Evaluation of righting reflex in cane toads
(Bufo marinus) after topical application
of sevoflurane jelly

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**Objective**—To evaluate the righting reflex after topical application of a sevoflurane jelly in cane toads (Bufo marinus).

**Animals**—8 cane toads.

**Procedures**—Toads were 6 to 8 months of age and weighed (mean ± SD) 142.0 ± 25.2 g. Sevoflurane jelly was applied to the dorsum of each toad at a dose of 25 µL/g in trial 1 and 37.5 µL/g in trial 2. Toads were placed in dorsal recumbency every 30 seconds until loss of the righting reflex. Jelly was then removed by rinsing the toads with tap water. Toads were then left undisturbed in dorsal recumbency until return of the righting reflex. Chamber sevoflurane concentration was measured to determine vaporization.

**Results**—6 of 8 toads in trial 1 and 8 of 8 toads in trial 2 lost the righting reflex. Mean ± SD time to loss of the reflex was 8.2 ± 1.3 minutes for trial 1 and 8.3 ± 0.9 minutes for trial 2; this difference was not significant. Mean ± SD time to return of the reflex was 25.6 ± 26.2 minutes for trial 1 and 84.4 ± 47.2 minutes for trial 2; this difference was significant. Chamber sevoflurane concentration did not change significantly, compared with baseline (time 0) concentration, at any time in trial 1; however, there was a significant change in chamber sevoflurane concentration from baseline (time 0) concentration in trial 2. Chamber sevoflurane concentrations were not significantly different between trial 1 and trial 2 at any time. Mean ± SD chamber sevoflurane concentration was 0.46 ± 0.2% for trial 1 and 0.57 ± 0.28% for trial 2.

**Conclusions and Clinical Relevance**—Sevoflurane jelly applied topically at a dose of 37.5 µL/g induced a more reliable loss of righting reflex and longer recovery time than when applied at a dose of 25 µL/g in cane toads. (Am J Vet Res 2013;74:823–827)

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A mphibians have been characterized as the evolutionary step between aquatic and terrestrial life.1,2 Animals of the amphibian order Anura (frogs and toads), in particular, have developed a respiratory system adapted for terrestrial life, and although larval forms have gas exchange mainly through gills, adult anurans respire through a combination of pulmonary and cutaneous routes.3 In addition, some species also have functional gas exchange in the buccal cavity.2 Pulmonary respiration in anurans occurs through simple saclike lungs, and ventilation of these structures is considered the most important factor in gas exchange.1,2 Cutaneous respiration is achieved via simple diffusion of gas across moist skin, acting as a supplement to pulmonary gas exchange.1 This cutaneous route has the potential to be used to deliver anesthetic agents in amphibian patients. In fact, isoflurane, tricaine methanesulfonate (MS-222), and eugenol have been applied topically to achieve anesthesia in a variety of amphibian species.4–12 Sevoflurane is a volatile ether anesthetic introduced into the veterinary market for general inhalation anesthesia in the late 1990s.13 Although there is a single report14 of the development of topical formulations of sevoflurane for use in anurans, to the authors’ knowledge, there are no reports describing the anesthetic effects of topically applied sevoflurane to provide anesthesia in anurans. The objectives of the study reported here were to develop and evaluate a topically applied compounded sevoflurane jelly as an anesthetic agent in cane toads (Bufo marinus).

**Materials and Methods**

**Animals**—This study was approved by the University of Illinois Institutional Animal Care and Use Committee. Animals were 6 to 8 months of age and weighed (mean ± SD) 142.0 ± 25.2 g. Sevoflurane jelly was applied to the dorsum of each toad at a dose of 25 µL/g in trial 1 and 37.5 µL/g in trial 2. Toads were placed in dorsal recumbency every 30 seconds until loss of the righting reflex. Jelly was then removed by rinsing the toads with tap water. Toads were then left undisturbed in dorsal recumbency until return of the righting reflex. Chamber sevoflurane concentration was measured to determine vaporization.

**Results**—6 of 8 toads in trial 1 and 8 of 8 toads in trial 2 lost the righting reflex. Mean ± SD time to loss of the reflex was 8.2 ± 1.3 minutes for trial 1 and 8.3 ± 0.9 minutes for trial 2; this difference was not significant. Mean ± SD time to return of the reflex was 25.6 ± 26.2 minutes for trial 1 and 84.4 ± 47.2 minutes for trial 2; this difference was significant. Chamber sevoflurane concentration did not change significantly, compared with baseline (time 0) concentration, at any time in trial 1; however, there was a significant change in chamber sevoflurane concentration from baseline (time 0) concentration in trial 2. Chamber sevoflurane concentrations were not significantly different between trial 1 and trial 2 at any time. Mean ± SD chamber sevoflurane concentration was 0.46 ± 0.2% for trial 1 and 0.57 ± 0.28% for trial 2.

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Committee. Eight individually housed cane toads (5 males and 3 females), approximately 6 to 8 months of age, weighing (mean ± SD) 142.0 ± 25.2 g were used in this study. Toads were housed individually in 48-L glass tanks in the same room that all procedures were performed. The room was kept at a temperature of approximately 26°C and 50% humidity, and toads were kept on a substrate of shaved coconut hulls, with a 12-hour photoperiod, and fed a diet of commercially raised, high-nutrition-fed crickets twice weekly during an acclimation period of at least 20 days.

**Anesthetic test chamber design**—An anesthetic chamber was built for this study on the basis of an invertebrate surgery chamber (Figure 1). The chamber was built as a 6.9-L, clear, airtight container. Two 10.2-cm-diameter round holes were cut into opposite sides of the container, and a 10.2-cm-diameter polyvinyl chloride male pipe connector was inserted into each hole. Silicone sealant was applied around the sides of the container, and a 14-gauge IV catheter, trimmed to 2.5 cm in length, was inserted from the outside with rubber washers to achieve an airtight seal. An airway gas sampling line was attached between the hub of the catheter and an airway gas monitor for evaluating anesthetic sevoflurane concentrations. Foam-lined kitchen gloves were placed through the PVC pipe connector openings, and the glove openings were stretched inversely around the outside of the PVC connectors and secured with 1-inch medical tape to achieve an airtight seal. This allowed for manipulation of the toads without opening the anesthetic chamber.

**Anesthetic protocol**—Anesthetic jelly mixture was composed of 3 parts liquid sevoflurane, 3.5 parts aqueous nonspermacidal jelly, and 1.5 parts distilled water. Each component of the mixture was added to a catheter-tip 60-mL syringe, and the syringe was shaken vigorously while the end of the syringe was occluded with a finger. When mixed thoroughly, the mixture changed from colorless to an opaque, white jelly of uniform consistency. Anesthetic jelly was mixed individually for each toad immediately prior to application. Other mixtures were evaluated with greater amounts of sevoflurane (between 3.5 and 4.5 parts sevoflurane) to make a more concentrated jelly; however, the additional sevoflurane would not mix into the jelly.

Toads were weighed immediately prior to anesthetic jelly application. Anesthetic jelly was applied with a 12-mL syringe after jelly was transferred from the 60-mL syringe at a dose of 25 µL/g of body weight to the dorsum of toads and then spread evenly with a gloved finger across the entire dorsal surface from just caudal to the head to the opening of the cloaca. No anesthetic jelly was applied to the limbs or abdomen. Toads were then placed into the anesthetic chamber, and the lid was closed. By use of the gloves attached to the anesthetic chamber, toads were placed in dorsal recumbency at 30-second intervals until loss of righting reflex was noted (loss of ability to return to sternal recumbency). Anesthetic concentration in the anesthetic chamber air was recorded from the gas analyzer at 30-second intervals. Upon loss of righting reflex, toads were removed from the chamber and rinsed clean of anesthetic jelly with tap water. Additionally, the anesthetic test chamber was rinsed clean of any residual anesthetic jelly that may have come from toads. Toads were then placed back into the anesthetic chamber in dorsal recumbency and the lid was replaced. Toads were left undisturbed until spontaneous return to sternal recumbency, and recovery time was noted. Anesthetic concentration was monitored at 30-second intervals until recovery. After completion of the study with anesthetic jelly applied at a dose of 25 µL/g of body weight, 2 of the toads did not lose righting reflex but had an observed loss of coordinated motor activity. Additionally, the anesthetic plane of the other 6 toads was considered to be light and unsuitable for practical clinical use. Therefore, a second trial, with the same toads, after a minimum of a 2-week washout period, was performed with a higher dose of sevoflurane jelly (37.5 µL/g of body weight). The methodology was the same as the first trial. During the second trial, all 8 toads lost their righting reflex. The first trial was considered treatment 1, and the second trial was considered treatment 2.

**Statistical analysis**—The distribution of continuous data was evaluated via the Shapiro-Wilk test. Paired t tests were used to compare induction time, room temperature, and recovery time. A Friedman test for repeated measures was used to evaluate sevoflurane concentration over time. If a difference was detected, a Wilcoxon paired-rank test was performed to determine when differences over time occurred. Values of $P ≤ 0.05$ were considered significant. A statistical software program was used to analyze the data. Data are reported as mean ± SD values.

### Results

Time to loss of righting reflex for trial 1 was 8.2 ± 1.3 minutes for the 6 toads that lost their righting reflex. Time to loss of righting reflex for trial 2 was 8.3 ± 0.9 minutes. There was no significant ($P = 0.87$) dif-
Sevoflurane is a volatile anesthetic that has a vapor pressure of 160 mm Hg at 20°C. The maximal vapor concentration of sevoflurane vapor in room air at sea level (760 mm Hg) is 21%. The elevation of the laboratory where this study was performed was comparable to sea level, so a similar maximal concentration of sevoflurane vapor could be expected to be released from sevoflurane liquid. However, the formulation of the compounded jelly minimized the amount of sevoflurane that vaporized during the testing period up to time of loss of righting reflex. Chamber air concentrations of sevoflurane only reached a maximum of 0.73 ± 0.31% in trial 1 and 0.84 ± 0.37% in trial 2. On the basis of these findings, use of this formulation of topical sevoflurane jelly should be performed in airtight containers where scavenging of vapor is possible or in an outdoor field setting where human exposure could be minimized. Test chamber sevoflurane concentration was significantly different, compared with baseline, in trial 2 but not in trial 1. Nonetheless, there was no difference between trial 1 and trial 2 at any time point.

Discussion

Originally, this study was designed to test loss and return of righting reflex in toads after a single application of a compounded sevoflurane jelly at a dose of 25 µL/g of body weight (trial 1). This dose was selected from a study7 that used compounded isoflurane jelly on African clawed frogs (Xenopus laevis). However, in the present study, only 6 of 8 toads lost their righting reflex, and those that did were not considered to be at a stable level of immobilization or anesthesia to be clinically useful; several of the toads had signs of arousal and movement when handled, and it was suspected that none of the toads would have tolerated any procedure that caused even minimal pain (eg, venipuncture). Therefore, a second trial was proposed that would use the same dose of sevoflurane jelly (25 µL/g of body weight) with a 50% increased sevoflurane concentration in the compounded jelly. This proved unsuccessful because the increased amount of liquid anesthetic would not mix into the jelly after multiple vigorous mixing attempts. Therefore, the dose of the successfully mixed sevoflurane jelly formula was increased by 50% to 37.5 µL/g of body weight, and a second trial was performed (trial 2).

Trial 2 had more consistent results than trial 1 and was subjectively considered to be more clinically applicable because the toads had no signs of arousal when handled. The times to loss of righting reflex in both trials (approx 8 minutes) were not significantly different. The main difference between the treatments was in the number of toads that lost their righting reflex (6/8 in trial 1 vs 8/8 in trial 2). The use of a topically applied compounded isoflurane jelly has been described for use in amphibians. A similar dose range of 25 to 35 µL/g of body weight was recommended in that study, with the lower dose being recommended for more aquatic species and the higher dose being necessary for more terrestrial species because of greater skin thickness. Those recommendations may also hold true with the compounded sevoflurane jelly used in the present study, given that cane toads are an almost exclusively terrestrial species and have a noticeably thick skin.

Time to return of righting reflex was significantly different between the 2 treatments, with that of trial 2 being approximately 85 minutes, or >3 times that of trial 1 (approx 26 minutes). This was likely the result of the toads having reached a deeper plane of anesthesia in trial 2 because of a greater tissue uptake of anesthetic from the greater dose, which prolonged elimination and thus time to return of righting reflex.

Other anesthetic agents used in anurans have a depressive effect on heart rate and gular movements. Cardiovascular and respiratory variables were not monitored for the toads in the present study because the act of placing a Doppler probe or other device on the toads could have interfered with the loss and return of righting reflex. However, isoflurane has cardiorespiratory depressant effects that are known to occur in anurans, and being that sevoflurane has similar properties in mammals, it is probable that these effects can be expected in anurans as well.

Sevoflurane is a volatile anesthetic that has an effective concentration of 0.73% at sea level. A similar dose range of 25 to 35 µL/g of body weight was recommended in that study, with the lower dose being recommended for more aquatic species and the higher dose being necessary for more terrestrial species because of greater skin thickness. Those recommendations may also hold true with the compounded sevoflurane jelly used in the present study, given that cane toads are an almost exclusively terrestrial species and have a noticeably thick skin.

Figure 2—Anesthetic test chamber sevoflurane concentrations (mean ± SD) after topical application of a compounded sevoflurane jelly at a dose of 25 µL/g (trial 1 [circles]) or 37.5 µL/g (trial 2 [triangles]) on cane toads. *Value is significantly (P < 0.05) different from baseline (time 0) concentration at individual time points for trial 2.
These findings could be interpreted that vaporized sevoflurane in the test chamber air did not contribute to the loss of righting reflex. However, these results must be interpreted with caution, considering that a lack of air movement in the test chambers may have resulted in stratification of air sevoflurane concentration and that the air sampling port was at the top of the chamber; therefore, the gas sampled for analysis may not have been representative of the air immediately surrounding the toads. Again, most important is that vaporized sevoflurane was recorded in the chamber air and that care should be taken to minimize human exposure. Sevoflurane has an MAC of 2.36% in dogs. Minimum alveolar concentration values of volatile anesthetics are typically conserved across mammalian species, so MAC in one species of mammal is similar in another species. In nonmammalian species, MAC is described as the ED50. The ED50 of sevoflurane in cane toads is unknown. The ED50 of halothane in toads is 0.76%, which is similar to the MAC of halothane in dogs of 0.86%. If an assumption that sevoflurane MAC in dogs is similar to the ED50 of sevoflurane in toads is made, on the basis of the findings of halothane, then, in this study, test chamber air concentration of sevoflurane did not approximate 1 MAC of anesthetic. Therefore, pulmonary uptake, if it occurred, could not be solely responsible for the loss of righting reflex observed in the toads.

The dorsum of the toads was chosen as the site of application of the anesthetic jelly because, physiologically, it plays the most important role in cutaneous gas exchange in anurans. Anurans have a dedicated blood supply to the skin from 2 large cutaneous arteries that originate from the same source as the pulmonary arteries (the pulmocutaneous arches), and the greatest concentration of capillaries associated with these arteries is found in the skin of the back and thighs. Additionally, the dorsum provided a convenient surface to apply the jelly while the toads were restrained and allowed for a large surface on which to apply and spread the anesthetic jelly. A limitation to the use of the anesthetic jelly was that, while the study was performed, some of the jelly would rub off onto the container. It is unknown whether this affected the study results.

Ambient temperature has an effect on the ratio of pulmonary to cutaneous respiration in anurans. As temperature increases, most anurans increase the amount of pulmonary oxygen uptake, compared with cutaneous uptake. For example, in American bullfrogs (Bufo catesbeiana), at 5°C, pulmonary uptake of oxygen accounts for 32.8% of total uptake, whereas at 25°C, pulmonary uptake increases to 66%. In the present study, there was a significant difference in room temperature between the 2 treatment groups. However, that temperature difference was only 0.5°C and was not considered to be biologically important. It is unknown whether change in ambient temperature affects the uptake of topically applied compounded sevoflurane jelly in amphibians.

Another limitation of this study was the use of the righting reflex. The inability of an amphibian to spontaneously move from dorsal to sternal recumbency may not be an indicator of anesthesia. However, the loss of righting reflex is a commonly used endpoint for testing the anesthetic properties of other agents in amphibians. Additionally, loss of righting reflex is not necessarily associated with loss of nociception. Further studies are warranted regarding the suitability of this agent for painful or surgical procedures. The acetate acid pain test has been used to test for pain response in anesthetized amphibians and future studies could incorporate that test to further define the efficacy of the sevoflurane jelly. Subjectively, after loss of righting reflex, toads were completely nonresponsive to handling and most likely would not have responded to a simple procedure such as venipuncture. Additionally, toads likely could have been easily intubated and administered inhalation anesthesia if necessary. In this study, the anesthetic jelly was removed immediately after loss of righting reflex, and it is unknown whether a longer application time would be safe.

In the present study, the toads were not randomly assigned regarding the order in which they received the 2 doses of sevoflurane jelly. It is unknown whether this affected the results of the study, and it is possible that the toads became conditioned to the handling associated with the procedure and thus were less excitable during trial 2, compared with trial 1. However, the toads were not handled during the washout period, and subjectively, the toads were no easier to catch and restrain during trial 2.

The development of a topically applied anesthetic jelly with sevoflurane has been attempted previously. Use of a topical sevoflurane jelly would have the potential benefits of reducing cost, reducing variability of anesthetic uptake and effect, bypassing the respiratory system, easy removal, and short induction and recovery time. Additionally, a topically applied anesthetic agent could be useful in field situations where water quality is questionable or could negatively affect the animal, where water quality analysis is unavailable, or, in the case of terrestrial amphibians, where the technique would be less stressful than an immersion technique. Ardente et al tested 5 formulations of a sevoflu-rane jelly and determined that sevoflurane mixed with pluronic lecithin organogel or pluronic acid gel best achieved their goal of a homogeneous, smooth, creamy mixture. They attempted to create a formulation with carboxymethyl cellulose sterile lubricant, similar to what was used in the present study, but were unable to achieve a homogeneous gel because the sevoflurane would separate out as soon as vigorous mixing was stopped. However, the ratio of sevoflurane to sterile lubricant and water they used was 50:50, unlike the jelly used in the present study, which contained only 38% sevoflurane. When we tried to increase the amount of sevoflurane, it remained separated. After complete mixing, our jelly turned to a homogeneous, white, opaque jelly. We are unsure why the jelly turned opaque after mixing 3 clear substances (sevoflurane, lubricant, and water) because we did not perform any analysis of the jelly; however, we speculate that the sevoflurane and carboxymethyl cellulose formed an emulsion that resulted in the opaque appearance.

This study determined the potential usefulness of a topically applied compounded sevoflurane jelly. Future studies are necessary to further define its use. Other
areas that could be investigated include the effect of ambient temperature, the site of application, different species, and different anesthetic agents.

a. Snapware, Mira Loma, Calif.
b. BD Angiocath, BD Medical, Franklin Lakes, NJ.
c. Expert DR-5300W,Datascope Corp, Mahwah, NJ.
e. Priority Care, First Priority Inc, Elgin, Ill.
f. SPSS, version 18.0, SPSS Inc, Chicago, Ill.

References

9. Calık Y, Strauch SM. Tricaine (MS-222) is a safe anesthetic compound compared to benzocaine and pentobarbital to induce anesthesia in leopard frogs (Rana pippins). Pharmacol Rep 2005;57:467–474.