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Assessment of Xylazine for Euthanasia of Anoles (*Anolis carolinensis* and *Anolis distichus*)

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Intracoelomic (IC) injection of xylazine was evaluated as a chemical euthanasia method for *Anolis* lizards (*Anolis carolinensis* or *Anolis distichus*). Lizards were allocated into 5 groups of 10 animals each. Each group was euthanized by one of these methods: 10 mg xylazine (100 mg/mL) IC; 10 mg xylazine and 0.5 mg acepromazine (10 mg/mL) IC; 10 mg xylazine IC followed by intracardiac injection of 0.1 mEq KCl (2 mEq/mL) once heart beats were no longer discernable by Doppler; 500 mg/kg 1% $NaCO_3$-buffered MS222 solution IC followed by IC injection of 0.1 mL unbuffered 50% (v/v) MS222 solution (experimental groups); and 1.95 mg sodium pentobarbital, diluted 1:10 in sterile water (38.9 mg/mL) given IC (control group). Compared with those given sodium pentobarbital or MS222, lizards euthanized by using xylazine showed prolonged persistence of purposeful movement after cardiac arrest. Therefore, xylazine is not an acceptable alternative euthanasia agent for use in anoles.

Abbreviations: IC, intracoelomic; MS222, tricaine methane sulfonate.

A euthanasia agent suitable for collection of *Anolis* lizards in the field must be humane, effective, able to maintain high-quality whole-animal specimens, easy to use, and cost-effective. Public Health Service Policy on Humane Care and Use of Laboratory Animals[17] requires that euthanasia of ectotherms be consistent with the *AVMA Guidelines on Euthanasia*.[1] According to these guidelines, euthanasia techniques "should result in rapid loss of consciousness followed by cardiac or respiratory arrest and ultimately lead to loss of brain function."[1] However, this objective may be difficult to achieve in many reptiles.[24] For instance, the *AVMA Guidelines* recommend intravenous, intraabdominal, or intrapleuroperitoneal injection of sodium pentobarbital (60 to 100 mg/kg) for euthanasia of ectotherms, including lizards. In fact, sodium pentobarbital is one of the only chemical agents listed in the *AVMA Guidelines* as acceptable for euthanasia of reptiles, and injection of this agent is considered the method of choice for euthanasia of these animals.[1,2]

However, use of sodium pentobarbital presents a number of practical challenges. First, even in the hands of trained personnel, this method can be difficult or impossible to apply to any animal in which vascular access is challenging. Sodium pentobarbital can be injected into the coelomic cavity of reptiles, but subsequent death may take 30 min or longer.[1,2,12,17] Second, concentrated sodium pentobarbital solutions that are formulated for intravenous use are highly alkaline and may cause irritation of tissues and pain when injected extravascularly.[2,14] Third, sodium pentobarbital is classified by the US Drug Enforcement Administration as a Schedule II controlled substance when formulated alone and a Schedule III controlled substance when formulated as a combination product.[19] As a result, many institutions require security checks before personnel can buy or use sodium pentobarbital. In addition, the use of Schedule II controlled substances necessitates strict documentation practices, and multiple layers of securely locked storage containers are required, making the use of sodium pentobarbital in the field particularly inconvenient.[6] Finally, sodium pentobarbital can cause postmortem 'kinking' near the point of injection in reptiles, making it difficult to position preserved specimens in a manner that will permit subsequent measurement and examination of key features.[6]

Other euthanasia methods have been proposed. Tricaine methane sulfonate (MS222) is effective intracoelomically (IC) for euthanasia of reptiles[6] but has disadvantages. For instance, MS222 must be made up in a 2-step process and is costly when used to euthanize large reptile species.[1] Another alternative to sodium pentobarbital is propofol, an anesthetic agent that has been used in a 2-step process for euthanasia in lizards.[1] However, the second step is a physical method of euthanasia, such as decapitation or cervical dislocation; this requirement is incompatible with the acquisition of whole-animal specimens. Moreover, propofol is relatively expensive and has a short shelf life (approximately 6 h) once the seal on the bottle has been broken.[20] In December 2010, the DEA proposed a rule to place propofol into Schedule IV of the Controlled Substances Act.[8] Once this rule is put into effect, propofol will become even less desirable for use as a euthanasia agent in reptiles.

Because pentobarbital, MS222, and propofol are not ideal for euthanasia of reptiles, we here evaluate an alternative agent for euthanasia of lizard species, especially those that are collected in the field and will be fixed as scientific specimens for future morphometric studies. Xylazine, an α2 adrenergic receptor agonist, is a CNS depressant that also has marked central muscular relaxant properties.[9] Xylazine does not cause tissue irritation when administered extravascularly, is inexpensive, and is not a controlled substance. In this study, we evaluated the use of xylazine as an intracoelomic euthanasia agent in lizards (*Anolis carolinensis* and *A. distichus*). Parameters studied included times to cessation of heartbeat and purposeful movements after injection of xylazine, MS222, or sodium pentobarbital. We hypothesized that xylazine would be an acceptable euthanasia agent for use when injected into the coelomic cavity of *Anolis* lizards. We also hypothesized that the use of xylazine as a euthanasia agent would eliminate the postmortem body rigidity

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commonly seen in reptiles after euthanasia by intracoelomic sodium pentobarbital.

Materials and Methods

A colony of wild-caught *A. carolinensis* and *A. distichus* lizards was maintained in an AAALAC-accredited facility and provided with food, housing, lighting, and environmental parameters according to established standard operation procedures for these species. Briefly, the room temperature and photoperiod were cycled to mimic natural seasonality. From April through November ('summer cycle'), the room temperature was maintained at 85 °F (29.4 °C) with a 14:10-h light:dark cycle. From December through March ('winter cycle'), the room temperature was maintained at 83 °F (28.3 °C) with a 10:14-h light:dark cycle. All anoles were kept in commercially available reptile enclosures (Lee's Kritter Keeper, L Schultz, San Marcos, CA) in breeding groups of 1 male to 3 female lizards. Each cage included potting soil for substrate, 2 sterilized sticks as perches, and artificial ivy for foliage. Each cage was misted twice daily to supply anoles with drinking water and to maintain humidity in the cage at approximately 85% throughout the year. Anoles were fed 3 times weekly during the summer cycle and twice weekly during the winter cycle. At every feeding, crickets were dusted with a multivitamin supplement (Herptivite, Rep-Cal, Los Gatos, CA) and once weekly with a 1:1 mix of the multivitamin supplement and a calcium additive (Calcium Powder, Rep-Cal).

The protocol under which these lizards are used was approved by the University of Rochester Institutional Animal Care and Use Committee (Rochester, NY). At the conclusion of the study to which they were assigned, 50 lizards were allocated to the current analysis and received intracoelomic xylazine (100 mg/mL) in various combinations, MS222 (10 mg/mL), or sodium pentobarbital (38.9 mg/mL). The current study was performed at ambient temperatures of approximately 27 to 33 °C, the preferred optimal temperature zone for anoles.[23]

Xylazine experimental groups. Groups of 10 lizards each received xylazine (0.1 mL IC; 10 mg, 3030.3 to 8333.3 mg/kg) only or combined with 0.05 mL (0.5 mg; 135.1 mg/kg to 454.5 mg/kg) acepromazine (10 mg/mL); another 10 lizards were given xylazine (0.1 mL IC) followed by intracardiac injection of 0.05 mL (0.1 mEq; 32.3 to 90.9 mEq/kg) KCl (2 mEq/mL) once the heart had stopped. KCl was administered by 'blind stick,' and aspiration of blood to determine correct placement was attempted but not confirmed in all cases. However, the target area (the chest cavity) in these 1- to 3-g lizards is small, increasing the likelihood that the KCl dose was injected appropriately.

MS222 experimental group. MS222 was administered to a group of 10 lizards according to a 2-stage reptile euthanasia procedure as described previously.[6] Briefly, a 50% (v/v) solution of MS222 was made by combining equal volumes of MS222 and sterile water. Then, a small volume of 1% MS222 solution was made by adding 0.2 mL 50% (v/v) solution to 5 mL (1 teaspoon) of water. The 1% solution was titrated to pH 6.6 by using sodium bicarbonate. Stage one of MS222 euthanasia involved injecting 0.1 mL (1 mg; 312.5 to 555.6 mg/kg) of the buffered solution intracoelomically. After loss of response to deep toe pinch, lizards each received 0.1 mL (50 mg; 15,625 to 27,777.8 mg/kg) of the 50% (v/v) MS222 solution intracoelomically.

Sodium pentobarbital control group. Each of 10 control lizards received 0.05 mL sodium pentobarbital (diluted 1:10 in sterile water; 1.95 mg; 780 to 1147.1 mg/kg) intracoelomically.

Experimental design. For all groups, a digital timer was used to record time to cessation of heartbeat. Lizard heartbeats were amplified (Ultrasonic Doppler Flow Detector, model 811B, Parks Medical Electronics, Aloha, OR) so that they were readily audible. A small amount of gel (Signa Gel, Parker Laboratories, Fairfield, NJ) was applied to the Doppler probe. Immediately after the euthanasia agent was administered, the lizard's chest was positioned on the dab of gel over the probe. The lizard was gently and easily held in place. In general, once positioned on the gel, the lizards ceased attempting to move off of the probe. Lizard reflexive, motor, and purposeful movement activity after injection of the euthanasia agent and heartbeat cessation was recorded for as long as 5 min after injection. Motor activity assessment included observation for spontaneous tail and toe movements. In addition, lizards were checked for palpebral reflex by tapping gently at the medial canthus using a 22-gauge, plastic needle cap. In addition, lizards were tapped on the tip of the nose with a 22-gauge needle cap to see whether the lizard would bite on the cap, indicating purposeful movement. Once death was confirmed in the study lizards by lack of heart rate, respiratory rate, and evidence of all reflexes, the euthanized animals were given to the investigator from whom the lizards were obtained for study. The subject's liver was removed, and the animal was positioned for morphologic studies.

Statistical analysis. Prior to model selection, data were inspected graphically by using q-q plots. Because of slight deviation from normal distribution, generalized estimating equations were used to permit robust estimation and limit concern about violation of normal distribution assumption in linear models.[7,13] The analyses modeled the association of time to purposeful movement cessation and time to cardiac arrest with type of euthanizing agent, adjusting for sex, baseline heart rate, and weight in grams. Pairwise assessments of euthanizing agents were made subsequent to the overall analysis; no adjustments were made for multiple comparisons.

With an estimated reflex cessation time of 60 s in the sodium pentobarbital group and an expected difference of 40 s in the xylazine group, sample sizes of 10 and 30, respectively achieved 82% power to detect that difference, assuming a standard deviation of 36 s in both groups and an α level of 0.05, using a 2-sided Mann–Whitney test. All statistical analyses were carried out by using SAS 9.2 (SAS Institute, Cary, NC) on a Windows 7 platform (Microsoft, Redmond, WA).

Results

Characteristics of the *A. carolinensus* and *A. distichus* lizards used in each study group are summarized in Table 1. The sodium pentobarbital and xylazine groups used more female than male lizards. However, the MS222 group used more male than female lizards (Table 1). Therefore, the average weight of the MS222 group that mainly included male animals was greater ($P < 0.0001$) than that of the sodium pentobarbital group that predominantly comprised female subjects. This weight difference between groups occurred because the investigator chose the animals that were to be euthanized and formalin-preserved. Depending on the group to be culled for the investigator's purpose, a preponderance of male or female anoles was available. Average anole heart rate did not differ significantly between the groups (Table 1). Injection of xylazine alone or in combination with acepromazine resulted in rapid cessation of heartbeat in less than 1 min in all lizards (Table 2). Injection of sodium pentobarbital resulted in loss of heartbeat in approximately 62.4 s to more than 2 min (Table 2). MS222 euthanasia resulted in cessation of heartbeat in 1 min or less (Table 2).

In addition, when sodium pentobarbital was used, reflexes were reduced greatly, and purposeful movements terminated in approximately 1.5 min or less (Table 2). When xylazine was

Table 1. Characteristics of euthanasia regimen study groups

				P	
	Pentobarbital	Xylazine	MS222	Pentobarbital versus xylazine	Pentobarbital versus MS222
Heartrate at baseline[a]	173.20 ± 27.33	167.87 ± 19.33	174.20 ± 12.87	0.5491	0.9121
Weight (g)[a]	1.79 ± 0.49	1.86 ± 0.74	2.71 ± 0.52	0.7295	<0.0001
Sex[b]				0.7148	0.0698
Female	7 (70)	18 (60)	2 (20)		
Male	3 (30)	12 (40)	8 (80)		

[a]Data given as mean ± SE; χ^2 test subsequent to linear modeling using generalized estimating equations.
[b]Data given as number (%); *P* value obtained by using 2-sided Fisher's exact test.

Table 2. Time to event (min; mean ± SE) under various euthanasia regimens

			Pairwise comparison *P* value[a]				
Event	Time to event	Euthanizing agent	Xylazine + acepromazine	Xylazine only	Xylazine + KCl	MS222	Pentobarbital
Cardiac arrest							
	0.14 ± 0.10	Xylazine + acepromazine	—	<0.0001	0.2139	0.0005	<0.0001
	0.34 ± 0.10	Xylazine only	—	—	0.0032	0.0005	<0.0001
	0.20 ± 0.12	Xylazine + KCl	—	—	—	0.0004	<0.0001
	0.65 ± 0.47	MS222	—	—	—	—	0.0003
	1.04 ± 0.58	Pentobarbital	—	—	—	—	—
Purposeful movement							
	4.63 ± 0.61	Xylazine + acepromazine	—	0.0422	0.1364	<0.0001	<0.0001
	>5.00	Xylazine only	—	—	0.0037	<0.0001	<0.0001
	4.08 ± 1.05	Xylazine + KCl	—	—	—	<0.0001	<0.0001
	1.16 ± 1.12	MS222	—	—	—	—	0.7519
	1.03 ± 0.58	Pentobarbital	—	—	—	—	—

[a]χ^2 test subsequent to generalized estimating equations analyses

used as the euthanasia agent either alone or in combination with acepromazine or KCl, reflexes and purposeful movement were evident for at least 3 min and, in most cases, for more than 5 min (Table 2). On average, absence of purposeful movement occurred within 10 to 20 min after injection in the xylazine-treated group. There was no significant difference between sodium pentobarbital and MS222 regarding cessation of purposeful movement (Table 2). MS222 and sodium pentobarbital euthanasia both lead to death, including cessation of purposeful movement, significantly ($P < 0.001$) more quickly than did any of the xylazine-based regimens (Table 2).

Table 3 displays the associations between the variables weight, heart rate, sex, euthanasia agent, and times to cessation of heartbeat and purposeful movement (time to event) when these independent variables are evaluated as individual predictors and as a group of predictors in which each variable is adjusted for all other variables in the model. Data were recorded during 5 min of observation; any lizard with purposeful movement at 5 min cessation was noted as having cessation of movement at 5 min, for purposes of analysis. The analysis in this table indicates a significant ($P < 0.0001$) difference between xylazine and sodium pentobarbital with regard to cessation of both cardiac cessation and purposeful movement in both univariate and multivariate analyses. In other words, once we adjusted for differences in subject weight, heart rate, and sex, time to cardiac cessation was shorter ($P < 0.0001$) when xylazine compared with sodium pentobarbital was used. In addition, once we adjusted for the difference in subject weight, heart rate, and sex, time to cessation of purposeful movement was significantly ($P < 0.0001$) shorter when sodium pentobarbital compared with xylazine was used (Table 3).

Postmortem kinking and rigidity occurred at an unacceptable level with the injection of full-strength sodium pentobarbital. Postmortem rigidity was not seen with intracoelomic injection of MS222 or xylazine (either alone or in combination with other agents). When diluted (1:10) sodium pentobarbital was injected intracoelomically, the specimen was deemed acceptable for morphologic study.

Discussion

The main objective of this study was to compare intracoelomic xylazine as a euthanasia agent in anoles with intracoelomic sodium pentobarbital, currently one of the only recognized, acceptable injectable agents for euthanasia in reptiles.[1,4,5,12,14,22] Xylazine was considered as a possible euthanasia agent candidate for use in lizards, in light of published accounts of its misuse having been the cause of death in other species, namely humans.[16,21,26] In addition, we chose xylazine because of its ease of use in veterinary medicine, as it is not a controlled substance and is easily transported for use in the field. Furthermore, the subjects used in the current study were collected as part of a taxonomic study for later use as museum specimens. Therefore, assessing xylazine for suitability as a sole agent for morphologic preservation of carcasses was important.

Postmortem rigidity often occurs when anoles are euthanized with sodium pentobarbital. We found that diluting the sodium pentobarbital and injecting a 0.05-mL volume helped to decrease postmortem rigidity to yield reasonably acceptable

Table 3. Univariate and multivariate associations with time to event

	Cessation of heart beat				Cessation of purposeful movement			
	Univariate		Multivariate		Univariate		Multivariate	
Variable	β weight	*P*	β weight	*P*	β weight	*P*	β weight	*P*
Weight (g)[a]	0.0835 ± 0.0846	0.3233	0.0801 ± 0.0751	0.2860	−0.5565 ± 0.3792	0.1422	0.0206 ± 0.1437	0.8858
Baseline heart rate[a]	−0.0066 ± 0.0056	0.9848	−0.0018 ± 0.0037	0.6300	−0.0178 ± 1.7670	0.0852	−0.0055 ± 0.0063	0.3840
Female compared with male	−0.0271 ± 0.1306	0.8354	0.0557 ± 0.0806	0.4897	0.4613 ± 0.5352	0.3888	−0.0212 ± 0.0063	0.9287
Xylazine compared with pentobarbital[b]	−0.8127 ± 0.1765	<0.0001	−0.8222 ± 0.1799	<0.0001	3.5340 ± 0.3796	<0.0001	3.5013 ± 0.3865	<0.0001
MS222 compared with pentobarbital[b]	−0.3870 ± 0.2248	0.0851	−0.4307 ± 0.1997	0.0311	0.1200 ± 0.3796	0.7519	0.0960 ± 0.3833	0.8023

[a]β weight (mean ± SE) expresses the change in the time (min) to event per unit increase in the independent variable.
[b]β weight (mean ± SE) expresses the difference in mean scores as per comparison.

study specimens. However, this method did not eliminate postmortem rigidity completely.

The study was done at temperatures between 27 to 32 °C, temperatures reflective of the anoles' preferred optimal temperature zone. Drug absorption, metabolism, and distribution vary with ambient temperature and are optimal at the specific reptile species' preferred optimal temperature zone.[3,11,22,24] In addition, these ambient temperatures reflect the field conditions from which these animals are collected, and we wanted to mimic these conditions in our study.

One group of lizards received intracoelomic xylazine in combination with acepromazine, and another was given intracoelomic xylazine followed by intracardiac KCl. These agents were added to xylazine to determine whether either combination (or both) provided additional benefits to xylazine given alone. Acepromazine is a phenothiazine neuroleptic agent. Phenothiazines block postsynaptic dopamine receptors in the CNS and are used in veterinary medicine principally for their tranquilizing effects.[18] We hypothesized that adding acepromazine to xylazine might 'quiet' the CNS, resulting in elimination of reflexes and purposeful movement after heartbeat cessation; however, this benefit was not realized (Tables 2 and 3). Use of Doppler ultrasonography to ascertain heartbeat is a recognized method in reptiles[10,15] and was used to verify the cessation of heartbeats before intracardiac injection of KCl injection. Although heart sounds rapidly ceased to be heard on Doppler ultrasonography once intracoelomic xylazine was administered, we hypothesized that weak cardiac beats, undiscernable by Doppler, may have been present, providing oxygenated blood to the brain and leading to prolonged purposeful movement in euthanized lizards. This hypothesis was disproven, because time to cessation of purposeful movement continued to be significantly ($P < 0.0001$) longer in the group that received KCl as compared with that given sodium pentobarbital. Although time to cessation of purposeful movement differed between lizards given xylazine and intracardiac KCl compared with xylazine alone, this time was still longer than that obtained with the use of intracoelomic sodium pentobarbital or MS222.

Reptiles can function at low respiration and heart rates and are able to withstand prolonged periods of induced hypotension and anoxia.[25] Furthermore, snakes and lizards have demonstrated consciousness after decapitation.[25] Compared with mammals, reptiles have lower metabolic rates and are better adapted to use anaerobic metabolism. As a result, reptilian tissues are more resistant to the effects of hypoxia. Therefore, methods of euthanasia that cause hypoxia but do not address lingering consciousness should not be recommended for use in reptiles.[2]

Intracoelomic xylazine resulted in rapid cardiac arrest, as evidenced by lack of heartbeat on Doppler ultrasonography.[10,15] However, lizards injected with xylazine, alone or in combination with acepromazine or KCl, appeared to remain aware and had purposeful movements for significant periods of time, often for more than 5 min. Purposeful movement was established in the xylazine-injected groups by the presence of a palpebral reflex elicited when the lizard was tapped lightly at the medial canthus and by inducing a bite reflex when the anole was tapped on the nose with a syringe cap. In addition, after intracoelomic xylazine injection, several lizards jumped off the Doppler probe after heartbeat cessation and ran a few steps prior to being caught. Several other lizards appeared to take breaths after heartbeat cessation. Once the 5-min time limit was reached, time to cessation of movement was no longer recorded. However, the lizards continued to be monitored closely until death. Once death was confirmed by absence of heartbeat, respirations, and all movement, the anoles were given to the investigator for liver removal and body fixation. Continued, careful monitoring of lizards until death once the 5-min recording limit was achieved and transferring the lizard to the investigator directly thereafter ensured that the investigator could use the carcasses for his study, which involves precise morphologic measurements.

MS222 compared favorably to sodium pentobarbital when examined as a possible euthanasia agent for use in anoles, although our sample size for this comparison was limited. Although time to cessation of purposeful movement did not differ significantly between these 2 agents (Tables 2 and 3), time to cardiac arrest was significantly shorter for MS222 in the multivariate analysis. Xylazine was an unacceptable alternative to sodium pentobarbital for euthanasia in lizards. Although intracoelomic xylazine caused rapid cessation of heartbeats, as evidenced by lack of heart sounds on Doppler ultrasonography soon after injection, purposeful movement continued intermittently in study animals until study completion. As found in a previous study,[6] MS222 is an acceptable alternative to sodium pentobarbital for euthanasia of lizards, resulting in a shorter time to cessation of heartbeats and a reasonable and similar

time to cessation of purposeful movement after intracoelomic injection.

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