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RESEARCH PAPER

Lack of postexposure analgesic efficacy of low concentrations of eugenol in zebrafish

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Abstract

Objective To test the postexposure analgesic efficacy of low doses of eugenol in zebrafish.

Study design Prospective experimental study.

Animals A total of 76 large adult zebrafish (*Danio rerio*).

Methods Fish swimming behavior (median velocity, freeze time, high-speed swimming and distance moved in the vertical direction) was recorded in a 1.6 L video arena before and after exposure to eugenol (0, 1, 2, 5, 10 and 20 mg L^{-1}). In a second experiment, fish were anesthetized with 2-phenoxy-ethanol and treated with an injection of 5% acetic acid (noxious stimulus), and then exposed to 0, 1, 2 and 5 mg L^{-1} eugenol. The fish swimming behavior was also recorded.

Results The higher doses (10 and 20 mg L^{-1}) reduced the median velocity, high-speed swimming and distance moved in the vertical direction, and increased the freeze time. Zebrafish behavior was not altered by eugenol (1, 2 and 5 mg L^{-1}) after noxious stimulation.

Conclusions and clinical relevance The change in the behavior of zebrafish associated with a noxious stimulus can be monitored and is a good model for studying analgesia in fish. Eugenol (10 and 20 mg L^{-1}) induced zebrafish sedation. The response after a noxious stimulus was not affected by postexposure to lower doses, and thus we cannot recommend its use as an analgesic.

Keywords fish, noxious stimulus, pain, sedation, swimming behavior.

Introduction

Although there is a controversy regarding the capacity of fish to experience pain (Braithwaite 2010; Rose et al. 2014), essentially all persons involved in the controversy agree that fish are capable of nociception, the perception of a noxious stimulus (Malafoglia et al. 2013; Curtright et al. 2015). Fish have nociceptors with properties that are very similar to those found in mammals (Braithwaite 2010). After neural encoding of a noxious stimulus, fish respond to it in a variety of ways depending on the stimulus and fish species. This response is referred to as a nocifensive response (Rose et al. 2014). Persons working with fish generally agree that it is appropriate to try to minimize these nocifensive responses.

A variety of anesthetic drugs have been investigated for their properties to reduce the nocifensive responses, to reduce the stressful impacts of handling, especially during routine procedures, such as weighing, vaccination, blood sampling, tagging, experimental surgery and veterinary procedures (Cunha et al. 2010a,b; Becker et al. 2012; Gressler et al. 2012; Parodi et al. 2014). Immersion anesthesia in fish is analogous to gaseous inhalant anesthesia in terrestrial animals. The fish ventilates the anesthetic dissolved in the water, which enters the bloodstream mainly through the gills.

However, there are few studies investigating or demonstrating analgesic activity of drugs used as anesthetics in fish. Most studies focusing on anesthesia and analgesia measure and report the complete immobility of the animal. However, the distinction between an anesthetic with analgesic properties and an immobilizing drug (anesthetic without analgesic properties) is not always clear

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(Harms 2005). In addition, anesthetics do not always provide analgesic effects, and can increase or have no effect on the stress response of fish (Weber et al. 2009) and/or on the nocifensive response of fish (Rose et al. 2014).

Eugenol [2 methoxy-4-(2-propenyl) phenol] is the principal active ingredient of clove oil, which is derived from the leaves, buds and stems of the clove tree (*Eugenia caryophyllata*), and has wide use as an anesthetic for aquatic organisms because of its low price and ready availability (Roubach et al. 2005; Hoseini et al. 2015). Several studies have evaluated its use to reduce fish hypermobility, thus reducing fish stress during handling (Palic et al. 2006; Cunha et al. 2010a). Eugenol has a peripheral local anesthetic (antinociceptive) action on axons (Markowitz et al. 1992), especially on specific voltage-gated sodium (Na^+) channels, at least in mammals (Wang et al. 2015). The aim of the present study was to analyze the swimming behavior of zebrafish to test the efficacy of postexposure to subanesthetic and subsedative doses of eugenol for analgesia in fish. Our hypothesis was that a noxious stimulus decreases zebrafish swimming activity and that subsedative doses of eugenol will prevent this behavioral change even after the fish are no longer exposed to eugenol.

Materials and methods

All experiments were performed in accordance with the Canadian Council on Animal Care guidelines, and were approved by the local Animal Care Committee at the University of Prince Edward Island, Atlantic Veterinary College (number 09-004).

Animals and housing

Large adult zebrafish (*Danio rerio*) were obtained from a local supplier, and maintained in 40 L tanks at 28.3 ± 0.4 °C (mean ± standard deviation). They were fed flake food three times daily (Nutrafin Max; Hagen Ltd., QC, Canada) with automatic feeders (model 3581; EHEIM GmbH & Co. KG, Germany), and maintained on a 12/12 hour photoperiod. Illumination was provided by fluorescent lights on a 24 hour cycle (on at 0600 hours/off at 1800 hours). Deionized water was supplemented with Nutrafin Aqua Plus (5 mL in 40 L; Hagen Ltd.), EasyBalance (5 mL in 40 L; Tetra Holding Inc., VA, USA) and sea salt (2 g in 40 L; D-D The Aquarium Solution Ltd., UK). The fish were acclimated to fish housing for 2 months prior to the experimental trials. Holding tank water was used to make anesthetic and recovery tank water.

Chemicals and eugenol

All chemicals were purchased from Sigma-Aldrich (MO, USA). Solutions of acetic acid (5%) were dissolved in deionized water. Eugenol was purchased commercially (Odontofarma, RS, Brazil), and the analysis of this product by gas chromatography/mass spectrometry (Varian Saturn 2200; Agilent Technologies Inc., CA, USA) revealed 99.03 ± 0.15% eugenol ($n = 3$) (Gomes et al. 2011). Q4

Behavioral variables

Behavioral testing was performed between 1100 and 1500 hours because the response of zebrafish to eugenol can change with the period of the day (Sánchez-Vázquez et al. 2011). Each fish was placed individually in a 1.6 L video arena (17 × 8 × 12 cm deep) supplied with tank water at 25 °C, pH 6.8–7.4 and constant aeration. The video arenas were covered with a black lid and with blue Styrofoam on all sides but the front to diminish the influence of external stimuli. After a 5 minute settling period, the swimming activity was recorded for 35 seconds (time pre) using a camera facing the front of the tank with a 17 × 12 cm view. Online records were stored on the computer and later analyzed for activity (LoliTrack Version 3.0.0 or 4.1; Loligo Systems, Denmark). Distance was calculated as the change in position in two dimensions, and velocity as this distance per time. Q5 Q6

The variables used to analyze the analgesic effect were based on those proposed by Cachat et al. (2010), and measurement time was 30 seconds: 1) median velocity during the trial (cm $second^{-1}$); 2) freeze time (time during the trial with no change in position): % of time that the fish moved with speed <0.5 cm $second^{-1}$; 3) high-speed swimming (time spent swimming at high speed): % of the time that fish swam with speed ≥10 cm $second^{-1}$; and 4) vertical distance (total distance moved in the vertical direction): total cm in a 30 second trial.

The difference between pre-exposure and post-exposure was calculated for each variable, such that zero would indicate no effect of eugenol or treatment.

Experiment 1: postexposure effects of low doses of eugenol

After recording the behavioral parameters in untreated fish (pre-exposure), each fish was transferred to a 100 mL beaker containing eugenol in tank water (0, 1, 2, 5, 10 or 20 mg L^{-1}, previously diluted in

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