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Comparison of Tricaine Methanesulfonate (MS-222) and Alfaxalone Anesthesia in Zebrafish (*Danio rerio*)

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The research use of zebrafish has risen exponentially over the past decade while anesthetic options have remained largely unchanged.[6] Tricaine methanesulfonate (MS-222) is widely accepted as an anesthetic for routine husbandry procedures, however it has limitations and safety concerns.[5,11] A greater variety of effective anesthetic options for surgical procedures would be advantageous for the research community. Adult zebrafish were randomly assigned to one of the following groups (*n* = 10, 5 males and 5 females): 200 mg/L MS-222; 6-, 10-, 13-, and 16-mg/L alfaxalone, and control. All zebrafish in the MS-222 group reached a surgical plane of anesthesia within 95 ± 32 s. By contrast, only 2 of 10, 1 of 10, 0 of 10, and 0 of 4 of the 6, 10, 13, and 16 mg/L alfaxalone groups, respectively, reached a surgical plane of anesthesia within the allotted 10-min period. Recovery time was also significantly slower in the alfaxalone groups as compared with MS-222, with some fish taking greater than 10 min to recover. In addition, 33 of 34 zebrafish (the 16 mg/L group was not completed due to safety concerns) in the alfaxalone groups lost opercular movements for greater than one minute during their anesthetic event and had to be removed to the recovery tank. The results demonstrated that alfaxalone was unable to provide a reliable and safe surgical plane of anesthesia at any of the drug doses tested. Therefore, we recommend alfaxalone not be used as an anesthetic for painful procedures on zebrafish and conclude that MS-222 remains a more viable anesthetic for immersion anesthesia in zebrafish.

Abbreviation and Acronym: MS-222, Tricaine methanesulfonate

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Introduction

Zebrafish are popular animals for biomedical research studies due to their high fucundity, small footprint and size, high throughput screening ability, availability of numerous transgenic strains, and diverse utility.[3] They are also often a much more affordable laboratory animal model than traditional warm-blooded species.[3] Perhaps most importantly, zebrafish embryos are transparent, easy to manipulate genetically, and can survive without active circulation at early stages of development.[19] These traits have helped drive this model's utility and acceptance as evidenced by its common use in translational biomedical research, toxicology, molecular genetics, neurophysiology, and drug discovery.[6,11,13,18,19]

The discovery and use of anesthetics that provide safe, reliable, and increasingly longer-duration anesthesia is critical to the continued expansion of the use of zebrafish in research and the development of novel procedures. The ideal surgical anesthetic for zebrafish should provide a reliable, rapid, and consistent surgical plane of anesthesia. It should be technically practical, provide sufficient time for invasive procedures, and immobilize the fish with little to no stress or negative physiologic alteration. The recovery period should be expedient and smooth. In addition, the anesthetic should be readily available, affordable, and safe for the user.[10,13]

Tricaine methanesulfonate (MS-222) is the most commonly used and only licensed anesthetic for finfish in the United States.[6,7,13,16] MS-222's is a sodium channel blocker.[1,13] The blockade of voltage-dependent Na^+ conductance of cellular elements comprising the neuromuscular system impedes action potentials subsequently blocking the sensation of pain and paralyzing the animal by muscle relaxation to exert a general anesthesia.[1,13,14] MS-222 is typically used for immersion anesthesia and has proven reliable for procedures such as fin clipping, transportation, gamete collection, and blood sampling.[4,8,18]

Overall, MS-222 has a long record of safe use but requires special handling due to the acidic nature of the compound. When mixed with water, MS-222 can have a pH as low as 2.8, therefore requiring a buffer (for example, sodium bicarbonate).[10,15] Incorrect handling of MS-222 may result in reported side effects, to include respiratory acidosis, cardiac depression, cardiac failure, gill bleeding, and death.[5,8,11] In zebrafish and other finfish, it can cause increases in blood glucose, plasma cortisol, lactate, and alterations in other blood chemistries.[5,13] Furthermore, it is not licensed for use in Spain, Greece, or France and it is restricted in several other countries.[17] MS-222 remains a viable option for achieving a surgical plane of anesthesia, however, due to side its side effects and the difficulties in handling the drug, other anesthetics are being investigated.

Other anesthetic approaches that are currently available for aquatic species include gradual cooling, isoflurane, isoeugenol,

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and metomidate hydrochloride. These have been identified as efficacious for sedation for *nonpainful* procedures.[5] As zebrafish research continues to expand, anesthetic protocols are increasingly important for management of painful procedures and for providing a variety of anesthetics to suit all varieties of research protocols. Alfaxalone is a neuro-active steroid that binds to γ aminobutyric acid-A receptors on the neuronal cell surface to produce sedation and anesthesia. Gamma aminobutyric acid-A is the primary inhibitory neurotransmitter in the brain and the major inhibitory neurotransmitter in the central nervous system. It causes the intrinsic ion channel to open which allows the influx of chloride ions that oppose depolarization and subsequently inhibit neuronal firing.[21] Alfaxalone is used in a variety of species through various routes of exposure (for example, injectables for koi and immersion for oscar and goldfish).[1,2,9] Recently, alfaxalone has been studied and shown to be efficacious in several fish species including goldfish, rainbow trout, black spot barbs, and peacock cichlids.[9,15,20] Side effects can include respiratory depression, prolonged anesthesia, and severe adverse effects such as death reported in koi and peacock cichlids.[1,2,9,20] A recent study compared 2 formulations of alfaxalone in zebrafish and found no difference between the preservative (Alfaxan Multidose) and nonpreservative form (Alfaxan).[6] Both formulations at 10 mg/L provided a surgical plane of anesthesia determined via utilization of a cotton-tip applicator swipe of the lateral surface and a von Frey filament to assess tactile responses.[6]

The purpose of our study was to optimize the dose of alfaxalone to obtain a surgical plane of anesthesia in zebrafish and to compare its anesthetic parameters, duration of anesthesia, and recovery with MS-222. We hypothesized that alfaxalone could provide a smoother induction and faster recovery for zebrafish than MS-222, with the added benefits of ease of preparation of the compound and an extended duration of the surgical plane of anesthesia.

Materials and Methods

Humane care and use of animals. Research was conducted under an approved Institutional Animal Care and Use Committee protocol. The facility is accredited by AAALAC International, has a Public Health Service Animal Welfare Assurance, and complies with the Animal Welfare Act Regulations and other federal statutes relating to research animals.

Animals and housing. Adult 19-mo-old Tűbingen *Danio rerio*, zebrafish, (n = 56) were produced inhouse and housed in custom built, flow through aquaculture racks (flow rate 2 ± 0.4 L/min) with the water temperature constantly maintained at 26.4 ± 1.2 °C. Zebrafish were housed at a stocking density of 4 fish per liter. Overhead, full-spectrum LED lighting provided illumination on a 14-h light, 10-h dark photoperiod. Water quality parameters were maintained within the following conditions: dissolved oxygen 60 to 100% saturation; pH 7.3 ± 0.3, alkalinity 110 to 180 mg/L as $CaCO_3$, hardness 150 to 210 mg/L as $CaCO_3$, conductivity 585 ± 10 µS/cm, and total ammonia less than 0.1 mg/L as NH_3. Adult zebrafish had 3 daily feedings on weekdays: 2 feedings of Gemma Micro 300 (Skretting Zebrafish, Westbrook, ME) and 1 feeding of live brine shrimp nauplii (Brine Shrimp Direct, Ogden, UT). On weekends, only 2 feedings were provided: 1 Gemma Micro 300 and one live brine shrimp nauplii. Feeding live brine shrimp nauplii provided both nutrition and environmental enrichment. Artificial plants were also included in every tank as an additional environmental enrichment.

Experimental design. Zebrafish were randomly assigned to one of 5 groups (n = 10, 5 males and 5 females) and one control group (n = 6, 3 males and 3 females). All tanks (including the anesthetic, recovery, and control group) were filled with fresh fish culture water that was obtained from the adult zebrafish recirculating system. Anesthetic and recovery tank water was changed between each anesthetic group (every 10 fish). Tricaine methanesulfonate (MS-222) and sodium bicarbonate were obtained from Sigma (St. Louis, MO) and alfaxalone (Alfaxan Multidose; 10 mg/mL) was obtained from Jurox (North Kansas City, MO). MS-222 was prepared at 200 mg/L in one liter of fresh fish culture water in a static 2-liter tank and stirred to mix. This dose was based on clinical experience and previous studies using the same or similar concentrations.[5,13] The pH was tested in all anesthetic tanks with an Orion Dual Star pH/ISE Benchtop meter (Thermo Scientific, Waltham, MA). The alfaxalone pH was 7.3 ± 0.2 and the pH in the MS-222 tanks was adjusted as needed with sodium bicarbonate to a final pH of 7.3 ± 0.3. Alfaxalone was prepared at 6, 10, 13, and 16 mg/L by adding the appropriate amount of the 10 mg/mL stock to one liter of fresh fish culture water and mixing. These concentrations were chosen based on previous studies and 1/2 log difference in either direction.[2,6,9,15] Zebrafish were fasted for 24 h prior to anesthesia.

Induction of surgical plane. The exposure tank was placed in a designated area that was taped off with an opaque white floor and background. A continuous video recording with a digital camera (GoPro Model: SPTM1) was used in addition to a stopwatch to accurately record times and reactions throughout each anesthetic event (Figure 1). Each zebrafish was monitored individually in a static tank for loss of righting reflex, startle reflex, and tactile response. Righting reflex was viewed as lost when zebrafish were no longer able to maintain buoyancy and were tilted greater than 90 degrees. Startle reflex was measured by a soft tap to the tank with a knuckle by an experienced technician. Tactile response was measured by lightly pinching the caudal fin with dissecting forceps (SS Biology Tweezers, Anti-ACID, item # 504748 by World Precision Instruments). Tactile response was ideally measured subsequent to the loss of the startle response because the tail pinch was the more noxious stimulus of the 2. However, even if a fish failed to lose the startle response, tactile response was nonetheless measured for completeness. The zebrafish remained in the anesthetic bath until startle and tactile responses were both absent or until opercular movements had ceased for more than one minute up to the allotted 10-min procedure period. If opercular movement was absent, startle reflex and tactile response were measured throughout the 1-min period. Anesthetic depth was measured by the same person throughout the study to minimize inconsistency of measured parameters; however, the observer was not blind to the anesthetic groups. The control group was treated and tested in the same way as other groups, but no anesthetic was added to the water. The control group was compared with only the groups in recovery to provide a reference for normal swimming behavior.

Anesthetic recovery. At completion of the anesthetic event, zebrafish were placed in a recovery tank containing fresh fish culture water from the recirculating system and recorded for 10 min, regardless of recovery time. Recovery time was measured as the time from placement of the zebrafish into the recovery tank until return of self-righting reflex and spontaneous swimming. If the zebrafish did not resume normal swim behavior within 10 min, it was placed in a temporary tank until normal behavior could be established and then it was returned to its home tank. The recovery tank was fitted to obtain a video of normally shoaling zebrafish on one side of the recovery tank; a digital video camera (Basler cA1920-155um USB2) mounted in

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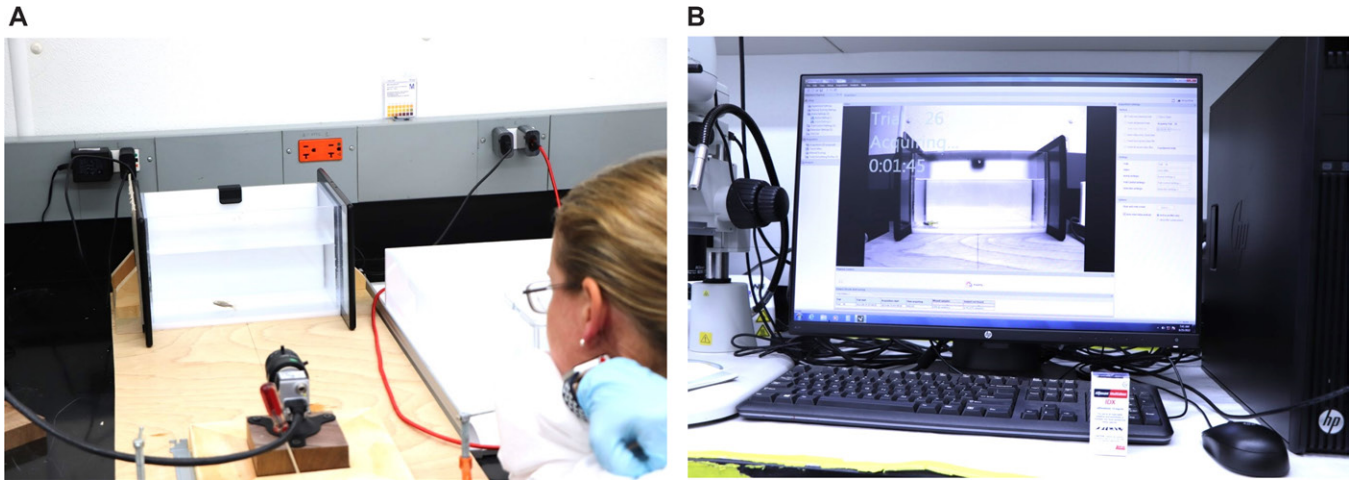

Figure 1. Experimental set-up. (A) Overview showing recovery tank on the left and anesthesia tank on the right. The pictured fish has lost his righting reflex and is anesthetized. (B) Image capture computer for recovery tank running Ethovision XT (Noldus) software. The fish is in the recovery tank.

front of the tank to record recovery. Normal shoaling behavior was measured based on shoaling zebrafish near the video screen; however, because we could not establish a normal baseline for this parameter, it was not used in the final analysis.

The zebrafish were monitored for 24 h after recovery from anesthesia, per Stoskopf's recommendation, to ensure that they survived and retained normal behavior.[16] They were then euthanized by immersion in MS-222 at 500 mg/L for a minimum of 30 min in accordance with *AVMA Guidelines for the Euthanasia of Animals: 2020 Edition*.[16] After euthanasia, the zebrafish were patted dry with a paper towel to ensure that minimal excess water weight was transferred into the weigh boat, and the fish were then weighed (Mettler Toledo analytical chemistry scale Model# XP204). Weights were similar across groups, but females weighed more than males. Statistical analysis was performed between sexes, but no statistical difference was detected. Data for males and females were therefore combined for further analysis.

Statistical analysis. Treatment group size was determined by a statistical power analysis. The primary outcome of interest was the main effect of alfaxalone treatment groups to MS-222 for the parameters explained above. The power analysis indicated that the smallest effect size for comparing alfaxalone and MS-222 at a Cohen $d = 3.4$ required sample sizes of 8 and 10, respectively. The power to find a difference between alfaxalone and MS-222 is over 99% (2-tailed test with Type I error controlled at 5%).

The video recordings were analyzed using Ethovision XT (Noldus) software that calculated swimming speed, distance moved and tank positional data during recovery, and time spent in defined zones to assess return to normal swimming behavior. The association between the binary outcomes of reaching the surgical plane, loss of opercular movement, and treatment group (excluding the control group) were evaluated using a Fisher Exact test. Each treatment group was compared individually to our standard anesthetic, MS-222. The time until regaining a righting reflex was analyzed in seconds using a 2-way ANOVA. The time for occurrence of the righting reflex was considered to be 0 s, indicating instantaneous righting. Mean velocity and mean distance moved were analyzed using a one-way ANOVA test. A 2-way ANOVA test was used to evaluate mean cumulative duration spent in either the top or bottom zones of the tank across the treatment groups (including the control group). Secondary outcomes of velocity and distance moved were analyzed using a one-way ANOVA. Each treatment group was compared pairwise with the control group. The cumulative times that fish spent in the top or bottom zones was assessed for any association between the treatment groups. *P* values were adjusted for multiple comparisons using the Sidak method. All tests were conducted in GraphPad Prism version 9 for Windows. Values of $P \leq 0.05$ were considered significant. Descriptive data are presented as mean and standard deviation.

Results

Surgical plane of anesthesia. Surgical plane of anesthesia (stage III, plane 2) parameters have been described previously to include loss of equilibrium, loss of reactivity, and shallow opercular movement.[4] The surgical plane of anesthesia in the current study was based on these parameters and was identified as loss of righting reflex, startle reflex, and tactile responses. All zebrafish in the MS-222 group reached a surgical plane of anesthesia within 95 ± 32 s, as measured by loss of all 3 parameters. By contrast, only 2 of 10, 1 of 10, and 0 of 10 of the 6, 10, and 13 mg/L alfaxalone groups, respectively, met all 3 of the above criteria within 10 min (Figure 2A). The alfaxalone 16 mg/L group was not completed due to the rapid loss of opercular movement for the first 4 fish of the group, creating concerns regarding recovery. The number of fish that reached a surgical plane of anesthesia for alfaxalone and MS-222 differed significantly ($P \leq 0.001$). None of the MS-222 group lost their opercular movements (< 1 bpm) during the anesthetic event (Figure 2B). However, nearly all zebrafish in the alfaxalone groups showed loss of opercular movements (<1 bpm) at 375 ± 126 s, 288 ± 137 s, and 245 ± 145 s for concentrations of 6, 10, and 13 mg/L alfaxalone, respectively; fish were removed from alfaxalone bath after 1 min of loss of opercular movement to prevent death. The time to loss of opercular movement differed significantly between alfaxalone and MS-222 groups ($P \leq 0.001$). Most of the fish in the alfaxalone groups did not reach a surgical plane of anesthesia as defined by above parameters. Often the fish that lost opercular movement and righting reflex continued to respond to stimuli by darting off across the water, which has been used previously to indicate that a fish is not deeply anesthetized.[4]

Recovery from anesthesia. Recovery from anesthesia was measured as time of resumption of the righting reflex and the return of normal swim behavior, including swim distance, speed, and tank position. The MS-222 group resumed significantly faster than did the 3 alfaxalone groups ($P \leq 0.001$; mean times of 80 ± 56 s, 318 ± 81 s, 394 ± 142 s, and 340 ± 100 s, respectively,

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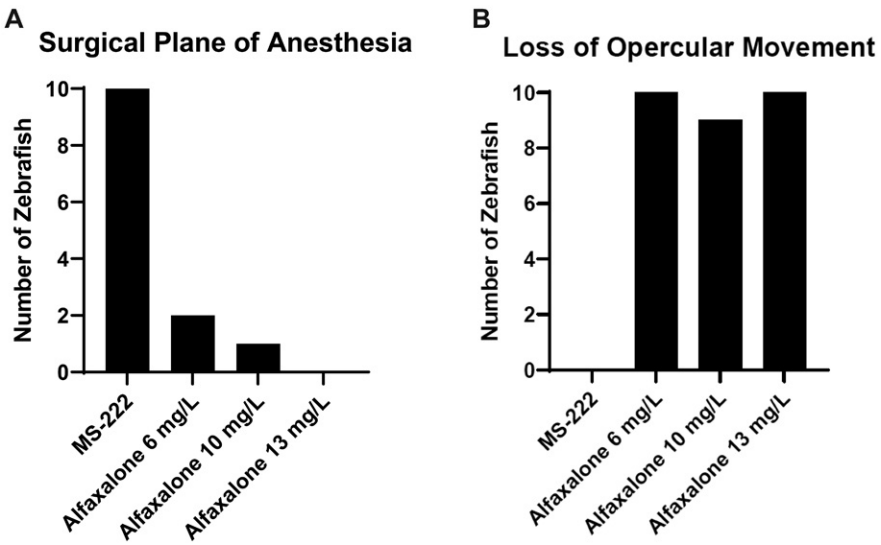

Figure 2. Plane of anesthesia and opercular movement. (A) All MS-222 exposed fish reached a surgical plane of anesthesia, while only 2 of 10, 1 of 10, and 0 of 10 alfaxalone exposed fish (6, 10, and 13 mg/L alfaxalone, respectively) met the criteria. (B) Among alfaxalone-exposed fish, 29 of 30 lost opercular movement (< 1 breaths per minute [bpm]), while none of the MS-222 treated fish exhibited opercular movements at less than 1 bpm.

for MS-222, and 6, 10, and 13 mg/L concentrations of alfaxalone, respectively) (Figure 3). Two zebrafish in the alfaxalone 6 mg/L group and one zebrafish in the 10 mg/L group did not regain a righting reflex within the 10-min observation period. Because their times fell outside the upper limit of observation, their data were not recorded or used in the statistical analysis.

We found no statistical difference between the control group and MS-222 for the mean distance moved or the mean cumulative time spent in the bottom of the tank during recovery (Figure 4A, B). However, all alfaxalone groups and the control group differed significantly for each recovery parameter ($P \leq 0.0001$) (Figure 4A, B). The alfaxalone groups spent significantly more time on the bottom of the recovery tank than did the MS-222 or control groups (Figure 4B). During the recovery period, the mean distance moved was significantly greater for the control and MS-222 groups as compared with the alfaxalone groups. All drug groups were individually compared with the control group for comparison to normal behavior (Figures 4 and 5). MS-222 exposed fish moved around the tank more than the control group, indicating a possible period of hyperactivity after MS-222 exposure (Figure 6).

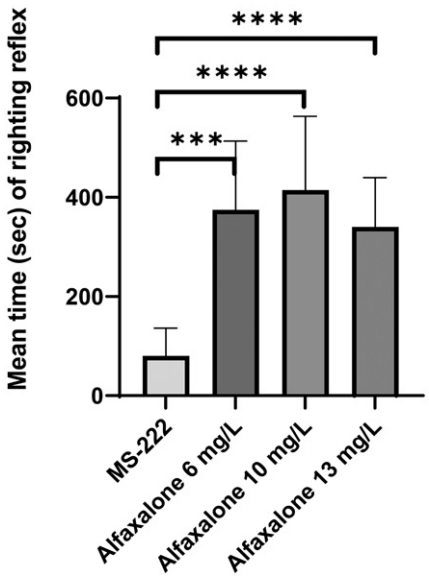

Figure 3. Mean time of return of the righting reflex. The time for the righting reflex to return was assessed as a measure of recovery. The MS-222 group had a significantly quicker return of the righting reflex than did any of the alfaxalone groups, ***$P \leq 0.001$, ****$P \leq 0.0001$. Error bars, 1 SD. $n = 10$ fish per treatment.

Discussion

The effectiveness of 2 anesthetics, MS-222 and alfaxalone, in reaching a surgical plane of anesthesia was measured by a loss of righting reflex, startle reflex (finger tap on tank), and tactile response (tail pinch with dissecting forceps), mirroring the criteria for surgical anesthesia in a variety of other fish studies.[4,5,9] A surgical plane of anesthesia is required for painful procedures. One study described the need to evaluate tail fin pinch and startle prior to any painful procedure to ensure zebrafish were deeply anesthetized.[4] If not deeply anesthetized, fish will dart off or wiggle in response to the pinch or respond to a tap on the tank.[4] If the opercula stop beating, the fish should immediately be placed in the recovery tank.[4]

In contrast to a previous study that used a cotton tipped applicator to test for a startle reflex (soft stimulus) and a 2 gram von Frey filament for to provide a tactile (hard) stimulus, we utilized startle and tactile responses mentioned previously.[6] The 2 gram von Frey filament, would likely have applied a similar force to the tail as our tail pinch, however, the study did not require nor discuss the use of their hard stimulus as a criterion for anesthesia.[6] In our study, 33 of 34 zebrafish in the alfaxalone groups lost opercular movements for over one minute during their anesthetic event and had to be placed in the recovery tank. The alfaxalone anesthetized fish without opercular movements often darted the tank during the tank tapping (startle response) and tail pinch (tactile response); therefore, they were not considered to have achieved a surgical plane of anesthesia.[4] Only one fish in the previous study had to be removed from the anesthetic bath due to cessation of opercular movements.[6] The higher number of zebrafish that experienced cessation of opercular movements in our study could be explained by our longer exposure to alfaxalone (average of 302 s as compared with 129 s in the previous study).[6]

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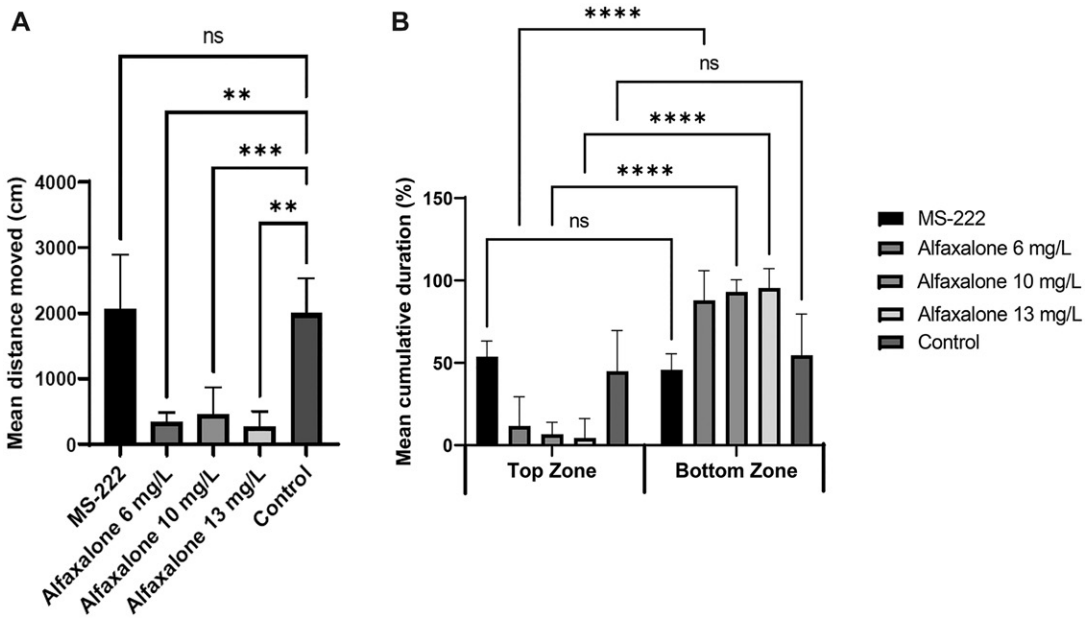

Figure 4. Movement and bottom-dwelling time of fish exposed to alfaxalone or MS-222. (A) Mean distance moved by the fish during recovery. The MS-222 and control groups have similar mean distances traveled, while the alfaxalone groups moved significantly less. (B) Cumulative fish duration in the top or bottom tank areas during recovery. The control and MS-222 groups spent about equal time in the top and bottom area of the tank while the alfaxalone groups spent significantly more time on the bottom. The similarity between MS-222 and the control demonstrated a much faster return to normal swimming than each of the alfaxalone groups. (A, B) $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ compared with controls. $^{****}P \leq 0.0001$. Error bars, 1 SD.

The method we used to determine a surgical plane of anesthesia included a hard stimulus whereas the previous study only used a soft stimulus; these difference study approaches could account for the differences in our results.[6]

The zebrafish in our MS-222 group (200 mg/L) reached a surgical plane of anesthesia within minutes. The MS-222 dose used in this study was based on clinical experience and previous literature using MS-222 in zebrafish and other teleost species, and our findings are consistent with other studies using MS-222 at lower and higher concentrations.[3,5,17] Only 3 of 34 fish in the alfaxalone groups reached a surgical plane of anesthesia based on our parameters (loss of righting reflex, startle reflex, and tactile responses within a 10-min period). The time until resuming normal swim was significantly longer with alfaxalone as compared with MS-222, with some fish taking greater than 10 min to resume swimming.

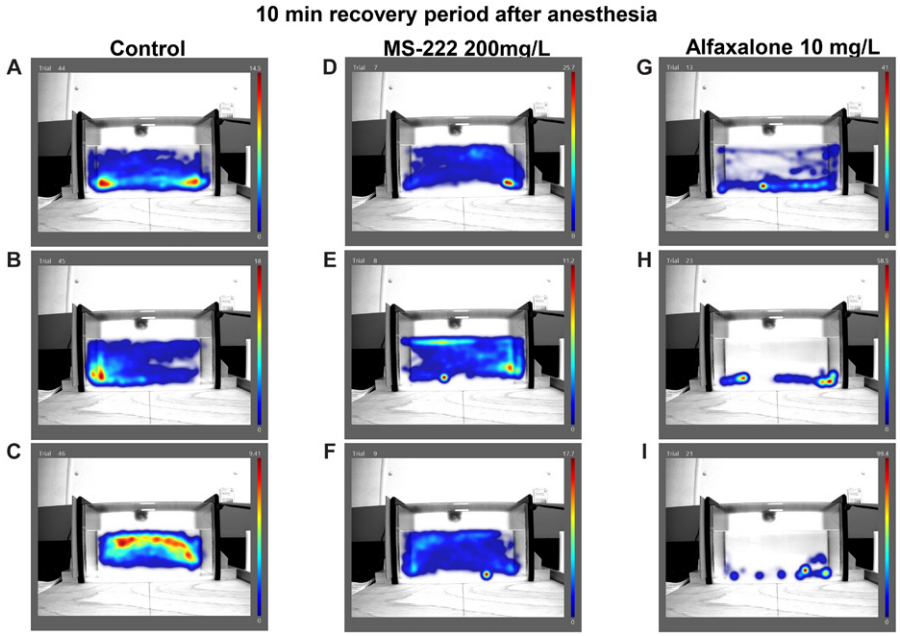

Figure 5. Representative heat maps demonstrating recovery movement. Representative heat maps of 3 different zebrafish in each group during the 10-min recovery period. Time spent swimming in different areas of the tank during the recovery period for control (A-C), alfaxalone (10 mg/L; D-F), and MS-222 (G-I). Heat maps were generated by the Ethovision XT (Noldus) software. The colored area (blue to red) represents where the fish were swimming and the time spent in those regions (red indicates a longer time). All zebrafish in all 3 alfaxalone groups had similar heat map images, as seen in D-F. The similarity between MS-222 and the control shows a much faster return to normal swimming than do the alfaxalone groups.

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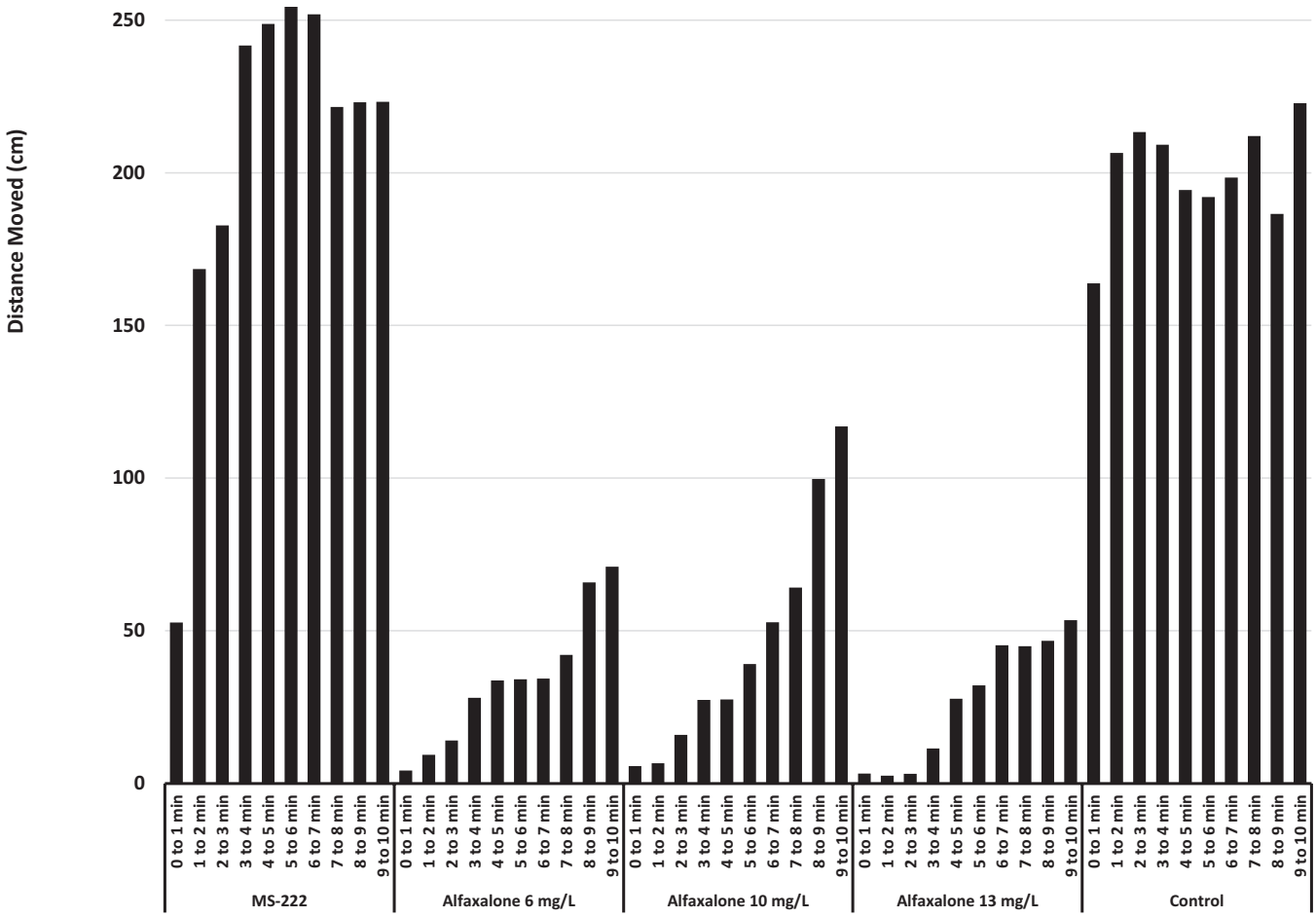

Figure 6. Mean distance moved during each minute of the recovery period. The MS-222 group showed rapid recovery from anesthesia and potentially some hyperactivity as compared with unanesthetized controls. Alfaxalone groups showed gradual increases in movement but did not travel as much as the control or MS-222 exposed groups during the 10-min recording period. n = 10 fish per treatment.

Alfaxalone is considered to be an effective immersion anesthetic for obtaining a surgical plane of anesthesia in a variety of teleost species.[2,9,12,20] The arrest of opercular movements that we observed raises concern about the use of alfaxalone in zebrafish. Similar respiratory side effects have also been reported previously in teleost species.[2,9] For instance, one study,that compared alfaxalone and MS-222 in black spot barbs and peacock cichlids reported that alfaxalone (5 mg/L) appeared to be adequate in obtaining surgical anesthesia for the majority of both species; however, the authors noted that starting at a dose lower than 5 mg/mL would be advisable due to loss of opercular movement for more than 30 seconds in 22 of 22 black spot barbs and 18 of 22 peacock cichlids.[20] In another study, goldfish were immersed in alfaxalone concentrations of 6, 7, and 9 mg/L, and a surgical plane of anesthesia was reached in all doses after an excitatory phase, but cessation of opercular movements occurred at both concentrations.[9] A study with koi found a similar cessation of opercular movement in 4 of 6 fish after immersion in alfaxalone at 2.5 mg/L; moreover 4 of 6 fish reacted to noxious stimuli (needle insertion into epaxial muscles) at 1 mg/L, suggesting alfaxalone may not be suitable for surgical procedures in fish.[12] Another study with koi that used intramuscular injection of alfaxalone at 10 mg/kg demonstrated prolonged apnea in 3 of 6 fish and 33% mortality.[1]

The largest number of zebrafish that reached a surgical plane of anesthesia in our study (2 of 10) were in the 6 mg/L group. However, achieving a surgical plane of anesthesia without cessation of opercular movements may not be possible at the lower concentrations tested in our study. We did not observe any deaths or other side effects prior to the 24-h euthanasia time point; however, all our zebrafish were removed from the alfaxalone anesthetic bath after one minute with no observed opercular movement. Two fish in the black spot barb and peacock cichlid study did not reach a surgical plane of anesthesia with MS-222, but the authors noted that their concentration of 100 mg/L may have been too low and a higher dose is commonly used.[20] This difference between the poor alfaxalone outcomes and the successful MS-222 anesthesia outcomes could be due to species variability and differences in MS-222 concentration.

We documented the recovery period for all groups with the Ethovision XT (Noldus) software. A control group was used for the recovery period to assess return to normal behavior. The control and MS-222 groups showed no significant differences in return of righting reflex, mean velocity, mean distance, and cumulative time spent in the bottom or top of the tank, indicating rapid recovery and resumption of normal swimming. In contrast, the alfaxalone groups differed significantly from the control group in all of these parameters, indicating that their resumption of normal swimming was significantly delayed when compared with the MS-222 group. A similar difference in alfaxalone and MS-222 recovery times was also reported for black spotted barbs and peacock cichlids, with recovery times for alfaxalone being significantly longer.[20] Average recovery times after alfaxalone immersion in other species were even longer than those reported for zebrafish (up to 37.5 min in Oscar fish).[1,2,9,12,20]

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In future experiments, measuring the drug concentration in the anesthetic immersion tank would validate the drug dosage that is delivered to each fish. However, the consistency of cessation of opercular movement and loss of righting reflex within groups leads us to conclude that the alfaxalone concentrations were accurate for each group and did not vary among fish within each group. Two studies recently reported the successful administration of alfaxalone via tube insertion in koi and rainbow trout, yet greater decreases in opercular movement and slower recovery were still observed with alfaxalone as compared with MS-222.[1,15] Tube insertion may not be a viable option in zebrafish due to their size, but it could be an option for other species if no other choice is available. Histopathologic analysis of the gills or other tissues could be performed to assess for and compare pathologic effects of MS-222 and alfaxalone.[6,11]

Overall, our study demonstrated that alfaxalone does not provide dependable transition to a surgical plane of anesthesia even at high doses. Further, it caused cessation of opercular movement and apnea for long periods even at low doses. Respiratory depression is a common side effect of alfaxalone reported in most studies, but the degree of depression may be related to species, which may explain the failure of the alfaxalone to produce a surgical plane of anesthesia in zebrafish due to their retained responsiveness to stimuli as compared with other teleost fish. Lower concentrations of alfaxalone could be tested in the future as well. We were unable to achieve our goal of optimizing alfaxalone doses to provide a safe alternative to MS-222 anesthesia in zebrafish. Given our results, we recommend alfaxalone not be used as an anesthetic for painful procedures on zebrafish, particularly at the concentrations we tested. Despite its reported side effects and handling limitations, MS-222 remains a more viable anesthetic for surgical procedures in zebrafish, and based on existing data, alfaxalone does not appear to be a safe alternative.

Acknowledgments

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