

Dose Finding Study for Treatment of Self-Injurious Behavior in Rhesus Macaques

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Introduction: The goal of this study was to develop treatment strategies for self-injurious behavior (SIB) in macaques that result in high remission rates. We used candidate compounds based on clinical efficacy in the short-term treatment of SIB and self-directed stereotypic behavior in macaques. Specifically, we performed a dose-finding study in order to establish optimal doses of fluoxetine (selective serotonin reuptake inhibitor) and venlafaxine (serotonin/norepinephrine reuptake inhibitor). We quantified the short-term effects on rates and duration of SIB and self-directed stereotypies associated with the range of doses of fluoxetine and venlafaxine examined.

Methods: The subjects were 17 male (aged 7 – 15 years) Indian origin rhesus macaques (*Macaca mulatta*) that were weaned at six months of age and housed in a single cage for at least 2 years. The subjects had a history of SIB and self-directed stereotypic behavior. The animals were housed individually in 0.56 m² cages and fed a commercially available primate chow twice daily. Water was provided *ad libitum*. Additional fresh fruit and foraging devices was provided at least five days per week for enrichment. All animals were provided with manipulanda including commercially available toys and wood in accordance with the UL Lafayette-NIRC Plan for Environmental Enhancement and Behavioral Management. The UL Lafayette-New Iberia Research Center operates in full compliance with ILAR Guide for the Care and Use of laboratory Animals and Federal guidelines and is accredited by AAALAC, International. The animals were matched based on baseline rates of self-biting and self-directed stereotypies and assigned to one of two of drug conditions as follows: Fluoxetine (n = 6): 0.5 – 8.0 mg/kg po q 24h and Venlafaxine (n = 6): 2.0 – 16.0 mg/kg po q 24h. We also included a placebo-treated control condition (n = 5). All medication was administered orally in a 5.0 gm fruit-flavored wafer (drug concentration 20 mg/wafer) prepared by a pharmacist. Following a four-week baseline period, animals entered a four-week placebo phase (fruit flavored wafer only). Animals then received the lowest dose at the beginning of the treatment phase of the study. Dose levels were increased at four-week intervals for four months. Twenty minute focal behavioral samples were collected from each animal twice per week balanced for time of day throughout the experiment using handheld computers with *The Observer* software (Noldus Information Technology).

Results: We found that animals consumed the fluoxetine-medicated wafer without difficulty at doses of 0.5 – 4.0 mg/kg. When fluoxetine was increased to 8.0 mg/kg, compliance decreased to 67 %. Venlafaxine-medicated wafers were well tolerated at doses of 2.0 – 4.0 mg/kg. Venlafaxine dosages of 8.0 – 16.0 mg/kg reduced compliance to 33 %. Heart rate, blood pressure, and body weight were evaluated at 4-week intervals and were not significantly affected by either drug treatment. Overall, we found that fluoxetine was significantly more effective in decreasing rates of self-biting compared to venlafaxine and placebo-treated conditions. The minimum dose of 0.5 mg/kg resulted in 50% in rates of self-biting. Doses of 2.0 - 8.0 mg/kg

resulted in 70 –73 % decreases in self-biting rates. Blood concentrations of fluoxetine and norfluoxetine were below assay detection at 0.5 mg/kg. Blood concentrations at higher doses were as follows: 2.0 mg/kg, fluoxetine = 18.1 ± 5.3 and norfluoxetine = 186.5 ± 31.0 ; 4.0 mg/kg, fluoxetine = 63.1 ± 19.2 and norfluoxetine = 369.6 ± 80.0 ng/ml; 8.0 mg/ml, fluoxetine = 182.2 ± 116.5 ng/ml and norfluoxetine = 654.9 ± 261.0 ng/ml. Venlafaxine produced a non-significant decrease of 43 % in rates of self-biting.

Conclusions: The results indicate that: 1) fluoxetine is superior to venlafaxine in reducing self-biting in rhesus macaques, and 2) fluoxetine at 2.0 mg/kg q 24 h should be used to evaluate the long-term effects of serotonin reuptake inhibition on self-biting in rhesus macaques.

Evaluation of tail biopsy collection in laboratory mice (*Mus musculus*): vertebral ossification, DNA quantity and acute behavioral responses

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Introduction: Our study was undertaken to rigorously evaluate institutional guidelines for mouse tail biopsy procedures promoted to research investigators and to develop science-based welfare standards. We wished to determine if mice of different genetic backgrounds vary in the maturation and ossification of tail vertebrae and to assess behavioral responses immediately following biopsy. We wanted to further determine if DNA isolation for genotyping is dependent upon the age of mice and length of biopsy sampled. Herein we demonstrate pronounced differences in caudal vertebral development between mice of differing genetic backgrounds, differences in effects on quantity of DNA harvested linked to varying age and biopsy lengths sampled, and strain- and age-dependent behavioral responses to tail biopsy.

Methods: Mice of five commonly used strains [C57BL/6 (B6), 129/SV (129), BALB/c, C3H, and FVB] and one stock [SW] were procured from an approved vendor or were transferred naïve from approved protocols for breeding purposes. Age-matched litters were combined to analyze ossification at twelve distinct time points; a total of 432 mice were assessed. Animals were assessed in age groups: *Cohort 1*. Pups developed to between 3 and 14 days of age; *Cohort 2*. Pups developed to between 17 and 28 days of age; *Cohort 3*. Pups developed to between 31 and 42 days of age. Animals were imaged by microradiography using Faxitron®, microcomputed tomography, and by histological tail sectioning for assessment of vertebral development in 2, 5, 10, 15 and 35mm tail lengths. Tail biopsy samples of three lengths (minimal (5mm), moderate (10mm) and maximal (15mm)) were taken from unanesthetized mice and animals were scored within the first hour for signs of response to the procedure. Statistical analyses were performed using Prism Graphpad software.

Results: Subtle strain/stock differences were noted in total coccygeal vertebral counts, ranging in number from 27 to 29 by day 42. In all mice, vertebrae developed in a proximal to distal direction as mice aged. The total vertebral counts in each biopsy sample increased with increasing length harvested, with a sharp increase in total tail growth within the first week after birth. Averaged across strains, mature vertebrae (MV) were first discernable by Faxitron at day 10 in the total tail; however, no MV were observed on radiographs in any 5 mm distal tail segment until after day 31. When comparing the two imaging modalities of microRad and microCT, there was approximately a two week difference in point of detection of MV across all genetic backgrounds, with microCT as the more sensitive imaging technique. Within 5 mm distal tail biopsy segments, B6 and C3H mouse strains had MV detectable prior to day 21. MicroCT verified that, although calcified vertebrae were present in the distal 2mm of tail, none of these contained endplates prior to 21 days of age. It is notable from graphs of both imaging modalities that those animals in Cohort 2 (aged 17-28 days) undergo the most dramatic developmental changes with maturation of vertebrae and the appearance of endplates. In 2, 5, 10 and 15 mm segments of tail there are no vertebrae with end plates prior to day 14, but between days 17 and 28 the number of vertebrae with end plates increases across all measurement lengths. Histological evaluation demonstrated a varying rate of development across genetic backgrounds of mice, for example: BALB/c do not show appearance of ossified MV until day 31, while B6, 129 and C3H mice have ossified MV as early as day 14 and have greater than 50% ossified MV by day 28. DNA was extracted from the tails and concentration of DNA determined spectrophotometrically by measuring absorbance at A260/A280. The relative yield (the amount of DNA relative to the weight of tissue) was highest in the shortest biopsy segment of 5mm across all cohorts. If the data is considered based on age cohorts, Cohort 1 animals (3-14 days) showed significantly higher DNA content and DNA yield than values calculated from Cohort 2 and 3 animals (17-42 days combined). It is likely that the increased DNA content and yield from 5mm biopsies in Cohort 1 animals reflects a higher degree of cellularity and lesser degree of mineralization in these samples. Acute behavioral responses as a function of genetic background, age, and biopsy length were evaluated and animals collectively were classified by the absence or presence of response(s) to the biopsy cut. Interestingly, biopsy length harvested did not have a statistically significant effect on behavioral responses. However, there were differences noted in percentage of responders between the various genetic backgrounds and age cohorts. For example, BALB/c mice had fewer responses at 10 and 60 minutes, while the B6, C3H, and FVB strains had a significantly higher percentage of responses. As well,

there was a statistically significant difference in percentage of responding animals between Cohort 2 and Cohort 3 animals, regardless of biopsy length harvested. Responses diminished over the observation period, but Cohort 3 (animals aged up to day 42) had a significantly greater number of positive responses at 60 minutes than did the younger Cohort 2 animals, which is expected due to the presence of greater numbers of mature tail vertebrae in older animals.

DISCUSSION: Our study elucidated distinct and functionally significant differences in tail vertebral development across six differing genetic backgrounds of mice through 42 days of age. There is evidence from the literature that additional vertebrae develop beyond this time point. We found that complementary imaging modalities were essential to accurately determine vertebral counts and to assess vertebral maturation. In our study, microradiography by Faxitron, while useful for whole body imaging of rodents up to 5X in magnification, was unable to provide the resolution needed to fully evaluate maturation and enumerate distal tail vertebrae typically harvested during biopsy. To enhance our ability to assess the most distal aspects of the tail, we imaged tail samples with microcomputed tomography (microCT). The 35 micron voxel resolution used in this study allowed for quantification of mineralized vertebrae within the distal 2mm of the tail across the six genetic backgrounds of mice. By day 21 of age, mature vertebrae with end-plates were present in 2 mm biopsy lengths in only two strains (C3H and B6). Within a larger biopsy length of 5mm, MV were visible in every analyzed strain and stock of mice at day 21. The tail biopsy procedure was performed according to institutional recommendations and was structured to emulate a minimal (5mm), moderate (10mm) and maximal (15mm) sampling technique. Interestingly, the youngest cohort of animals (Cohort 1) provided a higher yield of DNA and had increased DNA content in the distal tail compared to older cohorts. This greater DNA yield in younger animals is likely due to the presence of highly cellular cartilage, from which DNA can be more easily extracted than from bone. Between 17 and 31 days of age, we verified that mice undergo a marked developmental phase for the tail with maturation of vertebrae and the appearance of endplates. These findings support the harvest of the minimal biopsy lengths from pre-weaned animals at approximately 14 to 17 days of age to maximize DNA extraction from less developed tail tissues. All mice in our study were between 2.5 and 6 weeks of age when evaluated following conscious tail biopsy for behavioral changes within the first hour post-procedure. We noted strain- and age-associated differences which were associated with vertebral maturation. Surprisingly, length of biopsy did not have a statistically significant effect on behavioral responses. Our scoring system did not correlate to quantitative pain assessments, yet it did serve as an indicator of abnormal behaviors following the stimulus of biopsy. A difference in animal responses was observed between genetic backgrounds. We demonstrated that B6 mice had a higher percentage of animals responding at 10 and 60 minute across all ages than did the BALB/c mice. These findings were more readily explained once we elucidated the more rapid coccygeal vertebral maturation in B6 mice. BALB/c mice show the most delayed vertebral development and this strain had the lowest percentage of mice responding to the biopsy. We showed that younger animals in Cohort 2, which we documented to have less mature tail vertebrae, exhibited fewer responses to the procedure than Cohort 3 animals. Our findings support administration of appropriate anesthesia/analgesia for the tail biopsy procedure in mice older than 17 days of age. If responsiveness to tail biopsy is related to vertebral maturation, as supported by our study, the current institutional standards of anesthesia and/or analgesia required only for mice biopsied after 21 days of age may need to be reevaluated, particularly if greater than 2mm of distal tail is harvested. Regardless of genetic background, all mouse tail vertebrae have calcification and ossification within the distal 5mm of the tail at early ages, with mature endplates present prior to the typical age for weaning (21 days). Certain analyzed strains, including B6 and C3H, have mature vertebrae within 5mm of distal tail by day 17 of age. These findings unequivocally refute the common statements made in contemporary institutional guidelines that imply calcification has not occurred in the tail until day 21 of age. Current standards suggest that a range of 2-15mm of tail biopsy is adequate for DNA isolation for genotyping. This range can now be modified, and the suggestion be made that tail biopsies less than 5mm in length are sufficient for genotyping in mice no older than 17 days of age, unless anesthesia or topical analgesia is provided.

Restrained whole body plethysmography for measurement of strain-specific and allergen-induced airway responsiveness in conscious mice

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The use of mice in respiratory research is growing, in part due to the rapid development of new transgenic strains. Precise measurements of airway function may be obtained using invasive technologies that control for the confounding influences of lung volume (i.e. volume history, lung volume during measurement) and respiratory frequency. However, an important place remains for the *in vivo* study of conscious mice, where the influences and risks of anesthesia are absent, and longitudinal studies are desired.

Currently, a problem with the study of conscious mice is the lack of widely accepted techniques. The available methods each have advantages and disadvantages that relate to: (1) ease of the procedure, (2) animal tolerance, (3) precision and validation, and (4) their basis in known physical determinants of airway function (i.e., pressure and flow). Recently, concern has been expressed over the widespread use of unrestrained technology to characterize 'airway function' per se. These opinions were formed after several studies failed to corroborate data derived from unrestrained whole body plethysmography (UWBP) with more rigorous invasive techniques. Since UWBP is a commonplace application, this has left many users searching for practical alternatives. Several alternative methods been studied which do provide more direct measures airway mechanics. Mid-tidal expiratory flow (EF50) has been evaluated extensively for the ability to characterize bronchoconstriction in conscious mice. Outcomes of EF50 have correlated well with simultaneous invasive measures of pulmonary resistance and dynamic compliance during allergen, cholinergic agonist, and hyperoxia challenges. While EF50 permits the monitoring of tidal breathing flow limitation, one can only infer airway resistance from plethysmographically derived flow. Specific airway resistance (sRaw), the product of airway resistance (Raw) and lung volume could provide greater insight into airway mechanics. This variable can be measured using double chamber plethysmography, although there are practical limitations such as the use of a neck seal and complex restrainer. The reproducibility of airway reactivity derived from double chamber plethysmography (DCP) has also been challenged, and strain specific responses to methacholine were discordant with more invasive methods. For these reasons, the current method to measure sRaw using DCP is not widely cited. Transfer impedance (Ztr) is yet another conscious method, which is used to probe central (airway) vs. peripheral (tissue) contributions to bronchoconstriction and permit serial measurements over time. One limitation of Ztr is the need for acclimation to obtain acceptable coefficients of variation, although this can be obtained within a day. In sum, each available system provides a different level of user satisfaction and certainty regarding the status of the airways, and therefore the development of novel systems that improve upon this spectrum of technologies should continue.

We investigated the use of restrained whole body plethysmography (RWBP), an adaptation of the original method of body plethysmography (pressure plethysmography) in humans that was later used in guinea pigs. Mice are fast breathers (3-6 Hz) so special considerations concerning plethysmographic design and validation were addressed. The intent of this study was to provide an initial proof of concept for RWBP in conscious mice and thus stimulate future applications and comparisons with alternative systems. We hypothesized that 1)

baseline sRaw-RWBP and associated methacholine responses would be similarly reproducible to Raw-FOT, 2) values for sRaw measured with RWBP (sRaw-RWBP) would be comparable to sRaw derived using airway resistance from the forced oscillation technique (Raw-FOT) and by awake double chamber plethysmography (sRaw-DCP), 3) strain-specific airway responsiveness would be accurately characterized by RWBP and lastly, 4) RWBP could be used to characterize longitudinally the increase in airway responsiveness associated with chronic (10 wk) allergen challenge in a group of BALBc mice.

Methacholine (Mch) responses were compared using sRaw-RWBP versus airway resistance by the forced oscillation technique (Raw-FOT) in groups of C57, A/J, and BALBc mice. sRaw-RWBP was also compared to sRaw derived from double chamber plethysmography (sRaw-DCP) in BALBc. Finally, airway reactivity following allergen challenge in BALBc was measured using RWBP. sRaw-RWBP in C57, A/J, and BALBc mice was 0.51 ± 0.03 , 0.68 ± 0.03 , and 0.63 ± 0.05 cm*sec, respectively. Baseline measurement computed from Raw-FOT and FRC in C57 mice (0.48 cm*sec) was slightly lower than sRaw-RWBP (0.52 cm*sec). The intra- and inter-animal coefficients of variations were similar between sRaw-RWBP (6.8 and 20.1%) and Raw-FOT (3.4 and 20.1%, respectively). The order of airway reactivity employing sRaw-RWBP was AJ>BALBc>C57 and for Raw-FOT was AJ>BALBc=C57. There was no difference between the reactivity assessed by RWBP vs. DCP; however baseline sRaw-RWBP was significantly lower than sRaw-DCP. Allergen challenge caused a significant increase in Mch reactivity using RWBP. In conclusion, the technique of RWBP was rapid, reproducible, and easy to perform. Values of sRaw-RWBP were comparable to those measured using FOT. Airway responsiveness was measured equivalently for RWBP, DCP, and FOT. Allergen responses could be followed longitudinally which may provide greater insight into the pathogenesis of chronic airway disease.

The novel technology (RWBP) employed in this study offers a refinement to past techniques using conscious methods since the outcome measure (sRaw) can be obtained without the use of a neck seal which promotes stress and requires greater acclimation time. The restraint used in RWBP does not interfere with the measurement reproducibility as for transfer impedance (Ztr). The measurement is defensible using first principles in physiology, hence permitting one to publish data in top tier journals, an issue which has arisen as a significant limitation to unrestrained plethysmography (Penh). Finally, one does not have to resort to using an invasive techniques to obtain accurate measurements of sRaw and airway responsiveness.

ENVIRONMENTAL ENRICHMENT AND AGGRESSION IN MALE MICE

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Although provision of environmental enrichment to animals is assumed to be beneficial, in an earlier study we found that severe aggression was higher in groups of male CD-1 mice given a highly-preferred enrichment, a running wheel. It was unclear from that study whether this effect was specific to only one strain of mice, and whether other types of enrichments might have different behavioral effects. The purpose of this ACLAM-funded study was to determine the effects of several commercially available environmental enrichments on different strains of group housed male laboratory mice. Based on a review of previous literature, we hypothesized that rigid, non-destructible enrichments could partition the cage space in such a way as to create territories that male mice attempt to defend, while destructible enrichments might not have this effect. Thus, the enrichments used in this experiment were either destructible (Nestlets® and Shepard Shacks®) or rigid (perspex tunnels and Fasttrac and Igloo® running wheel and shelter combination). We further hypothesized that enrichment effects might be strain specific, and so observed two inbred strains of mice, Balb/cAnNCrI and C57/BL6NCrI, and one outbred strain, CrI:CD1(ICR).

The design of the experiment was a repeated measures Latin squares design, with all cages receiving all treatments for a period of two weeks, as follows:

CAGE	Period 1	Period 2	Period 3	Period 4	Period 5	Period 6	Period 7	Period 8
1	C	W	C	S	C	T	C	N
2	C	W	C	N	C	T	C	S
3	C	T	C	S	C	W	C	N
4	C	T	C	N	C	W	C	S
5	C	S	C	W	C	N	C	T
6	C	S	C	T	C	N	C	W
7	C	N	C	W	C	S	C	T
8	C	N	C	T	C	S	C	W

Table 2. Order of presentations of enrichment to each cage for each strain. Presentations will alternate between destructible and non-destructible items, with a control period of two weeks between each 2-week enrichment period. C=control (no enrichment), W=running wheel, S=Shepherd Shack, T=tunnel, N=nestlet

Mice were housed in polycarbonate cages in littermate groups each containing 5 mice. The study began when the mice were 60 days of age. At the end of each 2-week period we videotaped each

cage three times for 15 minutes each time: first hour after lights-on, first hour after lights-off, during the middle of the dark phase. From these tapes, we determined the rates of escalated aggression (serious aggression); mediated aggression (mild aggression); affiliative behavior (e.g. mutual grooming); and stereotypic behavior (e.g. bar-mouthing). Data were analyzed using the General Linear Model, with Tukey post-hoc tests for comparison of means. Fecal pellets deposited during the handling procedure were collected from each mouse for analysis of corticosterone (these data are still being analyzed and are not presented in this report). At the beginning, middle and end of the study the linearity of the dominance hierarchy (a measure of stability of social organization) was determined for each cage using the “tube test” of social dominance. The Landau’s index was used as the measure of hierarchy.

Strain Effects on Behavior

There were marked strain effects on behavior. Balb/c and CD-1 mice were more stereotypic (Figure 2) than C57/Bl mice (both $p < 0.0001$), while Balb/c and CD-1 mice did not differ from one another ($p = 0.8338$). There were significantly fewer affiliative behaviors in CD1 mice than C57/Bl mice ($p < 0.0001$), but no significant differences between either Balb/c and CD-1 or Balb/c and C57.

There was a significant effect of strain and time on the linearity of the social dominance hierarchy ($p < 0.001$). Balb/c mice had significantly more linear hierarchies than CD-1 and C57/Bl ($p < 0.0001$), and the hierarchy in CD-1 was more linear than in C57/Bl ($p < 0.0001$).

Treatment Effects on Behavior

There were no treatment effects, or treatment by strain interactions, on stereotypy, mediated aggression, hierarchy linearity, or affiliative behaviors (see figures). In CD-1 mice, there was significantly more escalated aggression during the tunnel enrichment than during control periods or periods with destructible enrichments ($p < 0.0001$ for all comparisons) and significantly more aggression during Tunnel than Wheel enrichment periods ($p = 0.05$). In C57/Bl mice, there was similarly more escalated aggression when the either the Tunnel or Wheel periods were compared to the control or destructible enrichment periods ($p < 0.0001$ for all comparisons). There was also significantly more escalated aggression in Tunnel than in Wheel ($p < 0.0001$).

Because we only measured dominance hierarchy linearity at three time points, we could not track changes in linearity associated with specific enrichments. However, in general social stability decreased somewhat over time. There was significantly higher linearity for the first social dominance measure compared to the second and third (both $p < 0.0001$) but no significant difference when comparing the second and third measurements ($p = 0.9760$). This decrease in linearity occurred in all three strains and so is likely to represent either time changes due to age or general changes in social behavior resulting from the changing environment associated with adding the various enrichments to the cages.

Summary

As predicted, the presence of rigid enrichments (Tunnel and Wheel) was associated with more serious aggression than was the presence of destructible enrichments (nestlet and Shepherd Shack). However, this effect was highly dependent upon mouse strain, with these effects shown

only for the C57Bl and CD-1 mice. The BALB/c mice in our study showed no evidence of serious aggression during the control periods, and their social behavior was unaffected by the enrichment regimen. They also had the most stable social structure, as reflected in the greater linearity of their dominance hierarchy. BALB/c mice are anecdotally reported to be aggressive, so this finding may be due to the particular substrain used in our experiment.

Surprisingly, enrichment did not lead to decreased stereotypy, although it is possible that our experimental design made it difficult to tease out these effects – in a previous study we found that stereotypy was decreased in male CD-1 mice given a running wheel (Howerton et al., submitted). Further studies will be necessary to evaluate the effects of enrichment on abnormal behavior in group housed male mice. However, the results of the present study indicate that providing male mice of aggressive strains/substrains with rigid enrichments alone is likely to increase potentially injurious aggression. Whether or not these effects could be mitigated by also adding destructible enrichments is unknown. The fact the Tunnel enrichment was associated with the highest levels of aggression supports our hypothesis that enrichments that partition the cage space create territories that male mice will try to defend.

Figure 1- Escalated Aggression

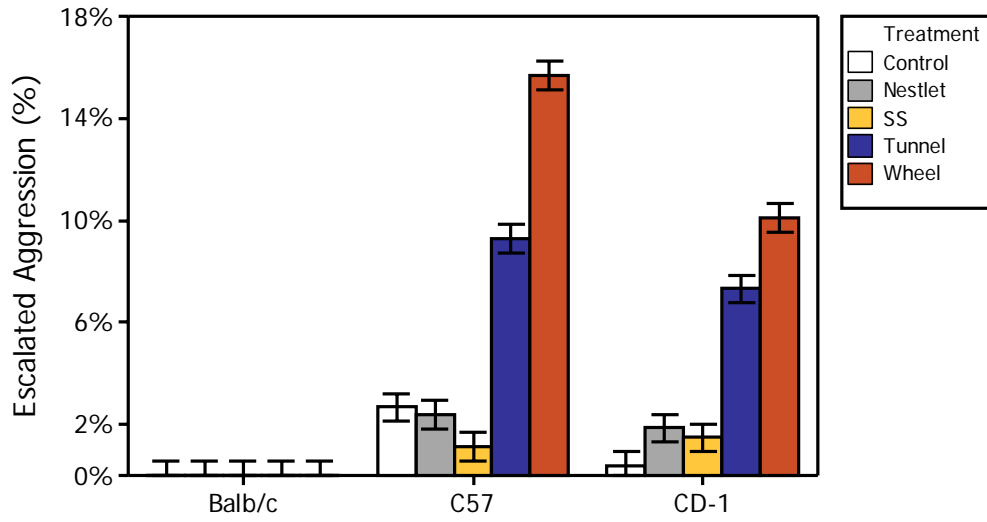


Figure 2- Stereotypies

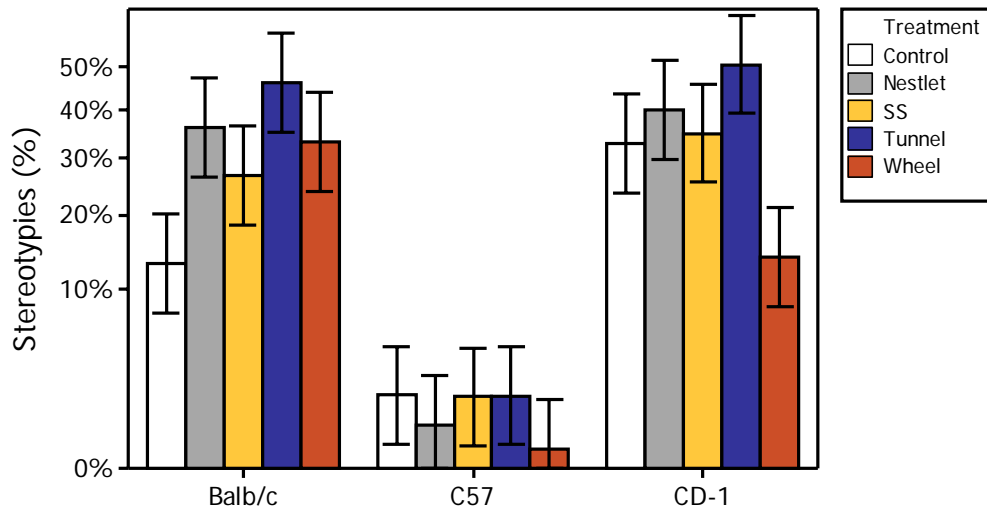


Figure 3- Affiliative Behaviors

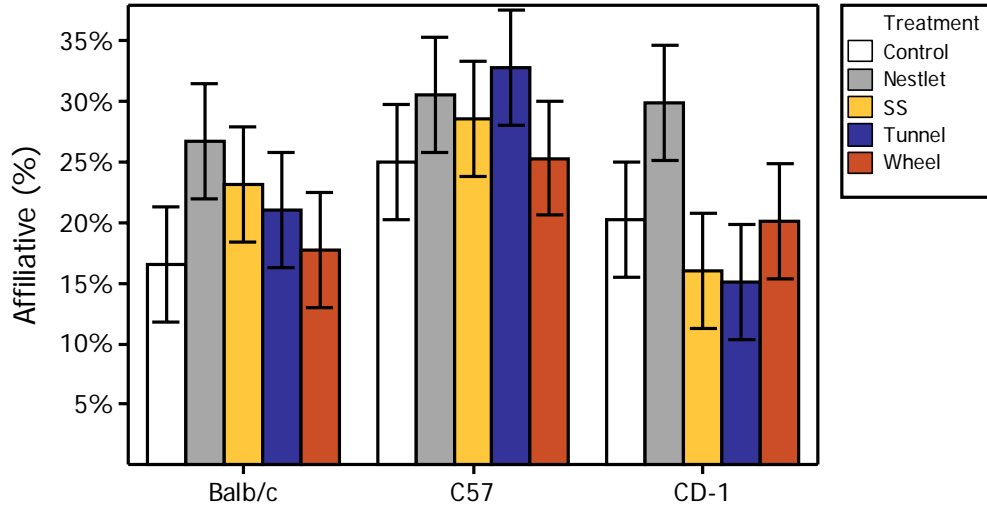
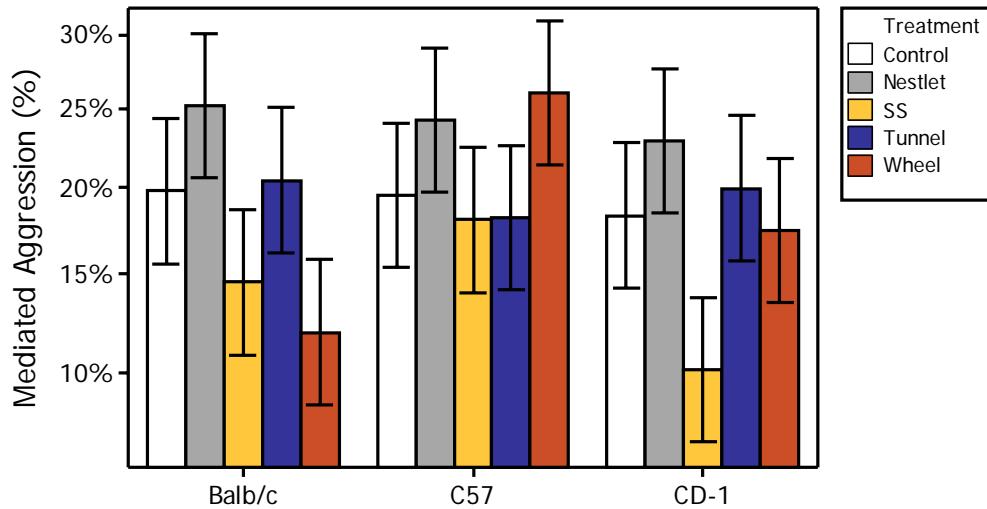


Figure 4- Mediated Aggression



Analgesic efficacy of butorphanol and morphine in bearded dragons (*Pogona vitticeps*) and corn snakes (*Elaphe guttata*)

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Abstract

Objective—To test the hypothesis that butorphanol or morphine produces antinociception in bearded dragons and corn snakes.

Animals—Juvenile and adult male and female bearded dragons (*Pogona vitticeps*, N=12) and corn snakes (*Elaphe guttata*, N=13).

Design—Prospective crossover study.

Procedure—Infrared heat stimuli were applied to the plantar surface of bearded dragon hindlimbs or the ventral surface of corn snake tails. Thermal withdrawal latencies were measured before and after subcutaneous administration of physiologic saline, butorphanol tartrate (2.0 or 20 mg/kg), or morphine sulfate (1.0, 5.0, 10, 20, or 40 mg/kg).

Results—For bearded dragons, butorphanol (2.0 or 20 mg/kg) did not alter hindlimb withdrawal latencies at 2–24 h post-injection. In contrast, morphine (10 and 20 mg/kg) increased hindlimb latencies to maximums of 2.7 ± 0.4 s and 2.8 ± 0.9 s above baseline, respectively, at 8 h post-injection ($P < 0.001$). For corn snakes, butorphanol (20 mg/kg) increased tail withdrawal latencies to a maximum of 3.0 ± 0.8 s above baseline at 8 h post-injection ($P < 0.001$); butorphanol (2.0 mg/kg) had no effect. Morphine injections (1.0, 5.0, 10, 20, and 40 mg/kg) did not increase tail withdrawal latencies between 2–24 h post-injection.

Conclusions and Clinical Relevance— Relatively large dosages of morphine (but not butorphanol) produce analgesia in bearded dragons. In contrast, relatively large dosages of butorphanol (but not morphine) produce analgesia in corn snakes. We conclude that there are significant differences in opioid effects across reptile Orders, and that potentially unsafe dosages of opioid drugs are required to produce analgesia in bearded dragons and corn snakes.

Interpretive Summary— Little is known with respect to providing safe and effective pain control in reptiles. Butorphanol is a widely used analgesic opioid drug for reptiles, but may

provide only marginal pain relief in corn snakes at high dosages, and is ineffective in bearded dragons and red-eared slider turtles. Morphine provides analgesia in bearded dragons and turtles, but provides no pain relief in corn snakes. Thus, opioid drugs that readily provide pain relief in mammals may not be as effective, and may have species-specificity in reptiles.

Introduction

Judicious use of analgesic drugs in all species is critical to the practice of clinical veterinary medicine.^{4,17} Conditions considered painful in humans and other mammals should be assumed to be painful across all other vertebrate species.^{1,10,17} While our understanding of pain control is considerable in domestic mammals and humans, methods for measuring pain and analgesia in nondomestic species require further development.^{10,17,20,21,22} Information regarding pain control is particularly scarce for reptiles, even though reptiles are frequently maintained as companion animals, and commonly represented in zoological and scientific laboratory facilities.^{1,10,18,24} In addition, reptiles are one of the most phylogenetically diverse animal Classes with three main Orders: Chelonia (turtles and tortoises), Squamata (lizards), and Serpentes (snakes). Thus, it is important and necessary to study ways to provide analgesia in representative species from each reptile Order.

Opioid-receptor agonists or partial agonists/antagonists are commonly administered, and considered the most effective drugs for controlling pain in mammals.¹⁶ However, the lack of experimentally derived data for opioid drugs in reptiles is a significant clinical disadvantage. The opioid receptor gene family (μ , κ , and δ) is highly conserved across multiple vertebrate orders, although specific information on reptilian endogenous ligands and opioid receptors is sparse.^{9,23} For example, two snake species have endogenous brain opiates,^{14,15} and red-eared slider turtles have both proenkephalin-derived peptides¹⁹ and functional μ - and δ -opioid receptors in the brain.^{6,25} Although opioid receptors are expressed in reptiles, opioid drugs that provide analgesia in reptiles are not known.

Anecdotal reports recommend administering butorphanol at mammalian-derived dosages.¹⁸ However, we recently demonstrated that butorphanol (2.0 and 20 mg/kg) had no analgesic efficacy in red-eared slider turtles using a noxious thermal stimulus paradigm.²² Consistent with these findings, butorphanol (1 mg/kg IM) had no antinociceptive effects using a thermal noxious stimulus in green iguanas,² and butorphanol (1.0 mg/kg IM) had no isoflurane-sparing effect in green iguanas.¹³ In contrast, morphine increased limb withdrawal latencies in turtles²² and crocodiles,^{7,8} and increased tail flick latencies in anole lizards,¹¹ thereby demonstrating that morphine provides antinociception in certain reptile species. Thus, there is a need for systematic evaluation of different opioid drugs and analgesia in several reptile species to test for species differences, dose-dependent effects, and duration of drug efficacy.

The objective of this study was to determine whether different dosages of butorphanol or morphine provides antinociception in bearded dragons (*Pogona vitticeps*) and corn snakes (*Elaphe guttata*) as representative species of the *Squamata* and *Serpentes*. Noxious thermal stimuli were applied to bearded dragon hindlimbs and the ventral tail surface of corn snakes before and after administration of saline or opioid drugs.

Materials and Methods

The Animal Care and Use Committee at the University of Wisconsin-Madison School of Veterinary Medicine approved all experimental procedures.

Subjects— Juvenile and adult bearded dragons (*Pogona vitticeps*, $n = 12$ [6 male, 6 female], initial weights = 57.9 ± 7.9 g) and corn snakes (*Elaphe guttata*, $n = 13$ [7 male, 6 female], initial weights = 72.5 ± 15.1 g) were obtained from commercial suppliers. All animals were considered healthy during the entire experimental period based on results of physical examinations and routine hematological and serum biochemical parameters. Room temperature was set at 28-29°C. Bearded dragons were housed individually in 38 L aquariums equipped with fresh water, a climbing branch, a hide box, and newspaper substrate. Bearded dragons were fed daily with a mixed salad composed of fresh dark, leafy greens, sliced carrots, and strawberries. Calcium carbonate powder^a was applied to the salad mix twice weekly and a multivitamin powder^b applied twice monthly. Bearded dragons were offered live crickets three times per week. A broad-spectrum ultraviolet light bulb^c was suspended above each lizard and kept illuminated 14 h/day. A ceramic heat bulb for basking was also suspended above each aquarium housing the lizards. Snakes were housed in standard rodent cages equipped with a hide box, fresh water, and newspaper substrate. Snakes were fed once per week with thawed mice.

Study Design—A crossover experimental design was used to evaluate opioid-dependent changes in thermal antinociception. The observer in the antinociceptive experiments was blinded to treatments.

Thermal analgesia experiments— Analgesimetry consisted of measuring withdrawal latency to a noxious infrared radiant heat stimulus applied to the plantar surface of bearded dragon hindlimbs or the ventral tail surface of corn snakes approximately 1-2 cm caudal to the cloacae using a standard apparatus^d. Animals were placed in plastic boxes (17 x 13 x 14 cm) on an elevated plexiglass surface with opaque barriers that prevented visual contact with each other. When infrared heat was applied, the rise in temperature caused the animal to withdraw and the time to withdrawal (*i.e.*, latency) was measured automatically. Stimulation strength was adjusted to produce baseline latencies of approximately 5–10 s (corresponds to 45-47°C). A maximum of 32 s was used to prevent prolonged heat exposure. Baseline mean withdrawal latency was established for each animal by applying one stimulus every 5 min for three times. Injections consisted of either physiologic saline (0.9% equivalent to opioid volumes); butorphanol^e (2.0 or 20.0 mg/kg), or morphine^f (1.0, 5.0, 10, 20, or 40 mg/kg). All drugs were given intramuscularly in the epaxial muscles, and in the cranial half of the body. At 2, 4, 8, and 24 hr post-injection, withdrawal latencies were obtained by applying one stimulus every 5 min for three times. All animals were conditioned to the chamber and tested with saline injections prior to being randomly assigned to receive drugs at different dosages.

Data Analysis—Withdrawal latencies at each time point were averaged together. Commercially available software^g was used to analyze all data and calculate two-way ANOVAs. If normality or equal variance assumptions were not satisfied, data were ranked and the two-way ANOVA recalculated on the ranked data. Post-hoc comparisons were made using the Student-Newman-Keul's test. All data are expressed as mean \pm SEM. P-values < 0.05 were considered significant.

Results

To establish baseline hindlimb thermal withdrawal latencies, saline injections were administered to bearded dragons ($n=12$) prior to starting the drug experiments. Baseline withdrawal latencies started at 7.7 ± 0.4 s and remained within 7.8 ± 0.4 s to 8.3 ± 0.4 s for the next 2-24 h post-injection (Fig. 1A). Mean baseline withdrawal latencies for butorphanol (2.0 mg/kg; $n=12$) were nearly identical to saline values over the 2-24 h period post-injection ($P= 0.86$; Fig. 1A). For higher dosages of butorphanol (20 mg/kg; $n=11$), mean baseline withdrawal latencies were 10.0

± 0.8 s, over 2.0 s greater than baseline values for saline and butorphanol (2.0 mg/kg), which produced a spurious significant drug effect ($P < 0.001$) since latencies did not change with time from baseline (Figs. 1A, 1B). It's possible that bearded dragons (and corn snakes) were actively shedding at certain times during this study. However, we avoided using individual reptiles actively shedding or preparing to shed in order to avoid the confounding influence of limited nociception during the shedding process. Thus, there is no obvious scientific, technical, or seasonal explanation for this increase in mean baseline withdrawal latency. Apparently, mean baseline hindlimb withdrawal latencies for bearded dragons exhibit considerable variability (*e.g.*, see baseline values in Fig. 2A). Nevertheless, 20 mg/kg butorphanol did not alter withdrawal latencies from 2–24 h post-injection ($P = 0.98$; Fig. 1B).

Morphine injections in bearded dragons at both 1.0 mg/kg ($n = 10$) and 5.0 mg/kg ($n = 11$) had significant drug effects ($P < 0.001$ for both dosages) compared to saline injections, but these effects were due to differences in baseline hindlimb values (Fig. 2A). When graphed as the change in latency, morphine injections at 1.0 and 5.0 mg/kg had no effect on latencies compared to saline injections despite an upward trend of nearly two seconds at 2–8 h post-injection before returning to baseline values at 24 h ($P = 0.13$; Fig. 2B). In contrast, higher dosages of morphine (10 mg/kg; $n = 11$ or 20 mg/kg; $n = 6$) produced nearly identical results with latencies increasing by 2.2–2.8 s for 2–8 h post-injection ($P < 0.001$ for drug effects; Figs. 2C, 2D). At 24 h post-injection of morphine (10 mg/kg), latencies were only 1.2 ± 0.7 s above baseline, indicating that morphine effects did not last 24 h at that dosage ($P = 0.14$; Fig. 2D).

For corn snakes, saline injections ($n = 7$) did not alter baseline tail withdrawal latencies of 5.6 ± 0.9 s over the next 2–24 h post-injection (Fig. 3A). Likewise, butorphanol (2.0 mg/kg; $n = 7$) injections did not alter tail withdrawal latencies in snakes ($P = 0.98$; Fig. 3A). With larger butorphanol doses (20 mg/kg; $n = 7$), baseline withdrawal latencies started higher at 8.3 ± 0.7 s and increased to a maximum of 11.3 ± 0.4 s at 8 h post-injection, which resulted in a significant drug effect ($P < 0.001$; Fig. 3A), even when graphed as the change in latency versus time (Fig. 3B; $P = 0.01$).

Morphine injections in corn snakes at 1.0 mg/kg ($n = 10$) or 5.0 mg/kg ($n = 11$) increased tail withdrawal latencies to a maximum of 2.3 ± 0.9 s above baseline at 4 h post-injection for both dosages (Fig. 4A). Since baseline withdrawal latencies were higher for the 5.0 mg/kg morphine injections, there was a spurious significant drug effect for these data ($P < 0.001$), but not for the 1.0 mg/kg data ($P = 0.65$; Fig. 4A). When the data were graphed as the change in latency, there were no significant drug effects ($P = 0.38$; Fig. 4B). Morphine injections (10 mg/kg, $n = 3$; 20 mg/kg, $n = 12$; and 40 mg/kg, $n = 3$) produced no significant drug effects ($P = 0.20$ for Fig. 4C; $P = 0.35$ for Fig. 4D).

Discussion

This is the first study to systematically evaluate the magnitude and 24-h time-course of the antinociceptive effects of butorphanol and morphine in lizards and snakes using a well-established thermal withdrawal latency test. Butorphanol, the most widely used analgesic opioid drug in reptiles, had no antinociceptive effects in bearded dragons, but provided antinociception in corn snakes. On the other hand, morphine provided antinociception in bearded dragons, but not in corn snakes. However, the antinociceptive dosages of morphine (10 and 20 mg/kg) in bearded dragons and butorphanol (20 mg/kg) in corn snakes were well above typical clinical doses and may cause severe respiratory depression. Thus, caution is warranted before

administering these opioid drugs to lizards and snakes to provide analgesia within a clinical setting.

The finding that morphine, but not butorphanol, provides analgesia in bearded dragons is similar to what was found in red-eared slider turtles.²² However, the magnitude of the hindlimb withdrawal latency increase in bearded dragons due to morphine is much smaller compared to turtles. For example, hindlimb withdrawal latencies increased nonsignificantly by 2.0 ± 0.9 s following morphine (5.0 mg/kg) injection in bearded dragons, while latencies increased significantly by 8.0 ± 2.8 s following morphine (6.5 mg/kg) injection in turtles.²² Even high dosages of morphine (10 and 20 mg/kg) produced hindlimb withdrawal latency increases of only 2-3 s in bearded dragons. Surprisingly, tail withdrawal latencies in corn snakes were not altered despite testing morphine dosages between 1.0 and 40 mg/kg. Thus, morphine efficacy appears to vary widely within reptiles. Since 1.5 mg/kg of morphine depressed breathing by 83% in red-eared slider turtles,²² high morphine doses given to bearded dragons may also depress breathing to a similar degree. In contrast, butorphanol produced mild analgesia in snakes, but had no effect on hindlimb withdrawal in bearded dragons. Based on these findings, it appears that the role of opioid receptor activation in providing analgesia against noxious thermal stimuli may not be conserved across reptile Phyla and species.

The ability to discriminate and quantify behavior indicative of pain from species-typical behavior is crucial to the study of pain and analgesia.⁴ Once pain-related behaviors are reliably quantified, it's possible to test antinociceptive drug efficacy. Unfortunately, there are few methods available for quantitatively assessing species- and context-specific pain-related behavior in reptiles and other non-domestic species.^{10,17,20} In this study, noxious thermal stimuli were used in an adaptation of a classic rodent paradigm for measuring withdrawal responses.⁵ Application of a noxious thermal stimulus provides a well-established behavioral model for assessing pain and analgesia. The advantages compared to other noxious stimulus paradigms include rapid application and decay of the noxious stimulus (so as not to cause lasting inflammation), instant latency quantification, and unambiguous behavior after stimulus exposure (either the animal withdraws its limb or it doesn't). Most importantly, the animal can escape the noxious stimulus by simply withdrawing its limb or tail.

Although we successfully adapted the noxious thermal stimulus model to bearded dragons and corn snakes, this model may not always be ecologically or physiologically relevant for all reptile species. This may explain the unexpected findings of this study, such as large variability in baseline withdrawal latencies, lack of consistent opioid drug effects on antinociception between species, and relatively small increases in withdrawal latencies with high doses of opioid antinociceptive drugs. For example, aquatic red-eared slider turtles respond consistently and predictably by withdrawing a hindlimb after application of noxious thermal stimuli, and morphine administration produces large increases in hindlimb withdrawal latencies.²² Since a typical aquatic ecosystem suitable for red-eared sliders may reach a maximum water temperature of 30°C (86°F), it is reasonable to assume that a hot stimulus would elicit avoidance. Likewise, bearded dragons live in different habitats, such as open woodlands and deserts in Australia, or arboreal habits, suggesting that exposure to hot environmental surfaces is not necessarily a constant part of bearded dragon ecology.¹² Also, bearded dragons demonstrate signs of discomfort after exposure to ambient temperatures greater than 40°C, suggesting that extreme heat may be aversive to this species.³ On the other hand, some bearded dragons are adapted to warm arid environments and prefer ambient temperatures ranging from 30-40°C.³ Thus,

adaptation to warm environmental temperatures may preclude this species from responding to opioid drug administration similar to certain turtle or mammalian species.

Since corn snakes inhabit a wide variety of temperate ecosystems, they would also be expected to withdraw their tails in response to hot noxious stimuli and exhibit antinociception following opioid drug administration. In this study, however, snakes had highly variable baseline tail withdrawal latencies, increased their withdrawal latencies only to very high doses of butorphanol, and were unresponsive to morphine. These findings were surprising and raise important questions as to how snakes process different nociceptive sensory afferent inputs. For example, captive snakes often receive thermal burns if allowed to bask on faulty overheated in-cage heating units. Although these are anecdotal observations, we hypothesize that snakes may process sensory inputs from noxious thermal stimuli differently than from noxious stimuli due to electric shock, surgical incision, visceral tissue damage, or inflammation. Alternatively, the evolution of limblessness in snakes may have dramatically altered spinal opioid receptor expression and function. However, there is no information in the literature regarding the presence or function of spinal opioid receptors in any snake species.

In conclusion, measuring pain in reptiles is complex and expansion of our methodology is critical for analyzing nociception and analgesia across species and contexts. When measuring withdrawal latencies to noxious thermal stimuli, μ -opioid agonists, such as morphine, appear to be more efficacious in bearded dragons and red-eared slider turtles than butorphanol, which is currently the analgesic drug of choice for all reptile species. The variability in response to noxious thermal stimuli in corn snakes precludes drawing a conclusion regarding the most effective opioid analgesic in this species. Application of multiple methods for measuring nociception and analgesia in reptiles may be more appropriate than just a thermal stimulus.

Footnotes

^a Rep-Cal Calcium, Los Gatos, CA

^b Rep-Cal Multivitamin, Los Gatos, CA

^c Reptisun 5.0, ZooMed Labs San Luis Obispo, CA)

^d Ugo Basile Plantar™ Analgesia Instrument (Hargreaves's Apparatus), Model 37370, Ugo Basile Company, Comerio VA Italy

^e Torbugesic-SA (Butorphanol tartrate, 10 mg/ml), Fort Dodge Animal Health, Fort Dodge, IA

^f Morphine sulfate (15 mg/ml), Baxter Healthcare Corp., Deerfield, IL

^g Sigma Stat, Jandel Scientific Software, San Rafael, CA

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