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Efficacy and Safety of 5 Anesthetics in Adult Zebrafish (*Danio rerio*)

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Although the safety and efficacy of tricaine methanesulfonate (MS222) for anesthesia of fish are well established, other anesthetics used less commonly in fish have been less extensively evaluated. Therefore, we compared gradual cooling, lidocaine hydrochloride (300, 325, and 350 mg/L), metomidate hydrochloride (2, 4, 6, 8, and 10 mg/L), and isoflurane (0.5 mL/L) with MS222 (150 mg/L) for anesthesia of adult zebrafish. The efficacy and safety of each agent was evaluated by observing loss of equilibrium, slowing of opercular movement, response to tail-fin pinch, recovery time, and anesthesia-associated mortality rates. At 15 min after anesthetic recovery, we used a novel-tank test to evaluate whether anesthetic exposure influenced short-term anxiety-like behavior. Behavioral parameters measured included latency to enter and number of transitions to the upper half of the tank, number of erratic movements, and number of freezing bouts. Behavior after anesthesia was unaltered regardless of the anesthetic used. Efficacy and safety differed among the anesthetics evaluated. Gradual cooling was useful for short procedures requiring immobilization only, but all instrumentation and surfaces that come in contact with fish must be maintained at approximately 10 °C. MS222 and lidocaine hydrochloride at 325 mg/L were effective as anesthetic agents for surgical procedures in adult zebrafish, but isoflurane and high-dose lidocaine hydrochloride were unsuitable as sole anesthetic agents due to high (30%) mortality rates. Although MS222 remains the best choice for generating a surgical plane of anesthesia, metomidate hydrochloride and gradual cooling were useful for sedation and immobilization for nonpainful procedures.

Abbreviation: MS222, tricaine methanesulfonate.

Several publications review anesthesia in fish.[1,16,20] Buffered tricaine methanesulfonate (MS222) is the anesthetic typically used to provide surgical level anesthesia in zebrafish (*Danio rerio*),[8,15] and most researchers use MS222 at a concentration of 164 mg/L.[15,25,26] However, reported side effects of MS222 that are dependent on dose and exposure duration include respiratory acidosis, cardiac depression, cardiac failure, and death.[10,19,21,24] MS222 increases blood glucose, plasma cortisol, lactate, and blood chemistry values in zebrafish and other species.[1,6,23,24] Because of these and other undesirable side effects of MS222, other anesthetics have been used to anesthetize adult zebrafish.[1,5,6,10,15,19,21,24]

The use of several less common anesthetic agents has been described in ornamental fish, including zebrafish.[16] Lidocaine hydrochloride is a local anesthetic that has previously been used for anesthesia in medaka (*Oryzias latipes*),[17,23] in which it produced a surgical plane of anesthesia. Metomidate hydrochloride is an imidazole-based nonbarbiturate hypnotic that is used to sedate fish for handling and to reduce the trauma and stress associated with transportation.[1,2,4,5,12,14,16,22] Isoflurane is a hydrocarbon that can be used as an anesthetic immersion bath.[16,23] Alone, isoflurane provides variable anesthesia and analgesia in fish, but when combined with MS222, it has been reported to produce a surgical plane of anesthesia for more than 20 min in zebrafish.[10,16] Gradual cooling has been shown to be useful for short-term procedures, such as intraperitoneal injections in adult zebrafish.[6,13,15]

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Little information is available regarding the suitability of these agents as anesthetics for invasive surgical procedures in the zebrafish. Therefore, we investigated the effects of lidocaine hydrochloride, metomidate hydrochloride, isoflurane, and gradual cooling on zebrafish because the anesthetic solutions require only a simple one-step preparation and because these agents potentially could provide a longer duration of surgical anesthesia than does MS222, with shorter induction and recovery times. This study is the first to compare multiple uncommon anesthetic agents in the adult zebrafish. We hypothesized that lidocaine hydrochloride, metomidate hydrochloride, and gradual cooling would be as efficacious as MS222 in providing surgical level anesthesia, but isoflurane alone would be ineffective, given that previous literature suggests this agent may be beneficial only in combination with MS222.[9]

In addition, we performed anxiety-like behavior tests to determine the effects of these anesthetic agents on the behavior of adult zebrafish. We sought to determine whether anesthesia-related behavioral effects in zebrafish are similar between anesthetic agents, and we hypothesized that anxiety-like behavior would be altered in fish recovering from isoflurane anesthesia, similar to what is seen in mice.[8]

Materials and Methods

Humane care and use of animals. Animals were housed in an AAALAC-accredited facility in compliance with the *Guide for the Care and Use of Laboratory Animals*.[11] All research procedures were approved by the IACUC at The Rockefeller University.

Animals and housing. Adult wildtype AB zebrafish (n = 110; male and female; age, 3 mo; Carolina Biologicals, Burlington, NC) were used for this study. Embryos were disinfected by soaking in 50 ppm bleach for 10 min, hatched, and reared in our facility. The fish were free of *Pseudoloma neurophilia*, *Pseudocapillaria*

tomentosa, *Mycobacterium* spp., and *Edwardsiella ictaluri* as determined by twice-yearly sentinel monitoring. The fish were housed at a density of 5 fish per tank in mixed-sex groups in 2.5-L tanks on a recirculating system (Marine Biotech, Apopka, FL) in 28 °C water (conductivity, 510 to 600 µS; pH, 7.3 to 7.7; hardness, 80 ppm; alkalinity, 80 ppm; dissolved oxygen, greater than 6 mg/L; ammonia, 0 ppm; nitrate, 0 to 0.5 ppm; and nitrite, 0 ppm) in a room with a 14:10-h light:dark cycle. System water was carbon-filtered municipal tap water, filtered through a 20-µm pleated particulate filter, and exposed to 40W UV light. The fish were fed twice daily with both a commercial pelleted diet (Adult Zebrafish Complete Diet, Zeigler Brothers, Gardners, PA) and an artificial *Artemia nauplii* replacement diet (Golden Pearls Reef and Larval Fish Diet 300 to 500 µm, Brine Shrimp Direct, Ogden, UT).

Experimental design. Each experimental group (n = 10, comprising 2 tanks of 5 fish each) was randomly assigned to a single anesthetic dosing regimen. The fish were fasted for 24 h prior to anesthesia to minimize the likelihood of regurgitation.[7] Each fish was tested individually between 1030 and 1300. Fish, holding tanks, anesthetic, and behavior tanks were brought to the testing room 1 h prior to the start of anesthesia to acclimate the fish to the new environment and to lower the water temperature slightly to the room temperature of 24 to 26 °C. Individual anesthetic agents were introduced into a static 2-L tank filled with system water obtained from the main recirculating system. At 15 min after anesthetic recovery, fish were evaluated for short-term anxiety-like behaviors by using a novel tank test. A behavioral control group of 10 unanesthetized fish housed under the same conditions as the experimental groups was tested in parallel. Experimental fish were monitored for 14 d after anesthesia to assess morbidity and mortality.

Anesthetic agent preparation and dosing regimens. With the exception of the gradual cooling group, anesthetic immersion baths were performed at temperatures between 24 and 26 °C, and sodium bicarbonate (catalog no. S233-500, Sodium Bicarbonate, certified ACS, Fisher Scientific, Fairlawn, NJ) was used to adjust the pH of the immersion baths to 7 to 7.5 as needed. All anesthetic solutions were prepared immediately before use. All of the water used was from the main recirculating system. Temperature and pH were measured by using a multiple-parameter portable meter (model HI 991300, Hanna Instruments, Woodstock, RI).

MS222 (Finquel Tricaine Methanesulfonate, Argent Chemical Laboratories, Redmond, WA) prepared under a chemical fume hood was mixed 1:2 with 95% sodium bicarbonate to make a stock solution of 10 mg/mL with a resulting pH of 7.3 in system water. The stock solution was added to the anesthetic tank containing system water to achieve a final concentration of 150 mg/L, to which the fish were added.

Lidocaine hydrochloride (Lidocaine Hydrochloride 10% for Injection USP, Hospira, Lake Forest, IL) was instilled beneath the surface of 1 L of system water in a 2-L tank by using a 25-gauge needle and stirred into solution to achieve final concentrations of 300, 325, and 350 mg/L at pH 6.9 to 7.4. Fish were added directly into the tank with the prepared lidocaine hydrochloride solution.

Metomidate hydrochloride (Aquacalm, Western Chemical, Ferndale, WA) was added to a fixed volume of system water to form a 10 mg/mL stock solution. The stock solution was titrated into a 2-L static anesthetic tank to achieve final concentrations of 2, 4, 6, 8, and 10 mg/L at pH 7.0 to 7.5. Fish were added directly into the tank containing the prepared solution.

Isoflurane (Aerrane, Baxter Healthcare, Deerfield, IL) was instilled beneath the surface of 1 L of system water in a 2-L tank by using a 25-gauge needle and stirred into solution to form a final concentration of 0.5 mL/L at pH 6.8. Fish were added directly into the tank with the prepared isoflurane solution. Anesthetic preparation and anesthesia was conducted in a certified chemical fume hood.

For the gradual cooling group, system water was placed into a plastic container (7.5 cm × 7.5 cm × 5 cm), which then was placed inside a larger, outer container (22 cm × 16 cm ×28 cm) containing cold tap water. Ice was added to the outer tank until a slurry formed and the temperature in the inner plastic container was 17 °C, at which point the fish were placed into the inner container.[13] More ice was added incrementally, reducing the water temperature of the inner container, until the fish were anesthetized as indicated by loss of response to tail-fin pinch, at which point, the water temperature was recorded. The water temperature was monitored continuously throughout cold-water exposure. The final water temperature achieved at the time of fish removal was recorded.

Estimating plane of anesthesia. A single designated person who did not observe the behavior trials monitored the fish throughout anesthetic exposure. The plane of anesthesia (Figure 1) was determined by using the criteria described previously.[20,23]

Anesthesia evaluation. The fish were placed in anesthetic solution and observed for loss of equilibrium, response to tail-fin pinch, slowing of opercular movement, recovery time, and mortality. Loss of equilibrium was recorded after fish remained inverted with their ventral abdomen oriented toward the surface of the water (upside-down swimming) for more than 3 s. Response to tail-fin pinch was tested by firmly applying pressure to the midsection of the tail fin with nonserrated tissue forceps (Integra Miltex, York, PA) every 10 s once loss of equilibrium occurred. A slightly anesthetized fish reacted to the pressure applied by twitching or attempting to swim away, whereas an anesthetized fish did not have an observable reaction. Opercular movement was observed, and the time at which the opercular rate slowed to a rate that was less than 25% of baseline or which opercular movement appeared uneven was recorded. In addition, fish were observed for signs of distress, such as rapid opercular movement, piping, twitching, regurgitation, and erratic swimming. Piping is defined as the fish rising to the surface of the tank to gulp air. If distress signs occurred, fish were removed from the solution immediately and placed into the recovery tank, which was a 2-L system tank filled with system water free from any anesthetic. The fish were maintained in the anesthetic solutions for as long as 4 min after achieving stage 3, plane 2 anesthesia; as long as 10 min when stage 3, plane 2 anesthesia was not achieved; or until opercular movement ceased for more than 3 s. If the fish was noted to have lost opercular movement for more than 3 s, it was removed from the anesthetic solution immediately by using a net and placed in a recovery tank. Time to recovery was recorded from the point at which an individual fish was placed in the recovery tank until the fish was able to swim upright for at least 5 s. All times were measured by using a stopwatch.

Novel-tank test. After exposure to an anesthetic agent, the fish recovered for 15 min in a recovery tank and were placed in a new tank to undergo a 6-min novel-tank test.[3] The test began immediately upon placement of fish into the novel tank (15.2 cm × 30 cm × 20 cm; 2.5 gal [9.5 L]; Aqueon, Franklin, WI), which was filled to within 1/8 in. below the top with system water that was 24 to 26 °C. Parameters measured included time until first entry into the upper half of the tank, the number of transitions to the upper half of the tank, the number of erratic movements, and the number of freezing bouts. Erratic movements were defined

Stage	Plane	Level	General behavior	Voluntary locomotor activity	Equilibrium	Rate of opercular movement	Reflex response	Heart rate	Muscle tone	Examples of procedures
0		None	Normal	Normal	Normal	Normal	Normal	Normal	Normal	
I		Sedation	Disorientation	Decreased	Difficult to maintain	Normal	Reduced	Normal	Normal	ENU mutagenesis, imaging
II		Excitation	Agitation	Increased	Lost	Increased	Increased	Increased	Normal	
III	1	Light anesthesia	Anesthetized	None	Lost	Decreased	Reduced	Regular	Decreased	Weighing, gill scrape, skin scrape
	2	Surgical anesthesia	Anesthetized	None	Lost	Shallow	None	Reduced	Decreased	Gill biopsy, tail fin clipping, recovery surgery
	3	Deep	Anesthetized	None	Lost	Rare movements	None	Reduced	Relaxed	Nonrecovery surgery
IV		Overdose	Apparently dead	None	Lost	None	None	Cardiac failure	None	

Modified from references 20 and 23.

Figure 1. Stages of anesthesia and potential associated procedures in adult zebrafish.

as sharp, rapid changes in direction (that is, darting). Freezing bouts were defined as periods of no body movement for at least 1s while the fish was on the bottom of the tank, although the eyes and gills might move. The behavior tests were recorded by using a video camera (Handycam DCR-SX60, Sony, Shenzhen, China), and the observer of the videos was blinded regarding the anesthetic group of the zebrafish, to avoid any bias during the behavior measurements. The metomidate groups were not included in the behavioral analysis because the anesthetic testing of this agent was performed before the behavioral component was added to this study. After behavioral testing, all fish were returned to their home tanks and were observed twice daily for 14 d for signs of morbidity or mortality.

Statistical analysis. Anesthesia and behavior data were analyzed for normality by using the Shapiro–Wilks normality test. The data were not normally distributed, and log transformation did not normalize the data; therefore, the Kruskal–Wallis test followed by pairwise analysis was used. To compare groups with MS222 only, the Mann–Whitney–Wilcoxon test with Bonferroni correction was performed by using statistical analysis software (STATA, StataCorp, College Station, TX). A P value of less than 0.05 indicated statistical significance.

Results

MS222. The average time to loss of equilibrium, lack of response to tail fin pinch, slowing of opercular movement, recovery time, and mortality for all groups is provided in Table 1. All fish exposed to MS222 achieved a surgical plane of anesthesia. No distressful behaviors were observed during induction or recovery. No fish died during anesthesia or during the 14-d period afterward.

Lidocaine hydrochloride. Fish exposed to 300 mg/L lidocaine remained in stage 1 (light sedation) throughout the observation period. Fish in the 325-mg/L group achieved stage 3, plane 2 (surgical plane of anesthesia). In the 325 mg/L group, the mean time until loss of equilibrium was significantly ($P \leq 0.001$) longer than for MS222 (365 s compared with 111 s), but lidocaine-treated fish lost the tail-fin pinch response more rapidly ($P \leq 0.001$) than did those anesthetized with MS222 (51 s compared with 252 s; Table 1). Recovery time was significantly ($P \leq 0.001$) longer in the 325-mg/L group than in the MS222 group (346 s compared with 140 s). No mortality was observed in the 300- and 325-mg/L groups during anesthesia or the 14-d observation period, however stage 3, plane 3 of anesthesia and stage 4 (death) were documented to occur in fish exposed to anesthetic doses of 350 mg/L. In fish exposed to 350 mg/L, time until lack of response to a tail-fin pinch (101 s, $P \leq 0.001$) and total time in solution (153 s, $P \leq 0.001$) were significantly shorter than with MS222 (252 s and 513 s, respectively). A 30% mortality rate was observed; 3 fish in the 350-mg/L group died during anesthesia; but no additional mortality occurred during the 14-d postanesthetic observation period.

Metomidate hydrochloride. Fish exposed to 2 and 4 mg/L metomidate achieved stage I anesthesia; the 6-, 8-, and 10-mg/L groups achieved stage III, plane 1 anesthesia. The slowing of opercular movement occurred significantly earlier (100 to 260s, $P \leq 0.001$) at all anesthetic doses when compared with MS222 (513s; Table 1). All fish, independent of anesthetic dose, remained in solution for 10 min without achieving a surgical anesthetic plane, as evident by persistent responsiveness to tail-fin pinching. Time to loss of equilibrium did not differ significantly from that for MS222. In the 6-, 8-, and 10-mg/L groups, the recovery time (625, 337, and 548 s, respectively) was significantly ($P \leq 0.001$ for all comparisons) longer than that for MS222 (140 s). No distressful behaviors were observed during anesthesia. Mortality did not occur in any of the metomidate-treated fish either during anesthesia or throughout the 14-d observation period.

Isoflurane. All isoflurane-exposed fish remained in stage II, the excitatory phase, as evidenced by various distressful behaviors including twitching, erratic swimming, and piping. Signs of disorientation and difficulty maintaining buoyancy, rolling, and swimming upside down occurred also; however, the study criteria for loss of equilibrium were never met. Time until opercular movement slowed and recovery time did not differ significantly from those for MS222 (Table 1). A 30% mortality rate was observed; one fish died during anesthesia, and 2 additional fish were found dead on day 1 of the 14-d postanesthetic observation period.

Gradual cooling. All of the fish that underwent gradual cooling achieved a surgical plane of anesthesia. When loss of equilibrium occurred, the average temperature of the water in the inner container was 11.9 °C (range, 10.9 to 12.5 °C). Time until loss of equilibrium was significantly ($P < 0.001$) longer for gradual cooling (313 s) than for MS222 (111 s; Table 1). The average temperature when opercular movement slowed and the tail-fin response was lost was 10.3 °C. Average recovery time was significantly ($P \leq 0.001$) less for gradual cooling (0 s) than for MS222 (140 s). No distressful behaviors were observed during induction or recovery. One fish died during the anesthesia test, when the temperature was around 8 °C; none of the remaining fish died during the 14-d post recovery observation period.

Novel-tank test. The number of freezing events, latency to surface, and number of transitions did not differ statistically between the various anesthetized groups and the unanesthetized control group (data not shown). Significant differences in erratic swimming behavior occurred between the unanesthetized

Table 1. Zebrafish response (seconds; mean ± 1 SD; n = 10 per group) to anesthetic agents

Agent	Dose	Loss of equilibrium	Slowing of opercular movement	Lack of response to tail-fin pinch	Recovery time
MS-222	150 mg/L	111 ± 92	513 ± 99	252 ± 89	140 ± 51
Lidocaine hydrochloride					
	300 mg/L	196 ± 95	323 ± 234	not applicable	206 ± 148
	325 mg/L	365 ± 137[a]	90 ± 190[a]	51 ± 162[a]	346 ± 310[a]
	350 mg/L	34 ± 20[a]	78 ± 30[a]	101 ± 18[a]	116 ± 104
Metomidate hydrochloride					
	2 mg/L	80 ± 136	100 ± 211[a]	not applicable	169 ± 14
	4 mg/L	159 ± 53	186 ± 242[a]	not applicable	313 ± 207[a]
	6 mg/L	113 ± 45	260 ± 55[a]	not applicable	625 ± 247[a]
	8 mg/L	40 ± 18[a]	166 ± 47[a]	not applicable	337 ± 150[a]
	10 mg/L	37 ± 19[a]	136 ± 25[a]	not applicable	548 ± 253[a]
Isoflurane	0.5 mL/L	not applicable	395 ± 136	not applicable	253 ± 166
Gradual cooling	—	313 ± 133[a]	313 ± 133[a]	313 ± 133	0 ± 0*

An entry of 'not applicable' indicates that the reflex was not lost.
[a]Value significantly ($P < 0.05$) different from that for MS222 after pairwise analysis with the Mann–Whitney–Wilcoxon test and Bonferroni correction.

control group and isoflurane group (55 and 28 events, respectively; $P \leq 0.01$) and between the unanesthetized control and MS222 groups (55 and 27 events, respectively, $P \leq 0.01$).

Discussion

We found that MS22 at 150 mg/L (0.57 mM) and lidocaine hydrochloride at 325 mg/L (1.39 mM) induced a surgical plane of anesthesia in zebrafish. Metomidate hydrochloride was useful for sedation. Lidocaine hydrochloride at 350 mg/L and isoflurane caused high levels of mortality during and 24 h after anesthesia. Gradual cooling was useful for immobilization. MS222 and isoflurane minimally altered postanesthetic behavior.

Zebrafish achieved a plane of surgical anesthesia faster with lidocaine hydrochloride at 325 mg/L, but recovery at this dose was longer than that for MS222. There was also no mortality associated with this dose. The 325-mg/L dose of lidocaine hydrochloride was the only one that could be safely used for surgical anesthesia in adult zebrafish. Opercular movement rapidly slowed and became difficult to visualize at 350 mg/L lidocaine hydrochloride, and some zebrafish removed from the 350-mg/L lidocaine solution within 3 s after opercular movement had ceased did not recover, demonstrating a narrow margin of safety in adult zebrafish and precluding lidocaine's use as an anesthetic agent at doses of 350 mg/L and greater. These results contrast with those reported for medaka (*Oryzias dancena*), in which concentrations as high as 800 mg/L could be used to induce anesthesia without side effects.[17] Overall, these results indicate that lidocaine hydrochloride at 325 mg/L may provide an alternative to MS222 anesthesia in adult zebrafish and may be preferred if rapid induction is needed.

At doses of 6, 8, and 10 mg/L, metomidate hydrochloride induced a more rapid loss of equilibrium than did MS222. Although doses of 2 and 4 mg/L were sufficient to slow swimming and induce loss of equilibrium, voluntary movement was often preserved at these doses. Recovery was prolonged (for example, to a mean of 625 s in the 6-mg/L group) but did not differ significantly from that for MS222. Perhaps higher doses of metomidate hydrochloride are needed to induce surgical anesthesia in adult zebrafish; doses as high as 16 mg/L have used in channel catfish (*Ictalurus punctatus*).[22] In that report, catfish achieved a surgical plane of anesthesia at doses of 4, 6, and 8 mg/L, contrary to what we observed for zebrafish. Similar to that in zebrafish, the recovery time of channel catfish increased as the dose of metomidate hydrochloride increased.[22] Metomidate hydrochloride may be a suitable alternative to MS222 for procedures such as ENU mutagenesis or imaging, where only immobilization or sedation is needed at the doses tested.

Our findings confirm previously published results indicating that isoflurane alone is not a suitable agent for the anesthesia of zebrafish.[10,16] Isoflurane induced distress behaviors in all fish; these behaviors were not observed with the other anesthetic agents. We do not recommend the use of isoflurane as a sole agent for anesthesia of adult zebrafish.

Our data on response to gradual cooling were similar to previously published results.[5,6,13] The response to fin pinching was abolished at an average temperature of 10.3 °C, which is lower than the previously reported mean of 12 °C;[13] however, the significance of this difference is unknown. It may be possible to lower the temperature even further, given that the temperature for euthanasia by rapid cooling is 4 °C,[25] but we did not evaluate this hypothesis in the current study. Using cool temperatures to maintain fish at a suitable plane of anesthesia for prolonged procedures might be difficult, because fish appeared to recover as soon as they came into direct contact with room-temperature instruments or surfaces. Zebrafish recovered instantaneously (0 s) when placed in 28 °C water. In many cases, fish responded when touched by the room-temperature net for removal from the cold solution. When using gradual cooling for anesthesia, all instrumentation and working surfaces must be maintained at cool temperatures (10 °C) to help prevent premature recovery during a procedure. Given the need to keep instruments at cool temperatures and to monitor water temperature very closely, we do not recommend this method of anesthesia for invasive surgical procedures. Gradual cooling should be used only when no alternative is available. Another disadvantage is that this

method of anesthesia is more labor- and time-intensive than are the other agents studied.

Our study did not test all of the possible anesthetics available for use in adult zebrafish. For example, one publication details the use of clove oil in zebrafish.[7] We also did not assess compounds such as benzocaine hydrochloride and quinaldine.[1,5,16,20] The fish mortality we observed was suggestive of death due to anesthetic stress, because the mortalities occurred within 24 h after anesthesia. The mortalities reported could have been due to other causes, but no additional mortality occurred during the remainder of the 14-d postanesthetic observation period.

Understanding the short-term effects of anesthetic agents on behavior is important because anesthetics may adversely influence certain studies, especially neurobehavioral studies. Anxiety-like behavior in mice increased when they were exposed to isoflurane anesthesia during tail biopsy.[8] The novel-tank test evaluated the effects of anesthetic agents on short-term anxiety-like behavior in adult zebrafish. Increasing values in the numbers of freezing events and erratic movements and in the latency to enter the top half of the tank suggest higher levels of anxiety in zebrafish.[3] An increasing number of entries to the top half of the tank suggests lower levels of anxiety.[3] We found no statistically significant differences between the unanesthetized control group and anesthetized groups in all variables except for the number of erratic movements. In this case, isoflurane and MS222 showed decreased numbers of erratic movements compared with those of the unanesthetized control group. The lack of statistically significant behavioral differences indicates that the anesthetics characterized had minimal effects on the behaviors evaluated in the novel tank test. We cannot exclude the possibility that the fish had not completely metabolized all of the anesthetics provided within 15 min. Therefore, reassessment at a later time point might clarify whether prolonged metabolism of the anesthetics plays a role in anxiety-like behavior.

Additional studies are needed to determine the effects of these agents on physiologic parameters in adult zebrafish and their effects on zebrafish embryos, larvae, and the reproductive efficiency of adults. A previously published study demonstrated high mortality in zebrafish larvae exposed to metomidate hydrochloride.[1,14] An alternative approach to improving anesthetic efficacy and surgical outcomes in zebrafish may be through the use of combinations of different anesthetic agents. The use of multiple anesthetic agents may produce a synergistic effect, thereby decreasing the dose of each drug needed and helping to reduce undesirable dose-related side effects. In addition, anesthetic combinations may help to safely extend the duration of anesthesia that can be achieved.[25] Potential anesthetic combinations, such as MS222 and metomidate hydrochloride, may produce stable long-term surgical anesthesia with faster induction and recovery times.[10] Furthermore, a related study explores the aversiveness of various anesthetic agents in zebrafish.[18]

In conclusion, the current study is the first to characterize the efficacy and safety of multiple anesthetic agents other than MS222 in adult zebrafish. We determined that MS222 remains a reliable and consistent anesthetic agent for invasive surgical procedures in adult zebrafish. Lidocaine hydrochloride at 325 mg/L is promising as an anesthetic agent for surgical procedures in adult zebrafish but has a low margin of safety at higher doses. Metomidate hydrochloride is suitable for nonpainful procedures that can be performed under sedation or immobilization without analgesia. Isoflurane is not suitable as a sole anesthetic agent for adult zebrafish. Gradual cooling should only be used as a last option and only when surfaces and instrumentation can be maintained at 10 °C. The current data can serve as a basis for evaluating the appropriateness of anesthetics used for zebrafish studies. Not all zebrafish strains may have the same responses to the anesthetics that we evaluated; a pilot study should always be performed on a small number of fish of different strains to ensure the safety and efficacy of the anesthetic used.

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References

1. **Ackerman PA, Moragn JD, Iwama GK.** [Internet]. 2005. Anesthetics. Canadian Council on Animal Care supplement to Guidelines on the Care and Use of Fish in Research, Teaching and Testing. [Cited 15 April 2013]. Available at: http://www.ccac.ca/en_/standards/guidelines/additional/add-info-fish
2. **American Veterinary Medical Association.** 2013. Guidelines for the euthanasia of animals, 2013 ed. Available at: http://www.avma.org/KB/Policies/Documents/euthanasia.pdf
3. **Cachat J, Stewart A, Grossman L, Gaikwad S, Kadri F, Chung KM, Wu N, Wong K, Roy S, Suciu C, Goodspeed J, Elegante M, Bartels B, Elkhayat S, Tien D, Tan J, Denmark A, Gilder T, Kyzar E, Dileo J, Frank K, Chang K, Utterback E, Hart P, Kalueff AV.** 2010. Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. Nat Protoc **5:**1786–1799.
4. **Crosby TC, Hill JE, Watson CA, Tanong RPE.** 2006. Effects of tricaine methansulfonate, hypno, metomidate, quinaldine, and salt on plasma cortisol levels following acute stress in threespot gourami *Trichogaster trichopterus.* J Aquat Anim Health **18:**58–63.
5. **DeTolla LJ, Srinivas S, Whitaker BR, Andrews C, Hecker B, Kane AS, Reimschuessel R.** 1995. Guideline for the care and use of fish in research. ILAR J **37:**159–173.
6. **Eames SC, Philipson LH, Prince VE, Kinkel MD.** 2010. Blood sugar measurement in zebrafish reveals dynamics of glucose homeostasis. Zebrafish **7:**205–213.
7. **Grush J, Noakes DLG, Moccia RD.** 2004. The efficacy of clove oil as an anesthetic for the zebrafish, *Danio rerio* (Hamilton). Zebrafish **1:**46–53.
8. **Hankenson FC, Braden-Weiss GC, Blendy JA.** 2011. Behavioral and activity assessment of laboratory mice (*Mus musculus*) after tail biopsy under isoflurane anesthesia. J Am Assoc Lab Anim Sci **50:**686–694.
9. **Harper C, Lawrence CL.** 2011. The laboratory zebrafish. Boca Raton (FL): CRC Press.
10. **Huang WC, Hsieh YS, Chen IH, Wang CH, Chang HW, Yang CC, Ku TH, Yeh SR, Chuang YJ.** 2010. Combined use of MS-222 (tricaine) and isoflurane extends anesthesia time and minimizes cardiac rhythm side effects in adult zebrafish. Zebrafish **7:**297–304.
11. **Institute for Laboratory Animal Research.** 2011. Guide for the care and use of laboratory animals, 8th ed. Washington (DC): National Academies Press.
12. **Kilgore KH, Hill JE, Powell JFF, Watson CA, Yanong RPE.** 2009. Investigational use of metomidate hydrochloride as a shipping additive for two ornamental fishes. J Aquat Anim Health **21:**133–139.
13. **Kinkel MD, Eames SC, Philipson LH, Prince VE.** 2010. Intraperitoneal injection into adult zebrafish. J Vis Exp **42:**e2126.
14. **Massee KC, Rust MB, Hardy RW, Stickney RR.** 1995. The effectiveness of tricaine, quinaldine sulfate and metomidate as anesthetics for larval fish. Aquaculture **134:**351–359.
15. **Matthews M, Varga ZM.** 2012. Anesthesia and euthanasia in zebrafish. ILAR J **53:**192–204.
16. **Neiffer DL, Stamper MA.** 2009. Fish sedation, anesthesia, analgesia, and euthanasia: considerations, methods, and types of drugs. ILAR J **50:**343–360.
17. **Park IS, Park SJ, Gil HW, Nam YK, Kim DS.** 2011. Anesthetic effects of clove oil and lidocaine-HCL on marine medaka (*Oryzias dancena*). Lab Anim (NY) **40:**45–51.
18. **Readman GD, Owen SF, Murrell JC, Knowles TG.** 2013. Do fish perceive anaesthetics as aversive? PLoS ONE **8:**e73773.

19. **Rombough PJ.** 2007. Ontogenetic changes in the toxicity and efficacy of the anaesthetic MS222 (tricaine methansulfonate) in zebrafish (*Danio rerio*) larvae. Comp Biochem Physiol A Mol Integr Physiol **148:**463–469.
20. **Ross LG, Ross B.** 2008. Anaesthetic and sedative techniques for aquatic animals, 3rd ed. Ames (IO): Blackwell Publishing.
21. **Sanchez-Vasquez FJ, Terry MI, Felizardo VO, Vera LM.** 2011. Daily rhythms of toxicity and effectiveness of anesthetics (MS222 and Eugenol) in zebrafish (*Danio rerio*). Chronobiol Int **28:** 109–117.
22. **Small BC.** 2003. Anesthetic efficacy of metomidate and comparison of plasma cortisol responses to tricaine methansulfonate, quinaldine and clove oil anesthetized channel catfish *Ictalurus punctatus.* Aquaculture **218:**177–185.
23. **Stoskopf M, Posner LP.** 2008. Anesthesia and restraint of laboratory fish, p 519–534. In: Fish RE, Brown MJ, Danneman PJ, Karas AZ, editors. Anesthesia and analgesia in laboratory animals, 2nd ed. San Diego (CA): Elsevier.
24. **Topic Popovic N, Strunjak-Perovic I, Coz-Rakovac R, Barisic J, Jadan M, Persin Berakovic A, Sauerborn Klobucar R**. 2012. Tricaine methane-sulfonate (MS-222) application in fish anaesthesia. Journal of Applied Ichthyology **28:**553–564.
25. **Wilson JM, Bunte RM, Carty AJ.** 2009. Evaluation of rapid cooling and tricaine methansulfonate (MS222) as methods of euthanasia in zebrafish (*Danio rerio*). J Am Assoc Lab Anim Sci **48:**785–789.
26. **Westerfield M.** 2000. The zebrafish book. A guide for the laboratory use of zebrafish (*Danio rerio*), 4th ed. Eugene (OR): University of Oregon Press.