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High dose propofol effectively euthanizes zebrafish (Danio rerio)

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Original Article

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Abstract

We evaluated propofol overdose for euthanasia of zebrafish (*Danio rerio*). We hypothesized that 1) propofol 40 mg/L at 10 min submersion euthanizes zebrafish as effective as 20 or 30 min submersion; 2) propofol 80 mg/L at 10 min submersion euthanizes zebrafish as effective as with 100 or 120 mg/L. Wild-type AB zebrafish were randomly submersed into: Exp.1 - propofol 40 mg/L for 10, 20, or 30 mins; or Exp.2 - 80, 100, or 120 mg/L for 10 mins. Criteria monitored: aversive behavior; time to loss of righting reflex (LORR); undulation cessation; operculation cessation; fish movement from tank tap reflex cessation; and euthanasia rate. Results: No aversive behavior noted; LORR 5 sec for both experiments; undulation cessation: 22 sec (Exp.1), 5 sec (Exp.2); operculation cessation: 66 sec (Exp.1), 51 sec (Exp.2); fish movement from tank tap reflex cessation: 13 min in the 20 and 30 min groups, and fish still moved in the 10 min group (Exp.1), no fish movement in any groups (Exp.2); euthanasia rate: 30% in 10 min group and 100% in 20 and 30 min group (Exp.1); 90% in 80 mg/L group, 50% in 100 mg/L group and 100% in 120 mg/L group (Exp.2). Propofol 40 mg/L at 10 min submersion does not euthanize zebrafish as effective as 20 or 30 min submersion; 2) propofol 80 or 100 mg/L at 10 min submersion does not euthanize zebrafish as effective as 120 mg/L. Propofol at 40 mg/L for at least 20 min or 120 mg/L for 10 min submersion effectively euthanizes zebrafish.

Keywords: Euthanasia, Propofol, Zebrafish

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Introduction

Zebrafish euthanasia was an important topic of a recent discussion (Kohler *et al*., 2017) highlighting recommendation gaps by organizations in their approaches to zebrafish euthanasia. Methods used at authors' institution includes tricaine methanesulfonate (MS222) and rapid cooling. However, it appears that overdose using MS222 is still the most widely used method in a typical zebrafish laboratory (Kohler *et al*., 2017) despite potential occupational hazards concerning human exposure to tricaine (Bernstein *et al*., 1997). Rapid cooling is a method to euthanize small, warm water teleosts (Leary *et al*., 2013; Kohler *et al*., 2017). The simple method encompasses direct transfer of zebrafish to a slurry ice bath between 2°C to 4°C. Rapid cooling of zebrafish leads to shorter euthanasia time and less distress oriented behaviors compared to tricaine (Wilson *et al*., 2009). Recently, rapid cooling was also shown to be an effective means to euthanize juvenile zebrafish within a reasonably short amount of time (Wallace *et al*., 2018) further highlighting this method's potential as a primary non-pharmacological means to euthanize zebrafish.

Despite these available methods, we are interested in determining whether propofol may be used to euthanize fish in light of a recent report that immersion in propofol water at 25 mg/L for 30 mins failed to euthanize 50% of red comet goldfish (Balko *et al*., 2018). Propofol is a hypnotic agent that binds to γ-aminobutyric acid receptors and at high doses can cause cardiorespiratory depression (Plumb, 2018). Its ability to induce unconsciousness prior to cardiorespiratory arrest is consistent with tenets of good euthanasia. Although the drug is not approved by U.S. Food and Drug Administration for use in fish, propofol has been demonstrated to successfully anesthetize cyprinids without appreciable negative animal welfare effects (Gholipourkanani and Ahadizadeh, 2013; Oda *et al*., 2014; Balko *et al*., 2017), including zebrafish (Valentim *et al*., 2016) although some mortality was noted at anesthetic doses. Despite the shortcomings to euthanize red comet goldfish using propofol by others (Balko *et al*., 2018), our aim is to demonstrate propofol as a euthanasia agent for zebrafish focusing on two criteria, namely time required to induce loss of consciousness and reliability/irreversibility.

Materials and Methods

Fish and Husbandry: Sixty 11 to 12-month old mixed sex wild-type AB zebrafish (Sinnhuber Aquatic Research Laboratory, Corvallis, OR) were used in this study. A small cohort of 5 fish was tested for a small panel of laboratory zebrafish diseases and was PCR negative for infectious spleen and kidney necrosis virus, *Edwardsiella ictaluri, Flavobacterium columnare, Mycobacterium spp., Ichthyopthirius multifillis, Pleistophora hyphessobryconis, Piscinodinium pillulare, Pseudocapillaria tomentsosa,* and *Pseudoloma neurophilia*. Adult fish were housed at 5-10 fish/L on a recirculating aquaculture system (Aquaneering Inc; San Diego, CA) with 10% daily volume exchange supplied by calcite-filtered reverse osmosis water. Recirculated water passes through a 50 µm pre-filter, a 25 µm mechanical filter, fluidized bed biological filter, carbon filters, and finally ultraviolet filtration by a lamp that provides a minimum of 100,000 mW/s/cm². This filtration system in combination with its 1000 W heater keeps water chemistry between 26-28°C, pH 7.2-7.6, conductivity 500-600 µS, ammonia <0.01 ppm, nitrite <0.01 ppm, and nitrate ≤50 ppm. Fish were fed twice daily with *Artemia* (E-Z Egg, Brine Shrimp Direct, Odgen, UT) and processed feed (GemmaMicro 300, Skretting USA, Tooele, UT). Stanford University is an AAALAC accredited institution and its Administrative Panel on Laboratory Animal Care approved all procedures described in our study, and care was taken to comply with the 3R concept.

Experiment #1 Propofol Euthanasia - Immersion Time: Ten zebrafish were randomly assigned to each of three groups with different immersion duration: 10 mins (10M), 20 mins (20M), and 30 mins (30M). Each were immersed in 40 mg/L propofol (Propoflo 1%; Zoetis Inc; Kalamazoo, MI) infused water. For each group, 20 mg propofol was diluted in 0.5L system water (temperature 27.3°C, pH 6.85) to a final concentration of 40 mg/L in three 1.4L polycarbonate tanks. Resultant water pH was measured with a pH meter. For all fish within each group, all ten fish were transferred from home tank to propofol tank as a group.

Experiment #2 Propofol Euthanasia – Dosage: Ten zebrafish were randomly assigned to each of three dosage groups: 80 mg/L, 100 mg/l, and 120 mg/L. Each group as a whole was immersed in propofol water for 10 mins. Behavioral indicators of aversion were noted. Time to loss of righting reflex, undulation cessation and operculation cessation was noted. Tank tap reflex was assessed every two mins. At the end of 10th min, all fish within a group were netted out, rinsed with system water while in net, and placed into fresh water for a 1-hour recovery observation. Number of fish that regained consciousness and ability to swim was noted.

For both experiments, criteria monitored were: 1) *aversive behavior* (Collymore *et al*., 2016) - erratic swimming, piping, twitching; 2) *time to loss of righting reflex (LORR)* - time to loss of upright plane of orientation within water column for the final fish within each group; 3) *undulation cessation* - time to loss of neuromuscular activity for the final fish within each group; 4) *operculation cessation* - time to loss of respiration for the final fish within each group; 5) *fish movement from tank tap reflex cessation* - startle reflex elicited by tapping side of tanks every 2 mins for the first 10 mins of immersion and continued every min from the 11th min until no fish within a group demonstrated reflex; 6) *euthanasia rate* - number of fish that regained consciousness and ability to swim at end of each immersion period in fresh water. At the end of immersion, all fish within a group were netted out, rinsed with system water, and placed into fresh system water for a 3-hr recovery observation (for experiment 1) and a 1-hr recovery observation (for experiment 2). All observations (1-6) were performed by visualization from the same experimenter DC; Fisher's exact test was used to determine the homogeneity of frequency

counts of live and dead fish across groups. *P*-value < 0.05 was considered statistically significant.

Results

***Experiment 1: Propofol euthanasia - immersion time*:** Water's pH after propofol infusion did not change significantly and remained between pH 6.70 and 6.75. 1) No aversive behavior was noted, 2) Fish in all three groups (Table 1), LORR was within 5 sec, 3) undulation cessation was within 22 sec, 4) operculation cessation was within 66 sec, 5) in 10M group, fish movement was elicited throughout the 10 mins, while in 20M and 30M fish movement from tank tap reflex cessation stopped at the 12th and 13th min, respectively, 6) euthanasia rates were 30% (10M group), 100% (20M group), and 100% (30M group), and this result revealed that a number of live and dead fish differed significantly across groups (*p*-value = 0.030).

Experiment 2: Propofol Euthanasia – Dosage: Water's pH after propofol infusion did not change significantly and remained between pH 6.70 and 6.75. 1) No aversive behavior was noted, 2) fish in all three groups (Table 2), LORR was within 5 sec, 3) undulation cessation was within 22 sec, 4) operculation cessation was within 49-50 sec, 5) in all groups, fish movement from tank tap reflex cessation was stopped at the 10th min, 6) euthanasia rates were 90% (80 mg/L group), 50% (100 mg/L group), and 100% (120 mg/L group), and this result revealed that a number of live and dead fish did not differ significantly across groups (*p*-value = 0.074).

Table 1 Propofol Euthanasia Assessment Results – Immersion Time (Experiment 1). Since all fish for each group were simultaneously immersed in propofol, data reflects the behavior and behavioral response of final fish within each group.

Group (mins)	10M	20M	30M
LORR†	≤ 5 sec	≤ 5 sec	≤ 5 sec
Undulation cessation	22 sec	20 sec	19 sec
Operculation cessation	66 second	55 sec	61 sec
Fish movement from tank tap reflex cessation	n/a	12 mins	13 mins
Euthanasia rate	30%	100%	100%

† Loss of righting reflex

Table 2 Propofol Euthanasia Assessments Results – Different Dosages, Same Immersion Time (Experiment 2). Data reflects the behavior and behavioral response of final fish within each group.

Group (mg/L)	80	100	120
LORR†	≤ 5 sec	≤ 5 sec	≤ 5 sec
Undulation cessation	≤ 5 sec	≤ 5 sec	≤ 5 sec
Operculation cessation	51 second	49 sec	50 sec
Fish movement from tank tap reflex cessation	10 mins	10 mins	10 mins
Euthanasia rate	90%	50%	100%

† Loss of righting reflex

Discussion

We assessed propofol's utility as a zebrafish euthanasia agent. Our data demonstrates that immersing zebrafish in propofol water at 40 mg/L for a least 20 mins or 120 mg/L for at least 10 mins effectively (100%) euthanizes adult zebrafish. Our data supports previous reports of propofol's potency. Zebrafish immersed in 2.5 mg/L to 7.5 mg/L propofol for 5 mins resulted in 10% to 33% mortality in a 48 hr time span during recovery (Valentim *et al*., 2016). Goldfish, a cold water cyprinid, exposed to 5 mg/L or 10 mg/L propofol resulted in immediate operculum cessation in nearly 70% of test subjects (Balko *et al*., 2018). Moreover, goldfish immersed in 16 mg/L for 30 mins resulted in 100% death rate (Gholipourkanani and Ahadizadeh, 2013). However and in contrast, goldfish exposed to 25 mg/L for 30 mins resulted in 50% recovery (Balko *et al*., 2018). We agree with those authors' conclusion that reaching irreversibility was a matter of identifying a good combination of dosage and exposure time. Therefore, we chose a dose for Experiment #1 that was at least 50% higher than previously reported (Balko *et al*., 2018) and chose three different immersion durations. In our study, presence of tank tap reflex by at least one fish in all groups by the 10th min clearly demonstrated that we did not reach 100% euthanasia and neural suppression. Whether the reflex was due to cerebral or spinal pathway is irrelevant because the aim of euthanasia was to suppress microcellular activities.

Typical recommendation for immersion euthanasia is exposure for at least 10 mins following cessation of opercular movement (Leary *et al*., 2013). Since all fish in all groups stopped operculating within one minute, a straight 10-min time period may be practical to ensure complete cease of respiration. Doubling the dosage from 40 mg/L to 80 mg/L and shortening the exposure period from 20 mins to 10 mins resulted in 90% euthanasia. However, at the next incrementally increased dosage of 100 mg/L resulted in only 50% euthanasia. One possible explanation could be due to the fact that propofol is a lipophilic drug, and in our study was diluted in an aqueous environment. This may lead to drug distribution that was not completely homogenous, possibly resulting in

variable pockets of drug concentration. This may explain why some fish lost tank tap reflex sooner than others. Although our data supports a 10 min immersion at 120 mg/L to ensure 100% euthanasia, the lower dose of 100 mg/L should be re-evaluated to rule out mixing or operator error as cause of euthanasia failure in half of the test subjects.

It should be noted that water pH dropped slightly from 6.85 to 6.70-6.75 after propofol infusion. Propofol stock 1% solution's pH was slightly acidic at pH 6.5 but can range from 6.0 to 8.0 and was manufactured to be neutral although we did not directly measure our stock propofol's pH. Like using ice bath, the operator does not need to add bicarbonate when using propofol immersion for euthanasia.

There are some practical drawbacks with using propofol for euthanasia. The obvious was the difficulty in seeing fish in propofol water beyond a certain concentration, which was similarly experienced by others (Oda *et al.*, 2014) using this drug in water. Further, propofol contains a number of inactive ingredients such as soybean oil, glycerol, egg lecithin, and oleic acid. It is unknown at this time what possible confounding effects, if any, may result as a consequence of peracute exposure to these inactive ingredients. A 10 to 20 min immersion time is greater than ice bath's 30 sec immersion time for adult zebrafish (Wallace *et al.*, 2018). Lastly, propofol must be discarded six hours after the vial's seal has been broken (Plumb, 2018).

A more recent pharmacological method uses high concentration lidocaine immersion (Collymore *et al.*, 2016). Although lidocaine has similar induction time as compared to MS222, the former has a much shorter onset of action with a seemingly higher reliability (Collymore *et al.*, 2016). Conversely, and similar to lidocaine (Collymore *et al.*, 2016), one great technical advantage of using a drug such as propofol is that it does not require special equipment or handling. While ice bath is an efficient method for zebrafish euthanasia, one must ensure ice is readily available and to ensure water temperature is below 5°C. Additionally, ice bath euthanasia may not be appropriate for non-tropical species or fish larger than 4 or 5 cm. Although euthanasia with tricaine is a popular method, potential occupational health concerns (Bernstein *et al.*, 1997) plus the drug label's warning not to inhale tricaine powder necessitates working under a fume hood when working with powder. Moreover, outside of the laboratory setting, operator may not have access to tricaine or to a fume hood. Lastly, tricaine's apparent lack of 100% efficacy as a goldfish euthanasia agent (Balko *et al.*, 2018) was somewhat alarming and may require a reevaluation of tricaine or exploration of other drugs for fish euthanasia.

In summary, we support the continued evaluation of propofol for fish euthanasia purposes. For zebrafish specifically, we recommend immersing in 40 mg/L for at least 20 mins or 120 mg/L for at least 10 mins.

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