Journal of the American Association for Laboratory Animal Science
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Vol 58, No 6
November 2019
Pages 810–816

Use of Rodent Sedation Tests to Evaluate Midazolam and Flumazenil in Green Iguanas (*Iguana iguana*)

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This study aimed to evaluate the applicability of rodent behavioral tests to assess the effects of midazolam and flumazenil in green iguanas. Four tests commonly used to assess sedation in rodents—the open field test, forced swim test, behavioral scale, and traction test—were conducted in 10 juveniles iguanas. The animals received midazolam (2 mg/kg IM) or 0.9% NaCl (0.4 mL/kg IM), and the tests were conducted between 0 and 300 min thereafter. To verify the effects of midazolam and flumazenil, the most informative tests from the evaluation stage and the limb withdrawal latency time (LWLT) were used. All 10 iguanas were tested under 4 conditions, as follows: MS, midazolam (2 mg/kg IM), followed 30 min later by 0.9% NaCl (0.4 mL/kg IM); FS, flumazenil (0.05 mg/kg IM), followed by 0.9% NaCl (0.4 mL/kg IM) 30 min later; MF, midazolam (2 mg/kg IM), followed by flumazenil (0.05 mg/kg IM) 30 min later; and CON, 0.9% NaCl (0.4 mL/kg IM). The behavioral scale and the forced swim test showed the best detection of the onset, peak effect, and the differences between the sedated and control iguanas, with testing done between 15 and 240 min after drug administration. The sedative effect of midazolam began at 15 min and persisted through 180 min when assessed on the behavioral scale and 240 min when assessed by the forced swim test; flumazenil administration reversed the sedative effect. An increase in the LWLT was observed in the midazolam treatment groups between 15 and 30 min after drug administration. Flumazenil decreased LWLT between 15 and 180 min in the FS and at 60 min in the MF. In conclusion, the best methods to assess sedation in iguanas were the behavioral scale and the forced swim test. A dose of 2 mg/kg of midazolam was effective at inducing sedation in these juvenile iguanas, and this effect could be reversed by flumazenil.

Abbreviation: limb withdrawal latency time, LWLT

DOI: 10.30802/AALAS-JAALAS-19-000005

The class Reptilia has gained notable importance due to its economic, social, and ecologic impacts. The green iguana (*Iguana iguana*) has a wide geographic distribution, from Central America to the northern regions of South America,[12] and it is essential for ecosystem maintenance.[16,27] Furthermore, sales of green iguanas are among the leaders in the reptiles marketplace;[33] they have gained great popularity as pets, and therefore, are important to the field of veterinary medicine. Sedation is necessary before any major clinical, surgical, or experimental procedures can be performed on iguanas. Including animal handling.[35] The sedation procedure for iguanas might be different from those used for other laboratory species, and little information is available regarding sedation in iguanas. Furthermore, differences in the anatomophysiologic and metabolic systems among reptiles can influence drug metabolism[26] and pharmacodynamics,[10] resulting in different outcomes during sedation.

Various tests have been used to evaluate the sedative, anxiolytic, and anxiogenic effects of drugs in experimental models. For example, the open-field test has been used to verify the effect of a drug on an animal's locomotion and exploratory activity.[25,32] Basically, this test involves an enclosure circular or rectangular arena, with surrounding walls that prevent escapes. Parameters commonly measured in this test include distance moved, time spent walking or grooming, and location on the field.[18] One way to assess an animal's movement is to count how many segments it crosses within a given time period.[25] Although the test was developed for rodents, it has shown reasonable results when used for reptiles.[9,19]

The forced swim test is also widely used in rodents. It was first applied to analyze antidepressant drug effects in mice[31] but also is used to assess the influence of sedatives on their swimming activity.[15] The test involves placing a mouse, for example, into a cylinder filled with warm water and then observing how long it swims, its behavior, or even the duration of immobility.[8] Untreated depressed animals show less swimming activity than treated depressed ones.[31] This method has been applied to reptiles to assess locomotor activity in a range of physiologic conditions.[1,2,17]

The traction test is another test used to assess drug effects in rodents.[20] This method consists of placing a mouse's forepaws on a suspended small-diameter wire. Normal rodents grasp the wire with forepaws and hindfeet to prevent falling.[22] Various traction tests might be compared for assess drug effects in iguanas in light of their arboreal behavior. However, to our knowledge, there is no information available regarding the use of traction tests in iguanas.

In addition to the tests described, behavioral analysis is commonly used in reptile studies. The coordination and head tonus present after sedative administration have already been

Received: 18 Jan 2019. Revision requested: 25 Feb 2019. Accepted: 19 Apr 2019.
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described in lizards,[7] chelonians,[14,36] snakes,[34] and alligators.[29] Reptiles' recovery from sedation has also been evaluated by using behavioral analysis.[10,21]

Midazolam is usually described as a good sedative drug for reptiles,[3] although its effect can vary among species. This drug has resulted in different outcomes among reptiles. For example, it is effective in *Salvator merianae*[7] and *Trachemys scripta elegans*[30] but ineffective in *Chelydra serpentina*,[6] *Chelonoides carbonaria*, and *Geochelone platynota*.[14] One of the main advantages of benzodiazepines, like midazolam, is the possibility of reversing their effects. Flumazenil antagonizes the CNS effects of benzodiazepines and facilitates recovery from sedation and muscle relaxation in animals.[39]

This study evaluated the applicability of tests commonly conducted on laboratory rodents, including the open field test, forced swim test, traction test, and behavioral analysis, in green iguanas. Furthermore, the study also focused on analyzing the effects of midazolam and its antagonist, flumazenil, in iguanas. We hypothesized that at least one of the tests would be effective in assessing sedation levels in iguanas; that midazolam would induce sedation in these animals; and that flumazenil would reverse the sedative effects of midazolam.

Materials and Methods

This study was approved by our IACUC (protocol 141477740) and by the Chico Mendes Biodiversity Conservation Institute (protocol 44767-2). Juvenile (age, 20 to 26 mo) green iguanas of unknown sex (n = 10; weight, 162 ± 13 g; length, 40 ± 6 cm) were used in this study. According to the established standards, their health statuses were verified through physical examinations and routine laboratory tests (CBC and plasma biochemic analysis).[35] Individual identification data and body surface temperature measurements were determined by using microchips (Destron Fearing, South St Paul, MN) implanted subcutaneously between the scapulae.

Beginning at 1 mo of age, the iguanas were allowed to acclimate to their environment in a room with controlled temperature (25° to 30 °C), humidity (50% to 60%), and photoperiod (12:12 h of light:darkness). They were maintained in round fiberglass tanks (diameter, 1.35 m; height, 0.73), with 5 animals in each tank, wood shavings as a substrate, twigs to provide enrichment, and plastic containers filled with water for bathing. Heat and light were provided by 250-W incandescent lamps, which provided increased temperatures (40° to 45 °C) at basking sites, and a 30-W fluorescent lamp with UVA (36%) and UVB (8%; JBL, Neuhofen, Germany) to stimulate activity and appetite. The iguanas were fed a diet of iron-rich vegetables supplemented with calcium and vitamin D3 (Zoo Med Laboratories, San Luis Obispo, CA) and water without restriction. Once each week, the iguanas received species-specific pet food (Iguvert, JBL GmbH, Neuhofen, Germany).

Sedation testing. To verify which test could most efficiently assessed sedation effects, we conducted open-field arena, forced swim, behavioral, and traction tests. Groups of 10 iguanas received either 2 mg/kg of midazolam (Cristalia Prod Quím Farm, Itapira, Brazil; SED group) or 0.4 mL/kg of 0.9% NaCl (Fresenius Kabi, Barueri, Brazil; CON group); the solutions were injected intramuscularly into the forelimbs. The study was done in a double randomization design, with one treatment and test each time and at least a 15-d washout period. Thus, each animal was used 8 times in total. Lots were drawn from 2 separate bags, one to select the test and the other for the treatment for each animal. The time points for evaluating the sedative effect were before treatment (0 min) and at 15, 60, 180, and 300 min after treatment. The observer was blinded to the treatment administered.

The apparatus for the open field test was built as described previously[25] and comprised a 1.2-m-diameter arena, which was divided into 20 segments and surrounded by a 0.4-m edge to prevent escapes. At each time point, an iguana was placed at the center of the arena, and locomotor activity was recorded for 15 min, without human presence, by using a video camera. The recording was later analyzed and scored by assigning 1 point for each complete (that is, all 4 limbs) segment crossed.

A nonvalidated scale was developed for this study to assess iguanas' behavior (Figure 1). Based on the authors' experience, 5 relevant behaviors were scored on a scale of 0 to 3. First, with the animal standing on an acrylic box, eye opening was verified without handling of the animal. Afterward, the iguana's head height was measured by using a ruler. We also scored the difficulty of manually removing the animal from the box. Finally, muscle tonus and the time to return to a ventral position were recorded. The sum of the scores was then used to classify sedation as absent (0 through 4), mild (5 through 9) or deep (10 through 14) sedation.

The forced swim test was performed in a tank (diameter, 0.45 m; height, 0.5 m) that was filled to create a 0.45-m water column (24 to 25 °C) to prevent the animal from touching the bottom of the tank. At each time point, an iguana was placed in the tank, and its swim activity was recorded for 120 s, without human presence, by using a video camera. The recording was later analyzed, and the total swimming time was calculated. The maximal duration of the test (120 s) was determined in a pilot study.

For the traction test, a 0.5 × 0.5-m metal grill, with a 0.5 × 0.5-cm grid was made. The iguanas were placed in the grill center, and their ability to grab the grill at 3 different slopes (0°, 90°, and 180° relative to the floor) for 15 s was assessed (Figure 2). The total score was classified as absent (0 through 4), mild (5 through 9), or deep (10 through 14) sedation.

Assessing the reversal effect of flumazenil. To verify the efficiency of flumazenil in reversing midazolam effects, all 10 iguanas received 4 treatments: 2 mg/kg IM midazolam, followed 30 min later by 0.4 mL/kg IM of 0.9% NaCl (group MS); 0.05 mg/kg IM flumazenil (União Química, São Paulo, Brazil), followed 30 min later by 0.4 mL/kg IM of 0.9% NaCl (group FS); 2 mg/kg IM midazolam, followed 30 min later by 0.05 mg/kg IM flumazenil (group MF); and 0.4 mL/kg IM of 0.9% NaCL (group CON). The sedation effect was assessed by using the tests found earlier to be most efficient for detecting sedation in iguanas.

The antinociceptive effects of the drugs were assessed as described previously.[23] Briefly, a noxious thermal stimulus was provided by exposing a hindlimb to a radiant heat source set (Ugo Basile, Gemonio, Italy) at an intensity of 70 (245 ± 7 mW/cm^2; 45 to 47 °C) for a maximum of 30 s to prevent tissue burns. The iguanas were restrained in an opaque acrylic box (60 cm × 13 cm × 14 cm) on the palmar test device. After 5 min of acclimation, the heat source was directed onto the plantar surface of one of the hindlimbs, and the noxious thermal stimulus was applied. The limb withdrawal latency time (LWLT) was automatically measured by the device. Data were collected before the first injection (0 min) and at 15, 30, 45, 60, 180, 240, and 300 min after treatment. The experimental design of and the washout period for this part of the study were as described above.

Statistical analysis. Statistical analysis was performed by using statistical software (GraphPad Software, San Diego, CA). The normality of data distribution was evaluated by using the Shapiro–Wilk test. Parametric data were compared by using

Behavior	Description	Score
Eye opening	Completely opened eyelids and follows the evaluator's movement	0
	Partially opened eyelids; does not follow the evaluator's movement	1
	Closed eyelids	2
Handling	Alert; on twigs; tries to escape when the evaluator tries to capture it; mild resistance to handling	1
	Alert; not on twigs; slowly tries to escape when the evaluator tries to capture it; no resistance to handling	2
	On shavings; flaccid; no escape attempts; no resistance to handling	3
Head height	Higher than 3 cm; same or higher than basal	0
	Between 2 or 3 cm; 1 or 2 cm less than basal	1
	Between 0 or 1 cm; 3 or 4 cm less than basal	2
	Does not lift head	3
Body righting reflex	Great resistance to rolling onto back, usually grabs the evaluator's hand or uses its tail to stop it	0
	Allows rolling onto back but immediately returns to quadrupedal position	1
	Allows rolling onto back takes 3 to 30 s to return to quadrupedal position	2
	Allows rolling onto back takes 31 to 60 s to return to quadrupedal position	3
Muscle tonus	Resistant to traction or turning of the limb	0
	Allows traction or turning of the limb but immediately returns to the original position	1
	Allows traction or turning of the limb but requires 3 to 30 s to return to the original position	2
	Allows traction or turning of the limb but requires 31 to 60 s to return to the original position	3

Figure 1. Sedation scoring for iguanas (*I. iguana*) according to 5 behavioral descriptions.

Slope	Parameter	Description	Score
0° from floor		Walks toward the edge in 5 s	1
		Stays in the center of the grill	2
	Body position	Abdomen far from the grill, support on limbs	0
		Abdomen close to the grill, no support on limbs	1
90° from floor	Escape attempt	Walks and gets to the edge in 15 seconds	0
		Walks and does not get to the edge	1
		Stays in the center of the grill	2
	No. of limbs on the grill	3 limbs or more holding the grill	0
		2 limbs holding the grill, possibly with nails	1
		Holding the grill with nails only (no paw contact)	2
	Body position	Abdomen close to the grill, support on the limbs	0
		Abdomen far from the grill, little support on the limbs	1
180° from floor	Escape attempt	Walks and gets to the edge	0
		Walks and does not get to the edge	1
		Stays in the center of the grill	2
	No. of limbs on the grill	3 limbs or more holding the grill	0
		2 limbs holding the grill, possibly with nails	1
		Holding the grill only with nails	2
	Body position	Abdomen close to the grill, support on the limbs	0
		Abdomen far from the grill, little support on the limbs	1
		Hanging from the grill, supported only by 2 or less limbs	2

Figure 2. Sedation scoring for iguanas (*I. iguana*) in the traction test conducted at various grill slopes.

a paired 2-way ANOVA. Intragroup test and baseline values were compared by using the Bonferroni post hoc test. For intergroup comparisons at each assessment point, 2-way ANOVA was followed by the Tukey post hoc test. Nonparametric data were compared by using the Kruskal–Wallis test with Dunn test corrections. Values are reported as mean ± 1 SD or median and interquartile range. Differences were considered statistically significant when the P value was less than 0.05.

Results

Sedation testing. The open-field test showed no difference between the SED and CON groups in the number of segments crossed (Figure 3 A). The iguanas did not display any particular behavior, especially in the CON group. That is, some iguanas ran away from the center to the border and then tried to climb the edge, whereas others ran to the border and then froze there, with no movement. Still other iguanas showed freezing behavior and stayed in the center of the field for the entire 15-min observation period. In comparison with baseline values, only the SED group showed significant ($P < 0.05$) differences at the 15- and 60-min time points.

The SED and CON groups differed significantly ($P < 0.05$) from 15 until 180 min in the forced swim test, during which SED iguanas swam less than CON animals (Figure 3 B). In this

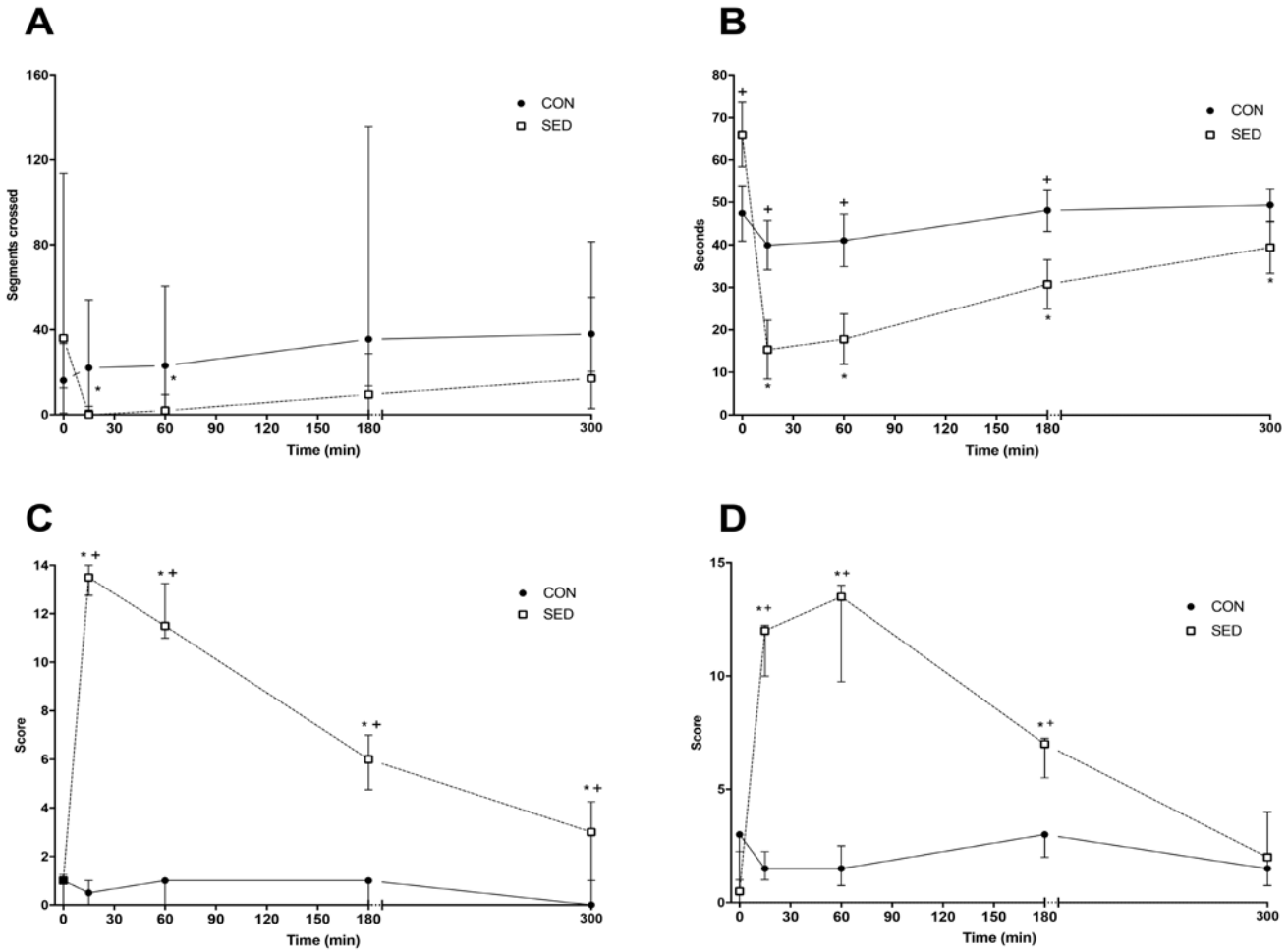

Figure 3. Results of testing of iguanas after administration of 0.9% NaCl (0.4 mL/kg IM; CON group) or midazolam (2 mg/kg IM; SED group). (A) Segments crossed over time during the open field test. (B) Time spent swimming in the forced swim test. (C) Total behavior score. (D) Total score in traction test. Values are expressed as median ± interquartile range, except for the forced swim test, for which data are expressed as mean ± SE. *, Value is significantly ($P < 0.05$) different from that at 0 min within the same group; +, Values at the same time point differ significantly ($P < 0.05$) between groups.

test, iguanas from the CON group showed vigorous swimming, trying to escape from the tank. However, the sedated iguanas only floated in the tank, just keeping their heads above the water; this pattern was most intense at 15 and 60 min after midazolam injection.

The behavioral scale also showed significant ($P < 0.05$) differences between groups. In particular, the SED group demonstrated deep sedation (score, >9) from 15 through 60 min and mild sedation at 180 min (Figure 3 C). According to the behavioral scale, animals in the CON group showed no sedation at any time point.

Regarding the traction test, sedation effect was observed until 180 min in the SED group compared within the CON group; however, peak sedation occurred at 60 min after treatment (Figure 3 D). In applying this test, we determined that it was not useful, mainly because of the need to manipulate the grill and because the handler could influence the evaluations.

Flumazenil reversal of midazolam effects. According to the results of the first stage of this study, the most efficient and practical tests to assess sedation in green iguanas were the forced swim test and behavioral scoring. These tests were practical to perform, showed clear differences between groups, and detected drug-associated effects early after administration. In the second stage of this study, behavioral scoring revealed deep sedation, beginning at the 15-min time point, in the iguanas of both of the midazolam groups (MS and MF). Furthermore, reversal of the sedative effect was observed in the MF group, in which animals received flumazenil 30 min after the midazolam injection. In this group, the sedation effect decreased after flumazenil administration and was no longer detected at 180 min (Figure 4 A).

Sedation and reversal were also observed in the forced swim test. The iguanas that received midazolam were unable to swim from the 15-min time point onward, displaying a deep sedation pattern. Animals from the MS group showed deep sedation at 45 min, which lasted for 240 min, when compared with the FS group. In the MF group, sedation was decreased at 30 min after the flumazenil administration, but values differed significantly ($P < 0.05$) from the FS group for as long as 60 min (Figure 4 B).

To analyze the antinociceptive effect of midazolam, the results obtained from each baseline were normalized to zero, and the data graphed as the change in latency. The iguanas that were given midazolam showed a longer LWLT at 15 to 30 min after drug administration. This effect lasted until 60 min in the MS group but decreased as soon as the flumazenil injection was administered in the MF group, and became lower than baseline at 60 min. In the same way, the LWLT of the FS group decreased

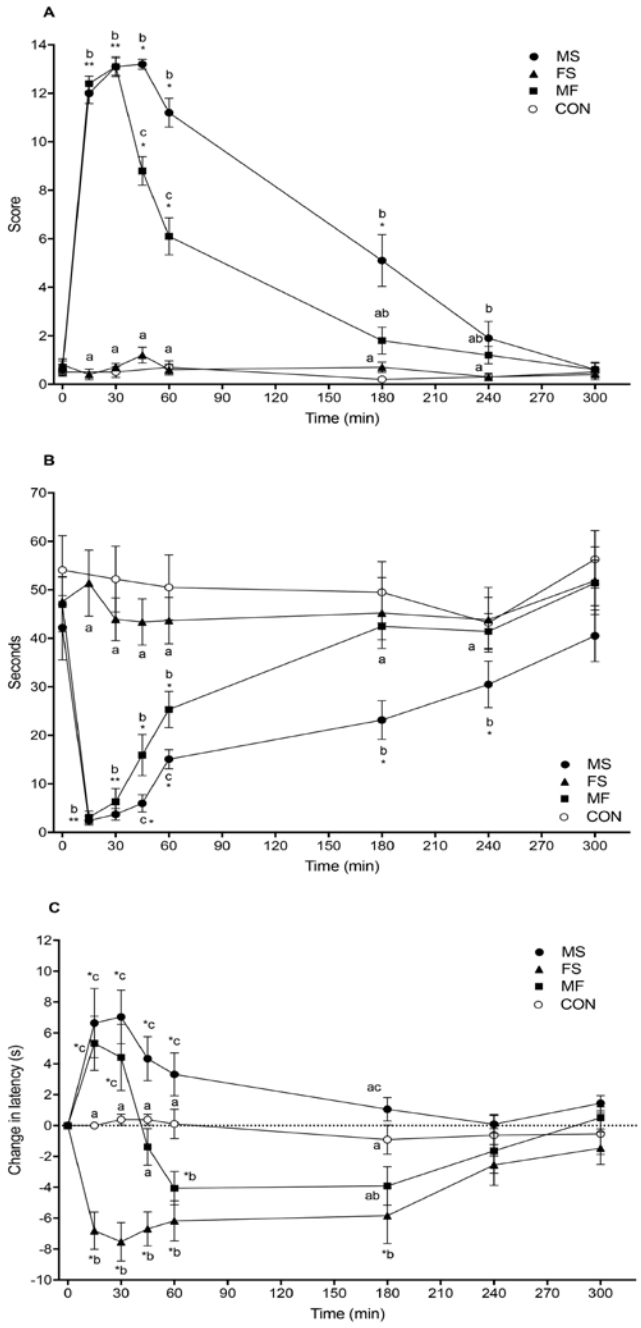

Figure 4. (A) Total behavioral score, (B) time spent swimming in the forced swim test, and (C) limb withdrawal latency at 30 min after iguanas were treated with midazolam (2 mg/kg IM) and 0.9% NaCl (0.4 mL/kg IM; MS group), flumazenil (0.05 mg/kg IM) and 0.9% NaCl (0.4 mL/kg IM; FS group), midazolam (2 mg/kg IM) and flumazenil (0.05 mg/kg IM; MF group), or 0.9% NaCl only (0.4 mL/kg IM; CON group). Values from the behavioral scale are expressed as median ± interquartile range; values from the forced swim and limb withdrawal latency tests are expressed as mean ± SE. *, Value significantly ($P < 0.05$) different from that at 0 min within the same group; different letters at the same time point indicate significant ($P < 0.05$) differences between groups.

between 15 and 180 min as compared with either baseline or the MS and CON groups (Figure 4 C).

Discussion

Our results demonstrated that the forced swim test and behavioral scoring were the best tests to verify the sedation effect of drugs in iguanas. The forced swim test was easy to apply, yielded quantitative data, and was sufficiently precise to distinguish differences between control and sedated animals. The forced swim test has been performed to verify the influence of many drugs on mouse behavior.[11] That is, mice treated with antidepressants showed longer swimming activity compared with control animals, which stopped swimming sooner. We adapted this test to iguanas because they are considered to be excellent swimmers.[4] Even though our current study was focused on assessing the opposite effect (that is, sedation), the forced swim test was quite feasible. In addition, this test was safe, because once they were sedated, the iguanas did not dive.

Although the behavioral scale could be considered a qualitative test and has not been validated for iguanas, the data identified a significant difference between sedated and control animals. Behavioral assessment to evaluate reptile sedation has been well described in lizards[7] and turtles.[6,30] This assessment can be easily applied even in clinical practice. Despite these advantages, specific behavior scales should be adapted for each species, correlating the scale with specific species-appropriate behaviors. Therefore, the behavioral scale that we proposed here is reasonably useful to verify the sedation of green iguanas under clinical conditions. However, a validation study of this scale is warranted.

The traction test has shown consistent results for assessing sedative effects in rats, which released their grip from the metal bars sooner than the control animals.[20] We considered this test because of iguanas' arboreal behavior. Although the traction test showed differences between groups, it failed to reveal the expected sedation peak until 15 min, as was observed with the other tests. Perhaps the sedated iguanas could hold themselves on the grill, even when they were under mild sedation. Therefore, this test was useful only for assessing deep sedation. Moreover, we decided to abandon the traction test because it was deemed impractical to apply as compared with the forced swim test and behavioral scoring.

The open-field test was not useful for evaluation of locomotor activity in iguanas, possibly because of the iguanas' arboreal life style. On the ground, the animals from the control group showed freezing during the test, whereas rats displayed different behaviors when given sedatives, central stimulants, or toxic substances.[18]

Based on the results of the sedative tests during the first stage of our study, we opted to evaluate the effects of midazolam in iguanas by using both behavioral scoring and the forced swim test. We also evaluated the antinociceptive effect of the sedative to a noxious thermal stimulus and the influence of flumazenil, a midazolam antagonist, with these measures. We then used both tests to assess the sedative effect of midazolam from 15-min after injection through almost 3 h. Finally, we tested the antagonistic characteristics of flumazenil by assessing its ability to counter the sedative effects of midazolam.

Midazolam is an effective sedative agent for reptiles, especially for minimally invasive procedures, such as biologic sample collection.[3,35] However, variations in the sedative effect have been reported depending on the species. The sedative effect of midazolam achieved in iguanas was quite stable, showing little variation in quality or intensity among animals. This effect was similar to that observed in tegus after administration of the same dose, which resulted in a moderate sedation in these lizards.[7] In contrast, in chelonids, such as *Chelydra serpentina*,[6] the use of midazolam did not induce satisfactory sedation when compared with its combination with ketamine. Furthermore, the seda-

tive effect did not follow the same pattern in *Trachemys scripta elegans*.[30] Another advantage of midazolam is the possibility to reverse its effects by using flumazenil. Data on the effects of flumazenil on reptiles are limited, but it has been effective in facilitating a rapid recovery from sedation in snakes,[5] turtles,[24] and lizards[28] that had been given midazolam. Furthermore, particularly fast and complete reversion of the sedation effect occurred in *Eublepharis macularius* that had been given flumazenil compared with others which were not.[13] These results are consistent with our findings in iguanas.

Flumazenil reduced the limb withdrawal latency in response to the noxious thermal stimulus. This unexpected effect might be due to a possible excitatory effect of flumazenil on iguanas; a similar effect occurs in mice.[38] Alternatively, a hyperalgesic effect might also be possible in iguanas, given that flumazenil was able to reverse the antihyperalgesic effect of midazolam in mice.[37] However, both of these possible effects should be explored further.

In conclusion, the best methods to evaluate sedation in iguanas are behavioral scoring and the forced swim test, but behavioral testing might be more feasible in a clinical setting. Furthermore, administration of 2 mg/kg midazolam effectively achieves sedation in iguanas, and this effect can be reversed by using flumazenil.

Acknowledgments

This study was funded by grant 2014/10452-9 from the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) and grant 304566/2015-2 from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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