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Analgesic Efficacy of Tramadol and Morphine in White's Tree Frogs (*Litoria caerulea*)

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Published data are sparse regarding the recognition of clinically relevant pain and appropriate analgesia in amphibians. The amphibian analgesia literature has primarily focused on nociceptive pathways in a single species, the northern leopard frog (*Rana pipiens*). The objective of the current study was to assess the analgesic efficacy and safety of oral tramadol and subcutaneous morphine in a commonly maintained zoo and pet species, White's tree frog (*Litoria caerulea*). We hypothesized that tramadol and morphine would provide dose-dependent antinociception, as measured by significant increases in hindlimb withdrawal latency after exposure to a noxious thermal stimulus. Two randomized, placebo-controlled, complete crossover studies were performed, with tramadol (*n* = 12) administered at 15, 25, and 40 mg/kg PO and morphine (*n* = 12) administered at 5 and 10 mg/kg SC. Hindlimb withdrawal latency was measured for a maximum of 72 h. No adverse side effects or signs of sedation were observed with any dose or drug evaluated. No significant difference in withdrawal latency was detected between the control and either tramadol or morphine. These negative results were surprising, suggesting that the thermal nociceptive model may not be biologically relevant in amphibian species.

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Additional validated models are needed for evaluation of pain and analgesic efficacy in amphibians. To date, studies of antinociceptive efficacy have been reported for only a few amphibian species, primarily the northern leopard frog (*Rana pipiens*) and other laboratory species.[5,8-12,16-18] Clinical studies that expand on the research models used in the laboratory are critical to ensure that appropriate and effective analgesics are provided to all amphibians, given that extrapolation from one species to another is inappropriate.

Previous antinociceptive research in amphibians focused on characterizing amphibian nociceptors, identifying and cloning opioid receptors, testing drug effects, and developing an alternative nonmammalian vertebrate analgesic model for research.[4,10-12,14,16,17,20] Few studies were aimed at determining effective clinical antinociception in amphibian species.[3,5,8,9] Moreover, methodologic differences between studies and over-reliance on a single nociceptive method, the acetic acid wipe test, make clinical interpretation of these results challenging. Currently, opioids are the foundation of antinociceptive research and pain management in amphibians.[9] Amphibians have a well-developed endogenous opioid system,[17] with documented presence of κ-, μ-, and δ-opioid receptors.[8,15] In addition, the relative potencies of various opioid-receptor–specific drugs are similar between amphibians and mammals, with μ-specific drugs more potent than δ-targeting compounds, which are more agonistic than κ-binding agents.[15] Therefore, μ-opioid agonists are likely to provide effective antinociception in amphibian species.

Tramadol and its active metabolite, O-desmethyl-tramadol (M1), contribute to analgesia by activating μ-opioid receptors and inhibiting serotonin and norepinephrine reuptake in the CNS.[6] Tramadol is a commonly used analgesic in mammals, and dosages used in dogs and cats typically range from 2 to 5 mg/kg, with some references as high as 10 mg/kg.[6] In aquatic turtle species, tramadol at 10 mg/kg PO provides thermal antinociception for longer than 92 h.[2] Although tramadol has not been systematically evaluated in any amphibian species, it—like morphine—may provide antinociception in amphibians due to its μ-opioid receptor-agonist properties. In addition, oral tramadol may provide a relatively simple and less stressful delivery alternative to injections and may have a longer duration of action in amphibians than mammals. Therefore, we hypothesized that oral tramadol and the positive control of subcutaneous morphine would provide dose-dependent antinociception in White's tree frogs, measured as a significant increase in hindlimb withdrawal latency after exposure to noxious thermal stimuli delivered by using a standard Hargreaves apparatus, and that neither drug would have clinically relevant adverse effects.

Materials and Methods

Animals. Young adult, captive-bred, White's tree frogs (*Litoria caerulea*; *n* = 21) were obtained from a commercial supplier (Reptile Rapture, Madison, WI) and used for these experiments. The sex of the frogs was not determined, because this species is not sexually dimorphic. The frogs were housed individually in transparent, amber-tinted plastic enclosures (10.75 in. wide × 19.25 in. deep × 10.75 in. high) with a floor space of approximately 143 cm^2 per frog. Each container was equipped with a water bowl, plastic plants for climbing and hiding, and a plastic hide box. The frogs were housed at 26 to 30 °C on a 12:12-h light:dark cycle, with light provided by using a UV bulb (Exoterra Repti Glo 5.0 UVB, 120 cm/48 in., 40 W, Rolf C Hagen, Mansfield, MA). Frogs had free access to fresh, dechlorinated water and were fed appropriately sized, gut-loaded (High-calcium Cricket Feed and Orange

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Cube Complete Cricket Diet, Fluker, Port Allen, LA) crickets every other day. Crickets were dusted with calcium carbonate powder (Repti Calcium without D3, Zoo Med Laboratories, San Luis Obispo, CA) once each week just before they were fed to the frogs. Baseline health testing included a complete physical exam and fecal parasite testing. All frogs received a single dose of oral fenbendazole (100 mg/kg PO; Fenbendazole 10% suspension, Intervet, Millsboro, DE) a minimum of 2 wk prior to the study. The frogs were weighed weekly, and food intake was determined after each feeding by counting the number of crickets consumed. During experimental phases, all frogs were fed the day after drug or saline administration. Before the experiments began, the frogs were acclimated for 4 wk to housing conditions and the room in which experiments were performed. All procedures were approved by the Animal Care and Use Committee at the School of Veterinary Medicine, University of Wisconsin–Madison (protocol number, V1623), an AAALAC-accredited facility.

Study design. This prospective study consisted of 2 phases for each drug tested; each drug was tested in a separate set of experiments. In phase 1, trial doses of the test drug were administered to groups of 3 frogs and assessed for antinociception by using the thermal hindlimb withdrawal apparatus in a noncrossover experimental design. In phase 2, the doses determined to be effective in phase 1 were evaluated for efficacy and adverse effects and compared with a saline control by using a randomized, complete crossover experimental design. The observer was blind to all treatments in both phases.

In the first phase of the tramadol study, randomly assigned young adult frogs (n = 9; weight, 13.6 ± 2.1 g [mean ± 1 SD; range, 10.7 to 18.2 g]) were assigned to cohorts of 3 and received a suspension of tramadol (Tramadol Hydrochloride, 50-mg tablets, Amneal Pharmaceuticals, Hauppauge, NY; crushed and mixed with distilled water to make a suspension) at doses of 5, 10, 15, 20, 25, 40, and 75 mg/kg PO by using a tapered, 5-French red rubber feeding tube. Each frog received a single dose per experiment. Randomization was accomplished by using a random number generator. In other words, 3 randomly assigned frogs were exposed to each dose over time, with a 2-wk washout period between treatments. The frogs were observed for dose-limiting adverse effects, including loss of righting reflex, loss of corneal reflex, loss of gular movement, and a decrease in heart rate of more than 50% of baseline at any time point after drug administration.

In the second phase of the tramadol study, a randomized, complete crossover experiment was performed. Frogs were given a suspension of tramadol at 15, 25, and 40 mg/kg PO. These doses were chosen in light of minimal hindlimb withdrawal responses in phase 1, based on 3 frogs per dose. Physiologic saline (0.9% sodium chloride, preservative-free, Hospira, Lake Forrest, IL) in a volume equivalent to that of the 25-mg/kg dose was used as a control. Frogs not previously used in phase 1 (n = 12; weight, 19.7 ± 3.0 g [range, 13.4 to 26.6 g]) were randomized to the treatment groups. Each frog received a single dose of either tramadol or saline control per experiment. A minimal washout period of 2 wk was provided between treatments.

In the first phase of the morphine study, young adult frogs (n = 12; weight, 23.9 ± 2.7 g [range, 19.6 to 28.5 g]) were randomly assigned to cohorts of 3 and received morphine sulfate subcutaneously just dorsal to the forelimb (Morphine Sulfate injection, preservative free, 10 mg/mL, Westward, Eatontown, NJ) at doses of 2, 5, 20, 40, and 75 mg/kg SC. Each frog received a single dose per experiment. In other words, 3 randomly assigned frogs were exposed to each dose over time, with a 2-wk washout period between treatments. The frogs were observed for dose-limiting adverse effects as described for the tramadol study.

The second phase of the morphine study comprised a randomized, complete crossover experiment. Frogs received morphine at 5 and 10 mg/kg SC just dorsal to the forelimb. Each frog received a single dose per experiment. These doses were chosen based in light of minimal hindlimb withdrawal responses in phase 1, on the basis of 3 frogs per dose. A volume of physiologic saline equivalent to that of the 10-mg/kg dose of morphine was used as a control. Frogs (n = 12; weight, 28.9 ± 2.8 g [range, 23.3 to 37.4 g]) were randomized to the treatment groups. There was a minimal 2-wk washout period between treatments.

Antinociceptive evaluation. All frogs were weighed shortly before the 0-h time point, so that accurate doses could be administered. At each time point (0, 1, 8, 12, 24, 48, and 72 h), heart rate was measured by using a Doppler flow probe placed on the ventral midline pectoral region for 15 s and was averaged for all frogs at each time point (mean ± SD). The frogs were allowed to acclimate to the testing chamber for 15 min. At that point, a gular rate (rate of respiration) was collected visually for 15 s at 0, 1, 8, 12, 24, 48, and 72 h after the frog had acclimated to the chamber but before the antinociceptive testing was started. The gular rate was averaged for all frogs at each time point. Antinociceptive experiments were conducted by using a Hargreaves apparatus (Plantar Analgesia Instrument, model 37370, Ugo Basile, Comerio, Italy), which applied an infrared thermal stimulus to the plantar surface of the hindlimb foot according to established methods.[2,7,13,14] The plantar surface of both hindlimbs were gently blotted with a paper towel to remove excess water before the frogs were placed in the testing chambers, because any water present between the infrared heat source and the frog can artificially increase limb withdrawal latencies. Frogs were placed into clear, ventilated plastic boxes (17 × 13 × 14 cm) that were elevated on a clear acrylic surface and contained dividers to prevent visual contact with other frogs. An infrared radiation source was activated directly below the surface on which the plantar surface of either hindlimb was rested (Figure 1). The infrared intensity was 90 mW/cm^2. Hindlimb thermal withdrawal latencies were measured by using a motion-sensitive timer, which stopped automatically when the hindlimb was removed from the noxious stimulus. To prevent tissue damage, a maximum exposure duration of 32.5 s was allowed.

The frogs were acclimated to the device chamber, the thermal heat intensity, and the presence of an observer for a minimum of 4 wk prior to the start of the study. During each testing period, frogs were allowed to acclimate to the testing chamber for 15 min before testing began. All withdrawal times were measured automatically (in seconds) at 0, 1, 4, 8, 12, 24, 48, and 72 h after drug administration. Three withdrawal latencies were measured on alternating hindfeet, 5 min apart, for each time point, and the latencies were averaged. When a measurement was suspected to be inaccurate due to movement, the measurement was excluded, and a fourth measurement was collected. The plantar aspects of the feet of the frogs were monitored daily for signs of reaction to the thermal heat stimuli throughout the 72-h testing period. All data were collected at the same time of day on each testing day, always between 9 AM and noon.

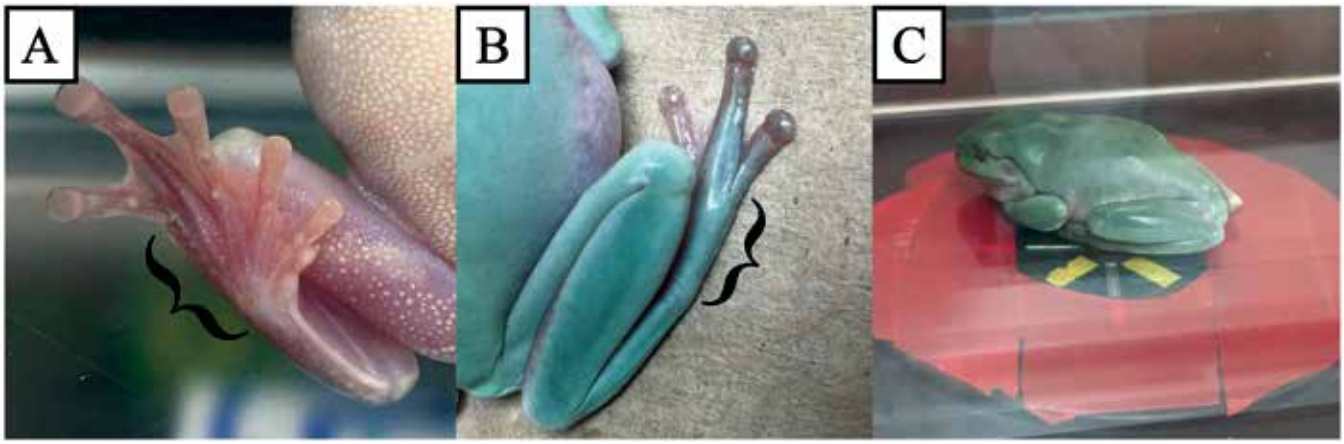

Figure 1. The region of the hindfoot of a White's tree frog from the (A) plantar aspect and (B) dorsal aspect under which the thermal noxious stimulus was centered for hindlimb withdrawal latency testing. (C) Frog within the testing chamber with the noxious thermal stimulus centered under the plantar surface of the left hindfoot.

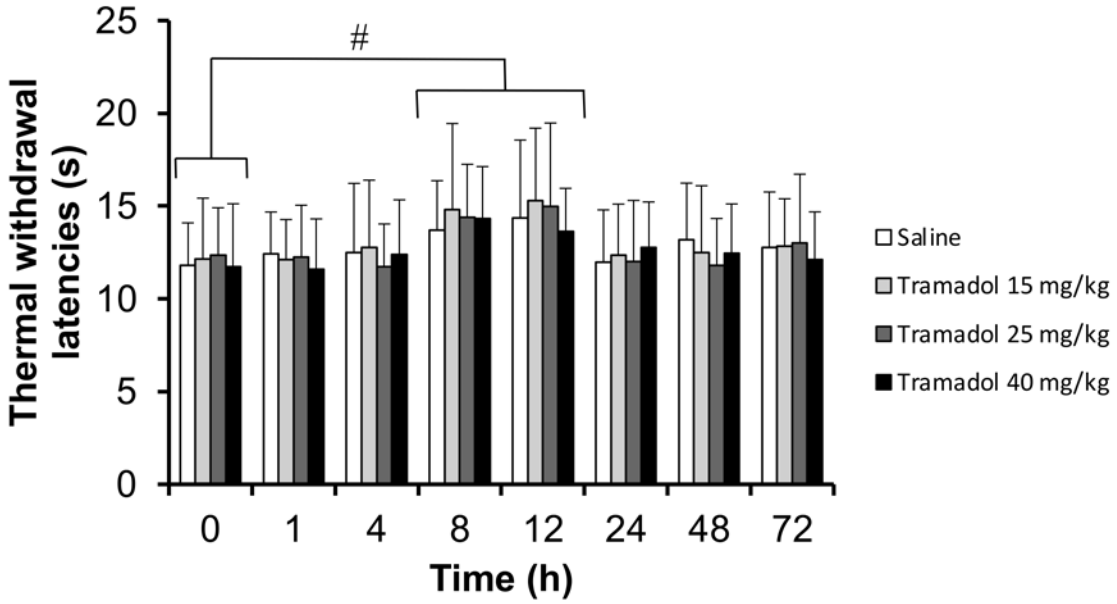

Figure 2. Hindfoot thermal withdrawal latencies (mean ± 1 SD) in White's tree frogs before and after tramadol administration at 15, 25, and 40 mg/kg PO. ‡, $P < 0.001$ for time effect for all 8-h compared with 0-h data and all 12-h data compared with 0-h data.

Statistical analysis. Data were analyzed by using commercial software (SigmaPlot 13.0, Systat Software, San Jose, CA). Data were evaluated for normal distribution by using the Shapiro–Wilk test and for equal variance by using the Brown–Forsythe test. The effects of drug dose and time on hindlimb withdrawal latency, heart rate, and gular rate were evaluated by using repeated-measures 2-way ANOVA. When normality assumptions were not satisfied, data were transformed before further analysis. Posthoc comparisons were made by using the Student–Newman–Keul test. All data are expressed as mean ± SD. P values less than 0.05 were considered significant.

Results

The baseline latency interval in untreated frogs ranged from 8.0 to 19.9 s (mean ± 1 SD, 12.0 ± 2.0 s) for the tramadol study and 8.8 to 22.0 s (mean ± 1 SD = 15.0 ± 3.2 s) for the morphine study (Figures 2 and 3). No significant differences in hindlimb withdrawal latencies were detected between saline and either oral tramadol or subcutaneous morphine at any dose tested ($P > 0.83$; Figures 2 and 3). Time-dependent changes were present for all data at the 8- and 12-h time points as compared with baseline (0 h; $P < 0.001$; Figure 2).

Tramadol decreased heart rate in a complex manner, with time-dependent effects for all data at 1, 8, 12, 24, 48, and 72 h as compared with baseline (0 h; $P < 0.023$) and significant decreases from baseline for 15 and 40 mg/kg at various times between 1 and 72 h ($P < 0.04$) but not for 25 mg/kg ($P > 0.36$; Figure 4). Morphine administration did not significantly change the heart rate ($P = 0.87$; Figure 5). Statistically significant changes were not detected in mean gular rate at any dose or time point after tramadol or morphine administration.

No adverse effects or signs of sedation were observed with either tramadol or morphine, even at trial doses of 75 mg/kg PO for tramadol and 75 mg/kg SC for morphine. No plantar foot lesions were observed at any time point during or after the study, and no deaths occurred during or after these studies.

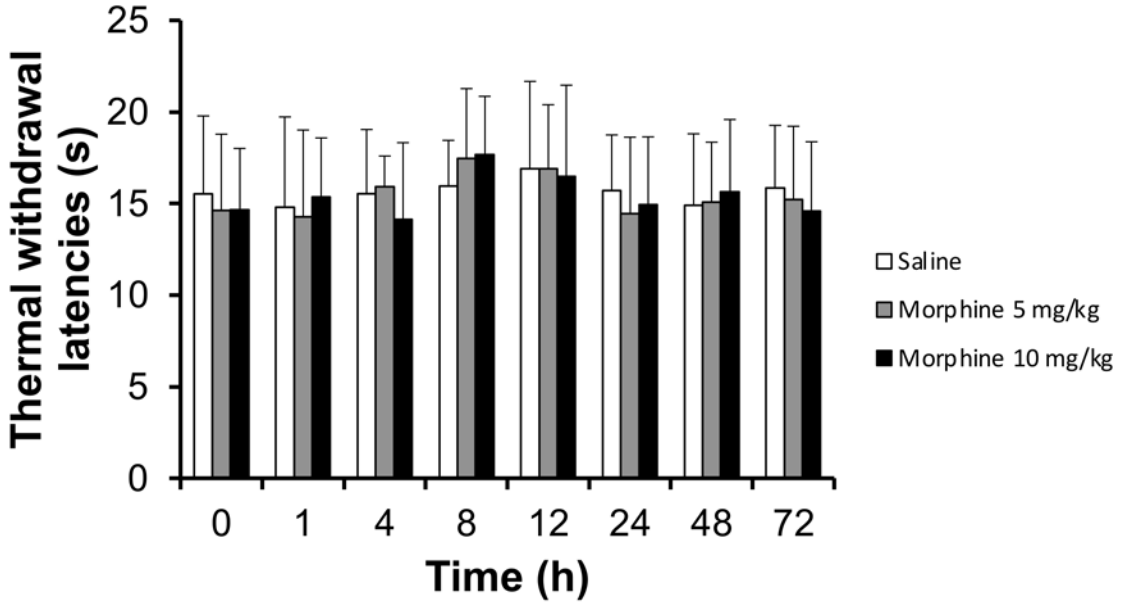

Figure 3. Hindfoot thermal withdrawal latencies (mean ± 1 SD) in White's tree frogs before and after morphine administration at 5 and 10 mg/kg SC.

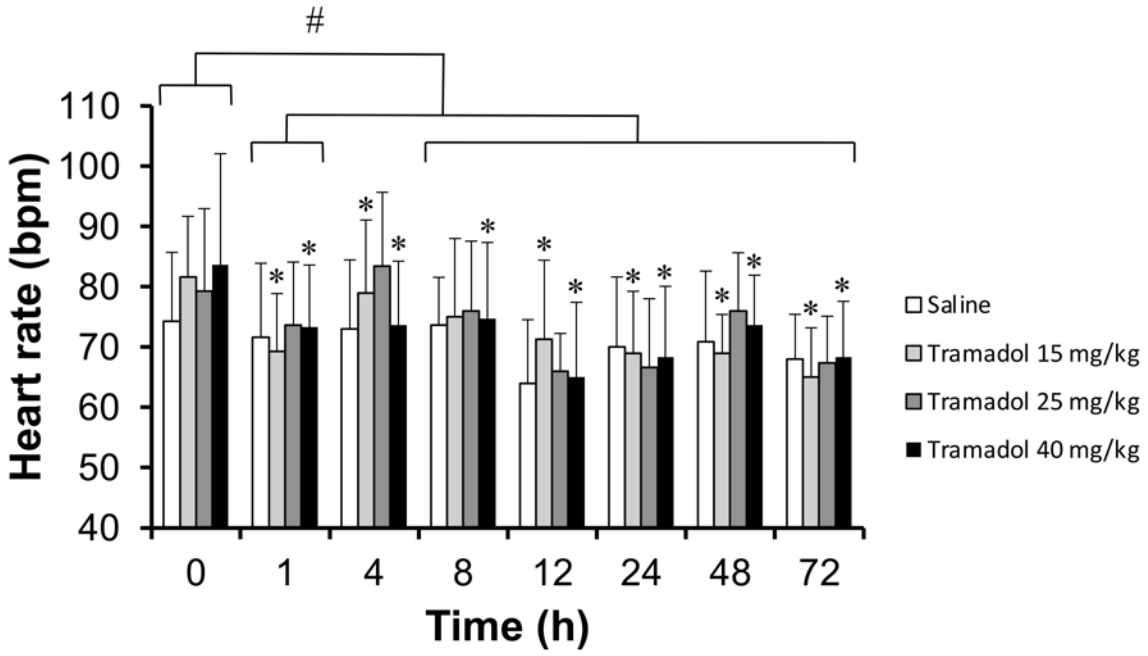

Figure 4. Heart rate (mean ± 1 SD) in White's tree frogs over time after administration of tramadol at 15, 25, and 40 mg/kg PO. **#**, Significant ($P < 0.023$) time effect compared with 0 h for the following time points: 1, 8, 12, 24, 48, and 72 h. *****, Significant ($P < 0.04$) difference compared with 0 h for various 15- and 40-mg/kg time points.

Discussion

This study is the first to systematically evaluate tramadol as a possible analgesic drug in amphibians and to assess doses of any analgesic drug in White's tree frogs. No statistically significant or biologically relevant differences in hindlimb withdrawal latencies were detected between control and either oral tramadol or subcutaneous morphine at the doses tested. This outcome may be due to experimental methodologic factors or species differences. For example, thermal nociceptive models may be inadequate for use in amphibian species, or the experimental doses of both morphine and tramadol may have been too low to achieve efficacy. The Hargreaves apparatus is a valuable research tool to screen for antinociceptive drugs in reptiles and mammals, but was recently shown to be less effective, or ineffective, in amphibian species.[19] Other antinociceptive models, such as noxious mechanical stimuli or a surgical model, might be a more ecologically appropriate method to detect analgesic drug efficacy in tree frogs.

This study used the Hargreaves apparatus instead of the acetic acid wipe test, a well-described model for amphibian nociception. We chose the Hargreaves apparatus because the

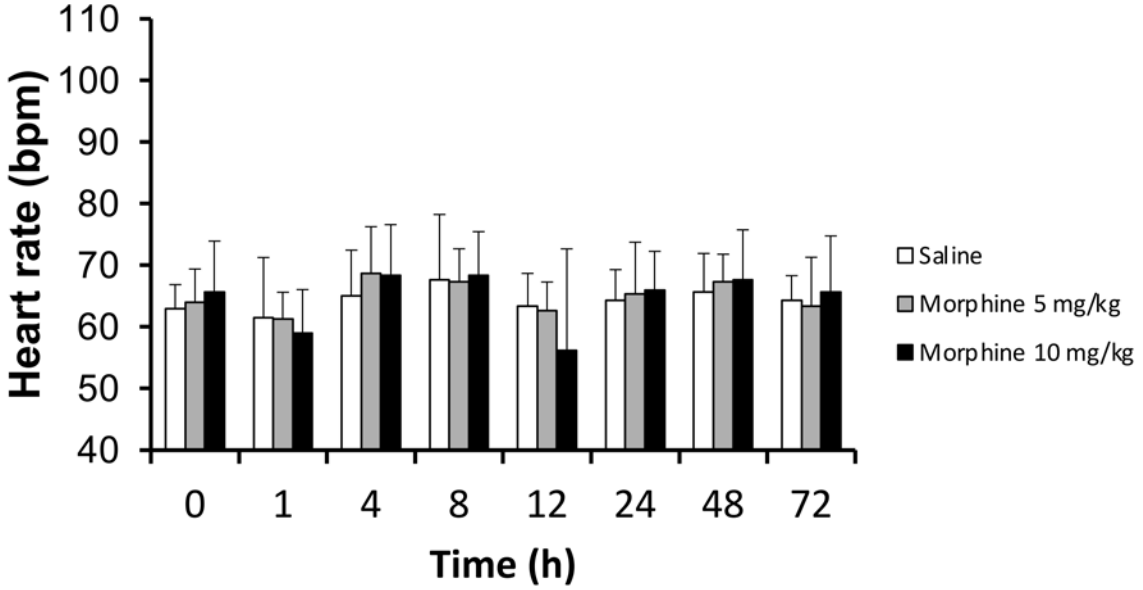

Figure 5. Heart rate (mean ± 1 SD) in White's tree frogs over time after morphine administration at 5 and 10 mg/kg SC.

application of acetic acid can cause both an inflammatory response and a nociceptive reaction; this is the major methodologic flaw with the acetic acid wipe test. After application of the acetic acid test, dermal lesions including ulceration and inflammation occurred in African clawed frogs (*Xenopus laevis*) and led to secondary bacterial infections and sepsis.[5] In another study using northern leopard frogs, similar dermal lesions developed after acetic acid tests, requiring euthanasia of affected frogs.[18] In smokey jungle frogs (*Leptodactylus pentadactylus*) exposed to NSAID, topical acetic acid was associated with epidermal necrosis.[3] However, the Hargreaves apparatus has been used to measure nociception in African clawed frogs and northern leopard frogs without causing an inflammatory response or tissue necrosis.[5,20]

Even at relatively high doses, both tramadol and morphine had no adverse effects. However, nothing is known about the pharmacokinetics or pharmacodynamics of opioid drugs in any amphibian species. In this study, we administered tramadol orally and morphine subcutaneously; both could demonstrate different pharmacokinetic profiles due to different absorption and metabolism rates. In aquatic turtles, tramadol administered orally and subcutaneously had similar effects in a thermal noxious stimulus model, with statistically significant initial hindlimb withdrawal effects at approximately 6 h after drug administration regardless of route.[2] This effect may or may not be similar in amphibian species. In the current study, oral tramadol changed in the heart rate of the frogs, implying drug absorption despite the lack of sedation or thermal antinociception. The depressive effect on heart rate occurred within 1 h of administration, supporting the hypothesis that oral absorption is relatively rapid and can induce a biologic effect.

Tramadol and morphine can cause respiratory depression in mammals and reptiles,[2,6,14] but our frogs showed no changes in gular rate at the doses evaluated. Because amphibians can respire percutaneously, monitoring respiratory depression may be difficult without spirometry. Previous studies have shown that amphibians generally tolerate high doses of μ-opioid agonists.[11] Therefore, adverse effects may not be observed except at exceptionally high drug levels.

In general, latencies tend to shorten throughout trials as the subjects learn to anticipate the noxious stimuli; this type of effect of learning also occurs in reptiles and rodents.[7,13,14] However, the tree frogs did not appear to anticipate the noxious stimuli over repeated trials, because the morphine study was completed after the tramadol study using the same frogs, and the mean latencies in the morphine study were actually longer than in the preceding tramadol study.

This study is the first to evaluate antinociception in White's tree frogs. Past studies have focused predominantly on northern leopard frogs.[10-12,15-18,20] Clinical studies that expand on research models are critical to ensure that appropriate and effective analgesics are provided to all species. This need is clearly demonstrated in reptile analgesia studies, in which extrapolation from one species to another, such as turtles and snakes, may not be appropriate.[13,14]

Data from previously published amphibian studies indicated that morphine should have acted as a positive control in the current study.[1,11,12,15,17] Tramadol, with its similar μ-agonist properties, should also have been an ideal candidate to produce an analgesic effect, but this was not observed. These negative results are unexpected given the known presence of endogenous μ-agonists and μ-opioid receptors in some amphibian species.[8,15] The lack of effect may be due to several issues, such as the application of a thermal noxious stimulus (for example, Hargreaves apparatus) rather than a mechanical stimulus, the study design, opioid tolerance, the inherent skin properties of amphibians, or species-specific effects.

The results of the current study indicate that White's tree frogs respond appropriately when placed in the Hargraves apparatus with regard to reliably withdrawing a hind limb in response to a noxious stimulus. Tramadol and morphine appeared safe but did not produce statistically significant changes in hindlimb withdrawal latencies. However, tramadol did reduce heart rate, implying that the drug or a metabolite were biologically active. These results provide a starting point for comparisons with nonopioids and use of different nociceptive models, such as other behavioral and surgical models in order to determine the most clinically effective analgesic medication and dosage.

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