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HOW WE TEACH | *Classroom and Laboratory Research Projects*

Anesthetic MS-222 eliminates nerve and muscle activity in frogs used for physiology teaching laboratories

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Medler S. Anesthetic MS-222 eliminates nerve and muscle activity in frogs used for physiology teaching laboratories. *Adv Physiol Educ* 43: 69–75, 2019; doi:10.1152/advan.00114.2018.—Frogs are routinely used in physiology teaching laboratories to demonstrate important physiological processes. There have been recent directives that promote the use of the anesthetic MS-222 (tricaine methanesulfonate), rather than lowering body temperature with a cold water bath to prepare reptiles and amphibians for physiological experiments or euthanasia. Indeed, the most recent edition of the American Veterinary Medical Association (AVMA) *Guidelines for the Euthanasia of Animals* proclaims that chilling in water is not an appropriate method and advocates for the usage of MS-222 or other anesthetics. However, prominent researchers have responded to this position by highlighting evidence that cooling ectothermic vertebrates is, in fact, an effective and appropriate method. Furthermore, MS-222 is a known voltage-gated Na^+ channel blocker, and this anesthetic's impact on the physiology of excitable tissues suggests that its use might be incompatible with experiments on nerve and muscle tissues. In the present study, I examined the effects of MS-222 at a concentration of 1.5 g/l on nerve, skeletal muscle, and cardiac muscle physiology of frogs. I found that immersion of frogs in this anesthetic blocked basic nerve and muscle physiology, making the frogs unsuitable for laboratory experiments. Applying MS-222 directly to the sciatic nerve dramatically blocked normal excitation-contraction coupling in skeletal muscle preparations, and direct application to the heart caused the organs to stop contracting. Based on these results, I conclude that MS-222 at the concentration studied may be incompatible with physiological preparations that rely on electrically excitable tissues for their normal function. Physiology educators who must use MS-222 with frogs should empirically determine an appropriate dosage and recovery time before using the anesthetic in the teaching laboratory.

cardiac muscle; frogs; MS-222; nerve; skeletal muscle

INTRODUCTION

Frogs are frequently used as experimental specimens in physiology teaching laboratories around the United States and have been studied by physiology students for decades. Frogs present robust physiological responses that are often maintained for hours at room temperature, making them preferable to mammals. They are also easy to keep and inexpensive to maintain with minimal laboratory animal care facilities. At the State University of New York (SUNY) Fredonia, those of us who teach physiology laboratories have used frogs in several different laboratory classes that teach physiological principles to undergraduate students. Frogs provide important models for several different laboratory investigations, including compound action potentials in peripheral nerves, skeletal muscle physiology, and cardiovascular physiology. It is our position that these laboratory experiences using real, living systems greatly enhance our students' understanding of physiological processes. We also enjoy sharing our students' excitement as they see the principles of physiology textbooks come to life before them.

There is growing concern from researchers and educators about the push to adhere to protocols that could ultimately restrict the use of ectothermic vertebrates (17, 21, 28). The most recent edition of the American Veterinary Medical Association (AVMA) *Guidelines for the Euthanasia of Animals* (1) proclaims that cooling of ectotherms like reptiles and amphibians is not an appropriate method of anesthesia or euthanasia. The AVMA guide goes on to recommend that MS-222 (tricaine methanesulfonate) be used as an alternate agent for these purposes. However, as pointed out by an interdisciplinary panel, these recommendations are not evidence based and actually fly in the face of the scientific data (21). A body of compelling evidence indicates that cooling of ectotherms is a normal component of their life history, and that it significantly reduces neurological activity (17, 21, 28). There is growing concern that the directives provided in the AVMA guide are becoming de facto policy because the National Institutes of Health and other oversight organizations look to the AVMA as an authority for guiding the methods involving animals in research and teaching (17, 21). Indeed, the most recent edition of the *Guide for the Care and Use of Laboratory Animals* states that, "unless a deviation is justified for scientific or medical reasons, methods should be consistent with the AVMA Guidelines on Euthanasia" (24).

In New York State, the Department of Health is mandated to oversee the care of animals in laboratories and research (25). We have routinely used ice water baths to anesthetize frogs before double pithing them for use in our teaching laboratories. However, we were recently directed by a Department of Health Inspector to change our methods and substitute MS-222 in place of chilling. Since MS-222 is a known antagonist of voltage-gated Na^+ channels, we have concerns that use of this anesthetic will interfere with the very physiological processes we are attempting to teach in these laboratories. A search of the literature reveals that very limited data are currently available about the effects of MS-222 on nerve and muscle physiology, but the data that are available suggest that MS-222 is likely incompatible with our laboratory preparations (9, 10, 13, 17,

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20, 27). In addition, we are concerned with the inevitable exposure of MS-222 to students, because it is known to be toxic to the retina in humans (4). In the present study, I investigated the effects of an intermediate concentration of MS-222 on nerve, skeletal muscle, and cardiac muscle physiology in leopard frogs. I also discuss considerations for MS-222 dosages, depending on whether the anesthetic is being administered for temporary anesthesia, such as for physiological experiments or surgery vs. that being administered for euthanasia.

MATERIALS AND METHODS

Animals. Leopard frogs (*Rana pipiens*) of medium size (snout–vent length ~7–8 cm; body mass ~40–50 g) were purchased from Wards Science (Rochester, NY) and housed at 25°C in aquaria with water and peat moss. They were fed crickets ad libitum. A guiding principle for using animals in research is the idea of the three R's (replacement, reduction, and refinement) to minimize the number of animals euthanized (24). This principle helped us decide on our sample size at the beginning of the study, and we reused frogs for both skeletal muscle and cardiac muscle responses. I began with 21 animals that were left over from teaching laboratories, knowing that we could add more animals if needed to increase statistical power. The procedures were consistent with those routinely followed in our teaching laboratories and were defined in detailed protocols reviewed and approved by the Institutional Animal Care and Use Committee at SUNY Fredonia. Briefly, after chilling in ice water for 15–20 min, frogs were grasped firmly and double pithed with a sharp steel probe. Frogs were subsequently checked for responsiveness through a toe pinch and by touching the eye. Physiologically responsive frogs typically exhibit a corneal reflex when the eye is touched, and this behavior is dependent on reflexive circuits through the cranial nerves (17, 20, 23). A firm pinch of the toe to elicit a withdrawal reflex is a common test of nociceptive responses in amphibians (6, 16, 18, 20, 31, 32). Animals were judged to be appropriately unaware of pain if they were unresponsive to both of these tests. Next, frogs were prepared for use in measurements of skeletal and cardiac muscle physiology. The procedures outlined here are the same as those we routinely follow in our physiology teaching laboratories. In an alternative procedure, frogs were immersed in a solution of 0.15% MS-222 (1.5 g/l; tricaine methanesulfonate; Sigma Chemical, St. Louis, MO) in 5% dibasic sodium phosphate, pH 7.0, for 20–30 min. This concentration was recommended by a colleague who routinely uses MS-222 to anesthetize axolotls and is within an intermediate concentration range recommended to anesthetize or euthanize frogs and toads (1, 6, 7, 9, 10, 16, 18, 19, 23, 30–33). Frogs were then double pithed and monitored as described above. All experiments were carried out in the physiology teaching laboratory with a temperature of 22°C.

Measurement of skeletal muscle contraction. Frogs were prepared by removing the skin covering the leg. The sciatic nerve was identified on the lateral surface of the thigh and separated from the adjacent muscles using a pulled glass probe. Care was taken not to touch the nerve with metal tools of any kind. The calcaneal tendon was separated from the foot and secured with a short length of dental floss. The leg was pinned to the bottom of a dissection pan with pins at the ankle and the knee, and the gastrocnemius muscle was attached to a force transducer (iWorx FT 104) with the floss attached to the calcaneal tendon. The force transducer signal was amplified with an iWorx 214 two-channel data recorder (iWorx, Dover, NH).

Muscle contraction was elicited by stimulating the sciatic nerve at the level of the thigh with a 1-V square-wave pulse of 10-ms duration. The resulting muscle twitch was recorded with the force transducer, calibrated to units of Newtons (N) using a known mass. The record of the stimulus and the corresponding muscle twitch were recorded using iWorx Laboratory Scribe version 3 software. The magnitude of peak muscle force elicited by stimulation was measured from recordings at a later time using tools within the Laboratory Scribe version 3 software.

Measurement of cardiac muscle contraction. Frogs were secured to a dissection pan in a position of dorsal recumbency, with pins attached to the dissection pan through the distal forelimbs. The skin and sternum were removed to expose the heart, and a metal hook attached to a short length of dental floss was secured through the apex of the heart. The heart was then connected to a force transducer (iWorx FT-104) using the floss connected to the heart. The force traces over time were recording using Laboratory Scribe software, as described above.

Experimental approach. Two different approaches were used to assess the effects of MS-222 on the nerve and muscle preparations. First, whole frogs were immersed in a 1.5 g/l solution of MS-222 in 5% dibasic sodium phosphate (pH 7.0). They were bathed in this solution for 20–30 min and then rinsed with deionized water. Frogs were then double pithed, as described above. Next, they were prepared as described above, first for assessment of skeletal muscle function, and then for cardiac muscle function.

In the second approach, frogs were immersed in an ice water bath for 15–20 min before being double pithed. Next, the frogs were prepared to measure skeletal muscle contraction elicited by stimulation of the sciatic nerve. After recording several baseline contractions,

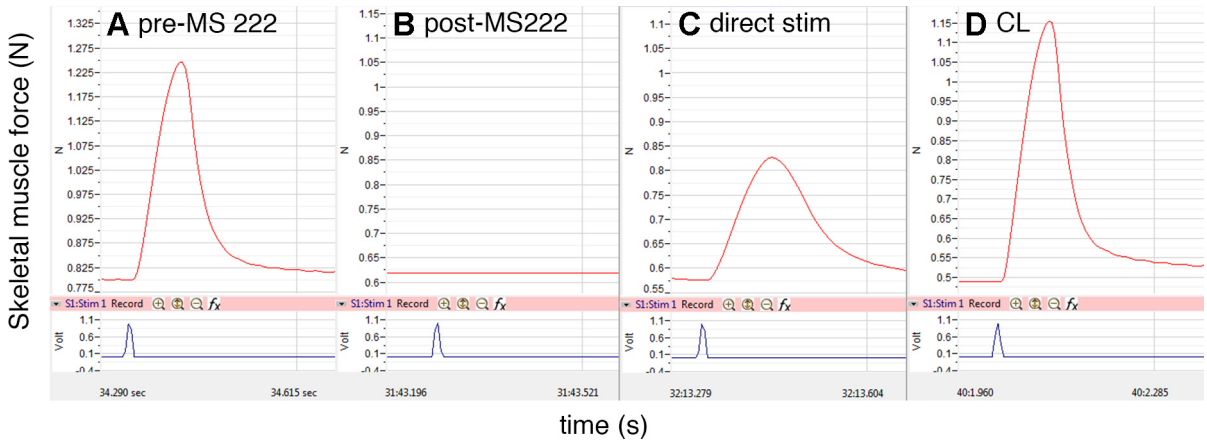

Fig. 1. Effects of MS-222 (tricaine methanesulfonate) on skeletal muscle contraction. *A*: gastrocnemius muscle twitch (red trace) following electrical stimulation (blue trace) of the sciatic nerve. *B*: after ~30 min of MS-222 application to the sciatic nerve, the muscle failed to respond. *C*: direct stimulation of the muscle produced a contraction. *D*: stimulation of the contralateral (CL) muscle through the sciatic nerve produced a strong contraction.

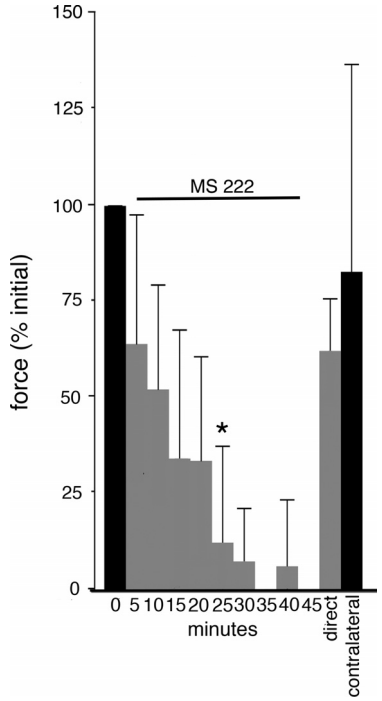

Fig. 2. Average effects of MS-222 (tricaine methanesulfonate) on skeletal muscle contraction. MS-222 was applied to the sciatic nerve over the indicated time period, and contractile force was assessed at 5-min intervals. After 25 min of MS-222 exposure, the average response was not significantly different from zero (*Wilcoxon signed-rank test: $P > 0.25$). After stimulation via the sciatic nerve failed, direct stimulation of the muscle continued to elicit muscle contraction. Following this testing period, the contralateral leg was prepared, and stimulation via the sciatic nerve consistently elicited contraction from the muscle. Solid bars identify responses from muscles never exposed to MS-222, whereas shaded bars are from MS-222-treated preparations. All values are means (SD).

MS-222 was applied to the sciatic nerve at intervals of ~5 min, and the contractile responses were measured periodically. After a period of time, the muscle failed to respond to these stimuli, and the muscle was then stimulated by a depolarization applied directly to the whole muscle. Following these recordings, the contralateral leg was prepared in the same way, and contractile responses were measured.

Following these experiments from the gastrocnemius muscle, the frog was prepared for cardiac muscle recordings. In every case, the heart was still contracting with a regular rhythm after the skeletal muscle experiments [mean heart rate 26.2 beats/min (SD 4.4); $n = 10$]. The heart was attached to a force transducer as described above, and baseline amplitude and rhythm were recorded for several minutes. Next, the MS-222 solution was dripped onto the exposed heart at intervals of ~5 min. The approach is similar to the way we apply pharmacologically active compounds (acetylcholine, atropine, and epinephrine) in our teaching laboratories. The recording was continued until the heart no longer contracted. In four control experiments, the hearts of frogs were permitted to contract without application of MS-222.

Data analyses. Force amplitude for skeletal and cardiac muscles and contractile frequency for the heart were quantified from recordings using measurement tools within the LabScribe version 3 software. Data were expressed in the form of Newtons for force and beats/min for heart rate. All of the values reported here are means (SD). For statistical analyses, forces from the skeletal muscles and from the hearts were standardized to percentage of initial contractile force to reduce variability among different preparations. Average responses were determined at 5-min intervals following the initial application of MS-222. All responses from preparations where MS-222 was applied directly to the nerve or heart muscle eventually failed to respond over time. The time points when average responses were not significantly different from zero were identified using a Wilcoxon signed-rank test. All statistical analyses were performed using JMP 10.0 software (SAS Institute, Cary, NC).

RESULTS

Skeletal muscle responses. Nine frogs were immersed in the MS-222 solution before attempting to stimulate the gastrocnemius muscle. In eight of the preparations, the skeletal muscles were completely unresponsive to stimulation, either through the sciatic nerve or when the muscle was directly stimulated. In one instance, the muscle contracted when stimulated through the nerve and after direct muscle stimulation. However, further stimulation of that preparation through the sciatic nerve failed after ~10 min. Direct stimulation of the muscle of that preparation produced only weak contractions.

In another eight preparations, frogs were chilled in ice water before double pithing. Skeletal muscles initially contracted

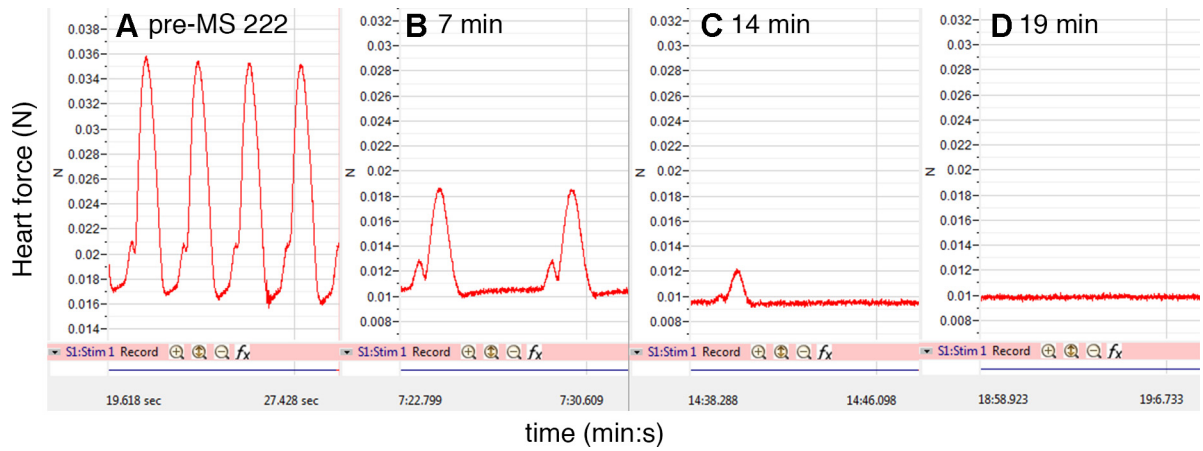

Fig. 3. Effects of MS-222 (tricaine methanesulfonate) on heart muscle contraction. *A*: the heart exhibited a strong, regular contractile rhythm before MS-222 was added [red trace shows heart force (N)]. *B*: ~7 min after applying MS-222, the amplitude and rate of contraction had declined. *C*: after 14 min, the amplitude had been reduced to a small fraction of the pre-MS-222 contractile force, and rate had also declined further. *D*: after 19 min of application, the heart completely stopped.

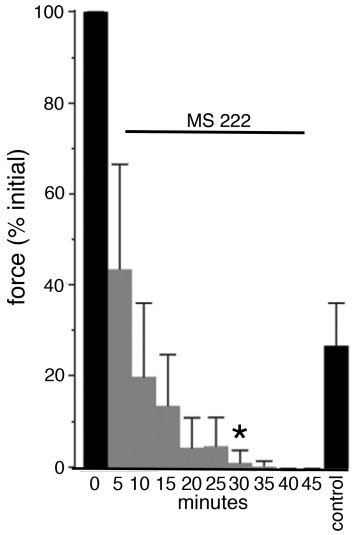

Fig. 4. Average effects of MS-222 (tricaine methanesulfonate) on cardiac muscle force. MS-222 was applied to the exposed heart over the indicated time period, and contractile force was assessed at 5-min intervals. The force of contraction in all hearts declined over time to zero. After 30 min of MS-222 exposure, the average contractile force was not significantly different from zero (*Wilcoxon signed-rank test: $P > 0.25$). Solid bars identify responses from hearts never exposed to MS-222, whereas shaded bars are from MS-222-treated preparations. Force of control hearts is shown after 1 h of monitoring, a time point when all MS-222-treated hearts had stopped. All values are means (SD).

strongly in response to stimulation through the sciatic nerve (Figs. 1 and 2). After recording several control contractions, MS-222 was applied to the sciatic nerve, and the contractile responses were recorded at ~5-min intervals. On average, muscles failed to respond to stimulation through the sciatic nerve after 25 min of MS-222 exposure (Fig. 2). After these muscles failed to respond, many of the muscles were stimulated directly, and each of these exhibited contraction. Furthermore, in several instances, the contralateral legs were prepared and demonstrated contraction when stimulated through the sciatic nerve (Figs. 1 and 2).

Cardiac muscle responses. Eight frogs were immersed in the MS-222 solution before their heart activity was investigated. In four of the frogs, the heart had stopped completely and was unresponsive on exposing the heart. In the other four animals, the hearts were still contracting, but with variable responsiveness. Two of the hearts were completely stopped, but being rinsed with Ringer solution and manual palpation of the hearts allowed them to develop a rhythm for a few minutes before stopping completely. The other two hearts were still beating with a regular rhythm that eventually became strong and regular after being rinsed with Ringer solution.

I used the hearts from 10 frogs that had been chilled in ice water, but not yet exposed to MS-222, to test the effects of applying the anesthetic directly on the hearts. In each of the preparations, the force of contraction and heart rate both declined steadily after dripping MS-222 solution directly to the heart (Figs. 3–5). On average, the force generated by the beating hearts was not significantly different from zero after 30 min of exposure to MS-222 (Fig. 4), while the average heart rate was not different from zero after 20 min (Fig. 5). All of the MS-222-treated hearts had stopped by 40 min. In four control frogs that were never exposed to MS-222, the hearts were still beating with a regular rhythm after 1 h (Figs. 4 and 5). In our teaching laboratories, frog hearts are highly robust and typically beat for several hours without stopping.

DISCUSSION

The results from all of the experiments clearly showed that MS-222 dramatically blocked normal nerve and muscle function (Figs. 1–5). When frogs were immersed in this solution before preparing them, we were unable to elicit responses from skeletal muscles in roughly 90% of the frogs tested, either through the sciatic nerve or by directly stimulating the muscle. In one-half of the frogs tested, the heart was completely inactive after the animals were immersed in MS-222. In the other one-half, the heart continued to beat after whole frogs were anesthetized in MS-222, but the heart activity soon stopped in one-half of these frogs.

Direct application of MS-222 to the sciatic nerve of frogs that had not already been exposed to this compound exhibited similar patterns of blocking muscle contraction (Figs. 1 and 2). Following nerve conduction block, the muscles still responded to direct stimulation of the muscle (Figs. 1 and 2). Furthermore, contralateral muscles continued to respond to nerve stimulation (Figs. 1 and 2). MS-222 application to the hearts of untreated frogs similarly abolished heart amplitude and rhythm in a predictable pattern (Figs. 3–5). The heartbeat of these frogs was completely blocked after <30 min on average. By comparison, those of us who teach in the physiology laboratories typically observe frogs that continue to have beating hearts after several hours in the teaching laboratory. The hearts of the control frogs used in the present study were still active after 1 h of study (Figs. 4 and 5). In fact, we have found that hearts that are completely removed from frogs at the end of a 3-h

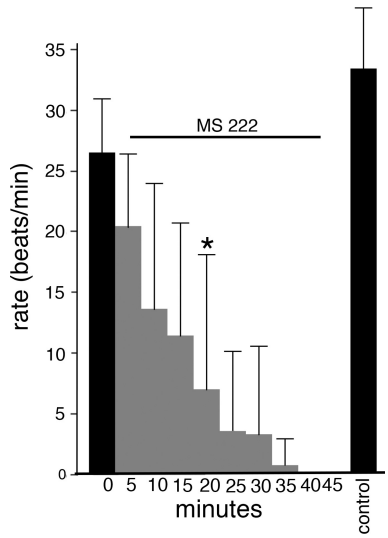

Fig. 5. Average effects of MS-222 (tricaine methanesulfonate) on cardiac muscle frequency. MS-222 was applied to the exposed heart over the indicated time period, and heart rate was assessed at 5-min intervals. The rate of contraction in all hearts declined to zero over time. After 20 min of MS-222 exposure, the average heart rate was not significantly different from zero (*Wilcoxon signed-rank test: $P > 0.125$). Solid bars identify responses from hearts never exposed to MS-222, whereas shaded bars are from MS-222-treated preparations. Rate of control hearts is shown after 1 h of monitoring, a time point when all MS-222-treated hearts had stopped. All values are means (SD).

laboratory will continue to contract if they are immersed in Ringer solution.

MS-222 is known to exert its effects by blocking voltage-gated Na^+ channels (1, 2, 5, 31), and the results observed in the experiments reported here are completely consistent with that mechanism of action. The action potentials within nerves, skeletal muscles, and cardiac muscles each depend on these voltage-gated Na^+ channels for their normal operation. Action potential conduction along neurons are dependent on voltage-gated Na^+ channel opening at the nodes of Ranvier (29). Action potential initiation and conduction within the muscle fibers are dependent on the same channels within the sarcolemma (29). The early depolarization phase of the cardiac action potential is also based on opening voltage-gated Na^+ channels (29). Consistent with this mechanism, the review by Downes (10) discusses peripheral neuromuscular inhibition as one of the key side effects of MS-222 as an anesthetic. A more recent study using zebrafish larva reported greater sensitivity of motoneurons to the effects of MS-222, compared with skeletal muscles (2). In that study, lower concentrations of the drug (0.16 g/l) completely inhibited movement, but direct depolarization of the muscles was able to elicit contraction. However, at a higher dose (0.84 g/l), the muscles no longer responded to direct stimulation. The authors inferred that differences in the precise voltage-gated sodium channel subunits might be responsible for these differences in sensitivity between motoneurons and muscles (2). Another recent study found that MS-222 administration significantly altered auditory brain stem activity from frogs, leading to changes in amplitude, threshold, and latency of brain stem responses to specific frequencies (9). In the present study, I found that immersion of frogs in 1.5 g/l of MS-222 abolished skeletal muscle contraction, whether stimulated through the sciatic nerve or through direct stimulation of the muscle. Given that MS-222 inhibits voltage-gated Na^+ channels in some fashion, it should come as no surprise that MS-222 interferes with the fundamental mechanisms that we are attempting to demonstrate to students in these teaching laboratories. All of the data collected within the present study support this interpretation.

An important point of consideration relates to the dosage and route of administration of MS-222. The AVMA guide to euthanasia recommends pithing as an adjuvant method for amphibians, only after the animals are fully anesthetized, and this is the procedure I followed in the present experiments. The AVMA guide further advises that prolonged immersion for up to 1 h may be required at dosages of 5–10 g/l (1). However, the dosages reported in the literature for amphibians vary widely (see Table 1 and references therein) (3, 6–12, 14–16, 18, 19,

Table 1. *Research papers, book chapters, and review papers focused on MS-222 immersion anesthesia for amphibians*

Dosage, g/l	Species	Context	Comments	Reference No.
0.025–0.5	*X. laevis* tadpoles	1, 4, 5	Blockage of nerve and motor activity	Ramlochansingh et al. (27)
0.1–0.8	*R. pipiens*	1, 4, 5	Examined dosages and compared with others	Cakir and Strauch (6)
0.1–1	*R. catesbeina*	1, 5	Inhibition of fictive respiratory; applied directly to central nervous system	Hedrick and Winmill (15)
0.15–1	Amphibians	4, 5, 6	General review of anesthetics for amphibians	Beckman (3)
0.2	*A. tigrinum*		Metamorphic vs. paedomorphic salamanders	Crook and Whiteman (8)
0.2–5	Amphibians	4, 5, 6	General review of anesthetics for amphibians	Mitchell (23)
0.2–1	Amphibians	4, 5, 6	Recommend 0.2 g/l for larva; 1 g/l for adults	Gentz (11)
0.5	*A. crepitans* *A. talpoideum* *B. fowleri* *D. fuscus*	1, 4	Refinement for anesthesia	Cecala et al. (7)
0.5–2	*D. monticola* *D. ocoee* *D. quadramaculatus* *E. wilderae*	1, 5, 7	Significant species differences; time to induction	Peterman and Semlitsch (26)
0.5–2	*P. elongatus* *P. cinereus* *E. eschscholtzii* *B. attenuatus* *D. ocoee*	1, 5, 7	Significant species differences; pH effects	Lowe (22)
0.5–5	Amphibians	4, 5, 6	Detailed review of anesthetics for amphibians	Wright (33)
0.5–5	*X. laevis*	2, 5	Recommend 5 g/l for at least 1 h to euthanize	Torreilles et al. (31)
1–5	Amphibians	4, 5, 6	Detailed review of MS-222 pharmacology	Downes (10)
1	*R. catesbeina*	3	Preparing animals for brain stem recordings	Hedrick and Morales (14)
1	*X. laevis*	1, 4, 6	Recommends 1 g/l with 30-min induction	Guenette et al. (12)
1	*B. alvarius*	1, 4	Mean induction time of ~20 min	Wojik et al. (32)
1–3	*X. laevis*	1, 5	No effect on heart rate, but respiratory depression	Lalonde-Robert et al. (19)
1–4	*A. mexicanum*	1, 5	Recommend 2 g/l for procedures lasting 20–30 min	Zullian et al. (34)
2	*B. daunchina*	1, 3	MS-222 impacted auditory brain stem physiology	Cui et al. (9)
2	*X. laevis*	4	MS-222 used as control for other anesthetics	Smith et al. (30)
3	*R. marina*	1, 4	Effects of MS-222 on hormonal stress response	Hernandez et al. (16)

Notes for context are as follows: [1]testing of anesthetic and physiological effects; [2]refinement for euthanasia; [3]anesthesia in preparation for research protocol; [4]comparison with other anesthetics; [5]compared or discussed dosages of MS-222; [6]review paper or chapter; and [7]compared species. Species are as follows: *Acris crepitans*, northern cricket frog; *Ambystoma mexicanum*, Mexican axolotl; *Ambystoma talpoideum*, mole salamander; *Ambystoma tigrinum*, tiger salamander; *Babina daunchina*, Emei music frog; *Batrachoseps attenuatus*, California slender salamander; *Bufo alvarius*, Sonoran toad; *Bufo fowleri*, Fowler's toad; *Desmognathus fuscus*, northern dusky salamander; *Desmognathus monticola*, seal salamander; *Desmognathus ocoee*, ocoee salamander; *Desmognathus quadramaculatus*, blackbelly salamander; *Ensatina eschscholtzii*; Ensatina salamander; *Eurycea wilderae*, Blueridge two lined salamander; *Plethodon cinereus*, red backed salamander; *Plethodon elongatus*, Del Norte salamander; *Rana catesbeina*, bull frog; *Rana pipiens*, leopard frog; *Rhinella marina*, cane toad; *Xenopus laevis*, African clawed frog.

22, 23, 26, 27, 30–34). Recommended dosages are influenced by the specific species being used and whether the animals are being prepared for surgery, being used for physiological study, or are simply being euthanized (Table 1). Dosages of ≤0.2 g/l have been recommended for anesthetizing *Rana pipiens*, due to prolonged recovery times, high mortality, and significant ECG changes at higher dosages (6). Similarly, Cecala et al. (7) administered MS-222 at a dosage of 0.5 g/l with a variety of amphibian species and found that concentration to be effective for anesthetizing the animals for surgery or other applications. At the other extreme is the recommendation by Torreilles et al. (31) of at least 1 h in 5 g/l to effectively euthanize *Xenopus laevis*, and this study is cited by the AVMA guide (1). For the purposes of physiology experiments in teaching laboratory, our results suggest that shorter induction times are better, and that exposure to MS-222 for 20 min or longer, with 1.5 g/l or higher concentrations, may make experiments untenable. It should also be noted that depth of anesthesia and recovery times are both positively correlated with the dosage of MS-222 administered (6, 20).

The most recent edition of the AVMA *Guidelines for the Euthanasia of Animals* (1) lists hypothermia as an unacceptable method for restraint or euthanasia for reptile and amphibians, unless they are very small (<4 g) (1). These recommendations are based on very little evidence, and recent papers have called the recommendations into question (17, 21, 28). A 2017 position paper published by an interdisciplinary group of highly accomplished experts from the field of herpetology has called the AVMA recommendations into question (21). These experienced researchers and veterinarians point out that the few references cited by the AVMA are speculative, and conclusions are drawn without empirical evidence. They also emphasize that the recommendations are based on extrapolation from our understanding of mammalian systems and reflect a lack of understanding of ectotherm physiology. For example, amphibians and other ectothermic vertebrates experience large variations in body temperature as a part of their natural history, both on a daily and seasonal basis (21). Recent studies have confirmed that cooling significantly impairs neurological activity in reptiles and amphibians (17, 28). We have used hypothermia as an accessory method to prepare frogs for double pithing for many years in the physiology laboratories at Fredonia. In our experience, chilled frogs appear to be calm and quiescent after the approximate 15–20 min of cooling in ice water. We are able to quickly and efficiently pith the frogs without any apparent signs of pain or suffering. Researchers with >25 yr of experience studying brain function in turtles concluded that hypothermia is currently the least stressful and most effective means to minimize discomfort to these animals preceding euthanasia (17). Based on the evidence presented by Lillywhite et al. (21) and from recent studies showing that thermal cooling blocks neurological function in reptiles and amphibians (17, 28), I propose that hypothermia can effectively be used as an accessory method before pithing frogs.

The reported experiments with the anesthetic MS-222 (tricaine methanesulfonate) demonstrate that this compound at the dosage examined (1.5 g/l) may be incompatible for use with frogs in preparations intended to demonstrate the physiology of nerve and muscle. MS-222 interfered with and abolished the very responses about which we intend to teach our students in the physiology laboratory (compound action potentials, skeletal muscle physiology, and cardiac muscle physiology). As noted previously, the *Guide for the Care and Use of Laboratory Animals* leaves discretion for deviations from the *AVMA Guidelines for the Euthanasia of Animals* "if justified for scientific or medical reasons" (24). The data presented in the present study clearly demonstrate scientific justification for not using MS-222 as an anesthetic when physiological responses are to be recorded. For instructors who must use MS-222 as mandated policy, I would encourage them to empirically determine the minimum dosage required to effectively anesthetize the frogs before pithing. I recommend beginning with a dosage of 1 g/l or lower and closely monitoring the frogs to gauge the progression of anesthesia. Instructors should remove frogs from anesthetic as soon as they become fully anesthetized and have been double pithed. They should then allow for adequate time for recovery of physiological responsiveness. Instructors should also be especially vigilant when using MS-222 with undergraduate students, since this compound is known to have toxic effects on humans (4). Indeed, this hazard is of particular concern when undergraduate students are involved, because they are less aware of hazards than experienced researchers, and gloves provide little protection from chemicals if students accidently touch their faces or other exposed areas while wearing gloves. I recommend preparing frogs before students are present and thoroughly rinsing the animals before being handled by students. Students should also be alerted to the potential hazards of MS-222.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author.

AUTHOR CONTRIBUTIONS

S.M. performed experiments; analyzed data; interpreted results of experiments; prepared figures; drafted manuscript; edited and revised manuscript; approved final version of manuscript.

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