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Quercetin, a natural product supplement, impairs mitochondrial bioenergetics and locomotor behavior in larval zebrafish (*Danio rerio*)

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ABSTRACT

Quercetin is a natural product that is sold as a supplement in health food stores. While there are reported benefits for this flavonoid as a dietary supplement due to antioxidant properties, the full scope of its biological interactions has not been fully addressed. To learn more about the mechanisms of action related to quercetin, we exposed zebrafish (*Danio rerio*) embryos to 1 and 10 μg/L quercetin for 96 h starting at 3 h post fertilization. Quercetin up to 10 μg/L did not induce significant mortality in developing fish, but did increase prevalence of an upward-curved dorsal plane in hatched larvae. To determine whether this developmental defect was potentially related to mitochondrial bioenergetics during development, we measured oxygen consumption rate in whole embryos following a 24-hour exposure to quercetin. Basal mitochondrial and ATP-linked respiration were decreased at 1 and 10 μg/L quercetin, and maximal respiration was decreased at 10 μg/L quercetin, suggesting that quercetin impairs mitochondrial bioenergetics. This is proposed to be related to the deformities observed during development. Due to the fact that ATP production was affected by quercetin, larval behaviors related to locomotion were investigated, as well as transcriptional responses of six myogenesis transcripts. Quercetin at 10 μg/L significantly reduced the swimming velocity of zebrafish larvae. The expression levels of both myostatin A (*mstna*) and myogenic differentiation (*myoD*) were also altered by quercetin. *Mstna*, an inhibitory factor for myogenesis, was significantly increased at 1 μg/L quercetin exposure, while *myoD*, a stimulatory factor for myogenesis, was significantly increased at 10 μg/L quercetin exposure. There were no changes in transcripts related to apoptosis (*bcl2*, *bax*, *casp3*, *casp7*), but we did observe a decrease in mRNA levels for catalase (*cat*) in fish exposed to each dose, supporting an oxidative stress response. Our data support the hypothesis that quercetin may affect locomotion and induce deformities in zebrafish larvae by diminishing ATP production and by altering the expression of transcripts related to muscle formation and activity.

1. Introduction

Quercetin is a highly abundant polyphenolic flavonoid found in many tissues of plants, including the bark of oak from the *Quercus* species. As such, this natural product is commonly found in important dietary sources of food including fruits, vegetables, and teas (Wach et al., 2007). Some studies suggest that quercetin is a potent antioxidant and scavenger of both reactive oxygen and reactive nitrogen species, in addition to serving as a compound that can bind and sequester transitional metal ions (D'Andrea, 2015; Nabavi et al., 2015). Quercetin has also been reported to be neuroprotective for oxidative stress in PC12 cells, a model for dopamine synthesis and neurodegeneration (Heo and Lee, 2004). In addition, studies in cancers have shown that quercetin reduces the growth of solid tumors in prostate xenograft rodent models by inhibiting angiogenesis (Pratheeshkumar et al., 2012). Due to such reports of beneficial effects for different human diseases, quercetin is a popular dietary supplement that is available in health food stores. However, evidence is not yet definitive and additional studies are needed to determine long term benefits of this compound on health.

From an environmental perspective, there is the potential for quercetin to be present in the aquatic environment from effluents of any industry that processes plant material, for example the pulp and paper industry (Hillis, 1962; Sjöström, 1993). To the best of our knowledge, this compound has not been actively pursued for measurements using analytical techniques in surface water, but studies have reported on the effects of quercetin in aquatic organisms. For example, female medaka (*Oryzias latipes*) treated with quercetin (100 ppb) in the water showed an increase in the number of atretic ovarian follicles (Weber et al., 2002) and quercetin at concentrations ranging from 50 to 200

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μM were shown to disrupt the formation of inter segmental vessels, the dorsal aorta and the posterior cardinal vein in transgenic zebrafish (*Danio rerio*) embryos (Zhao et al., 2014). Thus, quercetin may also have some negative impacts in fish. These studies in fish have relevance for aquaculture, as quercetin has been investigated as a fish feed supplement due to its antioxidant and beneficial properties (Pês et al., 2016). For example, it has been reported that >100 μg/L quercetin can prevent microcystin-LR-induced immunotoxicity to *Carassius auratus* lymphocytes *in vitro* by mitigating oxidative stress responses (Zhang et al., 2014).

As data suggest an interaction between oxidative stress, ROS production, and quercetin, it becomes important to more closely investigate the function of the mitochondria following treatments with quercetin. Polyphenols, such as quercetin, act on the mitochondrial permeability transition pore (MPTP), which can lead to depolarization, uncoupling, and ATP depletion (De Marchi et al., 2009). Mitochondrial dysfunction is a unifying factor underscoring toxicological responses (Dykens and Will, 2007) and based upon our investigations into the Tox21 dataset (EPA, 2013), there is evidence that quercetin reduces mitochondrial membrane potential in HepG2 cells (the concentration at half-maximal activity is 0.965 μM), supporting its role in regulating mitochondrial bioenergetics.

Most studies that measure mitochondrial function, such as mitochondrial DNA integrity and enzymatic activities of oxidative phosphorylation proteins, are generally slow and destroy the cell or tissue of interest (Chan et al., 2007). Recently, a new, physiologically relevant and non-invasive method has been developed to assay mitochondrial bioenergetics in developing zebrafish embryos utilizing the Seahorse Bioscience Extracellular Flux Analyzer, which can give accurate and reliable results for oxygen consumption (Stackley et al., 2011; Raftery et al., 2016). The mitochondrial toxicity of several chemicals, including triclosan (Shim et al., 2016), oxygenated polycyclic aromatic hydrocarbons (Knecht et al., 2013), and 2,4-dinitrophenol (Bestman et al., 2015), has been assessed in zebrafish with this established platform. To determine whether quercetin affected endpoints related to mitochondrial bioenergetics in developing embryos, we measured oxygen consumption rates in whole fish following exposure to the flavonoid. As we observed an increase in tail curvature and a reduced capacity for ATP production following quercetin treatment, we also measured endpoints related to locomotion and the expression of genes related to muscle function, apoptosis and oxidative stress to more completely evaluate the relationship between deformity, oxidative respiration, and behavior.

2. Materials and methods

2.1. Fish husbandry

Adult wildtype zebrafish (AB strain, *Danio rerio*, ~8 months of age) were housed in a laboratory at Animal Care Services in a room maintained at 28 °C with a 14/10 h light/dark cycle and fish were bred according to standard methods (Westerfield, 2000). Embryos were staged using the criteria of Kimmel et al. (1995). Experimental procedures were approved by the University of Florida Animal Care Committee and were carried out at the Center for Environmental and Human Toxicology at the University of Florida.

2.2. Animal exposures

Stock solutions of 0.1 and 1 mg/mL quercetin (purity ≥ 95%, Sigma-Aldrich; CAS Number: 117-39-5) were prepared in dimethyl sulfoxide (DMSO). The concentrations of DMSO (0.01%) and quercetin used in this study were based upon previous studies in fish (Weber et al., 2002). The exposure solutions were prepared by adding prepared stock solutions into embryo rearing medium (ERM) (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM Mg SO_4, 4.2 mM $NaHCO_3$, pH 7.2) to achieve final nominal concentrations of 1 and 10 μg/L of quercetin. Control embryos were treated with 0.01% DMSO only.

For endpoints related to development and survival, 72 embryos ($n = 24$ embryos/treatment) at 3 hpf were transferred to each well of a 96-well culture plate filled with 200 μL exposure solution. The three groups were 1 μM, 10 μM and the solvent control DMSO. Half of the exposure solution in each well was renewed daily as to not disturb the embryos. Development, survival rates and hatch time of the embryos were assessed using EVOS™ FL Auto Imaging System (Life Technologies) to acquire one bright field image per hour during a 96-hour exposure regimen. After 96 h, the larvae were anesthetized in buffered MS-222 (tricaine methanesulfonate, Sigma, USA) at a concentration of 10 mg/L and photographed using a digital microscope (Fisher Scientific) to obtain bright field images. Based upon the OECD guidelines for fish embryo testing and the description by others (Oliveira et al., 2009; Andrade et al., 2016), the following parameters were evaluated: somite formation, incidence of pericardial and yolk sac edema, body curvature, and absence of swim-bladder inflation. These experiments were performed four times, with six replicates conducted in each experiment, each with comparable results.

For mitochondrial and gene expression assays, 25 embryos were incubated in glass beakers with 5 mL of each exposure solution. Six replicates were performed for each exposure solution and for the solvent control. Thus, the beaker was considered the biological replicate. The chemical was renewed daily at 50% for all assays.

2.3. Oxygen consumption rate and respirometry

After exposure for 24 h, one embryo/beaker was collected from each of the selected replicates and it was used for measuring mitochondrial respiration using the XF^{e}24 Extracellular Flux Analyzer (Seahorse Bioscience, Agilent Technologies). Each well of an Islet Plate was filled with 1 mL of XF Calibrant fluid, and the Islet Plate was incubated with the sensor plate overnight at 26 °C. Approximately 1 h prior to experimentation, the injection ports on the sensor cartridge were filled with 75 μL of the following solutions: oligomycin (160 μM), carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP, 54 μM) and sodium azide (200 mM). The final concentrations per well were 20 μM oligomycin, 6 μM FCCP, and 20 mM sodium azide. The Islet Plate was then loaded into the XF24 instrument for calibration. Following the calibration, one embryo + 525 μL exposure solution ($n = 5$/treatment) was added to each well of a 24-well islet capture microplate plate. Four wells served as temperature control wells on each plate. During the assay, the oligomycin, FCCP and sodium azide were added sequentially following a published protocol for measuring oxygen consumption rates (OCR) (Stackley et al., 2011). The protocol consisted of the following time cycles: 2 min mixing, followed by 1 min of no activity, and then 2 min to measure oxygen levels. Twelve cycles were performed for basal respiration. Eighteen cycles were conducted following oligomycin injection to inhibit ATP production of embryos. Eight cycles were performed following FCCP injection. Sodium azide was introduced last and measurements were taken for 24 cycles to inhibit mitochondrial respiration of embryos.

2.4. Zebrafish locomotion

At 72 h of exposure, 2–3 individual larvae from each of the 6 biological replicates (total 16 individual larvae from each exposure) were transferred to wells of a 96-well culture plate and placed into the DanioVision™ Observation Chamber (Noldus Information Technology, Leesburg, VA). After 24 h acclimation in the instrument during which time quercetin exposure continued, an infrared analog camera in the Daniovision Observation chamber recorded a 50 min video consisting of alternating 10 min periods of light and dark, beginning with a dark period. This video was then analyzed using EthoVision® XT software