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Nitrate (NO_3^-) and nitrite (NO_2^-) are endocrine disruptors to downregulate expression of tyrosine hydroxylase and motor behavior through conversion to nitric oxide in early development of zebrafish

Meshkatul Jannat, Ratu Fatimah, Mitsuyo Kishida *

Graduate School of Science and Technology, Kumamoto University, 2-39-1 Kurokami, Chuo-ku, Kumamoto 860-8555, Japan

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ABSTRACT

With a view to consider the increasing concern over nitrogen pollution in the aquatic environment, we investigated effects of nitrate (NO_3^-) and nitrite (NO_2^-) on the activity of dopaminergic neuron in zebrafish embryos and larvae. Both nitrate and nitrite exposure decreased the expression of tyrosine hydroxylase (TH) in dopaminergic neurons at 48 hpf. Only nitrite decreased the response to tactile stimulation at 72 hpf, whereas both nitrate and nitrite decreased the swimming activity at 6 dpf. When the embryos were exposed to nitrate or nitrite together with an estrogen receptor blocker (ICI 182,780), the decreases in TH expression and motor behavior caused by nitrate or nitrite alone were reversed suggesting the effects of nitrate and nitrite were mediated through estrogen receptor (ER). The result of co-incubation with an oxidoreductase inhibitor, diphenyleneiodonium, indicated the conversion to nitric oxide (NO) is likely to be responsible for the effects of nitrate and nitrite, which was further supported by the increased staining for NO after exposure. The present study demonstrates that nitrate and nitrite are neurotoxicants acting as an endocrine disruptor possibly through conversion to NO to downregulate the activity of dopaminergic neuron in early development of zebrafish.

1. Introduction

Nitrate (NO_3^-) and nitrite (NO_2^-) are naturally occurring anions, which compose of the biological nitrogen cycle in the ecosystem [1]. However, excess nitrogenous substances entering natural aquatic system due to human activities such as agricultural runoff, industrial wastes and sewage effluents have been contributing to the elevated nitrate and nitrite concentrations in ground and surface water, and this pollution is becoming one of the most prevalent environmental problems on a worldwide scale. There are accumulated data showing that exposures to nitrate and nitrite from consumption of contaminated water as well as vegetables and meat products cause adverse effects on humans [1]. Ingestion of contaminated drinking water with nitrate causes methemoglobinemia in infants via the conversion of nitrate to nitrite [1]. Nitrate intake also has a potential role in developing cancers through formation of nitrosamines [2,3]. Deleterious effects on aquatic organisms have also been reported. Both acute and chronic exposures of nitrate and nitrite increase mortality and impair growth and reproduction [4,5]. Some studies in zebrafish at different life stages also reported toxic effects such as developmental defects, high mortality, and growth suppression [6,7]. Though some studies have shown the maternal exposure to high nitrate concentrations in drinking water and food may increase the risk of neuronal defects in infant [8], the information of specific mechanisms in the nervous system affected by nitrate and nitrite exposure is still limited.

In addition to the overall toxicity, endocrine disrupting role of nitrate and nitrite has been indicated in various species, and the possible pathways of altering steroidogenesis have been proposed [9]. According to the review by Guillette and Edwards nitrate could affect steroid by conversion to nitrite and nitric oxide in the mitochondria by altering chloride concentrations in the membrane transport or by altering action of steroidogenic enzymes by binding to the heme region of cytochrome P450 enzymes [9]. Veselik et al. demonstrated that nitrite is the active anion to bind to estrogen receptor to mimic the functions of estrogen in the breast cancer cell [10].

Nitric oxide (NO) is a ubiquitous signaling molecule, synthesized from of L-arginine by the action of nitric oxide synthases (NOS). However, a fundamentally different pathway of NO formation has been revealed over the past decades. It is now well established that the anions NO_3^- and NO_2^- can be converted to NO in blood and tissues [11]. Also several pathways are involved in reduction process

* Corresponding author. Fax: +81 96 342 3020.
E-mail address: mkishida@gpo.kumamoto-u.ac.jp (M. Kishida).

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of nitrite to NO by deoxygenated hemoglobin, xanthine oxidoreductase, deoxymyoglobin, mitochondrial enzymes, ascorbic acid, etc. [12]. NO is known to be involved in the cardiovascular, central nervous, and immune systems [13]. NO is also considered as a hormone and takes part in different metabolic/endocrine disorders such as diabetes and dysglycemia, thyroid disorders, hypertension, heart failure, and obesity [14]. Furthermore, NO plays an important role in regulation of synaptogenesis and neurotransmission in the central and peripheral nervous system [15,16].

Dopaminergic (DA) neuron secretes dopamine as a neurotransmitter and controls a number of important physiological functions such as locomotive activities, cognition, motivation, and emotion [17]. Impairment of DA neurons is known to be involved in some pathological conditions. Degeneration of DA neurons in substantia nigra in humans is a hallmark of Parkinson's disease (PD), and the malfunction of DA neurons in other brain regions is implicated in psychiatric disorders and neuroendocrine alteration [18,19]. Tyrosine hydroxylase (TH) is a rate limiting enzyme of dopamine synthesis, and thus changes in expression of TH will affect activity of DA neuron. In mammals, it has been known that DA neuron is one of the targets of estrogen [20]. Estrogen is known to regulate the expression of TH [21] and affect transcriptional regulation of TH by interacting with the cAMP pathway through ERα and ERβ [22]. In teleost fish, it has been known that dopamine plays a role in hypothalamus–pituitary–gonadal axis by inhibiting gonadotropin release, which is modulated by estrogen [23,24]. In ovariectomized trout, estradiol replacement increases both norepinephrine and dopamine levels in the hypothalamus suggesting possible modulations of catecholamine synthesis by estrogen [25]. In rainbow trout the immunoreactive cells to ER in preoptic area are also TH positive [26]. Thus, DA neurons can be considered as a target of endocrine disruptors which interact with estrogen receptor.

Therefore, in the present study, we tested the hypothesis that nitrate and nitrite act as an endocrine disruptor to interfere with activity of DA neuron using zebrafish embryos and larvae. The results showed that nitrate and nitrite downregulate the activity of DA neuron through estrogen receptor, and further suggested that nitric oxide converted from nitrate and nitrite is involved in regulation of dopaminergic system by acting through estrogen receptor.

2. Materials and methods

2.1. Fish maintenance and embryo culture

Adult zebrafish, *Danio rerio*, obtained from a local pet store, were reared in freshwater at 26–30 °C with the light regime of 14 h light and 10 h dark and fed with TetraMin (Tetra Japan). Fertilized eggs were washed in embryo medium (EM) (0.004% $CaCl_2$, 0.163% $MgSO_4$, 0.1% NaCl and 0.003% KCl) and incubated in a 6-well plastic plate (30 embryos in 8 mL EM per well) at 28.5 ± 0.5 °C.

2.2. Exposure experiments

For the stocks, potassium nitrate (Wako) and potassium nitrite (Sigma) were dissolved in distilled water at 1000 mg/L nitrate-N (NO_3-N) and nitrite-N (NO_2-N), respectively, and ICI 182,780 (Tocris) and diphenyleneiodonium (DPI) (Sigma) in dimethyl sulfoxide (DMSO) at 10 mM. Stock solutions were diluted with EM to the concentrations used in the experiments. For the controls EM with or without 0.1% DMSO was used. Exposure started at 2 h post fertilization (hpf) and the media were changed daily.

2.3. Whole-mount immunocytochemistry

Embryos at 48 hpf were fixed in 4% paraformaldehyde overnight at 4 °C and incubated in 3% H_2O_2/0.5% KOH to remove pigments. After dehydration and permeabilization, nonspecific binding was blocked in 1% normal goat serum and 3% BSA for 3 h at RT. Then embryos were incubated with mouse monoclonal anti-TH antibody (ImmunoStar) (1:1000) overnight at 4 °C. After washing the embryos were incubated in Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) (1:200) for 2 h. The fluorescence microscope (Leica M165 FC) was used for observation. For measurement focus was adjusted on the field with the largest positive area, and ImageJ software was used to quantify manually outlined areas.

2.4. Touch-response test

Response to tactile stimulation was measured at 72 hpf. Needlepoint simulation was applied at the caudal region of quiescent fish and the number of fish starting to swim was counted. Fifty fish were tested for each treatment, and the experiments were repeated three times with eggs collected from different spawns.

2.5. Crossline assay

Swimming activity was measured by the crossline assay [27]. Larvae at 6 days post fertilization (dpf) were transferred into a 12-well plastic plate (one fish per well containing 2 mL EM) with the bottom of each well divided with gridlines into four equal portions. After habituation for 5 min behavior was recorded with a video camera. Swimming activity was determined by the number of times each larva crossed a line within 1 min. Twenty-four larvae in each treatment were tested, and the experiments were repeated three times with eggs collected from different spawns.

2.6. DAF staining

Staining was carried out according to the protocol described previously [28]. Larvae at 5 dpf were incubated in 5 μM 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) (Molecular Probes) for 2 h in the dark at 28 °C and rinsed in EM. The larvae anesthetized in 0.003% tricaine and embedded in 1% agarose were observed under the fluorescence microscope (Leica M165 FC). Five larvae in each treatment were examined, and the experiments were repeated three times with eggs collected from different spawns.

2.7. Statistical analysis

All data were analyzed by one way ANOVA and followed by Tukey's post hoc test using SPSS 16.0 package for Windows to determine significant differences between the treatments. A significance level of $P < 0.05$ was used in all analyses.

3. Results

3.1. Effect of nitrate and nitrite exposure on TH

It has been known that TH expression in developing zebrafish is found in diencephalon (DC), locus coeruleus (LC) and arch-associated neurons (AAN) [18], but only the TH-positive neurons in DC are considered as DA neurons, because of co-expression of dopamine transporter [29]. Therefore, we measured TH expression in DC (indicated by the circle in Fig. 1Aa). Fig. 1Ab–h shows the representative micrographs of TH staining of different groups. Exposure to nitrate at 10 and 100 mg/L NO_3-N significantly decreased TH expression (Fig. 1B), and the decrease at 10 mg/L was completely reversed by addition of ICI (Fig. 1C). Similarly, exposure to nitrite at 1–100 mg/L NO_2-N significantly decreased TH expression

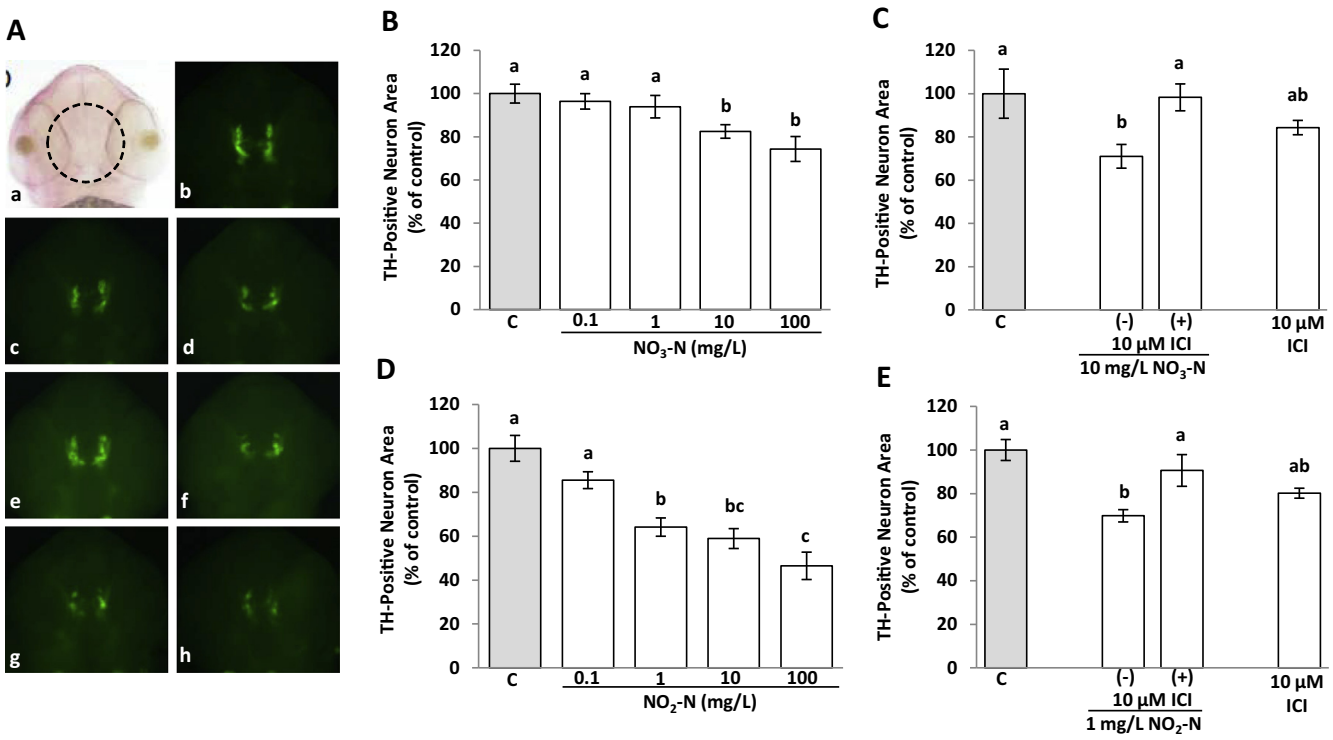

Fig. 1. Effect of nitrate, nitrite, and co-incubation with ICI on expression of TH. (A) Representative images of micrographs. Ventral view of a head with a dotted circle indicating the area where DA neurons are localized (a); control of the nitrate exposure experiment (b); 10 mg/L NO_3-N (c); 100 mg/L NO_3-N (d); control of the nitrite exposure experiment (e); 1 mg/L NO_2-N (f); 10 mg/L NO_2-N (g); 100 mg/L NO_2-N (h). (B–E) Measurements of the area of TH-positive neurons in the experiment of nitrate exposure, co-incubation of nitrate and ICI, nitrite exposure, and co-incubation of nitrite and ICI, respectively. Eight fish were examined in each group. Data are expressed as a mean ± standard error. Different letters in each graph indicate significant differences ($P < 0.05$).

(Fig. 1D), and the decrease at 1 mg/L was completely reversed by addition of ICI (Fig. 1E).

3.2. Effect of nitrate and nitrite exposure on motor behavior

As shown in Fig. 2A, there was no significant effect of nitrate exposure on response to tactile stimulation at any doses tested. On the other hand, nitrite exposure significantly decreased the response at 1 and 10 mg/L NO_2-N (Fig. 2B). When the embryos were co-incubated with nitrite at 1 mg/L NO_2-N and ICI, the decreased caused by nitrite was significantly reversed (Fig. 2C), suggesting that the effect of nitrite exposure was mediated through ER. In the crossline assay, nitrate exposure significantly decreased swimming activity at 1–100 mg/L NO_3-N (Fig. 3A), and the decrease caused by nitrate at 10 mg/L NO_3-N was completely reversed by co-incubated with ICI, indicating that the effect was mediated through ER (Fig. 3B). Similarly, nitrite exposure significantly decreased swimming activity at 1 and 10 mg/L NO_2-N (Fig. 3C), and the decrease caused by nitrite at 1 mg/L NO_2-N was completely reversed by addition of ICI, suggesting the effect was mediated through ER (Fig. 3D).

3.3. Effect of an oxidoreductase inhibitor, diphenyleneiodonium (DPI)

Addition of DPI significantly reversed the decreased TH expression caused by nitrate or nitrite but not completely to the level of controls (Fig. 4A). Effect of DPI on response to tactile stimulation was tested only for nitrite exposure, because nitrate did not show any significant effect as shown in Fig. 2A. DPI also significantly reversed the decreased percentage of the responding fish caused by nitrite (Fig. 4B). Similarly, addition of DPI to nitrate or nitrite completely reversed the decreased swimming activity to the level of controls (Fig. 4C).

3.4. Effect of nitrate and nitrite exposure on NO production

DAF staining using larvae at 5 dpf showed that nitrate exposure increased the staining slightly (Fig. 4Da–e), whereas nitrite exposure clearly increased the staining dose-dependently (Fig. 4Df–i).

4. Discussion

This study demonstrates the ability of nitrite and nitrate to perturb the activity of DA neuron by acting through ER in early development of zebrafish at around the concentrations of the safety limit for drinking water recommended by US EPA and WHO (10 mg/L NO_3-N and 1 mg/L NO_2-N) [30]. Nitrate exposure at 0.1–100 mg/L NO_3-N did not affect mortality, hatching rate, and gross morphological defects, whereas nitrite exposure significantly decreased the hatching rate at 100 mg/L NO_2-N but other parameters were unchanged (data not shown). According to other studies in zebrafish, developmental defects, growth suppression and high mortality occur at much higher concentrations (100–300 mg/L) [6,7]. Although few studies indicated that nitrate and nitrite exposure exerts adverse effect on nervous system [8,31], to our knowledge so far this is the first study to reveal the specific effect of nitrate and nitrite on the activity of DA neurons.

Both nitrate and nitrite exposures downregulated the expression of TH in diencephalon, and their effects were mediated through estrogen receptor. Although our finding is in accord with another study in breast cancer cells showing that nitrate and nitrite have the ability to mimic the effects of estradiol (E_2) due to nitrite interacting with ERα [10], our study indicates NO is the active molecule. It has been indicated that transcriptional regulation of TH by estrogen is through membrane receptor [32], and the putative membrane estrogen receptor homolog to ERα responds to E_2 and ICI [33]. It is also reported that E_2 regulates

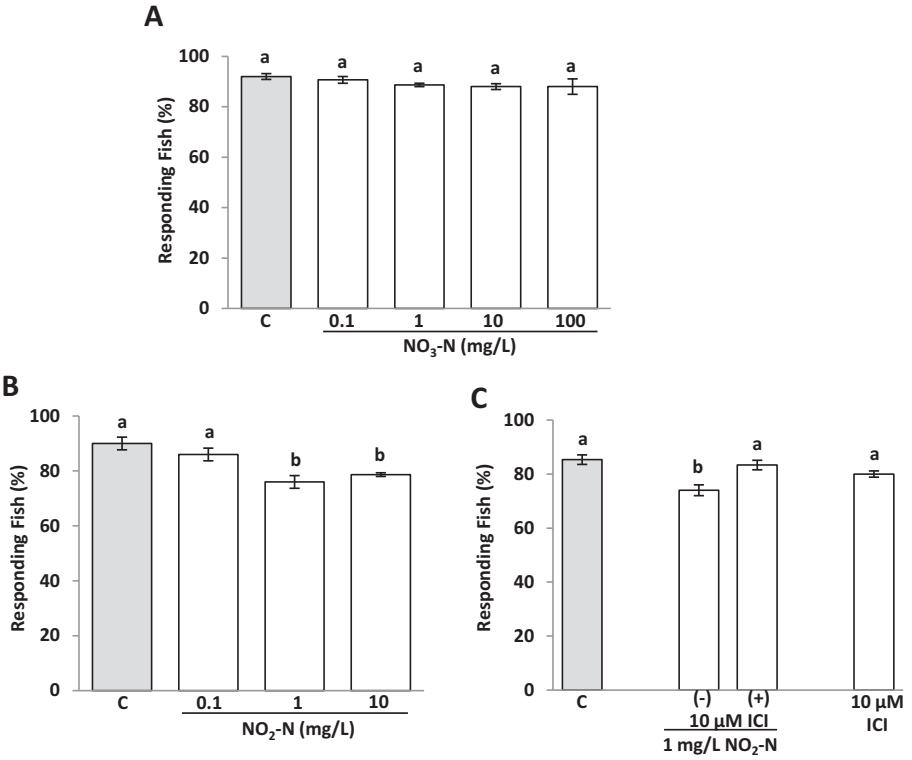

Fig. 2. Effect of nitrate, nitrite, and co-incubation with ICI on response to tactile stimulation. (A) Exposure to 0.1–100 mg/L NO_3-N. (B) Exposure to 0.1–10 mg/L NO_2-N. C: Co-incubation of 1 mg/L NO_2-N and ICI. Data are expressed as percentage of responding fish out of 50 fish. Different letters in each graph indicate significant differences ($P < 0.05$).

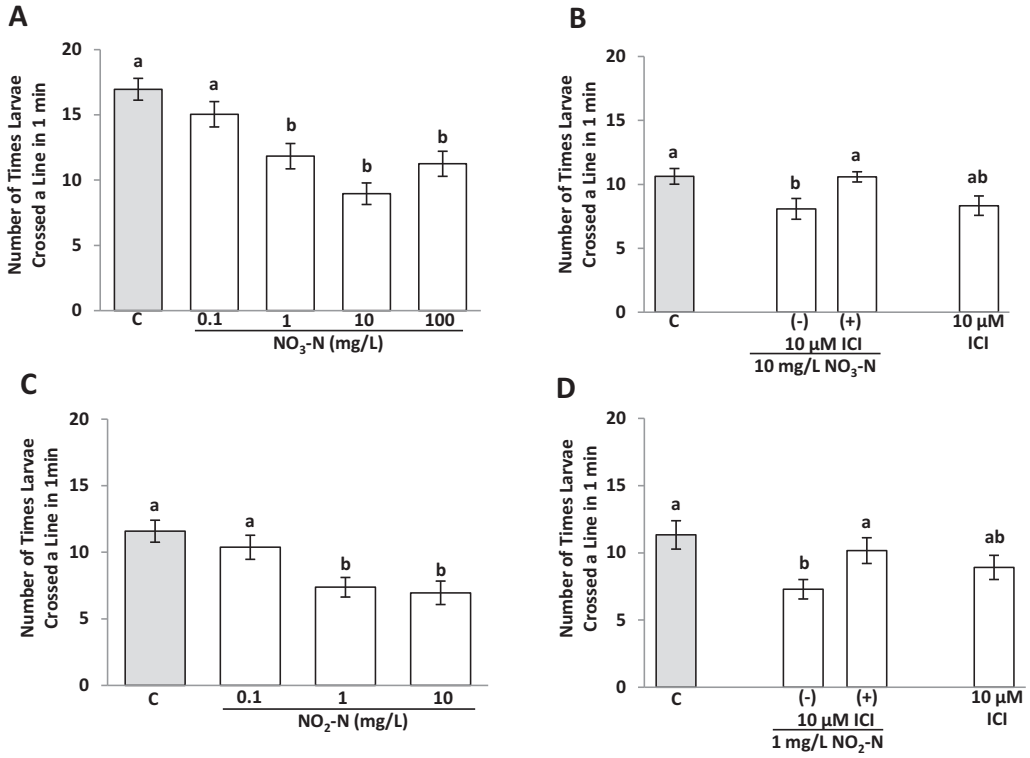

Fig. 3. Effect of nitrate, nitrite, and co-incubation with ICI on swimming activity. (A) Exposure to 0.1–100 mg/L NO_3-N. (B) Co-incubation of 10 mg/L NO_3-N and ICI. (C) Exposure to 0.1–10 mg/L NO_2-N. (D) Co-incubation of 1 mg/L NO_2-N and ICI. Data are expressed as the number of times each fish crossed a line in 1 min ($n = 24$). Different letters in each graph indicate significant differences ($P < 0.05$).

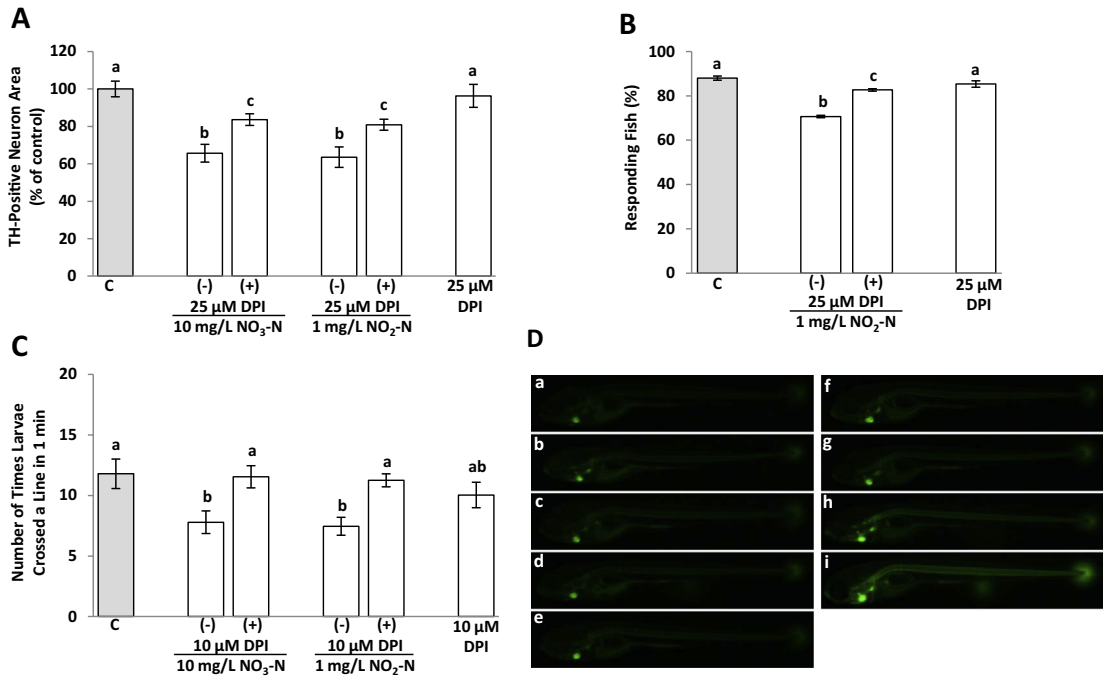

Fig. 4. Involvement of nitric oxide (NO) production in the activity of dopaminergic neurons of zebrafish exposed to nitrate and nitrite. (A) Effect of 25 μM DPI on TH expression at 48 hpf ($n = 8$). (B) Effect of 25 μM DPI on response to tactile stimulation at 72 hpf ($n = 3$). (C) Effect of 10 μM DPI on swimming activity at 6 dpf ($n = 24$). Data are expressed as a mean ± standard error. Different letters in each graph indicate significant differences ($P < 0.05$). (D) DAF staining at 5 dpf. Control of the nitrate exposure experiment (a); 0.1 mg/L NO_3-N (b); 1 mg/L NO_3-N (c); 10 mg/L NO_3-N (d); 100 mg/L NO_3-N (e); control of the nitrite exposure experiment (f); 0.1 mg/L NO_2-N (g); 1 mg/L NO_2-N (i); 10 mg/L NO_2-N (h).

transcription of TH by affecting through ERα and ERβ in PC12 cells [22]. Further studies are required to clarify the underlying molecular mechanisms for nitrate and nitrite to regulate TH expression.

Nitrate and nitrite decreased the motor activity at 72 hpf and 6 dpf acting through ER. It is likely that downregulated TH expression will result in the decreased dopamine production and release, which will impair the locomotor activity. In Parkinson's disease loss of midbrain DA neurons with reduction in dopamine content is the main cause to enervate motor activities [18,19]. Transient knockdown of TH during the early stages of development results in behavior alteration in adult zebrafish [34]. Dopamine receptor antagonist decreases the generation of motor neuron and reduces the response to tail-touch response test and swimming activity, suggesting those behaviors are under control of DA neuron [35]. On the other hand, it has been reported in zebrafish that NO signaling is involved in neuromuscular development and locomotor maturation [36], and perturbation of NO signaling affects the ontogeny of locomotor performance [37]. Although further studies are required to clarify whether NO produced by exposure to nitrate and nitrite acts directly on motor neurons, our data reveal that nitrate and nitrite act as an environmental toxicant on DA neurons.

Influence of NO signaling on nervous system is well established and the role of NO in the dopaminergic neurotransmission has also been reported [15,16]. Our data indicated that the effects of nitrate and nitrite exposures on DA neuron are due to NO production converted from nitrate and nitrite, which may directly activate ER. The nitric oxide synthase inhibitor, L-NAME significantly decreases dopamine release in rat indicating that endogenously produced NO may influence the activity of the DA transporter [38]. The same group also showed that the nitric oxide generator decreases the dopamine uptake [39]. Some studies provide evidence that estrogen induces NO production through activation of nitric oxide synthases (NOSs) mediating through ER [40,41], but the evidence of direct activation of ER by NO has not been reported yet. It has also been reported that the generation of NO and subsequent formation of $ONOO^-$ may contribute to the selective vulnerability of dopaminergic neurons through the oxidation of DA and modification of protein [42]. Thus, whether the increased NO production in this study alters dopamine signaling through estrogen signaling pathway together with or without the subsequent formation of $ONOO^-$ remains to be investigated.

In this study co-incubation with DPI blocked the effect of both nitrate and nitrite indicating that NO is the most active form to act on DA neurons. Although DPI significantly blocked the decreases in TH expression and in motor activity caused by nitrate and nitrite, the levels were still significantly decreased from the controls in some cases. These results suggest that both nitrate and nitrite may have some independent effect on DA neurons, which will require further clarification. It has been shown that in breast cancer cell, nitrite can directly activate ERα [10].

Increased endogenous NO production after nitrate and nitrite exposure was demonstrated by DAF staining, which is in accordance with another study in zebrafish [43]. Nitrate was much less potent in NO production after the exposure compared to nitrite, may be due to the fact that nitrate requires an extra step for reduction to NO than nitrite to NO and/or the cellular uptake of nitrite is more rapid in aquatic organisms through active uptake via Cl^-/HCO_3^- exchange mechanism [5]. Along with the presence of bacterial reductase in animals [44], the serial reduction of NO_3^- to NO_2^- to NO by xanthine oxidoreductase was detected in mammalian tissues [45] and in arthropod [46]. Though such enzyme has been known in zebrafish [47], the role in reduction of NO_3^- to NO_2^- to NO is not known.

In conclusion, this study demonstrates that nitrate and nitrite can act as an endocrine disruptor to perturb development of nervous system. The effect is likely to be mediated by resulting NO to bind to estrogen receptor. Although further studies are needed to understand specific mechanisms, considering the effective concentrations, contamination of nitrate and nitrite in the environment may pose a great impact on aquatic organisms.

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