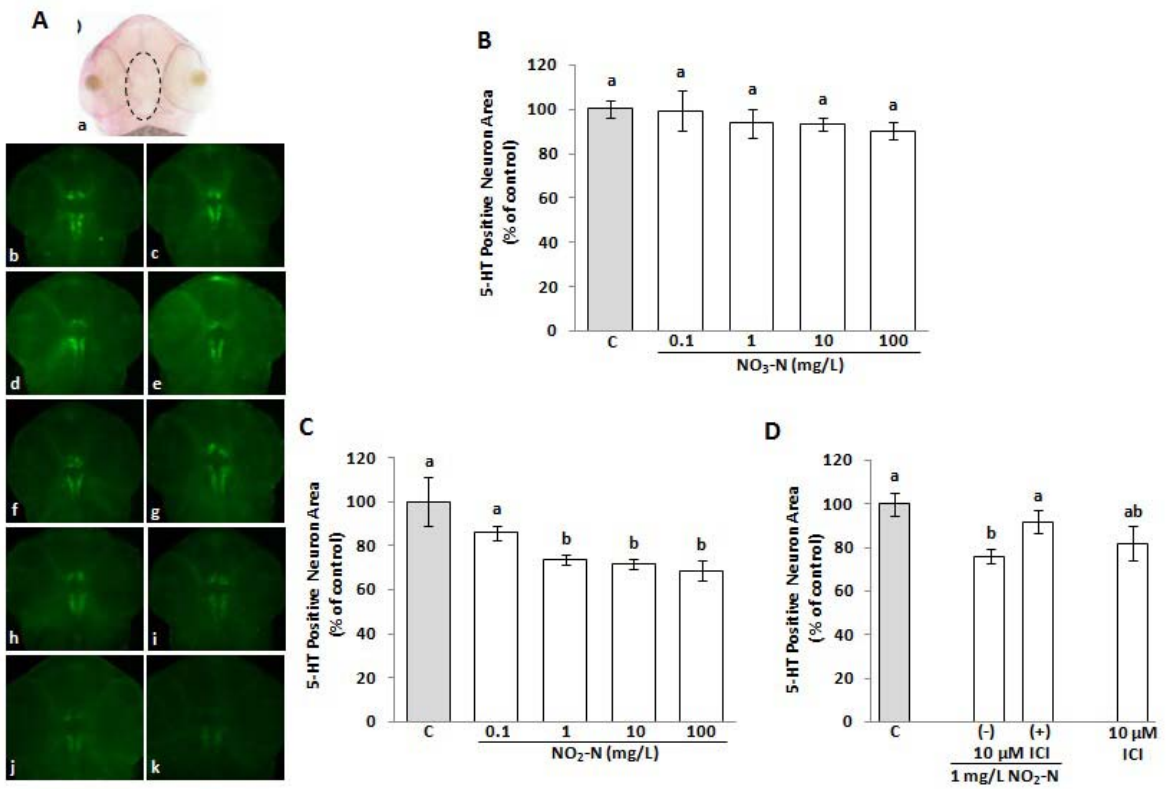
STATISTICAL ANALYSIS

All data were analyzed by one way ANOVA and followed by Tukey's post-hoc test to determine significant differences between the treatments. SPSS 16.0 package for Windows was used to conduct the analyses. A significance level of $P < 0.05$ was used in all statistical analyses. Each experiment was repeated at least three times.

RESULTS AND DISCUSSION

The results showed that nitrite decreased the 5-HT expression significantly at 1-100 mg/L NO_2-N, whereas nitrate had no significant effect at any of the doses (Figure 1A, B, C). It may be due to that nitrite can enter the body of aquatic organisms more rapidly than nitrate by the active cellular Cl^-/HCO_3^- exchange mechanism (Philips *et al.*, 2002), and that nitrate needs to be converted to nitrite, making nitrite more potent than nitrate. When the embryos were co-incubated with nitrite and the estrogen receptor blocker, ICI, the decreased 5-HT expression caused by nitrite was reversed significantly (Figure 1D), indicating that the effect of nitrite was mediated through estrogen receptor. It has been reported that the anion nitrite can activate the ERα through interaction with the ligand-binding domain of ERα (Veselik *et al.,* 2008). Estrogen is known to modulate the function of serotonergic neurons by affecting the release and reuptake of 5-HT and enzymatic inactivation for 5-HT production (Rubinow *et al*., 1998; McKuen and Alves, 1999). It has shown that estrogen has biphasic effect on the density of serotonin receptors in rat brain (Biegon and McEwen, 1982). In addition, nitrite can be converted to NO by enzymes like xanthine oxidoreductase (Zhang *et al.,* 1997; Godber *et al*., 2000), mitochondrial enzymes (Kozlov *et al.*, 1999). As NO is known to exert effects on synaptogenesis and neurotransmission in the central and peripheral nervous system (Godfrey and Schwarte, 2003; Kiss, 2000) and perturbation of NO signaling affects the ontogeny of locomotor performance in zebrafish embryos (Bradley *et al.*, 2010), it may be possible the effect of nitrite in the present study

Figure 1: Effect of nitrate (NO_3^-), nitrite (NO_2^-), and co-incubation with ICI on 5-HT expression of zebrafish embryos at 48 hpf detected by whole-mount immunofluorescence. A: Representative images of immunostains. Ventral view of a head in a bright field with a dotted circle indicating the area where serotonergic neurons are localized (a); untreated control of the nitrate exposure experiment (b); 0.1 mg/L NO_3-N (c); 1 mg/L NO_3-N (d); 10 mg/L NO_3-N (e); 100 mg/L NO_3-N (f); untreated control of the nitrite exposure experiment (g); 0.1 mg/L NO_2-N (h); 1 mg/L NO_2-N (i); 10 mg/L NO_2-N (j); 100 mg/L NO_2-N (k). B-D: Measurements of 5-HT positive neuron area in the experiment of nitrate exposure, nitrite exposure, and co-incubation of nitrite and 10 µM ICI, respectively (n=7). Data are expressed as a mean ± standard error. Different letters in each graph indicate significant differences (P < 0.05)



is due to NO production. In our previous study, effect of nitrite on dopamine neuron is likely to be caused by NO converted from nitrite (Jannat *et al.,* 2014). To elucidate the specific molecular mechanism further studies are necessary.

Heart rate was measured at 48 hpf as a marker of physiological activity controlled by serotonergic neurons. Nitrate exposure had no effect on heart rate at any of the doses (Figure 2A). Embryos exposed to nitrite significantly decreased the heart rate compared to the untreated control at 1 and 10 mg/L NO_2-N (Figure 2B). To investigate whether the effect of nitrite on heart rate was mediated through estrogen receptor, embryos were co-incubated with 1 mg/L NO_2-N and 10 µM ICI. The co-incubation significantly reversed the decreased heart rate caused by nitrite alone (Figure 2C), suggesting the effect of nitrite is mediated through estrogen receptor. It has been known that serotonin plays an important role in

cardiovascular development (Ni and Stephanie, 2006) and blood-pressure regulation (Stephanie *et al.*, 2012). It has been also reported that abnormal serotonin level impairs cardiovascular development (Koren and Boucher, 2009) and selective serotonin-reuptake inhibitors increase the risk of persistent pulmonary hypertension in newborns (Chambers *et al.*, 2006). Thus, it is likely that the decreased expression of 5-HT by nitrite subsequently decreased the heart rate in this study. In zebrafish embryos and larvae, it has been reported that excessive nitrite exposure affects the heart development (Li *et al.,* 2014).

Thigmotaxis assay was performed at 6 dpf to access anxiety behavior. Anxiety level was measured by the preference of the larva to stay in the outer zone of the well compared to the inner zone (Figure 3A). Nitrite exposure increased the level of anxiety significantly at 10 mg/L NO_2-N (Figure 3B). Thigmotaxis assay has been used

Figure 2: Effect of nitrate (NO_3^-), nitrite (NO_2^-), and co-incubation with ICI on heart rate of zebrafish embryos at 48 hpf. A: Exposure to 0.1 - 100 mg/L NO_3-N. B: Exposure to 0.1 - 10 mg/L NO_2-N. C: Co-incubation of 1 mg/L NO_2-N and 10 µM ICI. Heart beats were counted for 15 sec under the microscope (n=10). Data are expressed as a mean ± standard error. Different letters in each graph indicate significant differences (P < 0.05)

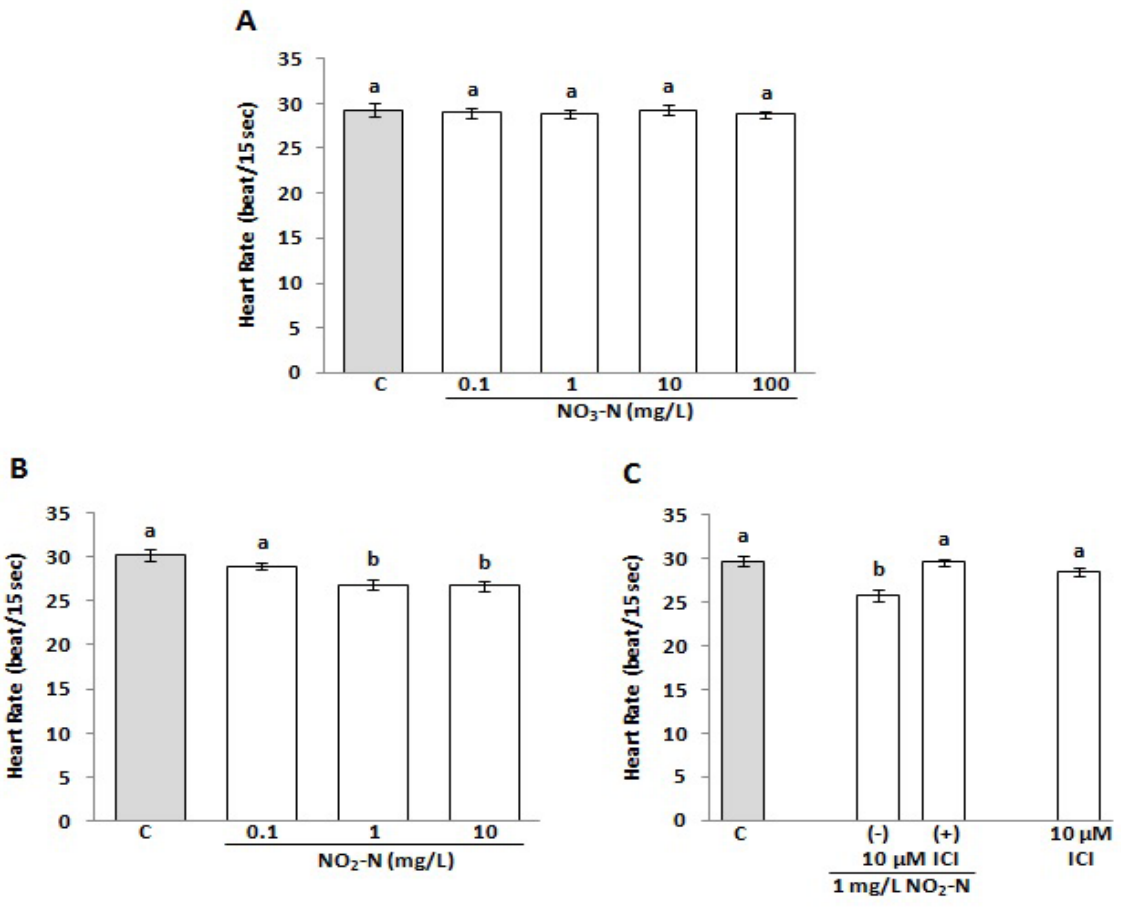
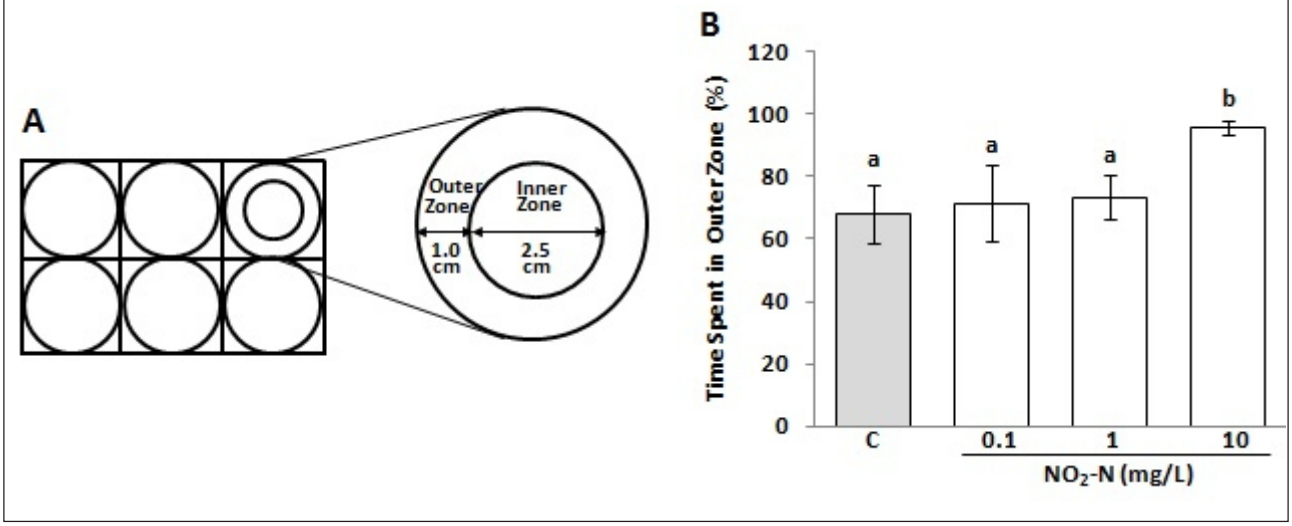


Figure 3: Effect of nitrite (NO_2^-) on anxiety of zebrafish larvae at 6 dpf tested by the thigmotaxis assay. A: The schematic diagram of a 6-well plate used for the assay. B: Thigmotaxis is expressed as the % of time spent in the outer zone in 5 min (n=12). Different letters in each graph indicate significant differences (P < 0.05)



in other studies to detect the anxiety level in zebrafish larvae (Rachel and Denis, 2012; Schnörr *et al*., 2012). The neurotransmitter 5-HT is involved in many behavioral functions, and defects in serotonergic system result in pathological outcome (Murphy, 1999; Parks *et al.*, 1998). The depletion in 5-HT level in the ventral striatum increases the anxiety in rat (Schwarting *et al.*, 1998), and the role of 5-HT in anxiety is also reported in zebrafish (Maximino *et al.*, 2013). Therefore, the present study suggests that the decreased 5-HT expression caused by nitrite exposure increases the anxiety level. It has been reported that the patients in depression state and with anxiety disorders have the plasma nitrate concentration higher than normal condition (Suzuki *et al*., 2001), and a possible role of nitric oxide in anxiety disorders is also revealed in mice and other animal models (Nakashima *et al*., 2003; Volke *et al*., 1997). Taking together, nitrite may have a role in regulating anxiety through acting on serotonin neuron.

CONCLUSION

Nitrite perturbs the activity of serotonergic neurons during early development of zebrafish mediating through estrogen signaling pathway, which indicates a possible endocrine disruptive role of nitrite. Although further studies are needed to elucidate underlying mechanisms, this study provides basic evidence for nitrite acting as an environmental neurotoxin and will give a new insight in the current environmental concern.

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