SHORT COMMUNCIATION

Comparison of two formulations of alfaxalone for immersion anaesthesia in laboratory zebrafish (*Danio rerio*)

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Abstract

Objective To compare two commercial formulations of alfaxalone for immersion anaesthesia in laboratory zebrafish.

Study design Prospective, blinded, randomized study.

Animals A total of 20 adult *Danio rerio* (Tuebingen strain).

Methods Zebrafish were divided into two groups of 10 (five female, five male) and placed in individual immersion baths containing 10 mg L^{-1} of unpreserved alfaxalone (group 1) or preserved alfaxalone (group 2). Anaesthetists blinded to treatment used a composite score scale (CSS) (range 0–12) to assess fish every 30 seconds until induction of anaesthesia. Anaesthetic induction occurred when equilibrium and response to stimulus were lost. Fish were then placed in a clean water bath and scored every 60 seconds. Recovery from anaesthesia was defined as a CSS of $\leq$ 1. Time variables recorded were anaesthetic induction time (AIT), anaesthetic recovery time (ART) and total procedure time (TPT). Fish were observed for evidence of roupgross external pathology during the procedure. Following anaesthesia, four fish from each group were randomly chosen and euthanized for gill histopathology analysis immediately after recovery criteria were met. Data are presented as mean ± standard deviation. An independent *t* test was used to compare the difference in average anaesthetic time variables between groups ($\alpha = 0.05$).

Results There were no statistical differences between groups in reported variables. TPT, AIT and ART were 10.2 ± 1.2, 1.9 ± 0.9 and 8.3 ± 1.2 minutes for group 1 and 10.8 ± 2.9, 2.4 ± 1.2 and 8.4 ± 2.7 minutes for group 2. No gross external pathology was evident, and no fish died during the experimental period. Histopathology showed normal gill pathology and no difference between the groups.

Conclusions and clinical relevance Immersion anaesthesia using 10 mg L^{-1} of either formulation of alfaxalone resulted in anaesthesia of similar quality and duration.

Keywords alfaxalone, alfaxan, immersion anaesthesia, zebrafish.

Introduction

Researchers annually use millions of zebrafish as transgenic, developmental, regenerative, behavioural, disease and toxicological models (Lidster et al. 2017). Anaesthetic techniques used in zebrafish have largely remained unchanged over the past decade, and the exponential rise in the use of these vertebrates as a research model has not been matched by evolution and development of anaesthesia techniques. Tricaine methanesulphonate (3-aminobenzoic acid ethyl ester methanesulphonate), more commonly known as MS222, is a sulphonated derivative of benzocaine and the most widely used anaesthetic agent for immersion anaesthesia in research laboratories (Lidster et al. 2017). Despite the widespread use of MS222 for anaesthesia in zebrafish, aversive behaviours and physiological effects such as gill bleeding, and dose-dependent ventilatory and cardiac depression, have been documented (Matthews & Varga 2012; Readman et al. 2013; Collymore et al. 2014).

Alfaxalone is a neurosteroid anaesthetic agent that is solubilized in 2-hydroxypropyl-beta-cyclodextrin and currently available in two commercial formulations. Alfaxan (alfaxalone 10 mg mL^{-1}) contains no preservative and has a limited shelf life after broaching the vial. In most countries, the product should be disposed of in less than 1 day. By comparison, Alfaxan Multidose (alfaxalone 10 mg mL^{-1}) is a preserved formulation containing chlorocresol 1 mg mL^{-1},

benzethonium 0.2 mg mL^{-1} and ethanol 150 mg mL^{-1} with a shelf life of 28 days after broaching the vial. Alfaxan is being replaced by Alfaxan Multidose in many counties because the two products are bioequivalent and Alfaxan Multidose has the benefit of a 28 day shelf life after broaching at room temperature. To the authors' knowledge, no studies have investigated the use of the preserved formulation of alfaxalone for immersion anaesthesia in fish. As the manufacturer may eventually discontinue the unpreserved formulation, it was deemed appropriate to perform a bridging study evaluating both formulations. The aim of this study was to compare the two formulations of alfaxalone in zebrafish and determine any differences in anaesthetic safety and quality.

Materials and methods

The zebrafish were bred and housed at the University of Queensland (UQ) Biological Resources zebrafish facility. This study was approved by the UQ Molecular Biosciences Animal Ethics Committee (2018/AE000384). The study was conducted in compliance with Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) 2.0 guidelines.

Data for estimating sample sizes for the two groups of anaesthetic agents were obtained from the studies reported by Collymore et al. (2014) and Martins et al. (2018).

Zebrafish were allocated to two groups of 10 adult Tüebingen (TU) (five female, five male) and placed in individual, single-use, static immersion baths containing 10 mg L^{-1} of either preservative free alfaxalone (group 1) or alfaxalone with preservative (group 2). Water was collected from the main recirculating system and the drug added immediately prior to immersion. Water quality was measured prior to the addition of any drugs. Treatment order was assigned using an online randomization tool (randomization.com, accessed 18 December 2018) with each fish assigned a number between 1 and 20 and subsequently anaesthetized in numerical order. One of two veterinarians, both blinded to treatment, used a composite score to assess each fish.

Data collection

The composite scoring scale (CSS) used in this study (Table 1) was a modified version of the scale described by Bauquier et al. (2013) used to evaluate the effects of alfaxalone in goldfish. Equilibrium, opercular movement, activity and tactile response baseline scores were recorded for each fish prior to anaesthesia immersion and then every 30 seconds until induction of anaesthesia criteria was met. Tactile response was assessed by gently swiping along the lateral surface of fish with a cotton tip as soft stimulus. Hard stimulus was assessed by application of a 2 g force with a von Frey filament (San Diego Instruments Inc., CA, USA) to the tail fin.

Fish were then removed from the anaesthetic bath, weighed and immediately placed in a clean, static, water recovery bath

Table 1 Composite scoring system used to assess immersion anaesthesia in 20 adult zebrafish, *Danio rerio* (Tuebingen strain) using a preservative-free formulation of alfaxalone (10 mg mL^{-1}) and a formulation of alfaxalone (10 mg mL^{-1}) with a preservative containing chlorocresol 1 mg mL^{-1}, benzethonium 0.2 mg mL^{-1} and ethanol 150 mg mL^{-1}

Category	Description	Score*
Equilibrium	Normal	0
	Slightly off vertical	1
	Leans or lateral or dorsal but can return to upright when stimulated	2
	Maintaining lateral or dorsal position	3
Opercular movement	Normal	0
	Reduced	1
	Markedly reduced $\leq$ 60 minutes	2
	Absent for > 30 seconds	3
Activity score	Normal	0
	Slow swimming	1
	Slow (very) but moves off in response to stimulus	2
	Stationary	3
Tactile response†	Normal to soft‡	0
	Reduced to soft	1
	Absent soft/positive to hard§	2
	No response to stimulus	3

*Range of scores 0–12.
†A response was defined as gross purposeful movement. Fin vibrations/tremors and changes in opercular movement were not considered a positive response.
‡Gently swiping along the lateral surface of fish with a cotton tip
§Application of a 2 g force von Frey filament to the tail fin.

and scored every 60 seconds until criteria for recovery from anaesthesia were met. The time between removing a fish from the anaesthetic bath and placing it into a recovery bath was < 20 seconds.

Fish were observed for adverse effects such as excitatory behaviour, increased ventilation, thrashing and jumping during the induction period.

The following anaesthetic time variables (measured in minutes) were recorded and defined as follows:

- Anaesthetic induction time (AIT) = time from when fish were first placed in anaesthetic bath (time zero) until loss of equilibrium (equilibrium = 3) and loss of response to soft stimulus (tactile score $\geq$ 2)
- Total procedure time (TPT) = time from when fish were first placed in anaesthetic bath (time zero) until fish met anaesthesia recovery criteria (CSS of $\leq$ 1) when observed in recovery bath.
- Anaesthetic recovery time (ART) = TPT − AIT.

Fish were assessed for evidence of gross external pathology from the effects of each drug solution during the procedure. Once recovered, the first two and last two male and female fish from each group were euthanized by rapid cooling. This occurred by submersion in an ice water slurry (0−4 °C) until cessation of opercular movements and visible cardiac movements. Fish were then immediately fixed in 10% neutral buffered formalin (Point of Care Diagnostics, NSW, Australia). Following fixation, the ventral gill arch was trimmed by cross-section at the caudal aspect of the head, and a parasagittal section medial to the operculum was made to view the lateral gill arch. A board-certified pathologist blinded to the allocated group inspected tissues for any morphologic tissue or cellular changes with a particular focus on gill pathology. The gill tissue was assessed for the presence of diapedesis of erythrocytes, overt haemorrhage, the presence of surface debris on the gills, and the presence of lamellar fusion and immune cell infiltrates. The severity of lesions was scored on a 5-point semi-quantitative scale: 0, normal; 1, minimal change; 2, mild change; 3, moderate change; 4, marked changes; and 5, severe change.

Statistical analysis

Data were analysed using descriptive statistics and summarized using tables and graphs. Initially, the distributions of the anaesthetic time variables were assessed using box plots for presence of outliers that may have affected the normality of the data. Shapiro Wilk test was performed to test the normality of the data of the two groups. Results were reported as mean ± standard deviation. Independent *t* tests were used to compare the difference in the mean anaesthetic time variables between the two groups. Data management and analysis were performed using the statistical package R Version 3.3.3 (R Core Team 2019; R Foundation for Statistical Computing, Austria). Values of $p < 0.05$ were considered significant.

Results

Anaesthesia via immersion, as defined by the fish meeting the AIT criteria, was achieved in 19 out of 20 zebrafish. A fish in group 2 was removed from the anaesthetic bath owing to cessation of opercular movement before anaesthetic induction criteria were met and went on to recover from anaesthesia uneventfully. No excitatory behaviour or modification of normal swimming behaviour were evident during the experimental period. There was no difference in the anaesthetic time variables between the groups.

The AIT, ART and TPT were 1.9 ± 0.9 and 2.4 ± 1.2 (p = 0.30), 8.3 ± 1.2 and 8.4 ± 2.7 ($p = 0.92$) and 10.2 ± 1.2 and 10.8 ± 2.9 minutes ($p = 0.55$) for groups 1 and 2, respectively. No morbidity or mortality were observed, and no evidence of gross external pathology was noted. Histopathological analysis revealed no difference in morphologic change in the gills between the two groups, with all observed changes considered background (incidental or spontaneous lesions not related to the protocol) or normal morphology.

Water quality from the main circulating system were recorded as: temperature (range 26.6−28.8 °C), pH (range 7.91−7.93), oxidation reduction potential (range 297−299 mV), conductivity (range 942.8−1042 uS cm^{-1}) and dissolved oxygen (range 6.07−6.22 mg L^{-1}).

Discussion

The ideal anaesthetic induction agent should result in rapid immobilization, rapid recovery and have a wide safety margin in the animal or patient. For immersion anaesthesia in fish, optimal times and responses reported in the literature describe an ideal anaesthetic agent resulting in anaesthesia in less than 3 minutes and a rapid recovery within 5 minutes with minimal changes in physiology and behaviour (Martins et al. 2016).

Although direct comparison between published fish studies is difficult, alfaxalone has been investigated for immersion anaesthesia in other species of fish and found to provide rapid and reliable anaesthesia (Bauquier et al. 2013; Minter et al. 2014; Bugman et al. 2016).

In this study, the administration of alfaxalone at 10 mg L^{-1} using both the preservative-free formulation and that containing preservative provided reliable and safe anaesthesia in zebrafish. Additionally, there was no evidence of aversive behaviour or physiological and histological effects (e.g., gill haemorrhage or pathology). Aversive behaviours have been documented in many alternative commonly used immersion induction agents in zebrafish (Readman et al. 2013).

There appears to be significant intra- and interspecies variability in the literature defining different stages and planes of anaesthesia in fish. Alfaxalone provided a slightly faster induction time in zebrafish than MS222 as previously reported in the literature (Collymore et al. 2014; Martins et al. 2018).

There was a longer recovery time in the alfaxalone group in this study, although the recovery definition used in this study appeared to be more comprehensively described than in previous studies. For example, Collymore et al. (2014) defined recovery from anaesthesia as 'able to swim upright for at least 5 secs' and 'recover equilibrium, adopting a ventral position'.

One of the limitations of this study were the criteria used to score anaesthesia. The induction of anaesthesia was described as an equilibrium score of 3 and tactile ≥ 2. Therefore, it is possible that some fish met the criteria for induction of anaesthesia, but the response to a physical stimulus may still have been present. In retrospect, complete loss of sensation would have been preferable, especially if invasive interventions (such as clipping caudal tail fins) were performed. Therefore, the development of a more complete, validated scoring system should be a priority when undertaking further studies.

Further improvement to the study would be the use of repeated measures of all the physicochemical water properties during the entire experimental period. This would include water temperature, pH, oxidation reduction potential, conductivity range, dissolved O_2 and measurement of alfaxalone by validated analytical methods.

Conclusions

This study showed that anaesthesia was achieved with both formulations of alfaxalone 10 mg mL^{-1}, and no significant differences were found when the preserved and unpreserved formulation were compared. Further studies comparing alfaxalone with currently utilized immersion anaesthetic drugs (e.g., MS222 and isoeugenol) are warranted. This would contribute to an improvement in the welfare and refinement of anaesthetic procedures in zebrafish which are a valuable research animal.

Authors' contributions

TF: study design, data management and interpretation, preparation and revision of manuscript. WG, HK, CL, KP: study design, data management and interpretation, revision of manuscript. SW: statistical analysis, revision of manuscript. RA: pathological analysis and revision of manuscript.

Conflict of interest statement

KP is an employee of Jurox Pty Ltd. The other authors declare no conflict of interest.

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