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Pharmacologic Parameters of MS222 and Physiologic Changes in Frogs (*Xenopus laevis*) After Immersion at Anesthetic Doses

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We evaluated the anesthetic efficacy of MS222 (dose, 1 or 2 g/L; pH 7) administered as an immersion bath (duration, 20 min) for nonbreeding female *Xenopus leavis* frogs (n = 33; average body weight, 103 ± 16 g). The acid acetic test, the withdrawal reflex, righting behavior, heart rate, respiratory frequency, and blood oxygen saturation were used to evaluate the level of anesthesia. Acetic acid and withdrawal reflex responses were present at 30 and 60 min following immersion for the 1- and 2-g/L doses, respectively. MS222 had no effect on heart rate or oxygen saturation, but caused pronounced respiratory depression, as expected. Microscopic observations of selected tissues (heart, lung, liver, kidneys, and skin) showed no evidence of lesions at 24 h after immersion. In addition, we calculated the pharmacokinetics of MS222 in plasma and analyzed the drug by HPLC–tandem mass spectrometry. The calculated half-life of MS222 is 3.2 h. We conclude that MS222 administered at 1 or 2 g/mL via immersion bath for 20 min is an effective anesthetic that can be used for surgical procedures of less than 30 or 60 min, respectively, in *Xenopus leavis*.

Abbreviations: $AUC_{0\text{-}t}$, area under the time–concentration curve from time 0 to the last measurable concentration; AUC_{inf}, area under the time–concentration curve extrapolated to infinity; MS222, tricaine methanesulfonate.

African clawed frogs (*Xenopus leavis*) have been used extensively in research because their eggs can be collected either naturally or surgically after hormonal stimulation.[13] Tricaine methanesulfonate, commonly known as MS222, is the primary agent used to induce anesthesia for surgical interventions in amphibians.[3,26,27] MS222 acts similarly to other local anesthetics (such as lidocaine and benzocaine) by blocking sodium currents,[4] and has widely been used as a general anesthetic in fish and amphibians. Other drugs that have been used as anesthetics in amphibians include systemic injections of ketamine or tiletamine combined with zolazepam,[12] benzocaine,[2,21,25] barbiturates,[7,27] methoxyflurane and isoflurane administered topically or via a water bath,[17,18,23,26] intraceolomic or intravenous injections or bath immersion of propofol,[8,11,22] and bath immersion in a eugenol-containing solution.[6,9] Most of these anesthetics produce variability in depth and duration of anesthesia, with differences seen both within and between species, between sexes, and with animal weight and route of administration.[11] MS222 appears to be one of the most reliable anesthetics for amphibians, but little is known about physiologic changes during MS222 anesthesia in *Xenopus leavis*, and no data are available regarding the plasma pharmacokinetics of this drug in frogs.

The primary goal of the present study was to determine the anesthetic effects of MS222 administered in a bath solution, as well as the pharmacokinetics of this drug in *Xenopus laevis* frogs when administered for 20 min at anesthetic doses of 1 and 2 g/L.

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Materials and Methods

Animals and husbandry. Nonbreeding female frogs (n = 33; body weight [mean ± 1 SD], 103 ± 16 g; *Xenopus laevis*; Xenopus I, Dexter, MI) were used: 12 frogs (n = 6 per MS222 concentration) for the evaluation of physiologic and histopathologic changes associated with MS222, and 21 frogs (n = 3 per time point) for the pharmacokinetic study. Frogs were housed in salted (final concentration, 0.5 g/L; Instant Ocean Synthetic Salts, Aquarium Systems, Mentor, OH) water-filled (more than 4 L per frog) polycarbonate cages (40 cm × 20 cm × 15 cm; Ancare, Bellmore, NY). The purified water was obtained by processing municipal tap water by filtering (0.5-µm filter) and processing it by reverse osmosis and then passing it through activated charcoal, UV treatment (S12Q, Gold Sterilight UV Water Sterilizer, Guelph, Canada). Water quality parameters were pH 6.8 to 7.3, less than 0.1 ppm chlorine and chloramines, less than 0.2 mg/mL ammonia (normal range, 0.4 to 0.6 mg/mL), no nitrites (normal, less than 1 mg/mL), no hardness (normal, 70 to 150 mg/mL), no copper (all PVC tubing) or other heavy metals (iron, manganese), and a conductivity of 10 MΩ. Water and room temperatures were kept at 21 ± 1 °C at all times. Water was changed and the containers were cleaned twice weekly. Frogs were fed every other day with commercial chow (Xenopus Express, Brooksville, FL). The experimental protocol was approved by the IACUC of the Faculty of Veterinary Medicine of the University of Montreal prior to animal use and is in accordance with the guidelines of the Canadian Council on Animal Care.[1]

MS222 administration. For the immersion bath, recommended MS222 concentrations of 1 and 2 g/L were used.[3] MS222 was added to purified water, and the solution was buffered to a pH of 7.0 ± 0.4 with sodium bicarbonate (Sigma Aldrich, St Louis, MO) and kept at room temperature. Approximately 250 mL of this preparation was then put in a metal container (diameter, 15 cm; depth, 10 cm), and frogs were placed in this solution for 20

min, which is less than the maximal recommended induction time (30 min).[3] The container was covered so that the frogs were in full darkness for the induction period. After the immersion bath, frogs were rinsed thoroughly with purified water and placed into polycarbonate cages with purified water at the bottom, such that their nostrils were in contact with air. Frogs were rinsed regularly to keep their skin moist.

Pharmacodynamic study. For the evaluation of anesthetic depth, 5 different tests were used: the acetic acid test, the righting reflex, withdrawal reflex, heart rate, and oxygen saturation. Frogs were tested prior to the bath immersion (−15 min) and until recovery of baseline values and observations (that is, at 0 [immediately after the immersion bath], 15, and 30 min and at 1 and 2 h after bath immersion). The experimenter was present throughout the pharmacodynamic study.

Unrestrained frogs were assessed first with the acetic acid test, which is an indicator of depth of analgesia.[19] This test consists in the application of single drops of incrementally increasing concentrations of acetic acid to the dorsum of the frog's thigh or leg. Volume was controlled by using an automatic pipette that delivered 20 µL. We used 5 concentrations of acetic acid (0%, 5%, 10%, 20%, and 50%) to test the frogs' pain sensitivity. A contact time of 1 to 2 s was allowed before we thoroughly rinsed the tested skin area with purified water. The test was considered positive when the frog exhibited the wiping response, which is a motor reflex where the frog dislodges the drop by using its other leg. If no reaction was observed, the next higher concentration of acetic acid was applied alternating between right and left legs and thighs. The test was considered positive when the frog reacted to 5% acetic acid or higher.

Righting and withdrawal reflexes then were evaluated. The withdrawal reflex was tested by pinching one phalangeal articulation of the pelvic limb with surgical forceps for a maximum of 2 s. The righting reflex was evaluated as the frog's ability to turn on its ventrum when placed on its back.

Last, heart rate and oxygen saturation were measured by using a pulse oximeter (CANL-425V, Med Associates, St Alban, VT). To minimize variability in the readings when frogs were not anesthetized, a handler applied a 2-hand restraint and the reading recorded after the value on the pulse oximeter remained constant for at least 3 s. For the cardiovascular parameters, frogs were placed in a sternal position, and the probe was placed under the sternum.

For the evaluation of surgical anesthesia, small (0.4 cm) incisions were made through the abdominal skin and muscles (without entering the abdomen) at the 15- and 30-min and 1- and 2-h time points after the acetic acid test, righting reflex, withdrawal reflex, heart rate and oxygen saturation had been assessed. No sutures were applied. Any movement from the frog was considered as a sign of insufficient surgical anesthesia.

Pharmacokinetic study. Frogs were placed individually in an immersion bath containing MS222 at 2 g/L. Terminal intracardiac blood samples (0.3 mL) were collected in heparin tubes at 15 and 30 min and at 1, 2, 4, 6, and 12 h after immersion. The experimenter was present throughout the pharmacokinetic study.

When frogs were anesthetized insufficiently for intracardiac blood sampling, a rapid anesthesia with eugenol (concentration, 350 mg/L; immersion for 15 min prior to the blood collection time point) was performed.[9] This intervention likely had no effect on the plasma concentration of MS222 as measured by HPLC–tandem mass spectrometry. Briefly, the HPLC system was a Perkin–Elmer liquid chromatography apparatus (Series 200, Boston, MA), and spectrometry system used was an API 2000 QTRAP (AB-Sciex, Concord, Canada). Data acquisition and analyses were performed by using Analyst 1.4 (Applied Biosystems/MDS SCIEX, Concord, Canada). Calibration curves were calculated by using the equation $y = ax + b$, as determined by weighted (1/x) linear regression of the calibration line constructed from the peak-area ratios of the drug and the internal standard. MS222 was extracted from frog plasma by using a protein precipitation method (50 µL plasma was mixed with 500 µL of internal standard solution (1 µg/mL phenylephrine in acetonitrile) in a 1.5-mL centrifuge tube). Samples were vortexed vigorously and allowed to rest 10 min at room temperature prior to centrifugation. Samples were centrifuged at approximately 12,000 × *g* for 10 min, and 300 µL of the supernatant was transferred into a 400-µL injection vial. Chromatographic separation was performed by using an isocratic mobile phase with a Thermo Aquasil C18 100 × 2.1 mm (3 µm) column (Thermo Scientific, Waltham, MA). The mobile phase consisted of acetonitrile, water, and formic acid at a ratio of 80:20:0.4, respectively. The flow rate was fixed at 250 µL/min. Five microliters of the extracted sample was injected, and the total run time was set to 3.5 min. The mass spectrometer was interfaced with the HPLC system by using a pneumatic-assisted electrospray ion source. The N_2 settings for ion source gas 1 was set to 20 units, ion source gas 2 was set to 50 units, the temperature was set to 400 °C, and the ESI electrode was set to 5000 V. The declustering potential was set at 50 V, the entrance potential was set to 10 V, and the collision energy was set to 30 V. The collision gas used was nitrogen (set to medium). Selected reaction monitoring transitions were m/z 166 → 138 and 168→ 91 for MS222 and phenylephrine respectively. The dwell time was set at 100 ms and the pause time at 5 ms.

The observed coefficient of determination was greater than or equal to 0.9922. The coefficient of variation (an indicator of precision) ranged from 0.3% to 5.2%, and the accuracy observed was 95.3% to 113.9%. The analytical ranges used were from 0.1 to 50 µg/mL.

Histology. Frogs used in the pharmacodynamic study were euthanized at 24 h after the experiment, and selected tissues (heart, lungs, kidneys, liver, and skin) were fixed in 10% formalin and embedded in paraffin. Sections (thickness, 5 µm) were stained with hematoxylin–eosin–saffron and evaluated by microscopy.

Statistical analysis. Statistical analysis of heart rate, respiratory frequency, and oxygen saturation was performed by using SAS (version 9.2, SAS Institute, Cary, NC) according to the repeated-measures linear model, with time as a within-subject factor. Statistical significance was set at a P value of less than 0.05.

Pharmacokinetic parameters of MS222 in plasma were calculated by using noncompartmental methods.[14] The area under the curve from time 0 to the last measurable concentration ($AUC_{0\text{-}t}$) was calculated by using the linear trapezoidal rule. A terminal rate constant of elimination was calculated by using a minimum of 3 measurable plasma concentrations, and the terminal elimination half-life was calculated as $0.693 \div k_{el}$, where k_{el} is the elimination constant. The AUC extrapolated to infinity (AUC_{inf}) was calculated by using $AUC_{0\text{-}t} + C_{last}/k_{el}$, where C_{last} is the last measurable plasma concentration.

Results

Pharmacodynamic study. For the acetic acid test at baseline, all frogs in both the 1- and 2-g/L groups reacted at the 5% acetic acid concentration. After immersion in MS222 at 1 g/L, all frogs lost the withdrawal reflex and response to acetic acid completely; the reflexes returned at 52 ± 13 min and 30 min for the

1- and 2-g/L groups, respectively. The righting reflex returned by 58 ± 4 min, and spontaneous movements were observed at 40 ± 14 min. For the evaluation of surgical anesthesia, no reactions (spontaneous movements) occurred before 30 min.

After immersion in the 2-g/L immersion bath, frogs lost the withdrawal reflex completely; it returned at 80 ± 23 min. The acetic acid response first occurred in 33 and 66 % of the animals at 60 and 120 min. The righting reflex was seen at 118 ± 25 min, and all frogs showed spontaneous movements at 83 ± 19 min. For the evaluation of surgical anesthesia, 40% of frogs first reacted at 60 min and the rest at 120 min.

Heart rate, respiratory frequency, and oxygen saturation after immersion in MS222 are presented in Figures 1 through 3, respectively. For the 1-g/L MS222 immersion, postexposure values were significantly ($P < 0.01$) different from baseline values, but no significant effects between the 2-g/L postexposure results, suggesting that the stress of restraint may have increased heart rate at baseline. MS222 induced significant ($P < 0.0001$) respiratory depression immediately after MS222 exposure that returned very rapidly to baseline values within 15 min after exposure; no significant changes were noted for oxygen saturation.

For the 2-g/L MS222 immersion, no significant changes were noted in heart rate and oxygen saturation. The variability in the baseline heart rate variability is important and suggests that restraint-associated stress may be a contributing factor; however, no decrease of heart rate after anesthesia was seen. Significant ($P < 0.001$) respiratory depression was present immediately and at 15 min after MS222 exposure but had started to return to baseline by 30 min after exposure. Even though respiratory depression occurred at both MS222 concentrations, oxygen saturation stayed within normal limits, suggesting that greater oxygen exchange may occur through the skin than the lungs.

Pharmacokinetic study. The pharmacokinetics of plasma concentrations of MS222 in frogs after a 2-g/L immersion bath were a maximal blood concentration of 38.7 µg/mL at 15 min, with an AUC_{last} of 58.9 µg/h/mL and an AUC_{inf} of 62.5 µg/h/mL (Figure 4). The calculated elimination half-life is 3.2 h.

Histology. Microscopic observations revealed a normal appearance of all tissues when compared with normal histology,[25] independent of the MS222 dose exposure. This is a common finding in many studies using MS222.

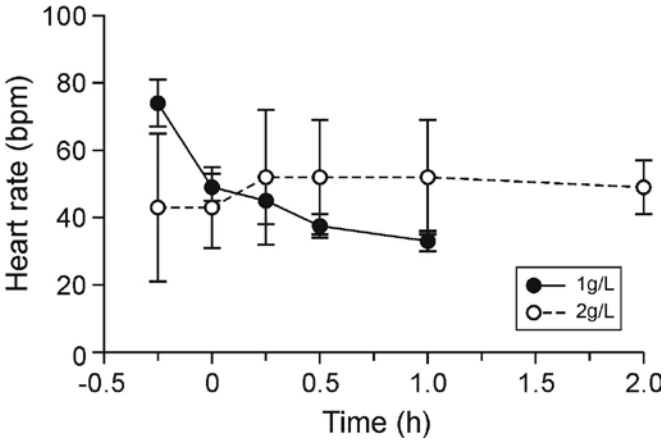

Figure 1. Heart rate (bpm; mean ± 1 SD) of *Xenopus laevis* frogs ($n = 6$/dose) after their immersion for 20 min in a solution of MS222 at either 1 or 2 g/L. The −30 min and 0 min time points respectively represent control values prior to and immediately after immersion.

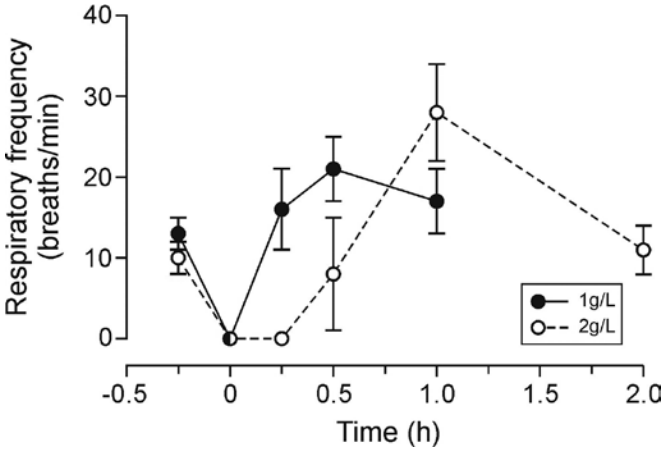

Figure 2. Respiratory frequency (breaths per minute; mean ± 1 SD) of *Xenopus laevis* frogs ($n = 6$ per dose) after their immersion for 20 min in a solution of MS222 at either 1 or 2 g/L. The −30 min and 0 min time points respectively represent control values prior to and immediately after immersion.

Discussion

MS222 produces an effective level of anesthesia in *Xenopus laevis* frogs when administered via bath immersion for 20 min at a concentration of 1 or 2 g/L. The surgical anesthetic evaluation and the acetic acid test both revealed that anesthesia duration is less than 30 and 60 min with 1- and 2-g/L MS222, respectively. For 2-g/L MS222, although the withdrawal reflex appeared in all frogs approximately 20 min later than did the acetic acid test response, the acetic acid response was present in only 33% of frogs at 30 min; therefore the acetic acid test and withdrawal reflex should be used concurrently to evaluate anesthesia depth after the immersion of frogs in MS222. This finding suggests that MS222 may provide some skin analgesia at increased concentrations. We previously showed that with the use of eugenol, the acetic acid test always occurred earlier that the withdrawal reflex, suggesting that the withdrawal reflex is the best indicator of anesthesia depth,[6,9] a result that was not seen in the current study. The righting reflex and spontaneous movements appeared approximately at the same time as did the withdrawal reflex for the 1-g/L MS222 concentration and later than did the withdrawal reflex for the 2-g/L MS222 concentration. In light of our combined results, we conclude that immersion of frogs in a bath of 1 or 2 g/L MS222 for 20 min is appropriate for surgical procedures lasting less than 30 or 60 min, respectively, and that 2 g/L is the better option. Our findings are in accordance with actual anesthesia practices in most research facilities working with *Xenopus leavis*. [26,27] However, we only evaluated the frogs at 15 and 30 min; more frequent checks during the first 30 likely would be useful for effectively monitoring the frogs.

Because MS222 primarily blocks sodium conductance[4] and can induce both sensory desensitization and motor blockade at high doses,[15] the use of this drug might be questionable, because a paretic or paralytic effect is possible. That is, MS222 could inhibit the sensory and motor functions of the peripheral nervous system without having an effect on the CNS. Regardless, MS222 remains a viable choice for general anesthesia, because all sensory input to the brain is lost; however because of the lack of loss of consciousness (a usual criterion for general anesthesia), subsequent events might be stressful for the frog. We know little about the blood–brain barrier in frogs, and if MS222 does cross into the brain, it likely acts as a central depressant. This hypothesis needs to be verified before any conclusion can be made regarding the central anesthetic effects of MS222 in frogs.

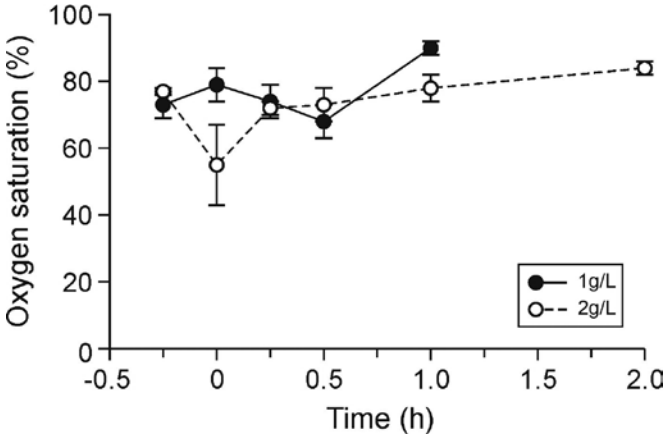

Figure 3. Oxygen saturation (%; mean ± 1 SD) of *Xenopus laevis* frogs (n = 6/dose) after their immersion for 20 min in a solution of MS222 at either 1 or 2 g/L. The –30 min and 0 min time points respectively represent control values prior to and immediately after immersion.

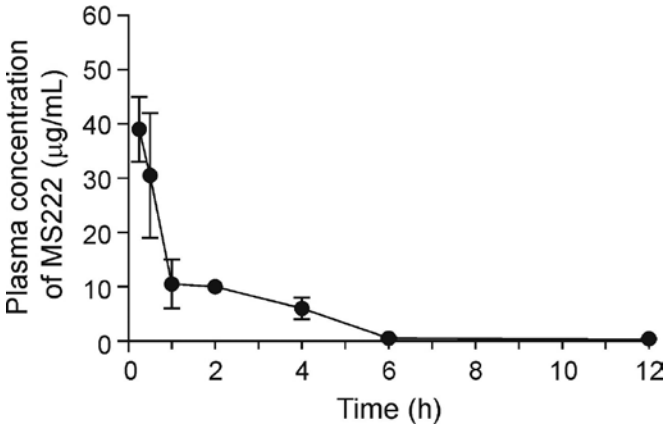

Figure 4. Plasma concentration (mean ± 1 SD) after the immersion of *Xenopus laevis* frogs (n = 6/dose) for 20 min in a solution of MS222 at either 1 or 2 g/L. The –30 min and 0 min time points respectively represent control values prior to and immediately after immersion.

Heart rate and blood oxygen saturation were relatively unaffected by anesthesia with MS222. However, the respiratory rate was depressed severely after bath immersion, and the duration of this response is not an indicator of surgical anesthesia. For 1-g/L MS222, mean heart rate differed significantly between baseline and after exposure. This difference may be explained by handling-associated stress before anesthesia, given that the frogs in the 1-g/L group struggled considerably while baseline values were being obtained. Under stressful conditions, hyperglycemia and tachycardia have been reported in frogs even during anesthesia with MS222.[10,16] In the context of its cardiorespiratory effects, MS222 is a good anesthetic for frogs, because oxygen saturation was well conserved throughout anesthesia. We obtained similar cardiorespiratory findings when we compared the present results with our previous findings from eugenol bath immersion of frogs similar in body weight to the current animals.[9] This result suggests that although *Xenopus* frogs are mouth breathers and although oxygen exchange through the skin has been considered less important in this species, the blood flow in the skin may increase to maintain oxygen saturation. This compensation could easily be verified by using a laser flow meter to measure capillary blood flow during anesthesia. However, different results are obtained in *Rana catesbiana*, in which MS222 causes severe apnea, thereby inducing a state of acidosis and hypoxia.[5,20] However, these previous experiments were performed in unbuffered solutions, and the acidity of the solution likely induced the acidosis and hypoxia in the frogs.[3] Although MS222 is still reported to cause hypoxia[3], this effect may be due to the preparation of the solution or to differences across species.

Amphibians require much higher concentrations of MS222 than do mammals;[3] the low plasma availability (AUC) of MS222 in frogs may explain this difference. Although the half-lives of MS22 (3.2 h) and eugenol (4 h) are similar, the elimination and availability of these 2 drugs differ somewhat. The elimination of MS222 dips markedly between 2 and 4 h, suggesting either slow hepatic metabolism or redistribution of the drug, possibly from fat tissue given that the drug is highly lipophilic.[9] Even though MS222 is readily soluble in water, it is highly lipophilic and nonionized in plasma, making MS222 a drug that will distribute well in living organisms.[3] The metabolism of MS222 is temperature-dependent, and in vitro studies with liver homogenate show very slow metabolism of MS222 in frogs.[24] However, our current study revealed that MS222 has a relatively short half-life and low drug availability (according to the AUC) in frogs—findings that suggest that this drug is nontoxic after repeated administration. However, the marked relaxant effect of MS222 on heart muscle is a possible reason for the reported muscle toxicity in poikilotherms.[23] At doses of 1 and 2 g/L, we did not see any effect of MS222 on heart rate, cardiac ECG tracings (not shown), or cardiac histology, thus suggesting that no significant heart toxicity occurred.

In conclusion, one-time immersion of African clawed frogs in MS222 at 1 or 2 g/L is effective and does not appear to induce toxicity. This treatment had no effect on heart rate or oxygen saturation, and all frogs recovered without any apparent side effects. The pharmacokinetics of MS222 do not suggest that daily administration at an anesthetic dose would be detrimental to the frogs, given that its half-life of 3.2 h supports a lack of drug accumulation with repeated administrations. Both MS222 and eugenol[6,9] showed similar results, and we therefore recommend MS222 as an anesthetic for *Xenopus leavis*.

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