Comparative Medicine
Copyright 2022
by the American Association for Laboratory Animal Science

Vol 72, No 4
August 2022
Pages 248–256

Original Research

Comparison of Alfaxalone-Midazolam, Tiletamine-Zolazepam, and Ketamine-Acepromazine Anesthesia during Plethysmography in Cynomolgus Macaques (*Macaca fascicularis*) and Rhesus Macaques (*Macaca mulatta*)

Brianna M Marion,[1,*] Jeanean M Ghering,[2] Benjamin C Dixon,[1] Amanda M Casselman,[1] Summer M Astleford,[1] Charles E White,[3] and Philip A Bowling[1]

Plethysmography is used in nonhuman primates (NHPs) to measure minute volume before aerosol exposure to an agent to calculate total time necessary in the exposure chamber. The consistency of respiratory parameters during the entire exposure time is paramount to ensuring dosing accuracy. Our study sought to validate an alfaxalone-midazolam (AM) anesthetic combination for use in aerosol studies. We hypothesized that AM would provide an adequate duration of anesthesia, achieve and maintain steady state minute volume (SSMV) for 20 min, and have anesthetic quality and side effects comparable to or better than either tiletamine-zolazepam (TZ) and ketamine-acepromazine (KA), the most common anesthetics used for this purpose currently. Two groups of NHPs, one consisting of 15 cynomolgus macaques and one of 15 rhesus macaques, received 3 intramuscular anesthetic combinations (AM, TZ, and KA), no less than one week apart. Anesthetized NHPs were placed in a plethysmograph chamber and their minute volumes were measured every 10 s to determine whether they had achieved SSMV and maintained it for at least 20 consecutive min. Achieving and reliably maintaining an SSMV for at least 20 min facilitates precise aerosol dosing of a challenge agent. Quality of anesthesia, based on the NHP's ability to achieve and maintain SSMV, was higher with AM compared with TZ and KA in both species, and AM had a longer duration of SSMV as compared with TZ and KA in cynomolgus macaques. Average SSMV was larger with AM compared with TZ in cynomolgus macaques, but larger with KA compared with AM in rhesus macaques. Duration of anesthesia was sufficient with all combinations but was longer for TZ than both AM and KA in both species. These results suggest that the AM anesthetic combination would produce the most accurate dosing for an aerosol challenge.

Abbreviations and Acronyms: AM, alfaxalone-midazolam; KA, ketamine-acepromazine; SSMV, steady state minute volume; TZ, tiletamine-zolazepam

DOI: 10.30802/AALAS-CM-22-000010

Introduction

When studying infectious disease in animals, the most accurate results are obtained by exposure that simulates natural exposure conditions to the extent possible. Inhalation is a common route of exposure for many infectious biologic agents, and nonhuman primates (NHPs) are used to model many infectious disease processes in humans. Infections that develop via an inhalational route of exposure (either naturally or experimentally) include Venezuelan equine encephalitis virus, pneumonic plague (*Yersinia pestis)*, anthrax (*Bacillus anthracis*), and monkeypox virus in cynomolgus macaques (*Macaca fascicularis*) and brucellosis (*Brucella suis)*, anthrax, and melioidosis (*Burkholderia pseudomallei*) in rhesus macaques (*Macaca mulatta*).[3,25,28,30,31,32]

Respiratory tract inoculation of infectious agents is often done in an exposure system in which the head of the NHP is placed in an aerosol chamber and an aerosolized agent is delivered directly into the chamber at a controlled rate.[1] Accurate measurement of the animal's respiratory parameters is critical to accurate aerosol dosing. These parameters can be measured using head-out plethysmography, in which the NHP's body is contained in a chamber with only the head exposed. In this set up, changes in chamber pressure caused by the NHP's chest movement in combination with flow rate are measured by a

Received: 09 Nov 2021. Revision requested: 03 Mar 2022. Accepted: 31 Mar 2022.
[1]Veterinary Medicine Division, [2]Aerobiology, Animal Clinical Pathology, and Telemetry Section, Veterinary Medicine Division, and [3]Statistics Section, Veterinary Medicine Division, United States Army Medical Research Institute of Infectious Diseases (USAMRIID), Frederick, Maryland
[*]Corresponding author. Email:brianna.m.marion.mil@mail.mil

pressure transducer. Plethysmography software then uses this information to calculate respiratory parameters such as tidal volume and minute volume (tidal volume × respiratory rate). Once an individual's minute volume is known, the NHP can be transferred to a head-only aerosol exposure system and an accurate dose of the agent can be delivered by calculating the exposure time based on the respiratory parameters.[5,24] Large fluctuations in an animal's minute volume during anesthesia may cause an incorrect dose to be delivered, as the minute volume cannot be measured or adjusted once the animal is in the exposure chamber. Thus, consistent anesthesia is highly desirable throughout the procedure.

A variety of anesthetics are available for use in NHPs, each with varying physiologic effects and durations of action. Ideal characteristics for NHP plethysmography anesthetic protocols include the following: subcutaneous or intramuscular administration to permit cage side injection, a minimum 45 min duration of action, rapid steady state minute volume (SSMV) achievement (defined as no more than a ± 10% change between minutes), and a greater absolute minute volume, thus minimizing the time needed in the aerosol chamber.[5,24] SSMV, which is defined to occur during a period of time in which the animal's minute volume changes minimally, has been used in a previous comparison of plethysmography anesthetics as a measure of consistency in breathing and therefore as a means to provide an accurate dosing period for an aerosolized agent.[6] In our facility, an NHP initially undergoes head-out plethysmography for 3 min before a challenge spray with an aerosol. If the minute volume for this period falls within a specified range, typically from 450 to 1,600 mL, the animal is transferred to the head-only exposure chamber in a Class III biosafety cabinet. The wide range of acceptable minute volumes is due to the wide range of NHP sizes (3 to 11 kg) used at our institution. A typical aerosol exposure takes 10 to 15 min.

Tiletamine-zolazepam (TZ) and ketamine-acepromazine (KA) are currently the most common anesthetics used with plethysmography in NHPs.[3,10,24,25,30,31] A previous study examined the effects of different anesthetics on rhesus macaques and found TZ to be the most consistent combination as compared with ketamine and KA. TZ could maintain NHP anesthesia for at least 45 min and provide a long period of SSMV, making it preferable for aerosol studies that use plethysmography.[5] However, at higher doses with longer durations of anesthesia, TZ cause a fall in rectal temperature as compared with KA.[20] TZ can also cause elevated body temperatures lasting for over 24 h after induction, which could potentially cause misinterpretation of data after challenge with infectious agents.[7] In addition, overheating as a result of TZ administration may cause convulsions, and animals may become ataxic during recovery, resulting in injuries.[12,23] At our institution, some NHPs have also exhibited a sensitivity to TZ, with clinical signs including rigid muscles, exaggerated movements of the limbs, torticollis, and bruxism.

The anesthetic agent alfaxalone has recently become available for intramuscular and subcutaneous use in NHPs in the United States. While approved by the Food and Drug Administration in 2012 for use in dogs and cats, in 2020 alfaxalone was added to the Index of Legally Marketed Unapproved New Animal Drugs for Minor Species, including use for sedation and anesthesia for NHPs.[2,8,9] Alfaxalone is a synthetic neuroactive steroid that produces anesthesia by acting on γ-aminobutyric acid subtype A (GABA A) receptors.[2,17] This drug has minimal cardiovascular effects and may be a potential alternative to TZ or KA, especially for NHPs that are known to be sensitive to these anesthetics.[16,29] A previous study examining the cardiorespiratory effects of intramuscular alfaxalone showed dose-dependent decreases in respiratory rate, noninvasive blood pressure, SpO_2, and rectal temperature in cynomolgus macaques.[29] In that study, a dose of 5 mg/kg produced a moderate to deep level of sedation, minimal cardiorespiratory depression, and deep sedation lasting greater than 20 min. Longer duration of sedation and anesthesia occurred at the higher doses, (7.5 and 10 mg/kg), but these doses also increased the incidence of clinically relevant hypothermia, hypoxemia, and hypotension. In addition, alfaxalone required larger injection volumes that could exceed 3.0 mL, requiring multiple injections.[29] Another study evaluated an alfaxalone-midazolam-medetomidine combination in rhesus macaques and found that a lower dose of alfaxalone was sufficient to induce anesthesia.[4] However, that study also found that medetomidine had depressive cardiorespiratory effects, without affecting total anesthesia duration. No anesthetic complications were observed and recovery was uneventful for all NHPs except for one that showed mild generalized muscular twitching for 1 min.[4] Muscle twitching has also been seen after alfaxalone administration in other species, including common marmosets, mice, pigs, dogs, and Egyptian fruit bats.[1,16,18,21,26,27] Overall, adverse effects related to alfaxalone appear to be minimal and thus it is a potential alternative to either TZ or KA. We evaluated alfaxalone in combination with midazolam only, due to the depressive cardiorespiratory effects of medetomidine.[4]

The goal of our study was to evaluate an alfaxalone anesthetic combination for use in aerosol studies by comparing it to the 2 anesthetics most commonly used for this purpose, TZ and KA. Based on a pilot trial, we chose to evaluate alfaxalone at a 5 mg/kg dose in combination with midazolam at a 0.3 mg/kg dose. We hypothesized that alfaxalone-midazolam (AM) would provide an adequate duration of anesthesia (minimum 45 min), achieve and maintain SSMV for a minimum of 20 min, and have overall anesthetic quality and side effects that were comparable to or better than either TZ or KA, making it a suitable anesthetic protocol for both rhesus and cynomolgus macaques undergoing plethysmography for aerosol challenge studies.

Materials and Methods

Animals. This research was conducted under a United States Army Medical Research Institute of Infectious Diseases (USAMRIID) IACUC-approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to the use of animals in research. Animals were housed in an AAALAC International accredited facility that adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*.[15] The study group consisted of 15 rhesus macaques (*Macaca mulatta;* 9 females, 6 males; age 5.8 to 8.8 y; weight 5.1 to 10.2 kg; body condition score ranging from 2.5 to 3 out of 5) and 15 cynomolgus macaques (*Macaca fascicularis;* 7 females, 8 males; age 7.1 to 12.8 y; weight 2.9 to 10.8 kg; body condition score ranging from 3 to 5 out of 5). Macaques were selected from the USAMRIID colony based on availability and matched on weight and gender when possible. All macaques used in this study were considered to be healthy based on physical examination and baseline bloodwork. Anesthetic events were conducted in ABSL2. While assigned to the study, the macaques received food twice daily (Teklad 2050, Envigo, Madison, WI) and water ad libitum via an automatic drinking valve. They were checked at least twice daily and given enrichment in the form of toys and diet. Macaques were socially housed whenever possible. They were housed in modular primate caging (Lab Products, Seaford, DE) in ABSL2 rooms at 64 to 84 °F (17.8 to 28.9 °C), 30 to 70% relative humidity, and 12:12-h light/dark cycle.

The macaques were procured from a breeder and acclimated at the facility for at least 90 d before the study.

Anesthesia and monitoring. Each macaque assigned to the study underwent 3 separate anesthetic events; once with TZ (Tiletamine–zolazepam [Telazol], Zoetis, Parsippany-Troy Hills, NJ) at a dose of 6 mg/kg of the solution (which contained 3 mg/kg of each component), once with ketamine (100 mg/mL; Dechra Veterinary Products, Overland Park, KS) in combination with acepromazine (10 mg/mL; Henry Schein Animal Health, Dublin, OH) in a 10:1 mixture at 0.12 mL/kg, and once with alfaxalone (Alfaxan; Jurox, North Kansas City, MO) in combination with midazolam (Heritage Pharmaceuticals, East Brunswick, NJ) at a dose of 5 mg/kg and 0.3 mg/kg respectively. Anesthetic trials for each macaque occurred no sooner than one week apart, which provides a washout period that is consistent with previous plethysmography comparison studies performed in NHPs.[5,10] All anesthetic agents were administered intramuscularly with a maximal injection volume of no more than 3 mL at a given site and a total injection volume for each episode of anesthesia that did not exceed 6 mL. If the total injection volume was greater than 3 mL, 2 separate injections were administered at different sites in quick succession. During anesthesia, the macaque's heart rate, respiratory rate, SpO_2, and temperature (using an esophageal probe) were monitored using a veterinary patient monitor (Bionet, Tustin, CA), or a detached SpO_2 monitor (Edan, San Diego, CA). Vital signs were recorded every 5 min. Adverse events during anesthesia were recorded. At the completion or termination of a trial, the macaque was returned to its home cage for recovery and was considered to be recovered when it regained a righting reflex and could maintain itself in an upright position. Macaques were randomized within species based on the order in which they would receive each anesthetic protocol (replicate), the study day within the series on which each macaque would be anesthetized, and the order in which macaques were anesthetized within a study day. Five macaques of the same species underwent a single trial each study day.

Plethysmography. Once anesthetized, macaques were placed into a head-out plethysmography system. Two sizes of plethysmograph chambers (large coffin type: width at shoulders (WS) = 10.75 in, width at feet (WF) = 6.75 in, Length (L) = 33 in, Height (H) = 8 in; extra large coffin type: WS = 14.75 in, WF = 10.75 in, L = 36 in, H = 8 in) were used for this study. The extra large chamber was used primarily for males weighing greater than 8 kg. Both chambers were calibrated daily using a known volume of air in accordance with the software manual and institute SOPs. In this setup, the macaque's torso and extremities were contained inside the plethysmograph chamber, with the head extended through a hole in the chamber. The chamber was then sealed with a dental dam (with a hole cut in the center, going over the head and fitting tightly around the neck), a closed-cell silicone rubber dam (mousepad, with a hole cut in the center), foam, and Plexiglas faceplates that were placed around the neck and latched into place. If necessary, a folded towel was used to support so that it was not kinked.

The pressure differential created by the macaque's chest movement was measured by a TRD 5700 pressure transducer (Data Sciences International (DSI), St. Paul, MN), and FinePointe v2.3.1.6 plethysmography software (DSI, St. Paul, MN) calculated respiratory parameters including tidal volume and minute volume. The plethysmography software generated a minute volume reading every 10 s. These values were manually transcribed in real time into a spreadsheet (Microsoft Excel 2016, Redmond, WA) on a separate computer that averaged the 6 readings recorded over each minute of the trial. The spreadsheet was used to calculate the percent change in minute volume between each minute and the previous minute. A macaque was considered to have reached SSMV when the percent change in minute volume between sequential minutes did not vary by ± 10% for 5 consecutive min. Plethysmography continued until the NHP achieved an additional 20 consecutive min in SSMV (change in minute volume between minutes did not vary by ± 10%), at which time the trial was considered complete. If the minute volume varied by more than ± 10% after achieving SSMV, but before achieving 20 consecutive min, SSMV was considered to have ended. The macaque remained in the plethysmograph chamber and a second attempt was conducted. If the macaque reached 20 consecutive min of SSMV during the second attempt, the trial was completed. If SSMV was not achieved in the second trial, the trial was terminated. The trial was also terminated if the macaque began to show signs of recovery (voluntary movement) or after 60 min if the NHP did not meet any other criteria for termination. Upon completion or termination of the trial, macaques were removed from the chamber and returned to their home cage for recovery.

Data and statistical analysis. The following parameters were analyzed to compare anesthetic combinations for each species: quality of anesthesia, duration of SSMV, duration of anesthesia, average volume of SSMV, time to SSMV, and time needed prior to beginning plethysmography. Quality of anesthesia was scored based on ranking criteria from 1 to 4, with 4 being the highest or best quality. A score of 1 was assigned if the NHP did not reach SSMV at all during the trial. A score of 2 was assigned if the NHP reached SSMV during the first attempt but failed to maintain it for a minimum of 20 min and did not reach SSMV again during the second attempt. A score of 3 was assigned if the NHP reached up to 20 min of SSMV during the second but not the first attempt. A score of 4 was assigned if the NHP reached SSMV during the first attempt and maintained it for 20 consecutive min. Duration of SSMV was calculated as the number of minutes the macaque remained in SSMV for a given anesthetic combination. For macaques that had 2 periods of SSMV, we used the average duration of the 2. Average SSMV was defined as the average minute volume during all periods of SSMV within a trial. Time to SSMV was defined as the number of minutes between trial initiation and achieving the first period of SSMV. Duration of anesthesia was defined as the number of minutes between administration of the anesthetic injection and recovery of the macaque. Time to start was defined as the number of minutes between injection of the anesthetic and the first vital sign recording after the macaque was secured in the plethysmography chamber. Side effects were documented for each trial, but they were not statistically analyzed.

Statistical analysis was performed using SAS version 9.4 (TS 1M5, SAS Institute, Cary, NC) in order to compare the effects of the 3 anesthesia protocols on the outcome variables of interest. Analysis of variance (ANOVA) was used to analyze outcome variables that were approximately normally distributed (time to SSMV, total duration of anesthesia, and time to start).[19] ANOVA allowed us to control for the relative influence of multiple study design factors (Macaque, Replicate, Day, and Order) on the results. Other data (duration of SSMV and quality of anesthesia) were analyzed using the nonparametric Friedman test, which does not assume a specific probability distribution.[13] However, the Freidman test only allowed an assessment of 2 design factors (Macaque and Anesthesia) at a time and using both factors allowed comparison of the same macaque under each anesthetic (every macaque was tested with all 3 anesthesia protocols. To minimize the risk of declaring an association to be statistically significant when it was not, statistical tests were conducted in

a hierarchy; Bonferroni and Scheffe post hoc tests were applied to the ANOVA and Freidman results, respectively, to control for multiple pairwise comparisons.[19] In general, the analytical hierarchy was to test the statistical significance of the full model, followed by testing the design and anesthesia factors within the model, and finally pairwise comparisons for the anesthetics. *P* values less than or equal to 0.05 were considered to be statistically significant. All descriptive statistics in the results section are presented with confidence intervals. Quality of anesthesia, total duration of anesthesia, and duration of SSMV also provide the mean.

Results

Three different anesthesia combinations were tested in 15 cynomolgus and 15 rhesus macaques to determine their effect on quality of anesthesia, time to anesthesia, duration of anesthesia, duration of SSMV, average SSMV, and time to SSMV. For these outcome variables, all reported *P* values indicate the probability that the results between combinations were significantly different, but not if the differences were due to higher or lower values. References to higher or lower values between anesthetic combinations are based on descriptive values associated with significantly significant test results ($P \leq 0.05$).

Quality of anesthesia was found to differ significantly across anesthetic combinations in both cynomolgus ($P = 0.0010$) and rhesus ($P = 0.0490$) macaques. The mean quality score in cynomolgus macaques was 3.7 for AM, 2.8 for TZ, and 2.3 for KA. AM had higher quality scores than did TZ ($P = 0.0008$) and KA ($P < 0.0001$), whereas TZ and KA quality scores were not significantly different ($P = 0.0707$). The mean quality score for rhesus macaques was 3.7 for AM, 3.3 for TZ, and 2.9 for KA. AM quality scores were significantly higher than those of TZ ($P = 0.0142$) and KA ($P = 0.0008$), whereas scores for TZ and KA were not different ($P = 0.3662$). Percentage values for quality scores are shown by species and anesthesia in Figure 1.

Total duration of anesthesia was different across anesthetic protocols in both cynomolgus ($P = 0.0003$) and rhesus ($P = 0.0002$) macaques. In cynomolgus macaques, the average duration of TZ was 110 min, AM was 87 min, and KA was 74 min. Duration was longer on TZ than either AM ($P \leq 0.05$, 95% CI: 6 to 41) or KA ($P \leq 0.05$, 95% CI: 19 to 54), with no significant difference in duration between AM and KA ($P > 0.05$, 95% CI: −4 to 30). In rhesus macaques, the average duration was 74 min with TZ, 61 min with AM, and 53 min with KA. TZ anesthesia lasted longer than either AM ($P \leq 0.05$, 95% CI: 2 to 23) or KA ($P \leq 0.05$, 95% CI: 10 to 31), with no significant difference in duration between AM and KA ($P > 0.05$, 95% CI: – to 19).

The cynomolgus macaques failed to achieve an SSMV at least once during 5 trials: 4 times with KA and once with TZ. None of the rhesus macaques failed to achieve at least one episode of SSMV except for one that never became fully anesthetized on KA. If an NHP failed to achieve an SSMV at least once during a trial, no values were obtained or used for analysis for average SSMV or time to SSMV for that trial. Duration of SSMV was considered 0 min in this scenario. The raw minute volume data recorded every 10 s for individual macaques in each trial is shown in Figure 2 for cynomolgus and Figure 3 for rhesus.

Duration of SSMV across anesthetic combinations differed significantly in cynomolgus ($P = 0.0002$) but not rhesus ($P = 0.0986$) macaques. In cynomolgus macaques, the mean duration of SSMV was 17.5 min for AM, 6.9 min for TZ, and 2.6 min for KA. Duration of SSMV with AM was longer than either KA ($P < 0$ 0.0001) or TZ ($P = 0.0001$), with no significant difference between TZ and KA ($P = 0.0528$). In rhesus macaques,

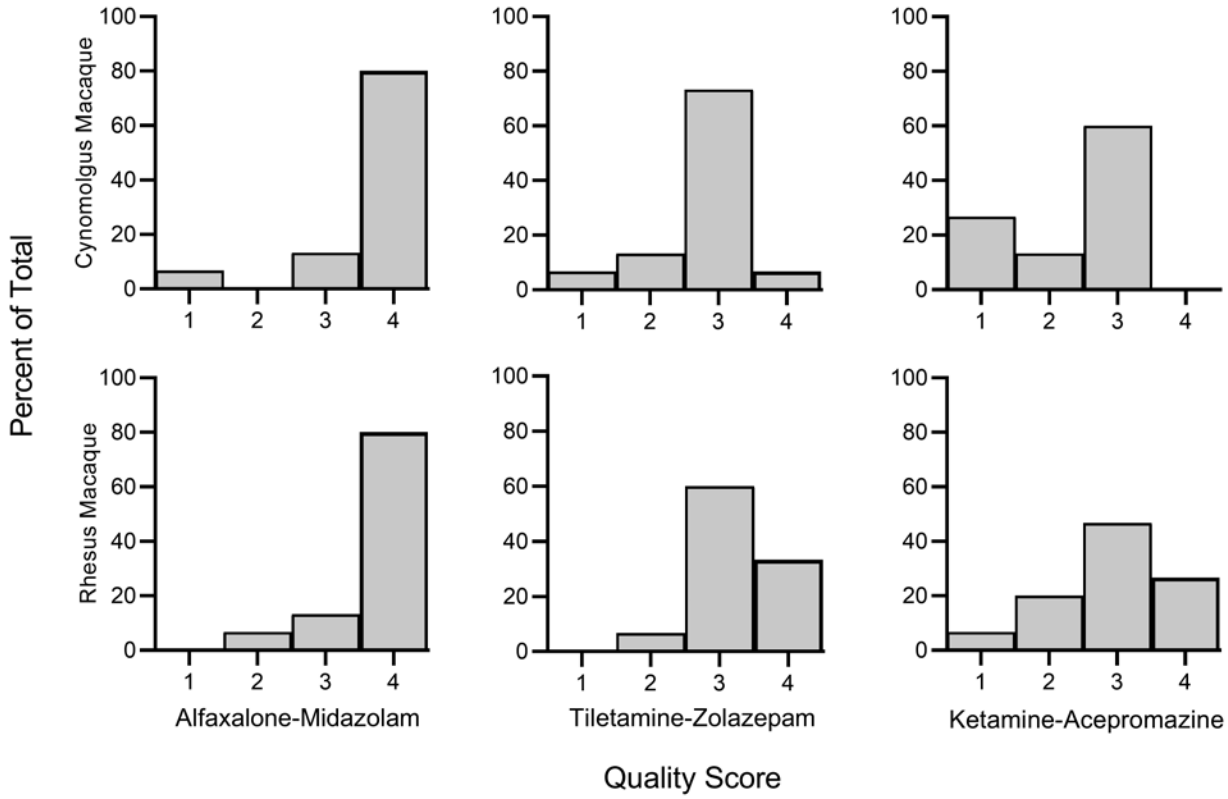

Figure 1. Percentage of 15 NHPs per species scored for quality of anesthesia. Quality scores are (1) no periods of SSMV achieved, (2) one period of SSMV achieved at less than 20 min, (3) 2 periods of SSMV achieved with the first attempt sustained for less than 20 min, and (4) first attempt at SSMV sustained for 20 min.

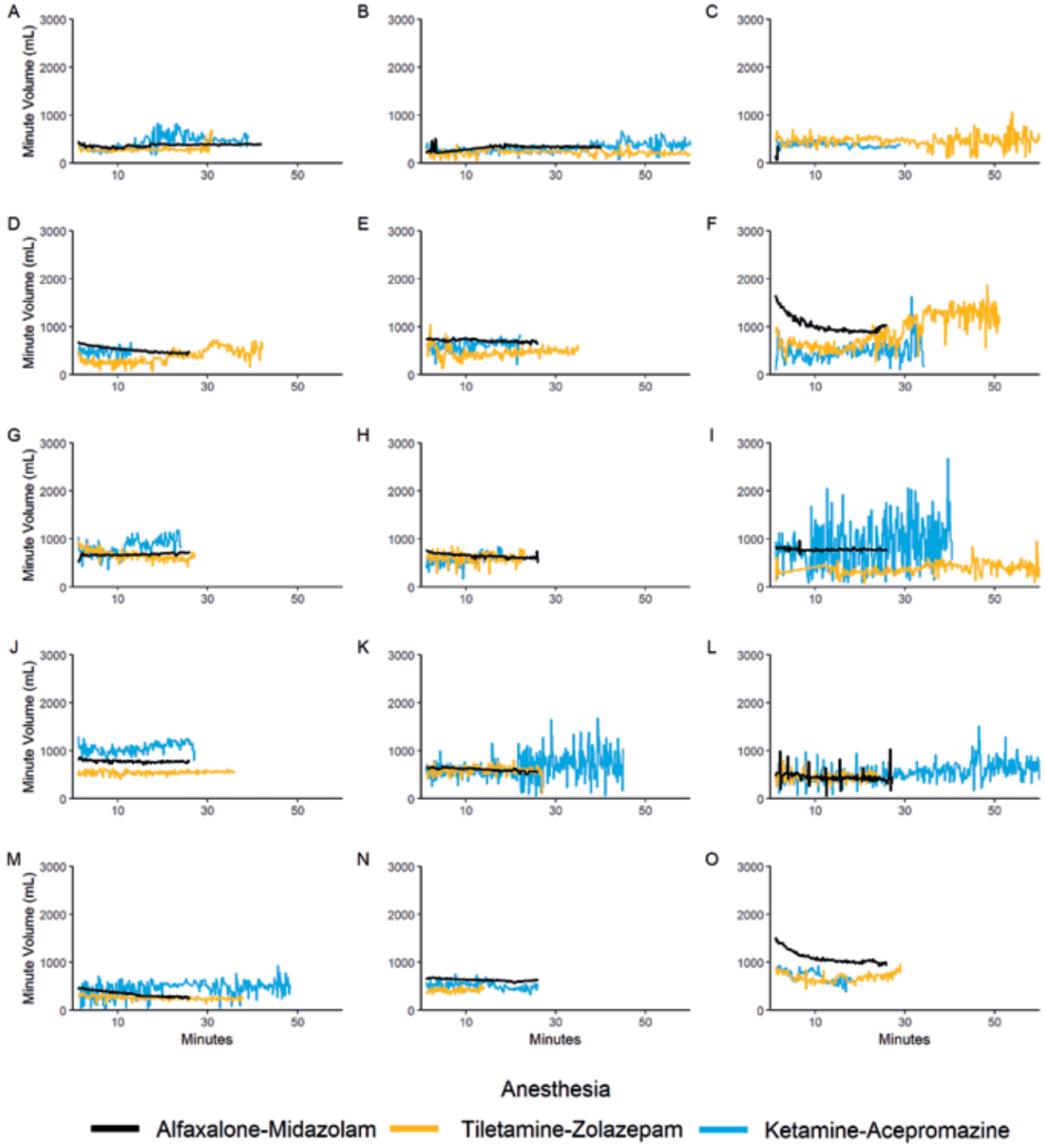

Figure 2. Minute volume as a function of time for the 15 cynomolgus macaques. Each frame (A-O) shows the anesthetic events for a single macaque colored by the type of anesthesia. Measurements of minute volume were taken every 10 s. One macaque (C) was unable to complete the AM trial due to apnea and therefore only a few values are recorded for this anesthetic.

the mean duration of SSMV was 18 min for AM, 12.8 min for TZ, and 10.8 min for KA. Individual durations by species and anesthetic are shown in Figure 4.

When controlling for the order in which individual macaques received each anesthetic combination, average SSMV differed across combinations for both cynomolgus ($P \leq 0.0001$) and rhesus ($P = 0.0004$) macaques. In cynomolgus macaques, the SSMV was approximately 114 mL larger for AM as compared with TZ ($P \leq 0.05$, 95% CI: 15 to 213), with no difference in average SSMV between AM and KA ($P > 0.05$, 95% CI: −164 to 111) or TZ and KA ($P > 0.05$, 95% CI: −286 to 5). In rhesus macaques, the average SSMV was approximately 175 mL smaller for AM as compared with KA ($P \leq 0.05$, −343 to −7), with no difference in average SSMV between AM and TZ ($P \leq 0.05$, −241 to 77) or between TZ and KA ($P > 0.05$, 95% CI: −263 to 77).

Time to SSMV did not differ across anesthetic combinations in either cynomolgus ($P = 0.1505$) or rhesus ($P = 0.6399$) macaques. Time to start was also not different among anes-

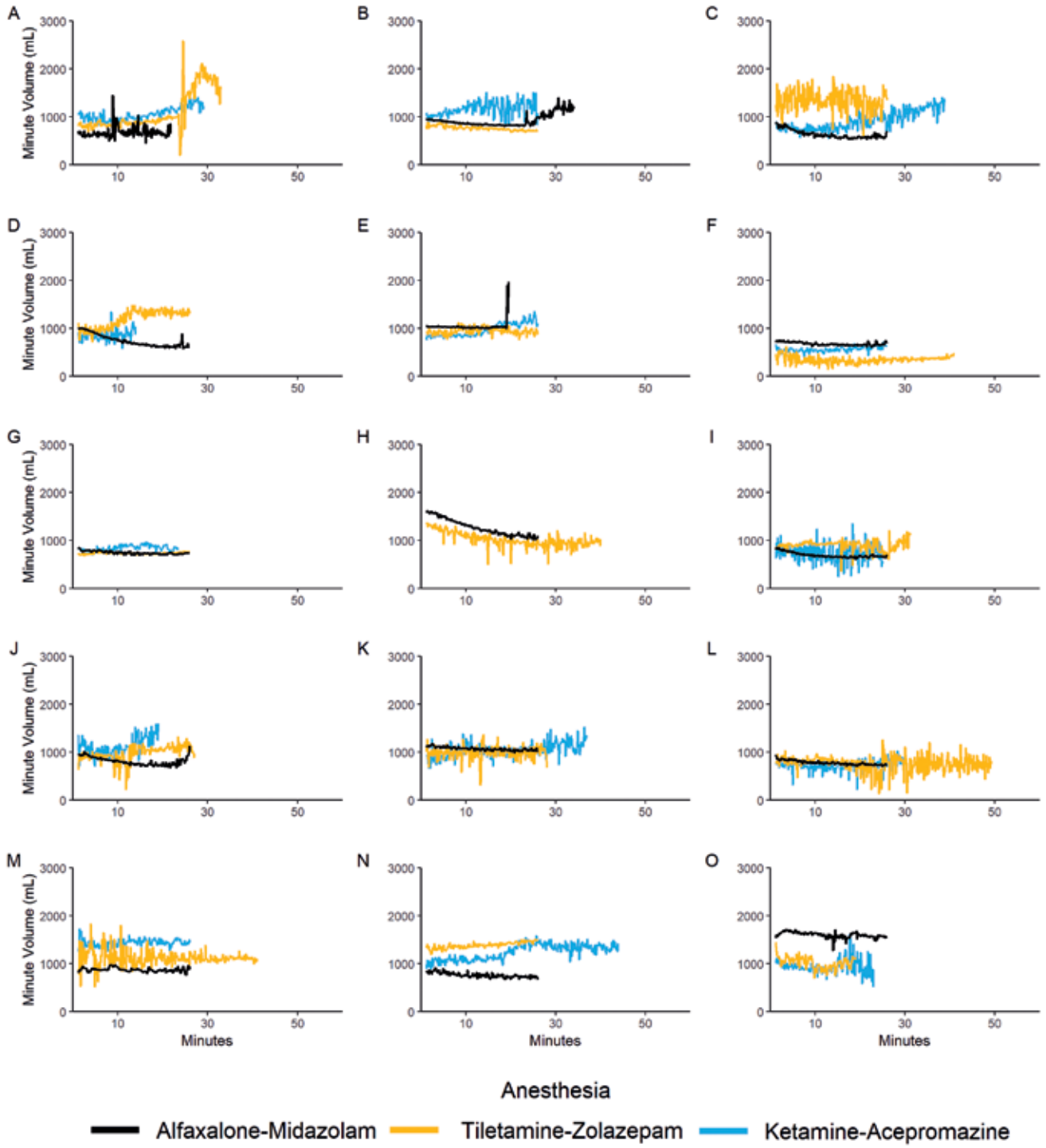

Figure 3. Minute volume as a function of time for the 15 rhesus macaques. Each frame (A-O) shows the anesthetic events for a single macaque colored by the type of anesthesia. Measurements of minute volume were taken every 10 s. One macaque (H) never became fully anesthetized on KA and therefore has no measurements.

thetic combinations in either cynomolgus ($P = 0.9435$) or rhesus ($P = 0.7804$) macaques.

Side effects are summarized in Table 1 but were not analyzed statistically. One cynomolgus macaque experienced apnea on AM when the dental dam was tight enough to create a seal in the plethysmography chamber. As a result, plethysmography could not be conducted for this individual on AM, and values were not obtained for duration of SSMV, SSMV average, or time to SSMV for this trial. One rhesus macaque never became adequately sedated while on KA for placement in the plethysmography chamber or measurement of vitals, and thus values for duration of SSMV, SSMV average, time to SSMV, and time to start were not obtained. Duration of anesthesia for this NHP was considered to be 0 min.

Discussion

This study sought to validate an AM anesthetic combination as a possible alternative for aerosol studies that use plethysmography in cynomolgus and rhesus macaques. Consistency of respiratory parameters directly impacts dosing accuracy of an

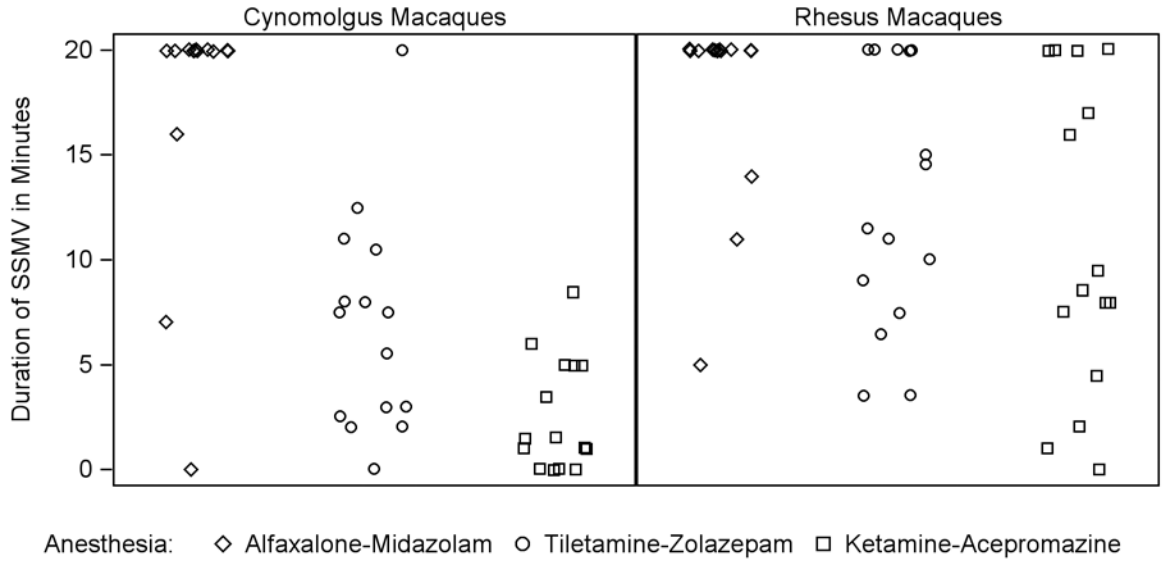

Figure 4. Duration of SSMV in minutes for 15 NHPs for each species by anesthesia. Data points on the horizontal axis are spread out to show otherwise overlapping measurements.

Table 1. Number of nonhuman primates experiencing side effects by species and anesthetic

	Cynomolgus macaques (n = 15)			Rhesus macaques (n = 15)		
Side effects	AM	TZ	KA	AM	TZ	KA
Vomiting	1	1	0	0	2	2
Rhythmic involuntary movements	0	2	4	0	5	2
Muscle twitching	2	0	1	6	0	0
Hypersalivation	0	1	2	0	0	0
Hiccupping	0	0	0	0	1	0
Elevated Heart Rate	0	0	0	1	1	3
Decreased Heart Rate	1	0	0	0	0	0
Constant Blinking	0	0	0	0	1	2
Raspy / Audible Breathing	2	0	2	2	0	0
Muscle Rigidity	0	0	0	0	0	1
Did not become fully anesthetized	0	0	0	0	0	1
Apnea when chamber was sealed	1	0	0	0	0	0
Injured self upon induction (minor)	0	0	0	1	1	0
Total	7	4	9	10	11	11

Abbreviations: AM = alfaxalone-midazolam, TZ = tiletamine-zolazepam, KA = ketamine-acepromazine

aerosol exposure because the minute volume measured initially with plethysmography is used to calculate the spray duration. At our facility, minute volume is no longer measured and spray duration is not adjusted after the subject has been placed in the aerosol exposure chamber.

In our study, AM was the most consistent combination, as evidenced by the higher quality of anesthesia in both species and longer duration of SSMV in cynomolgus macaques. Our data suggest that the values obtained with AM during initial plethysmography would likely remain consistent throughout the duration of an aerosol spray, thereby ensuring accurate delivery and dosing. TZ and KA were more likely to have sporadic changes in minute volume, as evidenced by lower quality of anesthesia. These changes in minute volume could lead to inaccurate dosing across a spray, which would have negative implications on study results and validity.

Although side effects were not analyzed statistically, the number of side effects were comparable with AM, KA and TZ. The most common side effect with AM was muscle twitching in response to touch. Muscle twitching after administration of alfaxalone has also been noted previously in macaques and other species.[1,4,18,21,26,27] For plethysmography or aerosol dosing, this side effect is not considered to be problematic, as the animal is generally not being manipulated in a manner that would stimulate muscle twitching during either of these activities. The most significant side effect seen with AM was that one NHP developed apnea when the dental dam in the plethysmography chamber was tight enough to create a seal. The NHP breathed normally when the dental dam was not around the neck or was loose. However, this particular NHP was considerably overweight and had a body condition score of 5 out of 5; this obesity could have contributed to the problem experienced during the trial, although this NHP did not exhibit apnea on TZ or KA. AM can induce apnea in dogs that receive this combination IV.[22] Although all NHPs had unremarkable recoveries on AM at the pilot dose of 2 mg/kg of alfaxalone and 0.3 mg/kg midazolam, the NHP used for the pilot trial showed a good plane of anesthesia with no movement or palpebral reflex until it abruptly recovered within the plethysmography chamber. Within 10 s, the macaque began to vigorously thrash about the chamber. While none of NHPs showed such an abrupt recovery at the higher dose of 5 mg/kg alfaxalone used in this study, they subjectively showed signs of a shorter interval before regaining a righting reflex as compared with TZ. Therefore, caution is necessary when using alfaxalone with midazolam at lower dosages in NHPs, as they may give little warning before regaining significant voluntary movement.

In rhesus macaques, the average SSMV was significantly larger for AM as compared with TZ in cynomolgus macaques, but was smaller than that of KA. Larger minute volumes are desirable because the animal would spend less time in the aerosol exposure chamber. However, all rhesus macaques on AM still had an average SSMV that fell within the ideal range at our institution (450 to 1,600 mL). The lower quality of KA anesthesia supports the use of AM, despite the larger minute volumes observed with KA.

No significant differences were noted in the time to reach SSMV, suggesting that if a NHP is going to achieve SSMV,

they will do so in approximately the same amount of time regardless of the drugs used in this study. Also, no differences were noted between anesthetics or species in the time from induction of anesthesia to the start of the procedure, suggesting that the times to sedation are approximately the same amount for all 3 combinations. Thus, using AM would not prolong the time needed for the animal to become sedate enough to handle.

Duration of anesthesia is also an important consideration when choosing an anesthetic for an aerosol study. Significant transportation time may be needed to move animals to the challenge area or to perform additional procedures such as physical exams or sample collection before plethysmography and aerosol exposure. In these instances, longer duration of anesthesia may be desirable. If procedures are not necessary before aerosol exposure, a shorter duration of anesthesia is preferable. At our facility, 45 min of anesthesia is considered the minimum for aerosol studies. All 3 anesthetic combinations met this requirement. The average duration of anesthesia for AM was 87 min in cynomolgus macaques and 62 min in rhesus macaques. While TZ anesthesia lasted significantly longer in both species, the intermediate but adequate duration of AM anesthesia, combined with its more consistent SSMV, would make it a better choice for many challenge studies.

The biggest drawback we found to using AM was the large injection volume. Although alfaxalone and midazolam have been administered subcutaneously in rhesus macaques (in combination with dexmedetomidine), we chose to evaluate intramuscular administration, which is consistent with the route of administration we currently use for plethysmography under TZ and KA anesthesia. Alfaxalone is currently available as an approved or indexed product only in a 10 mg/mL concentration.[17] In this study, NHPs weighing greater than 5.35 kg required 2 intramuscular injections to receive the full dose without exceeding 3.0 mL per injection. In studies using larger NHPs (> 5.0 kg), AM may be less desirable due to larger overall injection volumes and therefore the need for multiple injections. Ideal intramuscular injection volumes for NHPs range from 0.1 to 0.5 mL/kg, with volumes not to exceed 3.0 mL per site.[6,11,14,29] The injection volume of AM used in this study was 0.56 mL/kg. Using the maximum injection volume of 3.0 mL per site produced no obvious injection site reactions or deviances in behavior or locomotion. However, injection sites were not shaved and NHPs were only visually observed on the days after the procedure; therefore, minor injection site reactions may have been overlooked.

Limitations of this study include the small population size (n = 15 per species) and the exposure of each animal to only one anesthetic event per drug combination (3 events total per NHP). Another limitation in this study is that evaluators were not blind to the anesthetic that each NHP received, primarily due to the limited personnel available to support the study. Lack of blinding may have contributed to interrater variability and efficacy bias, particularly on more subjective parameters such as side effects. However, the 2 individuals concurred on the less objective parameters, and the principal investigator reviewed the numerical data for consistency in order to minimize potential bias. Furthermore, we did not evaluate some events that may occur during an actual aerosol challenge. One such event that warrants further investigation is the administration of additional anesthesia if the animal begins to recover before the procedure is complete. Anesthetic supplementation could significantly affect minute volume as the animal transitions through various depths of anesthesia.

Overall, our study showed that an AM anesthetic combination provides more consistent and higher quality anesthesia in cynomolgus and rhesus macaques as compared with TZ or KA. This makes it an acceptable, and in many cases preferred, anesthetic option for aerosol challenge studies that use plethysmography. An AM anesthetic combination is particularly attractive for studies in which the NHPs weigh less than 5 kg, so that total injection volume can be minimized.

Acknowledgments

The authors thank the USAMRIID leadership for their continued support of training and research programs that supported this study, and to James Writer for technical writing assistance in formatting and editing of this manuscript. Special thanks to the technical staff, including Nazira (Ashley) Alli, Stephanie Bellanca, Chase Cover, Ondraya Frick, Valencia Rolle, Melanny Rodriguez Vazquez, and Taleria Young, of the USAMRIID Veterinary Medicine Division for the assistance with conducting this study, and to Joseph Marion for his assistance with plotting minute volume data for this study.

Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

References

1. **Bakker J, Roubos S, Remarque EJ, Arndt SS, Kronen PW, Langermans JA.** 2018. Effects of buprenorphine, butorphanol or tramadol premedication on anaesthetic induction with alfaxalone in common marmosets (*Callithrix jacchus*). Vet Anaesth Analg **45:**309–319. https://doi.org/10.1016/j.vaa.2017.06.009.
2. **Barletta M.** 2019. Alfaxalone: An Old Drug in a New Formulation. https://todaysveterinarypractice.com/alfaxalone-an-old-drug-in-a-new-formulation/.
3. **Barnewall RE, Knostman K, Fisher D, Robertson A, Vales P, Bigger J.** 2012. Inhalational monkeypox virus infection in cynomolgus macaques. Front Cell Infect Microbiol **2:**117. https://doi.org/10.3389/fcimb.2012.00117.
4. **Bertrand HG, Sandersen C, Murray J, Flecknell PA.** 2017. A combination of alfaxalone, medetomidine and midazolam for the chemical immobilization of Rhesus macaque (*Macaca mulatta*): Preliminary results. J Med Primatol **46:**332–336. https://doi.org/10.1111/jmp.12315.
5. **Besch TK, Ruble DL, Gibbs PH, Pitt M.** 1996. Steady-state minute volume determination by body-only plethysmography in juvenile rhesus monkeys. Lab Anim Sci **46:**539–544.
6. **Diehl KH, Hull R, Morton D, Pfister R, Rabemampianina Y, Smith D, Vidal JM, Vorstenbosch CVD.** 2001. A good practice guide to the administration of substances and removal of blood, including routes and volumes. J Appl Toxicol **21:**15–23. https://doi.org/10.1002/jat.727.
7. **Fish R, Danneman PJ, Brown M, Karas Aeditors.** 2008. Anesthesia and analgesia in laboratory animals, 2nd ed. London (UK): Academic Press.
8. **Food and Drug Administration.** [Internet]. 2020. FDA adds Alfaxan Multidose IDX to the Index of Legally Marketed Unapproved New Animal Drugs for Minor Species. [Cited 20 October 2021]. Available at: https://www.fda.gov/animal-veterinary/cvm-updates/fda-adds-alfaxan-multidose-idx-index-legally-marketed-unapproved-new-animal-drugs-minor-species
9. **Food and Drug Administration.** [Internet]. 2021. The index of legally marketed unapproved new animal drugs for minor species. [Cited 20 October 2021]. Available at: https://www.fda.gov/animal-veterinary/minor-useminor-species/index-legally-marketed-unapproved-new-animal-drugs-minor-species
10. **Foster CD, Hunter TC, Gibbs PH, Leffel EK.** 2008. Whole-body plethysmography in African green monkeys (*Chlorocebus aethiops*) with and without jackets. J Am Assoc Lab Anim Sci **47:**52–55.
11. **Gad SC, Spainhour CB, Shoemake C, Pallman DRS, Stricker-Krongrad A, Downing PA, Seals RE, Eagle LA, Polhamus K, Daly J.** 2016. Tolerable levels of nonclinical vehicles and formulations used in studies by multiple routes in multiple species with notes on methods to improve utility. Int J Toxicol **35:**95–178. https://doi.org/10.1177/1091581815622442.

12. **Glander K.** 1993. Estudios Primatologicos En Mexico. Veracruz, Mexico: Universidad Veracruzana.
13. **Hollander M, Wolfe DA, Chicken E.** 2013. Nonparametric statistical methods. Hoboken (NJ): John Wiley & Sons.
14. **Hull RM.** 1995. Guideline limit volumes for dosing animals in the preclinical stage of safety evaluation. Hum Exp Toxicol **14:**305–307. https://doi.org/10.1177/096032719501400312.
15. **Institute for Laboratory Animal Research.** 2011. Guide for the Care and Use of Laboratory Animals. Washington (DC): National Academies Press.
16. **Jones KL.** 2012. Therapeutic review: alfaxalone. J Exot Pet Med **21:**347–353. https://doi.org/10.1053/j.jepm.2012.09.011.
17. **Jurox, Inc.** [Internet]. 2018. Alfaxan ® Multidose Brochure. [Cited 18 October 2021]. Available at: https://jurox.com/sites/default/files/resources/US - Alfaxan - Multidose Detailer - Digital.pdf
18. **Keates H.** 2003. Induction of anaesthesia in pigs using a new alphaxalone formulation. Vet Rec **153:**627–628. https://doi.org/10.1136/vr.153.20.627.
19. **Kirk R.** 1982. Experimental design: Procedures for the behavioral sciences. Belmont (CA): Brooks Cole Publishing.
20. **López KR, Gibbs PH, Reed DS.** 2002. A comparison of body temperature changes due to the administration of ketamine–acepromazine and tiletamine–zolazepam anesthetics in cynomolgus macaques. Contemp Top Lab Anim Sci **41:**47–50.
21. **Maddern K, Adams VJ, Hill NA, Leece EA.** 2010. Alfaxalone induction dose following administration of medetomidine and butorphanol in the dog. Vet Anaesth Analg **37:**7–13. https://doi.org/10.1111/j.1467-2995.2009.00503.x.
22. **Miller C, Hughes E, Gurney M.** 2019. Co-induction of anaesthesia with alfaxalone and midazolam in dogs: a randomized, blinded clinical trial. Vet Anaesth Analg **46:**613–619. https://doi.org/10.1016/j.vaa.2019.03.009.
23. **Murphy HW.** 2008. Get a hand on your patient: primate restraint and analgesia. Small animal and exotics. Proceedings of the North American Veterinary Conference, Volume 22, Orlando, Florida. Gainesville (FL): The North American Veterinary Conference.
24. **Obot Akata CJ, Blair LF, Barr EB, Storch S, Vigil G, Campen MJ.** 2007. Development of a head-out plethysmograph system for non-human primates in an Animal Biosafety Level 3 facility. J Pharmacol Toxicol Methods **55:**96–102. https://doi.org/10.1016/j.vascn.2006.04.002.
25. **Reed DS, Lind CM, Sullivan LJ, Pratt WD, Parker MD.** 2004. Aerosol infection of cynomolgus macaques with enzootic strains of Venezuelan equine encephalitis viruses. J Infect Dis **189:**1013–1017. https://doi.org/10.1086/382281.
26. **Siriarchavatana P, Ayers JD, Kendall LV.** 2016. Anesthetic activity of alfaxalone compared with ketamine in mice. J Am Assoc Lab Anim Sci **55:**426–430.
27. **Tuval A, Dror-Maman I, Las L, Bdolah-Abram T, Shilo-Benjamini Y.** 2021. Evaluation of alfaxalone and midazolam with or without flumazenil reversal in Egyptian fruit bats (*Rousettus aegyptiacus*). Vet Anaesth Analg **48:**239–246. https://doi.org/10.1016/j.vaa.2020.12.002.
28. **Vasconcelos D, Barnewall R, Babin M, Hunt R, Estep J, Nielsen C, Carnes R, Carney J.** 2003. Pathology of inhalation anthrax in cynomolgus monkeys (*Macaca fascicularis*). Lab Invest **83:**1201–1209. https://doi.org/10.1097/01.LAB.0000080599.43791.01.
29. **Wada S, Koyama H, Yamashita K.** 2020. Sedative and physiological effects of alfaxalone intramuscular administration in cynomolgus monkeys (*Macaca fascicularis*). J Vet Med Sci **82:**1021–1029. https://doi.org/10.1292/jvms.20-0043.
30. **Warren R, Lockman H, Barnewall R, Krile R, Blanco OB, Vasconcelos D, Price J, House RV, Bolanowksi MA, Fellows P.** 2011. Cynomolgus macaque model for pneumonic plague. Microb Pathog **50:**12–22. https://doi.org/10.1016/j.micpath.2010.10.002.
31. **Yingst SL, Facemire P, Chuvala L, Norwood D, Wolcott M, Alves DA.** 2014. Pathological findings and diagnostic implications of a rhesus macaque (*Macaca mulatta*) model of aerosol-exposure melioidosis (*Burkholderia pseudomallei*). J Med Microbiol **63:**118–128. https://doi.org/10.1099/jmm.0.059063-0.
32. **Yingst SL, Huzella LM, Chuvala L, Wolcott M.** 2010. A rhesus macaque (*Macaca mulatta*) model of aerosol-exposure brucellosis (*Brucella suis*): pathology and diagnostic implications. J Med Microbiol **59:**724–730. https://doi.org/10.1099/jmm.0.017285-0.