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A Comparison of Three Anesthetic Drug Combinations for Use in Inducing Surgical Anesthesia in Female Guinea Pigs (*Cavia porcellus*)

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Guinea pigs are often used in translational research, but providing them with safe and effective anesthesia is a challenge. Common methods like inhalant anesthesia and injectable ketamine/xylazine induce surgical anesthesia but can negatively affect cardiovascular, respiratory, and thermoregulatory systems and complicate the interpretation of research outcomes. Several alternative anesthetic regimens have been investigated, but none have consistently achieved a surgical plane of anesthesia. Therefore, identifying an anesthetic regimen that achieves a stable state of the surgical plane of anesthesia while preserving cardiorespiratory function would be a valuable contribution. To address this issue, we compared the efficacy of 3 anesthetic combinations in female Dunkin-Hartley guinea pigs: 1) alfaxalone, dexmedetomidine, and fentanyl (ADF); 2) alfaxalone, midazolam, and fentanyl (AMF); and 3) alfaxalone, midazolam, fentanyl, and isoflurane (AMFIso). We monitored anesthetic depth, heart rate, oxygenation, respiratory rate, respiratory effort, blood pressure, and body temperature every 15 min from injection to recovery. We also recorded the time to loss of righting reflex, duration of anesthesia, and time to achieve a surgical plane. The results showed no statistically significant differences in induction and recovery times among the groups. In the AMFIso group, 100% of the animals achieved a surgical plane of anesthesia, whereas only 10% of the animals in the AMF group reached that level. None of the animals in ADF group reached a surgical plane of anesthesia. Respiratory rate was significantly lower in the AMFIso as compared with the ADF group ($P < 0.001$) but was not different between the AMF and ADF groups. Temperature was significantly lower in the AMFIso group as compared with both the ADF and AMF groups ($P < 0.001$). In conclusion, both combinations of solely injectable anesthetics assessed in this study can be used for short, nonpainful procedures without significant cardiorespiratory depression. However, for mildly to moderately painful surgical procedures, the addition of an inhalant anesthetic like isoflurane is necessary for female guinea pigs.

Abbreviations and Acronyms: ADF, alfaxalone, dexmedetomidine, and fentanyl; AMF, alfaxalone, midazolam, and fentanyl; AMFIso, alfaxalone, midazolam, fentanyl, and isoflurane; bpm, breaths per minute; GP, guinea pig; HR, heart rate; KX, ketamine and xylazine; LORR, loss of righting reflex; MAP, mean arterial pressure; RR, respiratory rate

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Introduction

Guinea pigs (GPs) have been used as models for over 200 y in various fields of research, including nutrition, otology, immunology, and hypersensitivity.[11–13,21] However, GPs can be difficult to anesthetize and require chemical restraint or anesthesia even for minor procedures like blood collection or surgery because of their tough skin and few accessible peripheral vessels, which make venous access difficult.[12,13,21] Furthermore, their elongated soft palate and the presence of palatal ostium can obstruct the airway during intubation, and they often retain remnants of food in their mouths, which complicates the intubation process.[13,21]

Inhalant anesthetics can also be used in GPs. In GPs, isoflurane allows rapid onset and recovery, minimal cardiovascular and respiratory depression, and precise control that supports safe and effective sedation for various procedures.[11,12,16,18] Isoflurane also enhances the anesthetic and analgesic properties of injectable anesthetics and helps maintain a consistent and reliable surgical plane.[11,12,16,18] However, exposure of GPs to isoflurane in an induction chamber is not recommended due to the tendency of GPs to hold their breath.[12,19] Breath holding can result in a sudden deep inhalation of the anesthetic gas, potentially causing respiratory and/or cardiac arrest. The use of ketamine/xylazine (KX) combination can prevent this breath-holding behavior.[19] KX can be administered intraperitoneally, intramuscularly, or subcutaneously to GPs, and this combination is the industry standard for injectable anesthesia in this species. KX also provides some short-term postoperative pain relief.[3,13,14] The duration of anesthesia ranges from 30 to 90 min in a dose-dependent manner, but supplementation with injectable or inhalant anesthesia may be necessary to maintain a surgical plane of anesthesia.[3,7,23] Moreover, the use of KX has been associated with cutaneous irritation and muscle necrosis in GPs, making it suboptimal for GP anesthesia.[7,13,23]

Procedures involving GPs typically are performed in rodent procedural space in which injectable anesthesia may be the

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most practical choice. While KX is considered superior among many anesthetic regimens, the search for alternatives has been increasing.[12] A combination of midazolam, the α-2 agonist medetomidine, and an opioid can provide approximately 30 min of surgical anesthesia with fewer side effects as compared with KX.[18,19] Alfaxalone is also a potential alternative in combination with either a benzodiazepine or an α-2 agonist.

Alfaxalone is a neuroactive steroid that can produce anesthesia with a rapid onset of action and short duration of effect, making it a good option for procedures requiring brief periods of anesthesia in many species.[12] Alfaxalone is generally considered safe and well-tolerated by animals, with minimal side effects and excellent relaxation.[25,26] Notably, it has minimal cardiovascular effects despite the associated respiratory depression.[6,10] Alfaxalone is FDA-approved for intravenous administration in cats and dogs and has been used off-label through other routes such as intramuscular, intraperitoneal, and subcutaneous.[10,25,26] Previous studies report that the subcutaneous administration of alfaxalone to GPs has a higher safety margin than XY and provides adequate short-term sedation for nonpainful procedures.[10,23] Although alfaxalone induces excellent relaxation and sedation in GPs,[10] the large volume required can make certain routes of administration impractical and may exceed recommended substance administration guidelines for GPs.[9] As a result, benzodiazepines like midazolam and diazepam are often combined with alfaxalone to increase the duration of sedation and reduce the volume of drugs needed.[1,9,10] Benzodiazepines have minimal cardiovascular effects, yet they provide only limited analgesia.[12] Because of this lack of analgesic properties, alfaxalone has been combined with opioids or α-2 agonists.[1,9,10] However, those studies found that adding opioids increased the duration of sedation and immobility without achieving general anesthesia. Although partial *mu*-opioid agonists may have fewer undesirable effects as compared with full agonists, they also diminish the desirable analgesic effects and provide less sedation.[10] Therefore, some studies have evaluated full *mu*-opioid agonists like fentanyl for sedation and anesthesia.[17] Opioids and α-2 agonists used in combination can act synergistically to produce greater sedation.[18,19]

Based on these findings, we tested whether a combination of alfaxalone with a full mu-opioid agonist and either a benzodiazepine or an α-2 agonist would induce a surgical plane of anesthesia in GPs. We also tested whether supplementing the injected regimen with inhaled anesthesia would further enhance surgical anesthesia. We compared subcutaneous administration of alfaxalone in combination with dexmedetomidine and fentanyl, midazolam and fentanyl, and midazolam and fentanyl combined with inhaled isoflurane in female GPs.

Materials and Methods

Animals, ethical approval, and husbandry. Nine 11-mo-old healthy female Dunkin-Hartley GPs (Charles River Laboratories, Montreal, Canada) with a mean weight of 1,080 ± 109 g were used in each treatment group. Before this study, the GPs had been used on an IACUC-approved research project that involved immunization with tick proteins and subsequent challenge with pathogen-free nymphs. None of the animals were experimentally exposed to *Borrelia*, Anaplasma, or any other pathogens. All GP had negative screening at the vendor for a range of pathogens, including *Sendaivirus*, *Reovirus*, *Lymphocytic Choriomeningitis Virus*, *Pneumonia Virus* of mice, *Guinea Pig Adenovirus*, *Bordetella bronchiseptica*, *Helicobacter bilis*, *H. hepaticus*, *Klebsiella pneumoniae*, *K. oxytoca*, *Pasteurella multocida*, *Pseudomonas aeruginosa*, *Mycoplasma pulmonis*, *Streptococcus pneumoniae*, *S. zooepidemicus*, ectoparasites, endoparasites, and *Eimeria cuniculi*.

Our facility predominantly uses female GPs for research projects due to their relatively calm demeanor, which is advantageous in long-term experiments that house animals together. The lower aggression seen in female GPs minimizes the likelihood of injury, thus providing a safer and more controlled research environment. The GPs used in this study had body weights and body condition scores that were higher than those of GPs usually enrolled in research studies because they had previously been part of another study. All the animals chosen for this study were originally slated for euthanasia but were subsequently reassigned to a new protocol for use in this study.

The GPs were housed in groups of 3 to 5 in open-top Allentown GP caging units (Allentown, Allentown, NJ) following the Yale University housing density standard with paper chip bedding. The GPs received a Teklad global high-fiber GP diet (Inotiv, Boston, MA) ad libitum; daily servings of hay, fresh fruits, and vegetables; and unrestricted access to water via an automated system. The room was on a 12-h cycle (on at 0700 and off at 1900 h). Temperature was maintained at 22 ± 2 °C with a relative humidity between 30 and 70%. Cages were enriched with plastic tunnels.

All procedures were performed in accordance with guidelines and approved by the Institutional Animal Care and Use Committee of Yale University. Yale University is AAALAC-accredited and is compliant with all federal regulations overseeing the use of animals in research in the United States.

Premedication. GPs were weighed on a digital scale (Tanita, Arlington Heights, IL) the evening before sedation. The mouths of all animals were flushed and cleaned using 10 mL saline (normal saline; Hospira, Lake Forest, IL) at the time of sedation. Combinations of alfaxalone (A: 10 mg/mL; Alfaxan Multidose; Jurox), dexmedetomidine (D: 0.5 mg/mL; Dexdomitor; Zoetis, Parsippany, NJ), midazolam (M: 5 mg/mL; Heritage Pharmaceuticals, East Brunswick, NJ), and fentanyl (F: 50 µg/mL; Hospira) were tested. Between 0800 and 1000, a 27-gauge needle was used to inject GPs subcutaneously between the scapulae with the desired drug combination. GPs were then placed in a covered cage with minimal tactile, auditory, or visual stimulation while sedation was assessed. Drugs were undiluted for use; final volumes ranged from 2 to 2.5 mL depending on the weight of the animal.

Sedation depth and duration. Induction time was measured as the time of drug administration until the loss of righting reflex (LORR) with no attempt to regain recumbency. All GPs were continuously monitored, with LORR and sedation depth recorded every 5 min. Because determining the depth of sedation can be difficult in GP, we used sedation monitoring criteria derived from previously described methods for GP as described in Table 1.[19]

A surgical plane of anesthesia was defined as loss of the pedal withdrawal and inguinal reflexes. These reflexes were, respectively, assessed by clamping a mosquito hemostat to the first notch on a toe for a maximum of 2 s at the level of the distal interphalangeal junction and by applying digital pressure to the inguinal region.[19] The toe used to assess the pedal withdrawal reflex alternated between the hind feet at each time point. The duration between the loss and return of the pedal withdrawal reflex was considered the length of the surgical plane of anesthesia. Once the GPs regained the pedal reflex, they remained in a lateral recumbent position for monitoring until fully recovered. They commonly showed a persistent sedation-like state with

Table 1. Sedation depth monitoring criteria

Criteria evaluated	Sedation depth score
Normal coordinated movement with immediate sternal recumbency.	0
Coordinated movement but takes 5–10 s to regain sternal recumbency. Decrease response to manipulation (movement/noise).	1
Uncoordinated movement with attempted, but failed efforts to regain recumbency. Drowsy, floppy, and response to manipulation. Pedal/inguinal reflex present.	2
Lateral recumbency. Animal does not attempt to reposition and some response to manipulation. Infrequent and uncoordinated movement. Pedal/inguinal reflex present.	3
Dorsal recumbency. Infrequent, weak, uncoordinated movements with minimum response to manipulation. Pedal/inguinal reflex present.	4
No movement observed, lies in dorsal recumbency, and is unresponsive to manipulation. Pedal/inguinal reflex present or absent.	5

intermittent resumption of the righting reflex and subsequent relapse into mild sedation characterized by a lack of locomotion with responsiveness to external stimuli. Therefore, time to recovery was defined as the GP righting itself at least twice and showing normal ambulation.

Monitoring physiologic parameters. Once LORR occurred, the GPs were placed on a towel-covered veterinary warming system with a 50-W heating pad warmed to 40 °C (105 °F) (Hot Dog Warming; Augustine Surgical, Eden Prairie, MN) and remained on the heating pad until recovery. A warm towel was also draped over the top of the animals. Eye lubrication (Dechra, Overland Park, KS) was applied after LORR. All animals received 100% oxygen, and the alfaxalone, midazolam, fentanyl, and isoflurane (AMFIso) group also received isoflurane via a nose cone, using the Somnosuite Low-Flow Anesthesia System (Kent Scientific, Torrington, CT), which has a digital vaporizer integrated with a syringe filled with isoflurane and a digital dial for precision control over the delivery of anesthetic gas. Within 5 min of LORR, cardiopulmonary parameters and body temperature were recorded every 15 min, and for noninvasive blood pressure measurement, a size 1 cuff was placed on the right thoracic limb and connected to Jorvet Vital Signs Monitor (Jorvet, Loveland, CO). Respiratory rate (RR) was measured by visually counting breaths per minute (bpm) and a passive pneumatic respiratory sensor (model 1030; Small Animal Instruments, Stony Brook, NY) was placed underneath the animal. A pulse oximeter probe (Physiosuite; Kent Scientific) was placed on the interdigital space of a hindlimb to monitor SpO_2 and heart rate (HR). A lubricated fiberoptic rectal temperature probe was used to measure core body temperature. The investigator monitoring these anesthetic parameters was blind to the injected anesthetics the GP had received. Anesthesia parameters were recorded by the same individual until the GP regained the righting reflex.

Pilot study. Initially, 2 separate groups of GPs (2 per group) received subcutaneous injections with one of the following: 15 mg/kg of alfaxalone and 2 mg/kg of midazolam (A15 + M2) or 20 mg/kg of alfaxalone and 2 mg/kg of midazolam (A20 + M2). The GPs were then closely monitored for the induction and the depth of sedation to determine whether they reached a surgical plane of anesthesia. After we identified the doses that could achieve sedation, they were supplemented with 0.015 or 0.025 mg/kg of fentanyl (A20 + M2 + F0.015 and A20 + M2 + F0.025, respectively). The specific fentanyl doses were determined based on published data.[9] The dexmedetomidine dosages were selected based on previously established ranges suitable for GP (A: 15 mg/kg + D: 0.25 mg/kg).[16] These combinations were then combined with the 2 fentanyl dosages (A15 + D0.25 + F0.015 and A15 + D0.25 + F0.025) in 2 additional groups of animals (n = 2) to test their efficacy. Antagonistic agents were omitted to minimize variables between groups. Although higher doses might have resulted in a surgical plane, they might also extend the recovery time. We therefore added isoflurane to the most promising drug combination. After GPs that had received a subcutaneous injection of A20 + M2 + F0.025 showed LORR, they were given isoflurane through a nose cone. The concentration of isoflurane was increased every 5 min in 0.25% increments with a 0.7 L/min gas flow rate and 100% oxygen. Based on the manufacturer's recommendations, the flow rate was based on the weight of the animal.[8] Isoflurane was discontinued once the animal reached a surgical plane of anesthesia as determined by a negative response to noxious stimuli. For safety considerations, both protocols underwent a stepwise evaluation, starting from the lowest dose and progressing to the highest (Table 2). Each animal underwent a minimum of 2 sedation events involving 2 distinct sedation protocols for the pilot study. Subsequently, all these animals were incorporated into the main study for analysis. To ensure the animal's well-being and to mitigate any potential cumulative effects of the substances, a 7-d washout period was used between each dosage trial.

Experimental design. A priori sample size estimates using G*Power software (ver. 3.1.9.7; Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany) revealed that a total of 22 subjects were necessary to achieve power of 80% and 7 to 8 GPs per group were needed based on the variable of time to sedation. Therefore, we allocated 9 GPs to each sedation protocol, and each animal received the following combinations: A20 + M2 + F0.025 (AMF), A15 + D0.25 + F0.025 (ADF), and

Table 2. Pilot study data for all drug combinations used

Drug combination (n = 2 per group)	Induction time (min)	Sedation depth score	Surgical plane	Recovery time (min)
A15 + M2	18 ± 4	3	0	107 ± 14
A20 + M2	14 ± 2	4	0	138 ± 6
A20 + M2 + F0.015	10	5	0	187 ± 6
A20 + M2 + F0.025	6 ± 1	5	1	223 ± 29
A15 + D0.25	16 ± 7	4	0	180 ± 5
A15 + D0.25 + F0.015	14 ± 2	5	0	196 ± 7
A15 + D0.25 + F0.025	13	5	1	226 ± 15

Each dose was tested on 2 animals. Surgical plane was determined by pedal withdraw reflex and inguinal reflex. Time to recovery was defined as time between LORR and the animal regaining the righting reflex twice and then ambulating normally.

A20 + M2 + F0.025 + Iso (AMFIso) for final analysis, with a washout period of 7 to 10 d between each protocol. One GP was euthanized on the day after sedation with AMF due to suspected stress-induced enterotoxemia. As a result, the AMFIso group had only 8 animals. Each animal underwent sedation 3 times, with each protocol administered in a crossover fashion with a washout period of at least 14 d. Drug combinations that were used were selected in a blind manner to provide randomization. These combinations were labeled numerically by an individual who prepared the drugs but was not involved in the assessment of sedation.

Statistical analysis. Data were analyzed using commercially available SPSS software (SPSS 28; IBM, Armonk, NY) and Matlab R2023b distribution of the Statistics and Machine Learning Toolbox (MathWorks, Natick, MA). Any data point that falls beyond the whiskers of a box plot or is situated at a substantial distance from the main peak in a Kernel Density Estimate is considered an outlier. Outliers were included in the analysis because they did not impact statistical significance. The Welch ANOVA was used to analyze the time to sedation and recovery across the 3 doses, with post hoc GAMES-Howell procedure when appropriate. All the physiologic parameters were measured in 15-min time intervals. Physiologic variables were treated as repeated measures for each animal. A repeated ANOVA was used to identify significant differences as a function of time and significant treatment-time interactions. Although measurements were collected for 255 min, data were analyzed only through 150 or 180 min to ensure sufficient data points for each time interval and to have full column rank for the repeated measures ANOVA, as follows: 0 to 180 min for SpO_2, temperature, HR, and RR, and 0 to 150 min for systolic, diastolic, and mean arterial pressure (MAP).

If the null hypothesis was rejected based on the ANOVA, pairwise comparisons were performed using either the Games-Howell procedure or Tukey honestly significant difference procedure with unequal sample sizes (Tukey-Kramer) to identify differences between the 3 drug groups on time to recovery as well as the physiologic parameters HR, temperature, MAP, and SpO_2. The Gaines-Howell method was used if the homogeneity of variances or the sphericity assumptions failed. Otherwise, the Tukey-Kramer procedure was used. For comparing paired samples from the same group to assess time relationships, the Wilcoxon signed-rank test was used.

The Fisher exact test was conducted to determine whether there was a statistically significant association between the drug groups and whether a surgical plane was achieved. For those individuals who reached our definition of a surgical plane, descriptive statistics such as mean, median, and SD were employed to examine the distribution of the data. All data are reported as the mean ± SD, and a P value < 0.05 was considered to indicate significant effects.

Results

Pilot dose determination. In all dose groups, every animal became sedated with a smooth and relatively rapid induction. LORR occurred within a span of 7 to 21 min after the subcutaneous injection. The A20 + M2 + F0.025 combination had the shortest induction time (6 and 7 min), and A15 + M2 had the longest induction time (18 and 21 min). The depth of sedation varied among groups, with only the fentanyl combination groups achieving deep sedation. Among these, only 50% of the animals in the A20 + M2 + F0.025 reached a surgical plane, which lasted for 20 min. In the A15 + D0.25 + F0.025 group, one animal had a questionable surgical plane, with intermittent lack of response to noxious stimuli for 5 to 10 min (Table 2). Using the Somnosuite low-flow anesthesia system set at a flow rate of 0.7 mL/min allowed all animals sedated with the A15 + M2 + F0.025 drug combination to reach a surgical plane of anesthesia within 36 ± 3 min, starting at 2% isoflurane and incrementing by 0.25% every 5 min. Animals that received the 0.0025 mg/kg fentanyl combinations had a longer recovery time than the other groups. Considering these findings, we used A20 + M2 + F0.025 (AMF), A15 + D0.25 + F0.025 (ADF), and A20 + M2 + F0.025 + Iso (AMFIso) as our primary treatment groups for the main study. We based this decision on the hypothesis that isoflurane might not prolong the recovery period.

Sedation/anesthetic depth and duration. For every combination of drugs administered, all 9 animals reached LORR in an average of 10.3 ± 5.0 min. The induction times for the AMF and AMFIso groups were similar because isoflurane was administered after sedation with AMF. The average induction times were as follows: AMIso, 8.4 ± 3.6 (95% CI = 5.1 to 11.8) min; AMF was 8.4 ± 2.3 (95% CI = 6.7 to 10.2) min; and ADF, 14.2 ± 6.0 (95% CI = 5.1 to 11.8) min. These values were not significantly different [$F(2;13.467) = 3.638$, $P = 0.055$].

All GPs in the AMFIso group achieved a surgical plane of anesthesia with 2% isoflurane at a 0.7 mL/min flow rate. However, none of the GPs in the ADF group and only 10% of the AMF group reached this planet ($P < 0.001$). All others retained a positive response to noxious stimuli. On average, GPs in the AMFIso group required 29 ± 13 min to reach a surgical plane. After stopping the supplemental isoflurane, the average duration of the surgical plane was 14 ± 6 min. In the AMF group, only one individual reached a surgical plane; this occurred after 24 min of sedation, and the duration was 60 min. The average times for recovery were as follows: AMF, 217 ± 61 min; AMIso, 249 ± 37 min; and ADF, 258 ± 38 min, but these values were not significantly different ($P = 0.185$).

Physiologic parameters. The GPs received supplemental oxygen, and their mucous membranes were pink throughout the study. The oxygen saturation levels (SpO_2) ranged from 92% to 100% at all time points. The Mauchly test for the repeated measures ANOVA found no significant effect of time on SpO_2 and no significant treatment-time interaction ($P = 0.3443$ and 0.3326).

The heart rates of all groups were below normal physiologic levels, ranging from 163 to 245 bpm based on reference values for unanesthetized GPs.[20] HR values were as follows: AMFIso, 2,312 ± 14 bpm; AMF, 2,007 ± 15 bpm; and ADF, 173 ± 9 bpm. The overall analysis revealed a statistically significant difference in HR among the groups ($P < 0.05$) (Figure 1), but adjusted P values indicate did not show a significant effect of time on HR or a significant treatment-time interaction for HR, with P values of 0.2469 and 0.3976, respectively.

The Games-Howell analysis revealed that the RR of the AMIso group (56 ± 6 bpm) was significantly different from that of the ADF group (71 ± 3 bpm) but not from the AMF group (54 ± 9 bpm) ($P < 0.001$). RR remained within the normal physiologic range of 42 to 104 bpm (Figure 1).[20] The Wilcoxon signed-rank test indicated that the RR of the AMFIso group decreased over time for the AMFUso group but ADF and AMF did not affect RR.

The AMFIso group exhibited a subnormal physiologic body temperature of 36.8 °C, while the other groups maintained temperatures within the normal range of 37.1 to 39.8 °C. Temperature was significantly lower in the AMIso group (36.3 ± 0.8 °C) as compared with either the ADF (37.6 ± 0.7 °C) and AMF

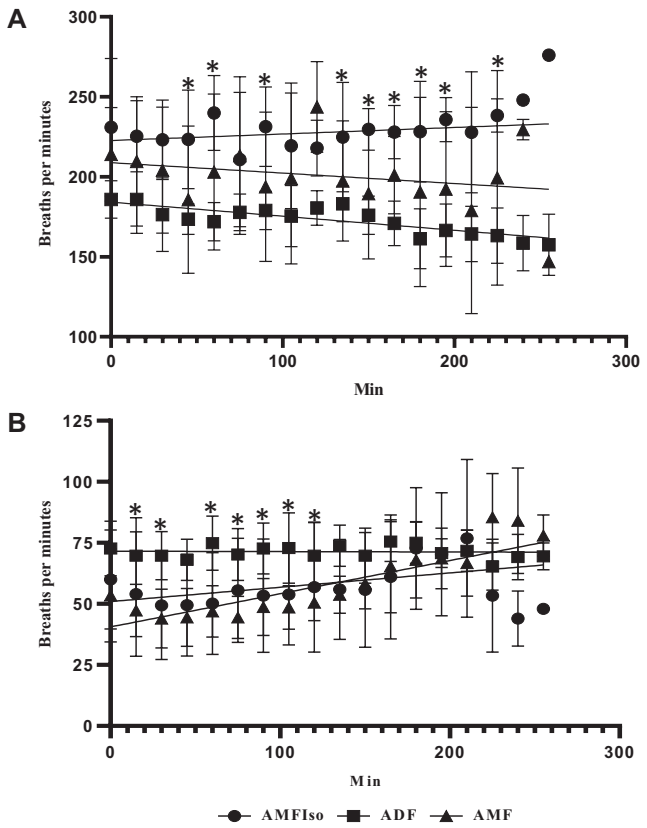


Figure 1. (A) Effect of each anesthetic combination on heart rate over time. (B) Effect of each anesthetic combination on respiratory rate over time. Rates were measured as beats or breaths per minute. Triangle: AMF; square: ADF; circle: AMFIso. Whisker bars represent mean and SD changes from baseline to recovery at different time points in response to different drug combinations. Lines were drawn based on the linear regression model that was been fitted to the data. *, $P < 0.05$ in comparisons between groups as specific time points were compared at each time point.

(37.7 ± 0.6 °C) groups ($P < 0.001$), but the ADF and AMF groups were not different ($P = 0.917$).

The effects of sedation on physiologic parameters were analyzed using ANOVA. Sedation accounted for a significant proportion of the variation in HR (79.5%), RR (62.7%), and temperature (70.9%).

In all groups, the MAP and systolic pressure remained within the normal physiologic range (systolic: 80 to 94 mmHg; MAP: 55 to 58 mmHg).[20] However, the diastolic pressures were below the normal range. Group values were not significantly different (Figure 2). The repeated measures ANOVA did not reveal a significant effect of time on the MAP or a significant treatment-time interaction for MAP (*P* values 0.5367 and 0.0888, respectively). The Wilcoxon signed-rank test suggested a probable decrease in both systolic and diastolic pressures in the AMFIso and ADF groups as compared with their initial measurements. However, AMF appeared to have minimal effect on both systolic and diastolic pressures.

Adverse events. One animal displayed signs of respiratory distress after receiving AMFIso and was subsequently euthanized. Another animal was found dead the day after the procedure, with a suspected cause of stress-induced gastrointestinal stasis and enterotoxemia.[20] One GP developed an erosive lesion approximately 1.5 cm in diameter at the injection site; the lesion resolved without intervention.

Discussion

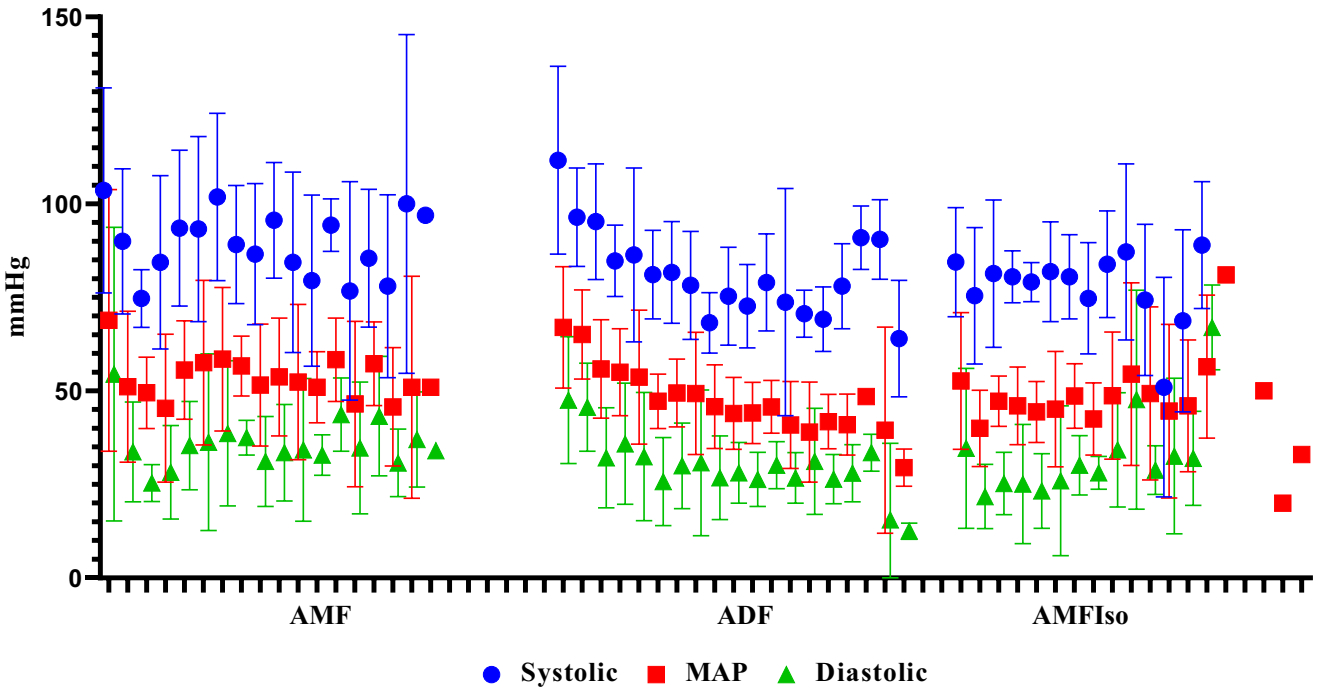
Female Dunkin-Hartley GPs are widely used in research due to their gentle temperament and low aggression.[21] In our facility, we acquire juvenile GPs for short-term studies in which half of them serve as control subjects. Once the study concludes, these GPs are either euthanized or used for other purposes or experiments to align with the principles of the 3 Rs (Replacement, Reduction, Refinement). Our objective in the current study was to develop an injectable anesthesia protocol that consistently induced a surgical plane of anesthesia in female GPs.

Our data showed that while none of the injectable regimens eliminated the pedal withdrawal reflex, they did provide immobilization and muscle relaxation suitable for nonpainful procedures. Furthermore, induction was rapid and consistent with published data on comparable drug combinations.[1,9,10] The time required for induction varied across the different dose groups, with the ADF group taking an average of 14 min as compared with 8 min in the AMF and AMFIso groups. The comparable induction times observed in the latter 2 groups can be attributed to the use of the same induction agent before the use of isoflurane. Nonetheless, the ADF group experienced a notably longer induction time, potentially due to the gradual uptake of dexmedetomidine subcutaneously and the reduced alfaxalone dosage relative to the AMF and AMFIso groups. All drug combinations in this study were administered through the subcutaneous route. While alfaxalone and fentanyl have previously been administered subcutaneously, this route is off-label for midazolam and dexmedetomidine.[1,5,8,12] Subcutaneous administration can result in slower absorption and an extended duration of action. During the pilot study, we noticed that animals subjected to sedation in the later part of the day, using different protocols, exhibited longer induction times, likely a consequence of stress despite our efforts to mitigate it by maintaining a low-stress environment with minimal noise and handling in the company of conspecifics.

The treatment groups showed no significant differences in recovery time. However, our recovery times were longer than those reported in other studies, including studies of KX.[3,7,9,22] Considering the use of different definitions of recovery in GPs, different studies cannot be compared directly; some authors have defined recovery as righting to sternal recumbency but not necessarily ambulating with normal coordinated movements.[8] We defined recovery as requiring 2 displays of regaining the righting reflex twice and subsequent normal ambulation. Our GP often appeared to remain sedated from which they might regain their righting reflex and then revert to a semisedated state in which they did not ambulate but responded to manipulation. The obesity of these animals might have altered their metabolic rates in a manner that could have extended the recovery period. We did not use reversal agents to promote consistency in comparing all drug combinations. Reversal agents for α-2 agonists and benzodiazepines should be used to shorten the recovery period.

The effects of sedative drugs on physiologic parameters can vary significantly when an anesthetic is administered to the same subject at different times. This intraindividual variability in response must be considered when comparing the effects of sedatives and anesthetics. In our study, the physiologic responses of individual animals were different in response to sedation and anesthesia. However, variation can also occur when inhalational anesthesia is used in conjunction with injected anesthetics. Thus, AMF and AMFIso groups both received the same combination of injectable anesthetics, yet the inclusion of isoflurane significantly altered the physiologic effects.

A



B

Parameter	AMF	ADF	AMFIso
MAP	49±14 mmHg	54±18mmHg	47±15mmHg
Diastolic	31±14 mmHg	35±14mmHg	30±16mmHg
Systolic	82±19 mmHg	89±21mmHg	78±17mmHg

Figure 2. (A) The effects of different drug combinations on systolic, diastolic, and MAP at various time points. The *x*-axis represents time in 30-min increments, starting from the baseline until recovery for each drug combination. Triangle: diastolic; square: MAP; circle: systolic. Whisker bars represent mean and SD. (B) Average and SD values for each group. No statistically significant differences were detected for systolic, diastolic, and MAP values among groups at any time point ($P > 0.05$).

RR was significantly lower in the AMFIso protocol, possibly due to the respiratory depressive effects of isoflurane.[12,13,17,19] The AMFIso group also had significantly lower temperatures. Isoflurane is known to induce hypothermia,[12,13] and because all the GPs reached a surgical plane of anesthesia under this regimen, their thermoregulation was apparently affected. Therefore, supplemental heat support must be provided during anesthetic procedures, particularly in small rodents with a high surface area to body weight ratio.[13] In our study, sedated or anesthetized animals were placed on a veterinary warming device covered with a towel. Other warming devices such as heat lamps, warm air devices, or towel-wrapped warming devices can also be used; avoiding direct contact between the animal and the device should help to prevent thermal injury.

The HR varied across all groups. Dexmedetomidine causes vasoconstriction of peripheral vessels, leading to reflex bradycardia.[15] Consistent with this, the ADF group had a significantly lower HR than the other groups. MAP was not significantly different among the groups, although isoflurane lowers blood pressure by causing peripheral vasodilation in a dose-dependent manner.[27] However, despite significant differences in some physiologic parameters among the groups, the values observed were still within the normal range for GPs; therefore, none of the drug combinations we administered significantly affected their physiologic parameters.

In our study, the injectable regimens alone did not achieve a surgical plane of anesthesia in female Dunkin-Hartley GPs. A combination of KX and high levels of isoflurane is often used to achieve a reliable surgical plane of anesthesia in GPs.[3,7,13] Similarly, combining dexmedetomidine, buprenorphine, and alfaxalone prolonged the duration of sedation and immobility but did not induce general anesthesia.[8] We tested fentanyl, a full *mu* receptor agonist, in conjunction with alfaxalone, dexmedetomidine, and alfaxalone with midazolam. While previous research showed that fentanyl in combination with another agent provided a surgical plane of anesthesia, only 10% of our animals reached this level with AMF in our study.[18,19] However, both AMF and ADF combinations produced effective sedation with minimal side effects and with no significant effects on physiologic parameters. We administered fentanyl via the subcutaneous route, which has been shown to be safe and effective for analgesia with a low incidence of adverse effects.[5,16,18,19] The GPs used in this study were moderately overweight due to ad libitum feeding, and the presence of excess fat may have contributed to slower absorption of fentanyl. Fentanyl has a short half-life and rapid liver metabolism.[5] Therefore, the dose of fentanyl may have been metabolized before the beginning of anesthesia. Administering a second dose of fentanyl after an initial dose might achieve and maintain a surgical plane for surgical procedures. Other full *mu* opioids such as hydromorphone or

morphine have longer half-lives and may be useful. The fentanyl combinations used in this study produced deep sedation but did not achieve a surgical plane of anesthesia in this species, warranting further investigation into more effective methods to measure conscious nociception. The nociceptive withdrawal response employed in this study was intended to signify conscious pain perception in the animal, but positive spinal reflexes may present even without conscious nociception.[15] In this study, we also used the inguinal reflex to differentiate pain perception from spinal reflexes; this distinction is important because differentiating sedation and surgical plane can be difficult in GPs. Assessing responses to genuinely noxious stimuli, such as a skin incision, together with physiologic parameters like heart rate and blood pressure could provide a more precise indication of the actual anesthetic depth.

Because our initial drug combinations did not achieve a surgical plane of anesthesia, we added isoflurane, a commonly used supplemental inhalation anesthetic agent, to induce a surgical plane of anesthesia.[11,20,21] Isoflurane is relatively safe, and numerous environmental controls are used in animal research facilities to prevent occupational exposure of staff.[11,24] In our study, all animals in the AMFIso group reached a surgical plane of anesthesia under 2% isoflurane with a flow rate of 0.7 mL/min and requires an average of 29 ± 13 min to achieve a surgical plane. The use of a higher flow rate of isoflurane might have reduced the time required for induction, but using a low-flow-rate rodent anesthesia system has distinct benefits. A low-flow system can administer anesthetic gas in accordance with the animal's weight as compared with a conventional vaporizer. A low-flow vaporizer also releases a minimal amount of waste anesthetic gas, remaining under the threshold detectable by dosimeters even during extended periods of use.[8] Nonetheless, using a low-flow rate anesthesia system should be tested in GPs due to their propensity for breath holding.[12,19] Future research could examine the combination of KX and a low-flow rate of isoflurane to determine its ability to induce anesthesia in this species.

Subcutaneous injection of alfaxalone, both alone and combined with midazolam, was effective in producing high-quality sedation in pet GPs of both sexes, with minimal side effects.[1] These studies used 5 mg/kg of alfaxalone in various pet GP strains, whereas a higher dose (10 mg/kg) was effective for Dunkin-Hartley GPs in previously published studies.[1,7] These findings suggest strain variations in sedation and anesthetic response among GPs, as occurs in other rodents.[2,13,26] Moreover, sex is an independent factor that affects the response to anesthesia.[4] Sex effects have been reported in GPs subjected to various sedation and anesthetic protocols.[23] Several potential explanations for this sexual dimorphism include sex hormone cycles and variations in adiposity and drug metabolism.[11] Therefore, testing male GPs in our study could have been informative. However, we used female GPs who were already present at our institution and had been scheduled for euthanasia, allowing us to repurpose these animals rather than purchasing more, in adherence with the 3R principle aimed at minimizing animal usage.

During the use of isoflurane anesthesia, one animal exhibited symptoms of suspected aspiration and respiratory distress. Isoflurane can irritate the respiratory mucosa, trigger excess mucus production, and stimulate vomiting.[17,19] GPs are typically not fasted before short anesthetic events, as they have a high metabolic rate and require a constant source of food to maintain gut health.[21] In our study, GPs were not fasted, which raises the possibility that this GP, which exhibited increased RR and effort, may have aspirated. GPs commonly have food particles trapped in their mouths due to their numerous cheek folds; some authors recommend flushing out these particles before surgical procedures.[12,13,21] Given this unforeseen death, despite having flushed the mouth as a precaution, we thereafter meticulously swabbed the cheeks and thoroughly suctioned the mouths of the remaining animals before sedation. Another animal died on the day after a procedure, likely due to stress-induced gastrointestinal stasis. This condition may have led to the accumulation of harmful bacteria and the release of toxins into the bloodstream, potentially causing enterotoxemia.[20] Minimizing stress and the duration of anesthesia in GPs can promote animal well-being.[21] One GP developed a mild injection site reaction, characterized by an approximately 1.5-cm diameter erosive lesion that healed on its own. Administering subcutaneous injections to GPs requires restraint by a handler. The injection site reaction may be due to intradermal rather than subcutaneous drug administration.

Based on induction time, recovery time, and anesthetic parameters, none of the injected anesthetic regimens had clear advantages over the others. Drug dosing is typically based on population-based estimates of concentration-response relationships, but these algorithms can lead to over- or underdosing at the individual level. Therefore, customization of anesthetic protocols to the unique physiologic needs of each animal rather than using generalized population-based standards could be beneficial. We observed that individual reactions to a fixed drug concentration varied significantly. Our data emphasize the need to customize anesthesia for GPs to balance between effective anesthesia and the preservation of essential bodily functions. Solely relying on the injectable regimens evaluated in this study was not adequate for attaining a surgical plane of anesthesia in female Dunkin-Hartley GPs. However, these regimens could be useful for noninvasive procedures that require immobilization (for example, radiography or blood sampling). Including an inhalant agent like isoflurane gas can allow the transition from sedation to anesthesia.

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