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Elevated nitrate alters the metabolic activity of embryonic zebrafish☆

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ABSTRACT

Nitrate accumulation in aquatic reservoirs from agricultural pollution has often been overlooked as a water quality hazard, yet a growing body of literature suggests negative effects on human and wildlife health following nitrate exposure. This research seeks to understand differences in oxygen consumption rates between different routes of laboratory nitrate exposure, whether via immersion or injection, in zebrafish (*Danio rerio*) embryos. Embryos were exposed within 1 h post fertilization (hpf) to 0, 10, and 100 mg/L NO_3-N with sodium nitrate, or to counter ion control (CIC) treatments using sodium chloride. Embryos in the immersion treatments received an injection of 4 nL of appropriate treatment solution into the perivitelline space. At 24 hpf, Oxygen Consumption Rates (OCR) were measured and recorded *in vivo* using the Agilent Technologies XF^{e}96 Extracellular Flux Analyzer and Spheroid Microplate. Immersion exposures did not induce significant changes in OCR, yet nitrate induced significant changes when injected through the embryo chorion. Injection of 10 and 100 mg/L NO_3-N down-regulated OCR compared to the control treatment group. Injection of the 100 mg/L CIC also significantly down-regulated OCR compared to the control treatment group. Interestingly, the 100 mg/L NO_3-N treatment further down-regulated OCR compared to the 100 mg/L CIC treatment, suggesting the potential for additive effects between the counter ion and the ion of interest. These data support that elevated nitrate exposure can alter normal metabolic activity by changing OCR in 24 hpf embryos. These results highlight the need for regularly examining the counter ion of laboratory nitrate compounds while conducting research with developing zebrafish, and justify examining different routes of laboratory nitrate exposure, as the chorion may act as an effective barrier to nitrate penetration in zebrafish, which may lead to conservative estimates of significant effects in other species for which nitrate more readily penetrates the chorion.

1. Introduction

Anthropogenic sources of reactive nitrogen enter the environment primarily through agricultural activity, particularly the application of fertilizers, as well as through the burning of fossil fuels (Galloway et al., 2003; Fields, 2004). A plant utilizes only 2-10% of the nitrogen from applied fertilizers (Galloway et al., 2003) with the remaining 90-98% being lost to the environment. As nitrogen accumulates in heavily farmed regions, soil runoff, accompanied by specific drainage factors, sewage, and erosion, allows this excess nitrogen to enter both groundwater stores and aquatic ecosystems (Matson et al., 1997). It is estimated that anthropogenic sources of nitrogen have nearly doubled natural background levels (Galloway et al., 2003; Fields, 2004).

The United States Environmental Protection Agency (1995) has set a Maximum Contaminant Level (MCL) for nitrate in drinking water as part of the Safe Drinking Water Act (SDWA) at 10 mg/L nitrate-nitrogen (NO_3-N) for the protection of human health. This MCL for nitrate applies solely to public water supplies where standards can be regularly monitored. It is estimated that approximately 70% of the global population has a higher intake of nitrate

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than recommended (Erisman et al., 2013), as up to 20% of groundwater sources in the United States may exceed the 10 mg/L MCL even after SDWA regulations (Townsend et al., 2003). Environmental concentrations of nitrate vary considerably, and generally range from non-detectable to 100 mg/L NO_3-N (as reviewed by Rouse et al., 1999).

The association between nitrate ingestion and adverse thyroid anatomy and physiology in vertebrates is compelling (Guillette and Edwards, 2005). Nitrate competes with iodide in the production of thyroid hormones, and animal studies investigating elevated nitrate exposure have shown an effect on normal iodide metabolism of the thyroid gland (Bloomfield et al., 1961; Eskiocak et al., 2005), a decrease in thyroid hormone triiodothyronine (Eskiocak et al., 2005; Zaki et al., 2004), an increase in thyroid gland weight (Eskiocak et al., 2005; Zaki et al., 2004), and histomorphological changes that include vacuolization of thyroid follicles, goiter, and an increase in the size of the follicles (Eskiocak et al., 2005; Morris et al., 2011; Zaki et al., 2004). Human epidemiological studies investigating elevated nitrate exposure and thyroid dysfunction have found an increased risk of goiter in pregnant women (Gatseva and Argirova, 2008), an increase in thyroid volume leading to the development of thyroid hypertrophy (Tajtakova et al., 2006; van Maanen et al., 1994), and an increased rate of phenotypic evidence for subclinical thyroid disorders (Tajtakova et al., 2006).

Disruption of normal thyroid functioning may likely lead to abnormal metabolic activity, as thyroid hormones play critical roles in regulating heat production, metabolism, and oxygen consumption. Although thyroid hormones do not play a significant role in heat production in fishes as they do in endotherms, they are nonetheless thought to play a role in regulating tissue respiration (Little and Seebacher, 2014). The relationship between normal thyroid functioning and metabolism in ectothermic teleost fish relates back to the evolutionary role of thyroid hormones regulating peripheral and intermediary metabolism in poikilotherms (Eales, 1985; Pliestskaya et al., 1983).

The zebrafish (*Danio rerio*) serves as an excellent *in vivo* laboratory model system for investigating endocrine disruption (Segner, 2009), toxicology and chemical screening (Dai et al., 2014; Yang et al., 2009), and cell metabolism (Santoro, 2014). Interest in analyzing metabolic profiles of individual developing zebrafish embryos has led to novel instrumentation and tools to measure mitochondrial function and oxygen consumption rates (Gibert et al., 2013; Huang et al., 2013; Shim et al., 2016; Stackley et al., 2011; Zhang et al., 2017). Embryonic zebrafish utilize cutaneous gas exchange by diffusion through the boundary layer of the chorion (Pelster and Bagatto, 2010). Zebrafish metabolic and bioenergetic profiling has revealed significant variation in metabolic rates of developing embryos, which suggests implications for early life-history characteristics (Bang et al., 2004). Zebrafish embryo basal respiration has also been shown to increase linearly with increasing developmental stage and embryonic age (Huang et al., 2013; Stackley et al., 2011).

More recently, scientific interest has grown surrounding nitrate research due to many of the factors outlined here, and most of this research used the more commonly available compound sodium nitrate. Many of these studies do not include a carrier ion control exposure treatment group to account for the sodium component of the sodium nitrate compound. While solvent controls are routinely included in experimental designs, the conjugated salts of water-soluble compounds are often largely ignored, especially in laboratory nitrate research studies.

The research objectives of this study are (1) to investigate the effects of nitrate, using sodium nitrate, on the metabolic activity of embryonic zebrafish by measuring oxygen consumption rates *in vivo*, (2) to compare differences in oxygen consumption rates between different routes of laboratory nitrate exposure, specifically immersion versus injection techniques, and (3) to explore the role of the sodium carrier ion in the primarily available laboratory chemical compound, sodium nitrate, on oxygen consumption rates.

2. Materials and methods

2.1. Chemical treatments

Egg water was prepared with Instant Ocean Sea Salt (Instant Ocean) and reverse osmosis water to a final concentration of 60 μg/ml. After egg water preparation, water soluble sodium nitrate at 0, 10, and 100 mg/L nitrate-nitrogen (NO_3-N) was added to egg water to make nitrate treatment solutions. Counter ion control (CIC) treatments were made with sodium chloride, where the concentrations of sodium were equivalent to the sodium found in the 10 mg/L NO_3-N and 100 mg/L NO_3-N treatments, and are annotated as 10 mg/L CIC and 100 mg/L CIC, respectively. A positive control treatment of 0.05 μM carbonyl cyanide- 4- (trifluoromethoxy) phenylhydrazone (FCCP) was also added to egg water. FCCP is considered an uncoupling agent because it disrupts ATP synthesis by collapsing the proton gradient and altering mitochondrial membrane potential. After FCCP exposure, hydrogen ion flow through the electron transport chain is uninhibited and oxygen is maximally consumed.

2.2. Immersion trial experiment

Adult AB wild-type zebrafish were housed and bred according to standard laboratory husbandry methods (Westerfield, 2000) in the Zebrafish Facility at the University of Maine. Embryos were collected within 1 h post fertilization (hpf), and were transferred to chemically treated egg water. Approximately 25 embryos per 100 mL of treatment egg water solutions were maintained at 28.5 °C in a 14/10 light-dark photoperiod for 24 hpf. At 24 hpf, dead embryos were removed and treated egg water solutions were replaced.

2.3. Injection trial experiment

AB wild-type zebrafish embryos were collected as described for immersion trials, and were microinjected within 1.5 hpf with 4 nL of treatment solution through the chorion into the perivitelline space, prior to transfer into nitrate treatment solutions. Approximately 50 embryos were microinjected per treatment group, and were maintained at 28.5 °C in a 14/10 light-dark photoperiod for 24 hpf at densities of 50 embryos per 100 mL of treatment egg water solution. A negative control treatment group of 0 mg/L NO_3-N (60 μg/ml Instant Ocean, egg water) un-injected embryos was added to this experiment to determine whether the microinjection technique alters zebrafish embryo OCR.

2.4. Experimental design

At 24 hpf, embryos were removed from the incubator. One zebrafish embryo was loaded into each well of an XF^{e}96 Spheroid Microplate (Seahorse Bioscience, Agilent Technologies) with 150 μL of fresh treatment solution. Under a microscope, embryo health, position, and developmental stage were assessed using the established criteria of Kimmel et al. (1995). If an embryo was not in the correct position at the bottom of each well, a blunt-ended aligning rod was used to gently adjust positioning. Each experiment was repeated with triplicate assay plates, and sample sizes per plate were as follows: Immersion trial experiment: 0 mg/L NO_3-N – 14 embryos, 10 mg/L NO_3-N, 10 mg/L CIC, 100 mg/L NO_3-N, and