A Comparison of Buffered Tricaine Methanesulfonate (MS-222) and Isoeugenol Anesthesia for Caudal Fin Clipping in Zebrafish (*Danio rerio*)

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The purpose of this study was to compare the safety and efficacy of buffered tricaine methanesulfonate (MS-222) and isoeugenol for the anesthesia of zebrafish undergoing caudal fin clipping. Eighty 9 mo *Danio rerio* (AB strain) zebrafish were allocated to one of 2 equal groups: buffered MS-222 (168 mg/L, $n = 40$) or isoeugenol (20 mg/L, $n = 40$). The time to induction of anesthesia was significantly shorter in the isoeugenol group (141 ± 70 s) than in the MS-222 group (207 ± 103 s). The time to recovery from anesthesia was also shorter in the MS-222 group (373 ± 125 s) than in the isoeugenol group (491 ± 176 s). No obvious displays of distress or aversion to anesthesia were observed in either group. No difference was detected in the proportion of zebrafish that became anesthetized with either drug. One male zebrafish in the buffered MS-222 group was found dead at the 1-h post-procedural monitoring time point, but there was no difference between groups in the proportion of fish that survived anesthesia to the end of experiment. In conclusion, the safety and efficacy of buffered MS-222 (168 mg/L) and isoeugenol (20 mg/L) was similar for zebrafish undergoing anesthesia for caudal fin clipping.

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General anesthesia is defined as the controlled and reversible intoxication of the central nervous system where noxious stimulation does not cause arousal.¹² This definition infers that drugs used for anesthesia must be both efficacious and safe, so animals do not respond to procedures that are likely to cause pain, but can then regain consciousness, without any short- or long-lasting side effects. Finding a drug that meets these criteria is difficult, particularly in zebrafish, as there are few drugs to choose from, few methods of administration, and limited capacity to monitor animals during anesthesia. Nevertheless, anesthesia mitigates pain and suffering and maybe beneficial during the performance of invasive technical procedures, such as caudal fin clipping of zebrafish.

The most commonly used immersion anesthetic agent in fish is tricaine methanesulfonate, also known as MS-222.⁶ This drug is considered safe for zebrafish, despite the occurrence of adverse side effects such as aversion, epidermal and corneal lesions, hypoxemia, decreased heart rate, and death.⁶ MS-222 is classified as a local anesthetic, which functions via blockage of sodium channels and therefore membrane action potentials. However, its mechanism of action as a general anesthetic is not fully understood.⁶ Despite its popularity for zebrafish anesthesia, further refinement of its use is required as aversion to this anesthetic is reported,⁸ and the solution of the drug requires buffering to avoid exposing fish to a potentially toxic acidic environment.¹ Furthermore, MS-222 may be hazardous to personnel, as it is retinotoxic and irritating to mucous membranes like the upper respiratory tract.¹

Isoeugenol is a constituent of clove oil and is also used as an immersion anesthetic for fish. Isoeugenol is soluble in water and is approved in some countries for anesthesia of fish for human consumption, with no withdrawal period.⁷ The mechanism of action of isoeugenol is unknown, but may be similar to that of other local anesthetics such as MS-222.¹¹ As a compound structurally similar to eugenol, isoeugenol may also inhibit sodium, potassium and calcium channels, inhibit NMDA receptors and potentiate GABA_A receptors in the same way as eugenol.¹³ The side effects of isoeugenol have not been extensively reported, although more evidence is available for eugenol (also a constituent of clove oil).⁷ Eugenol may have a wider safety margin and be associated with a longer induction time and a slower recovery than does MS-222.^{3,9}

The ideal anesthetic for fish should be easy to administer, effective at a low dose, able to induce anesthesia in less than 3 min with minimal stress, provide immobilization and effective analgesia throughout the procedure, allow recovery from anesthesia within 5 min, and induce no or minimal changes in physiology and behavior.⁸ In addition, the ideal anesthetic should be affordable, readily available, practical to use and safe for personnel.⁸

This study aimed to compare the safety and efficacy of single doses of buffered MS-222 and isoeugenol for zebrafish undergoing anesthesia for caudal fin clipping. The hypotheses were that induction of anesthesia and recovery from anesthesia would be longer with isoeugenol than with MS-222, that the safety of the 2 drugs would be comparable, as assessed by morbidity and mortality, and that preparation of isoeugenol solutions would be simpler than the preparation of buffered MS-222 solutions.

Materials and Methods

The study was approved by the Animal Ethics Committee of the University of Western Australia in accordance with the

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Code of Practice for the Care and Use of Animals for Scientific Purposes.¹⁰

The zebrafish were housed in the Western Australia Zebrafish Experimental Research Centre, an AAALAC accredited facility. The tanks were 3.5 L with a maximum housing density of 8 fish per L as a recirculating system (ZEBTEC Centralized Aquatic System, Tecniplast, Milan), with a water exchange rate (system volume) of 10% per 24 h and water exchange within tanks 9 times per hour. The municipal water supply was processed through a reverse osmosis system and activated carbon (100 microns), rotating drum (42 microns), biologic (fluidized bed with biochips) and UV (200 000 uWs/cm²) filtration. Rooms were maintained at approximately 26 °C with a 14:10 h light:dark cycle. The Tecniplast system automatically doses for conductivity, 800 µS ± 100 µS (salts— Aquarium Systems Instant Ocean Aquarium Salt, France), pH 7.4 ± 0.6 (sodium bicarbonate) and heating elements for the water temperature, with a set point 28.5 °C ± 1.5 °C. System water is tested weekly using a water testing kit (API Liquid Test Kits, API) for general hardness (GH, 71.6 mg/L - 89.5 mg/L), carbonate hardness (KH, 17.9 mg/L - 35.8 mg/L), ammonia (<0.05 mg/L), nitrates (<100 mg/L) and nitrites (target 0 mg/L) and a colorimeter (DR 900, HACH) for pH, ammonia (<0.05 mg/L), nitrates (<100 mg/L) and nitrites (target 0 mg/L) and oxygen (>6 mg/L).

The health testing regime for the zebrafish colony involved testing of whole fish, housed both pre- and postfiltration, twice a year. *Edwardsiella ictaluri*, *Flavobacterium columnare*, *Ichthyophthirius multifiliis*, Infectious spleen and kidney necrosis virus (ISKNV), *Mycobacterium abscessus*, *Mycobacterium chelonae*, *Mycobacterium fortuitum*, *Mycobacterium haemophilum*, *Mycobacterium marinum*, *Mycobacterium peregrinum*, *Mycobacterium* spp., *Myxidium streisingeri*, *Piscinoodinium pillulare*, *Pleistophora hyphessobryconis*, *Pseudocapillaria tomentosa* and *Pseudoloma neurophilia* were not detected by PCR in fish samples. *Mycobacterium abscessus*, *Mycobacterium chelonae*, *Mycobacterium fortuitum*, *Mycobacterium haemophilum*, *Mycobacterium marinum*, *Mycobacterium peregrinum*, *Mycobacterium* spp., *Myxidium streisingeri*, *Pseudocapillaria tomentosa* and *Pseudoloma neurophilia* were not found by PCR in mulm (tank detritus) or rotifer samples. The general health of the zebrafish was checked twice daily Monday to Friday and once daily on the weekends. These checks included visual observation of each tank for cleanliness, water flow, and normal fish behavior and general appearance. The fish were manually fed twice daily with a commercial pellet (2 clicks of 70 mg each morning and one click of 70 mg in the afternoon, NRD 300 to 500 µm, NRD, Thailand) and daily with *Brachionus plicatilis* (L-type) enriched roti grow plus (one squirt [5 to 10 mL] from a bottle of 200 to 400 rotifers/mL).

Eighty *Danio rerio* (AB strain) zebrafish (embryos sourced from Zebrafish International Resource Centre, University of Oregon) at 9 mo of age were allocated to one of 2 equal groups: buffered MS-222 ($n = 40$); or isoeugenol ($n = 40$). Within each group, there were equal numbers of male and female fish.

Buffered MS-222 was prepared in a biologic safety cabinet by an operator wearing gloves, gown, and a respirator. The concentration of buffered MS-222 was 168 mg/L, from a stock solution of 8.4 g/L. The stock solution was created by dissolving 1.68 g of MS-222 (Ethyl 3-aminobenzoate methanesulfonate, Sigma-Aldrich, China) in 200 mL of reverse osmosis water. Aliquots of this stock solution were wrapped in foil and frozen prior to use. The anesthetic bath was made by adding 10 mL of thawed stock solution to 490 mL of tank water and gently dispersing the drug through the solution. The solution was then

buffered using drops of 1 M sodium bicarbonate until the pH of the anesthetic bath was around 7.0 to 7.5.

Isoeugenol (Aqui-S, Lower Hutt, New Zealand) was prepared in accordance with the manufacturer's instructions to a concentration of 20 mg/L by adding 0.01 mL of Aqui-S to 500 mL of tank water and gently dispersing it into the bath. The pH of this bath was also measured and recorded.

The order of anesthetics was determined by alternating male and female with a randomized allocation of drugs. The anesthetic baths were prepared in a room adjacent to the procedural space, and the personnel performing procedures and observations were blinded to the drug being used. The respective anesthetic baths were replaced every 20 anesthetic exposures to ensure that the efficacy of the anesthetic was not affected by ongoing use. Anesthesia was performed in the morning, between 0800 and 1100, with either 20 or 30 fish anesthetized each day for 3 d.

The fish were prepared for anesthesia in their home tank, with only male or female fish in any given tank. One fish was selected at a time and placed in the anesthetic bath (500 mL). The fish were observed until stage 5 anesthesia was apparent, as previously described,⁸ and the time taken to reach this threshold was recorded by a single investigator. Stage 5 anesthesia is associated with complete loss of equilibrium and muscle tone, no reaction to stimuli (gentle touching with a plastic spoon), and a decreased respiratory rate (opercular movement). Signs of aversion to the anesthetic were considered to include erratic breathing, twitching, thrashing, or gasping. The fish was then gently removed from the water with a slotted plastic spoon, placed on a paper towel and dried carefully. Clipping of the caudal fin was performed in a culture dish with a scalpel blade by a second investigator. The time out of the water was recorded and was limited to 30 seconds. The fish was then placed in an aerated drug-free recovery tank and observed by a third investigator until it was swimming normally. Up to 2 fish could be placed in a subsection of the recovery tank (one male and one female), which was separated into 2 areas with a fenestrated tank divider.

Postprocedural monitoring was performed 1, 2, 3, 4, and 24 h after the procedure. These observations included attributing a score of 0, 1 or 2 (0 = normal, 1 = slightly or intermittently abnormal, 2 = moderately or consistently abnormal) to 8 parameters: physical appearance, food consumption, respiratory pattern, swimming behavior, activity, social behavior, wound site and any other presenting signs. The observations were performed by watching the fish within the tank for one minute. The morning after the procedure, the fish were euthanized in an ice slurry and weighed. The use of an ice slurry is a standard operating procedure in the facility and is used as a method of euthanasia as it is simple, reliable, and predictable. The anesthetic bathwater was disposed of through the facility drainage after dilution with mains water.

Data are expressed as mean (± SD) and where relevant were compared with a *t* test (GraphPad Prism, Prism 7 for MAC OS X). Tests for the difference in proportions (efficacy of anesthesia and morbidity) were performed by a proportion test, a χ^2 test and a Fisher test (R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>). A *P* value < 0.05 was considered significant.

Results

At the end of the study, the fish were euthanized and their sex confirmed. Despite the goal of including equal numbers of

male and female fish in each group, 18 males and 22 females were exposed to buffered MS-222, and 21 males and 19 females to isoeugenol. Female fish were heavier than males (534 ± 91 mg and 405 ± 50 mg respectively; $P < 0.0001$). The pH of the tank water was 7.72 ± 0.07 , and the pH of the buffered MS-222 and isoeugenol baths were 7.66 ± 0.09 and 7.74 ± 0.07 respectively ($P = 0.977$).

The time to reach stage 5 anesthesia was shorter ($P = 0.0015$) in the isoeugenol group (141 ± 70 s) than in the MS-222 group (207 ± 103 s) (Figure 1). The time to recovery from anesthesia was shorter ($P = 0.001$) in the MS-222 group (373 ± 125 s) than in the isoeugenol group (491 ± 176 s) (Figure 2). Male and female fish did not differ with regard to time to reach stage 5 anesthesia or time for recovery (Table 1). No apparent displays of distress or aversion to anesthesia and no movement or response to the procedure was observed for either group.

Two female zebrafish in the buffered MS-222 group did not achieve stage 5 anesthesia within 10 min, so the procedure was abandoned for these animals and they were removed from the experimental group. There was no difference in the number of zebrafish that reached stage 5 anesthesia with either drug ($P = 0.474$). One male zebrafish in the buffered MS-222 group was found dead at the 1-h post-procedural monitoring time point, but there was no difference between groups in the proportion of fish that survived anesthesia to the end of experiment ($P = 1$). The post mortem examination of the male fish that died was inconclusive. Otherwise, postprocedural monitoring scores were 0 for 79 zebrafish.

Discussion

The goal of this study was to compare the safety and efficacy of single doses of buffered MS-222 and isoeugenol for zebrafish undergoing anesthesia for caudal fin clipping. The results of the study suggest that although induction of anesthesia was faster with isoeugenol, and recovery from anesthesia was slightly longer, the safety of isoeugenol may be superior to buffered MS-222 (though efficacy and mortality were not significantly different). In addition, drug preparation was simpler for the isoeugenol solution than for buffered MS-222. Based on the criteria of an ideal anesthetic for fish, neither buffered MS-222 nor isoeugenol fulfilled all requirements.⁸

Both buffered MS-222 and isoeugenol were easy to administer by immersion and effective at the doses chosen for this study. The doses were selected from the literature and the manufacturer's directions, respectively. Two fish in the buffered MS-222 group failed to reach the required plane of anesthesia for the procedure, but this reduction in the MS-222 cohort was not significant. For the 78 zebrafish that were anesthetized, the conditions during anesthesia and the procedure seemed suitable, but no physiologic monitoring was performed in this study, other than observation of opercular movement. When anesthetized with isoeugenol, the time to onset of stage 5 anesthesia was less than the ideal 3 min⁸ and recovery took longer than the expected 5 min for both groups. Finally, the preparation of isoeugenol was considerably easier than for buffered MS-222. Based on proposed criteria,⁸ isoeugenol may be closer to the ideal anesthetic than buffered MS-222. However, further work is needed to optimize the dose of this drug for other laboratory procedures and for different ages of zebrafish. A recent study comparing the safety and efficacy of various anesthetic agents and concentrations in adult zebrafish, including buffered MS-222 and isoeugenol, concluded that buffered MS-222 was the most reliable anesthetic for zebrafish, as it produced a quick induction of deep anesthesia and a quick recovery with

Figure 1. Time (s) to stage 5 anesthesia in zebrafish anesthetized with MS-222 (black) or isoeugenol (gray). ** $P < 0.05$.

Figure 2. Time (s) to recovery from anesthesia of zebrafish anesthetized with MS-222 (black) or isoeugenol (gray). ** $P < 0.05$.

high survival rates.³ Others used buffered MS-222 at a slightly higher concentration than we used in this current study (200 mg/L compared with 168 mg/L).³ However, the lowest dose of isoeugenol used was 40 mg/L,³ which is higher than the 20 mg/L used in the current study. The higher dose of isoeugenol was associated with poor survival and variable recovery from anesthesia, and was therefore considered unsuitable for anesthesia of adult zebrafish.³ Our study suggests that the dose of isoeugenol we used warrants further investigation and refinement, especially as our findings appear to challenge the earlier results suggesting that isoeugenol is not practical for use in adult zebrafish at higher doses.³

Some features of this study must be considered when interpreting the results. A single observer made all observations. Although the observer was technically blind to the treatment group, the odor of isoeugenol was apparent, so the observer was not truly blind to treatment. No physiologic monitoring was performed during anesthesia (for example, monitoring heart rate or respiratory rate) so anesthetic morbidity could only be assessed by postprocedural behavioral observations or mortality. Some other studies of anesthesia in zebrafish have investigated smaller treatment groups ($n = 10$ per group,² $n = 5$ per group,⁵ $n = 8$ per group⁶), so our treatment groups were relatively large. This study investigated only single doses of drugs, and further investigations are necessary to optimize dosages and to refine the physiologic monitoring of fish during anesthesia. Single doses of each anesthetic were used in this study to keep the design simple and to compare the safety

Table 1. Time to stage 5 anesthesia and time to recovery from anesthesia (seconds) for zebrafish anesthetized with MS-222 or isoeugenol.

	Male	Female	P value
Time to anesthesia (s) buffered MS-222	190 (±109)	222 (±97)	0.345
Time to anesthesia (s) isoeugenol	128 (±49)	155 (±87)	0.562
Time to recovery (s) buffered MS-222	360 (±142)	384 (±110)	0.235
Time to recovery (s) isoeugenol	470 (±135)	515 (±215)	0.417

and efficacy of buffered MS-222 and isoeugenol specifically for adult zebrafish undergoing caudal fin clipping. Further work investigating the optimal doses of these drugs will be important to continue refining anesthetic practices for zebrafish. The context of such studies is important, and comparison of this current work to previous studies is limited, as few studies focus on adult zebrafish undergoing such a short procedure. However, the results of this study are somewhat comparable to previous work with buffered MS-222 in 1-mo old zebrafish in which the time to induction of anesthesia was similar with a dose of MS-222 of 160 mg/L.⁴ Similarly, recovery from anesthesia with buffered MS-222 was comparable between these 2 studies.⁴

The concept of balanced anesthesia in which anesthetics and analgesia are combined to achieve safe and effective anesthesia can be applied to zebrafish. The dose of individual drugs can often be lower when they are used in combination with other drugs, and therefore the risk of adverse side effects is lowered. One group investigated the combination of MS-222 and isoflurane and concluded that this combination of drugs holds promise for use in zebrafish, especially for prolonged anesthesia.⁵ When used in combination with isoflurane, the effective dose of MS-222 was less than half the dose used in our current study.⁵ Another group also investigated various anesthetic drug combinations for zebrafish using MS-222 as the benchmark anesthetic.⁶ The dose of MS-222 used in the current study was almost 1.7 times the dose used in that study, which indicates the range of doses that may be appropriate for zebrafish anesthesia.⁷ Further refinement to the use of MS-222 is required along with further exploration into alternative drugs, which may be safer for both fish and personnel.

In both the current study, and in a previous investigation, isoeugenol was associated with a longer recovery period than buffered MS-222.³ This feature of isoeugenol anesthesia suggests that the elimination half-life of the drug is relatively long as compared with MS-222. If anesthesia is only needed for short procedures, the recovery period may be acceptable, but if anesthesia is required for longer procedures, the pharmacokinetic profile of isoeugenol may not lend itself to readily titratable anesthesia, and some accumulation of the drug may occur over time. Drug administration by bath immersion is equivalent to inhaled anesthesia in mammals. The processes of absorption and elimination are determined by the diffusion rate through the gill epithelium and depend on lipid solubility, ionization, and polarity of the drug.¹³ Isoeugenol is known to transfer rapidly from plasma to tissues and then to be slowly eliminated from the plasma.¹³ These features support the clinical observations of a faster induction and slower recovery with isoeugenol as compared with buffered MS-222, which is rapidly cleared and eliminated.¹³ Species-specific pharmacokinetic profiling of isoeugenol may be warranted to complement ongoing studies using this compound for long anesthetic procedures in zebrafish. The criteria for the ideal anesthetic for fish are likely to be influenced by the pharmacokinetics and pharmacodynamics of the drug, and for isoeugenol, these profiles may compromise its capacity to be considered ideal.

In the current study, the anesthetic baths were replaced after every 20 exposures. This approach was somewhat arbitrary, as detail on the duration of action or efficacy of baths is sparse. Neither the manufacturers nor the published literature clearly state how frequently the baths should be replaced or refreshed. Potentially baths could be used for much longer than they were in the current study, but a better understanding of the rate of uptake of the anesthetic by fish, and therefore the rate of decrease of drug concentration in the bath is necessary to define this parameter.

This simple study aimed to address a practical dilemma to support the refinement of zebrafish anesthesia. The use of buffered MS-222 involves multiple steps in preparation and various safety risks for personnel. To optimize conditions for predictable and reliable anesthesia, the preparation of anesthetic drugs should be failsafe and straightforward. Isoeugenol offers those attributes. Finally, in conclusion, the safety and efficacy of buffered MS-222 and isoeugenol for adult zebrafish undergoing anesthesia for caudal fin clipping was similar, suggesting that while the pharmacokinetics of isoeugenol and MS-222 differ, the two compounds are not significantly different in efficacy at this dosage.

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