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Use of Ketamine or Xylazine to Provide Balanced Anesthesia with Isoflurane in C57BL/6J Mice

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Balanced anesthesia—the use of a combination of drugs to achieve a desired anesthetic plane—offers many benefits, including smoother induction and recovery and fewer adverse effects than occur with individual drugs. Although premedication prior to inhalant anesthesia is routine in other species, mice are commonly induced with gas anesthesia alone. The hypothesis of this study was that premedication with ketamine or xylazine would safely reduce the stress of isoflurane induction and lower the minimum alveolar concentration (MAC) of isoflurane. Young adult male and female C57BL/6J mice were premedicated with ketamine (100 mg/kg), xylazine (4 mg/kg), or isotonic crystalloid (0.1 mL) and were used in 4 experiments. First, isoflurane induction was video recorded under all test conditions, and the videos were scored according to a behavioral ethogram to identify signs of distress. Mice in the ketamine group experienced tremors and ataxia before and during induction. Therefore, ketamine was given after induction with isoflurane in subsequent experiments. Second, the MAC value for each anesthetic protocol was determined by using quantal and bracketing analysis. Third, mice were anesthetized according to the 3 protocols, and vital parameters were monitored for 60 min. Finally, anesthetized mice were challenged with hypoxia and hypovolemia, and vital parameters were monitored. Premedication with xylazine significantly reduced the stress scores for isoflurane induction (control, 7.3 ± 1.5; ketamine, 6.0 ± 3.0; xylazine, 3.1 ± 1.0). Ketamine and xylazine both reduced the MAC of isoflurane (control, 1.89%; ketamine, 0.96%; xylazine, 1.20%). All mice survived 60 min of anesthesia and the hypoxia-hypovolemia challenge. Premedication with xylazine reduced the stress of induction and lowered the necessary dose of isoflurane in C57BL/6J mice to maintain a surgical plane of anesthesia. We recommend administering xylazine before isoflurane induction and anesthesia of healthy mice that are undergoing procedures in which 100% oxygen is provided and anticipated blood loss is less than 10% to 15% of the total blood volume.

Abbreviations: HR, heart rate; LORR, loss of righting reflex; LRS, lactated Ringer solution; MAC, minimum alveolar concentration; RR, respiratory rate; SpO_2, peripheral oxygen saturation

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Introduction

Although murine anesthesia is a common procedure in biomedical research, anesthetic standards for mice lag behind those for other species in multiple areas, including options for anesthetic protocols, monitoring, and supportive care. Balanced anesthesia is widely used in human and veterinary medicine. Also known as multimodal anesthesia, balanced anesthesia is defined as the use of a combination of drugs and techniques to achieve a desired level of anesthesia. Previous studies in multiple species have evaluated balanced anesthesia protocols using a combination of injectable and inhalant anesthetics and have found them to have many benefits, including improving the quality of induction and reducing the amount of inhalant anesthetic required to keep animals at a surgical plane of anesthesia.[9,11,12,19,23,41,47]

Premedication with a sedative or injectable anesthetic is one example of a balanced technique and has been used to reduce the stress associated with gas anesthesia induction.[9,26,33] Although this practice is common in other mammalian species, mice are routinely induced with gas anesthesia alone.[14,17] Isoflurane offers many advantages in murine anesthesia, including easy titration of the dose and depth of anesthesia and rapid induction of and recovery from anesthesia.[9,21] However, despite isoflurane's utility, challenges associated with using it as a sole anesthetic agent include aversiveness to animals, dose-dependent cardiovascular and respiratory depression, and absence of enduring analgesia after surgery.[14,15,24,26,27,37] Premedication with an injectable anesthetic prior to isoflurane induction and anesthesia can be used to mitigate some of these concerns, improving both the safety and reducing the stress of anesthetic events.[17,25]

In 1847, a veterinarian studying ether anesthesia in dogs reported that their actions—including excitement, high muscle tonus of the tail, and vocalizations—were extremely distasteful and indicative of suffering.[42] Since then, gas induction without sedation has largely fallen out of favor for most species and is used only when specifically indicated. More than 150 y later, other researchers described the behaviors of mice induced with sevoflurane to include "defecation, urinating, shaking the head or limbs, jumping, and locomotion."[9] Despite the fact that mice experience similar behaviors to those identified as distressful in dogs in 1847, the use of isoflurane without sedation is still considered the "preferred anesthetic for all rodents when

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equipment for gas anesthesia and scavenging is available."[17] Reducing the stress of inhalant-only anesthesia would be a significant improvement in the welfare for mice in research, because minimizing stress is an important part of fear-free handling throughout veterinary medicine.

The current study evaluated how the administration of intraperitoneal ketamine or xylazine prior to isoflurane anesthesia affected the induction and maintenance of anesthesia in mice. Ketamine and xylazine are 2 commonly used anesthetics in mice. Ketamine is a N-methyl-d-aspartate receptor antagonist that produces anesthesia and analgesia with minimal depression of the cardiovascular system and has been included in balanced anesthetic protocols in multiple species.[3,7,10,20,25,41] Xylazine is an α2 adrenergic receptor agonist commonly used in balanced anesthetic protocols; this drug causes sedation and muscle relaxation and has analgesic properties.[36] We chose these 2 drugs because of their potential to significantly reduce the amount of isoflurane necessary to maintain a surgical plane of anesthesia and to decrease the overall distress associated with the induction of inhalant anesthesia. Distressing events associated with different aspects of the induction process include the injection of premedications, the period between the injection and the start of induction with the inhalant agent, and the actual induction with inhalant. Administration of ketamine prior to inhalant induction has been associated with distress in some species but has also been shown to reduce the stress of induction in the anesthetic chamber.[9,26] No study to date has objectively evaluated cumulative stress during the induction of inhalant anesthesia.[9,20,26]

The hypothesis of the current study was that premedication with xylazine or ketamine would reduce the stress of induction with isoflurane, lower the minimum alveolar concentration (MAC) of isoflurane, and improve the physiologic response to pathologic conditions under anesthesia in mice. Another goal of this study was to provide dosages and data for the expected vital parameters commonly monitored in anesthetized mice for the 3 protocols tested. These monitoring data are helpful for identifying abnormalities in mice under anesthesia and can potentially signal the need for the anesthetist to intervene before critical clinical issues arise. Finally, we designed a protocol to evaluate these anesthetic combinations under pathologic conditions by inducing hypoxia and hypovolemia in anesthetized mice.

Materials and Methods

Overview of experiments. This project consisted of 4 experiments. Experiment 1 examined the effect of premedication with ketamine or xylazine on the stress of anesthetic induction with isoflurane. Experiment 2 evaluated the effect of premedication with ketamine or xylazine on the MAC of isoflurane in mice. Experiment 3 measured the vital parameters over 60 min of anesthesia for the 3 anesthetic protocols. Experiment 4 tested whether premedication with ketamine or xylazine improved physiologic responses to combined hypoxia and hypovolemia under isoflurane anesthesia.

Mice. Male and female C57BL/6J mice (*Mus musculus*; $n = 50$; age, 8 to 20 wk; weight, 17 to 27 g; Jackson Laboratories, Bar Harbor, ME) were used in this study. Mice were housed in an AAALAC-accredited facility under a 12:12-h light:dark cycle, fluorescent lighting, 315 Lux, in same-sex groups of 5 mice per cage in static polycarbonate microisolation cages with 75 $in.^2$ (484 cm^2) of floor space (Max 75, Alternative Design, Siloam Springs, AR) containing disposable bedding (0.12-in. Bed-O-Cobs, The Andersons, Maumee, OH) with cotton squares (Nestlets, Ancare, Bellmore, NY). Mice were fed standard pelleted laboratory rodent chow (no. 5001, LabDiet, St Louis, MO) without restriction and received municipal water supplied by bottle. Sentinel mice in the facility were tested routinely and were considered to be free of fur mites, pinworms, and contagious pathogens, including mouse hepatitis virus, mouse parvoviruses, rotavirus, ectromelia virus, Sendai virus, pneumonia virus of mice, Theiler murine encephalomyelitis virus, reovirus, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, mouse adenovirus, and polyomavirus. Mice were given at least 7 d to acclimate to the housing facility and cage environment prior to the start of the study. Each mouse in this study underwent no more than 2 anesthetic events with at least a 10-d washout period between experiments. All procedures were approved by the University of Pennsylvania's IACUC.

Premedication. For all experiments, mice were weighed on a digital scale (model KD-160, Tanita, Arlington Heights, IL) prior to dosing. Mice were randomly assigned to experimental groups prior to each experiment. Mice received either isotonic crystalloid fluid (0.1 mL IP; lactated Ringer solution [LRS], Hospira, Lake Forest, IL), ketamine HCl (100 mg/kg IP; 100 mg/mL diluted to 10 mg/mL; Ketaset, Zoetis, Kalamazoo, MI), or xylazine (4 mg/kg IP; 20 mg/mL diluted to 0.8 mg/mL; AnaSed, Akorn, Lake Forest, IL). Ketamine and xylazine were diluted in 0.9% sodium chloride and used within 12 h. Mice were picked up by the base of the tail or with a cupped hand and then briefly scruffed for injections. All injections were performed by the same experimenter (EMD) to limit variability in technique. Injections were administered intraperitoneally in the lower left or right quadrant of the abdomen by using a 1-mL syringe and 27-gauge needle. For experiment 1, mice in all groups were injected 5 min before anesthetic induction. Based on an objective assessment of the quality of induction in experiment 1, mice in subsequent experiments received ketamine injections immediately after induction with isoflurane, whereas mice that received xylazine or LRS were injected 5 min before anesthetic induction.

Anesthetic induction, maintenance, and monitoring of anesthesia. Mice in all groups were induced with 4.0% isoflurane (Isoflurane, Piramal Critical Care, Bethlehem, PA) in either 100% oxygen (experiments 1 through 3) or 21% oxygen (experiment 4) in an anesthetic induction chamber. The gas flow rate for induction was 2.0 L/min. The induction chamber was not filled with isoflurane prior to putting the mouse in the chamber. After loss of the righting reflex (LORR) occurred, mice were moved to a nose cone and received isoflurane in either 100% oxygen (experiments 1 through 3) or 21% oxygen (experiment 4). For maintenance of anesthesia, the gas flow rate was reduced to 0.6 L/min. Mice were placed in dorsal recumbency on a circulating water blanket (Stryker T/Pump, Kalamazoo, MI) under an adjustable heat lamp, which was adjusted as needed to maintain mice at a body temperature of 35 to 37 °C throughout all experiments. Eye lubricant (Akorn, Lake Forest, IL) was applied, a rectal temperature probe (9 mm; RET3, ThermoWorks, American Fork, UT) connected to a thermocouple (TW2-193, MicroTherma, ThermoWorks, American Fork, UT) was inserted, and a mouse-specific pulse oximeter (MouseSTAT Jr; Kent Scientific; Torrington, CT) was placed on the left or right hindfoot; the oximeter was removed briefly when the depth of anesthesia (described in experiment 2) was assessed. Respiratory rate (RR) was measured by visually counting thoracic excursions. Heart rate (HR), RR, peripheral oxygen saturation (SpO_2), body temperature, and depth of anesthesia were recorded every 5 min. Throughout all experiments, the isoflurane concentration at the fresh gas outlet was measured in real time by using an anesthetic gas monitor (Poet IQ2, Anesthetic Gas Monitor Criticare

Systems, Waukesha, WI). Because the isoflurane concentrations were measured from the inspiration gas stream, they were only estimates of the alveolar isoflurane concentration.

Postprocedural analysis. Body temperature was measured until the return of righting reflex. All mice were normothermic when return of righting reflex occurred. Heat support was provided until mice were fully ambulatory, at which point mice were returned to the home cage. No anesthetic reversals were given. Any mice that died under anesthesia were evaluated by necropsy for gross and histopathologic abnormalities. All statistical analysis was performed using SigmaPlot 12.3 (Systat Software, San Jose, CA). Statistical significance was set at $P < 0.05$. The specific tests performed are described for each experiment.

Experiment 1: Scoring the stress of induction. Individual mice were randomly assigned to an experimental group; injected as described above with either ketamine ($n = 7$; male mice, 3; female mice, 4), xylazine ($n = 7$; male mice, 2; female mice, 5), or LRS ($n = 7$; male mice, 3; female mice, 4); and returned to the home cage for 5 min. Anesthesia was then induced by using 4.0% isoflurane in 100% oxygen. A videorecording device (Apple, Cupertino, CA) directed along the long axis of the cage was used to record mice in the home cage starting 4 min after injection and in the induction chamber until LORR. Each videorecording was assigned a random number and scored according to a behavioral ethogram (Figure 1) to identify signs of distress during induction. The mice were evaluated during the minute before transfer to the induction chamber and throughout the time they were in the chamber. Videos were scored throughout induction for the presence of frantic behaviors, tremors, and ataxia. Frantic behavior was defined as rapid exploratory behavior, abnormal alteration in activity level, flipping, spinning, pacing, or rearing onto back legs. Tremors were defined as repetitive, involuntary muscle movements (localized or generalized). Ataxia was defined as uncoordinated attempts to walk or stand, loss of balance, or postural abnormalities. Ataxia was scored as mild, moderate, or severe. Mild ataxia was defined as mild gait abnormalities but full ambulation. Moderate ataxia was defined as poor ambulation with pronounced gait abnormalities. Severe ataxia was defined as a complete loss of balance with at least one fall. Videos were scored by 2 veterinarians who were experienced in evaluating rodent behavior and who were blind to the anesthetic protocol the mouse had received. The correlation between the 2 scores was 0.91 ($P < 0.001$). The scores of the 2 reviewers were averaged for statistical analysis.

Statistical analysis of experiment 1 used a block design, with sex as the blocking factor. The effect of sex on induction stress was compared by using a *t* test (SigmaPlot 12.3), Systat, San Jose, CA). The scores for the behavioral ethogram were compared by ANOVA, using the anesthetic protocol as the main effect.[32,35] Tukey posthoc analysis was performed to further define actual statistical differences in stress between groups. The analysis was then extended to compare the effects of the different protocols on stress before the mice were moved to the induction chamber and during the time in the induction chamber, again by using one-way ANOVA and Tukey posthoc testing when significant effects were detected. Data are reported as mean ± 1 SD.

Experiment 2: MAC determination. Individual mice were randomly assigned to 3 groups and injected intraperitoneally with ketamine ($n = 11$; male mice, 5; female mice, 6), xylazine ($n = 11$; male mice, 5; female mice, 6), or LRS ($n = 8$; male mice, 4; female mice 4) as described above. The videos recorded during experiment 1 showed that mice in the ketamine group experienced tremors and ataxia both before and during induction, in addition to distress associated with the injection. Because of these observations, mice in the ketamine group were injected immediately after induction with isoflurane, whereas mice in the xylazine and control groups were injected 5 min before anesthetic induction.

All mice were induced with 4.0% isoflurane and 100% oxygen in an anesthetic induction chamber until LORR. Mice were then transferred to a nose cone and instrumented and monitored as described above (*Maintenance and Monitoring of Anesthesia*). After instrumentation, isoflurane delivery was set to the first experimental concentration. Mice remained at the first of 3 randomly selected and increasing experimental isoflurane concentrations for 15 min to allow equilibration. Mice were tested at increasing isoflurane concentrations in each experiment to prevent neural inertia from affecting their response.[16,23,46] This strategy differs from the traditional up–down technique of MAC determination and helps prevent the previous state of consciousness and responsiveness from affecting subsequent testing.[23] Mice were given 10 min to equilibrate to the 2nd and 3rd experimental concentrations.[10,23,45,47] Isoflurane concentrations evaluated ranged from 1.5% to 2.1% for control mice, 0.7% to 1.6% for the mice receiving ketamine, and 0.7% to 1.9% for the mice receiving xylazine. Because of the differences in these ranges, the experimenter was aware of the premedication that the mouse received.

After the equilibration period, the depth of anesthesia was assessed at each isoflurane concentration by using a 300-g noxious stimulus (Touch Test, North Coast Medical, Gilroy, CA). The device is a handheld filament that delivers 300 g of force when depressed manually and bends when this force is

	Preinduction		In induction chamber			
Points	Ataxia	Tremors	Time to last movement	Ataxia	Frantic behavior	Tremors
0	Absent	Absent	<30 s	None	None	None
1	Absent	Absent	30 to <60 s	Mild	<15 s	<15 s
2	Present	Present	60 to <90 s	Moderate	15 to <30 s	15 to <30 s
3	Present	Present	>90 s	Severe	>30 s	>30 s or rapid tremors

Figure 1. Ethogram used to score anesthesia induction. Frantic behavior was defined as rapid exploratory behavior, abnormal alteration in activity level, flipping, spinning, pacing, or rearing onto back legs. Tremors were defined as repetitive, involuntary muscle movements (localized or generalized). Ataxia was defined as uncoordinated attempts to walk or stand, loss of balance, or postural abnormalities. Ataxia was scored as mild, moderate, or severe. Mild ataxia was defined as mild gait abnormalities but full ambulation. Moderate ataxia was defined as poor ambulation with pronounced gait abnormalities. Severe ataxia was defined as a complete loss of balance with at least one fall.

reached, limiting the amount of force delivered. In previous studies, application of the touch test device reliably replicated the response to a firm toe pinch, delivering consistent force each time without causing lameness or pain in the mouse after the procedure.[23] The stimulus was delivered 4 times, alternating between hindfeet, with at least 30 s between tests. When a vigorous response to the touch test occurred on the first test, no further tests were performed to prevent testing from altering the plane of anesthesia. The response was defined as either a positive or negative response to the 300-g noxious stimulus. Any movement in any of the 4 trials was considered a positive response, and a lack of movement in response to any of the noxious stimuli was considered a negative response. After testing at 3 isoflurane concentrations, anesthesia was discontinued, and mice were monitored until fully recovered.

In the statistical analysis of experiment 2, the isoflurane MAC value was determined for each anesthetic protocol by using both quantal analysis and a bracketing technique. For the quantal analysis, the percentage of mice that did not respond to the noxious stimulus at each isoflurane dosage was graphed and compared among isoflurane concentrations. These data were then fit to a sigmoidal curve by using logistical regression. The isoflurane percentage on this curve at which 50% of the mice were not responsive to the stimulus was determined to be the isoflurane MAC value for that anesthetic protocol. For the bracketing analysis, the transition point for each mouse was determined as the average of highest isoflurane percentage with a positive response to the noxious stimulus and the lowest isoflurane concentration with no response to the noxious stimulus. Because of the random isoflurane concentrations tested for each mouse, a mouse was not included in the bracketing analysis if all of its tests were either positive or negative. The average values were then compared by using one-way ANOVA, with drug protocol as the main effect. When significant differences were detected, Tukey posthoc analysis was performed.

Experiment 3: 60-min trials. Individual mice were randomly assigned to experimental groups and injected intraperitoneally with ketamine (n = 6; male mice, 3; female mice, 3), xylazine (n = 6; male mice, 3; female mice, 3), or LRS (n = 4; male mice, 2; female mice, 2). Based on the results of experiment 1, mice in the xylazine and control groups were injected 5 min before anesthetic induction, whereas mice in the ketamine group were injected immediately after induction. Mice in all groups were induced with 4.0% isoflurane and 100% oxygen until LORR. For experiments 3 and 4, the maintenance isoflurane percentage for each group was determined based on the results of experiment 2; mice were maintained at 0.2% isoflurane above the first percentage at which 100% of the mice had lost the response to the noxious stimulus in experiment 2. This isoflurane concentration was expected to reliably keep essentially 100% of C57BL/6J mice at an anesthetic plane in which they would not respond to a noxious stimulus. Therefore, experiments 3 and 4 were conducted by using 2.2% isoflurane for control mice, 1.6% isoflurane after xylazine premedication, and 1.3% isoflurane after ketamine premedication. Due to the different isoflurane concentrations for each group, the scorer was aware of the experimental group. Vital parameters (HR, RR, SpO_2, temperature) and depth of anesthesia were recorded every 5 min. After 60 min, isoflurane anesthesia was discontinued, and mice were monitored until fully recovered.

Statistical analysis in experiment 3 used a block design for HR, RR, and SpO_2, with sex as the blocking factor. The effect of sex on each of the 3 dependent variables was measured by using a t test; 2-way, repeated-measures ANOVA, with time and anesthetic protocol as main effects for the 3 dependent variables. When significant differences were detected, Tukey posthoc analysis was performed.

Experiment 4: Pathologic conditions under anesthesia. Mice were injected intraperitoneally with ketamine (n = 4; male mice, 2; female mice, 2), xylazine (n = 4; male mice, 2; female mice, 2), or LRS (n = 4; male mice, 2; female mice, 2), with mice in the xylazine and control groups injected 5 min before anesthetic induction and those in the ketamine group immediately after induction. Mice were induced with 4.0% isoflurane in 21% oxygen in an anesthetic induction chamber until LORR. Mice were moved to the nose cone and instrumented as described for previous experiments. In addition, in experiment 4, a continuous electrocardiogram monitoring system (ECGenie and eMouse 11 Analysis Software, Mouse Specifics) was used to monitor for cardiac arrhythmias and HR variability. Due to technical difficulties, ECG was not recorded in 2 of the mice in the control group. HR variability was calculated as the standard deviation between differences in sequential heart beats for the complete set of ECG signals and was evaluated at 5 time points: 5 min after induction, just before the acute blood loss, just after acute blood loss, 5 min after blood loss, and just before discontinuing isoflurane.[11] The isoflurane concentration was set according to the results of experiment 2 (control, 2.2%; xylazine, 1.6%; ketamine, 1.3%) and delivered in 21% oxygen. As in experiment 3, due to the different isoflurane concentration for each group, the researcher was aware of the experimental group. Mice anesthetized with 21% oxygen as a carrier gas have been shown to be hypoxemic.[4] Mice were monitored every 5 min as described in the section *Maintenance and Monitoring of Anesthesia*. After 20 min, retroorbital bleeding was performed to collect approximately 10% to 15% of blood volume, with the goal of making mice hypovolemic in addition to hypoxemic. To ensure hemostasis, firm pressure was applied to the eye for approximately 10 s after blood collection. After 60 min of anesthesia, isoflurane was discontinued, and mice were monitored until recovered.

Data from experiment 4 were analyzed using a block design performed on HR, RR and SpO_2, with sex as the blocking factor. The effect of sex on each of the 3 dependent variables was measured by using a t test. Two-way, repeated-measures ANOVA was then performed, with time and anesthetic protocol as main effects for each dependent variable. In addition, 2-way repeated-measures ANOVA was performed to test the effects of time and anesthetic protocol on HR variability. When significant differences were detected, Tukey posthoc analysis was performed.

To compare the effect of using 21% or 100% oxygen as the carrier gas, 3-way ANOVA was performed on data from experiments 3 and 4 collected during the first 20 min of anesthesia for each anesthetic protocol. In the first 20 min—the period before blood loss—the only difference between experiments 3 and 4 was the use of 100% oxygen (experiment 3) compared with 21% oxygen (experiment 4) as the carrier gas for isoflurane delivery. The dependent variables analyzed were SpO_2, HR, and RR, and the main independent variables tested were anesthetic protocol, oxygen concentration, and time. When significant differences were detected, Tukey posthoc analysis was performed to identify specific differences.

Results

Experiment 1: Induction scoring. Sex did not have a significant effect on the stress of the mice during this experiment (P = 0.87). The sexes were then pooled for subsequent analysis. In contrast,

premedication significantly (P = 0.002) influenced induction scores (mean ±1 SD; control, 7.3 ± 1.5; ketamine, 9.0 ± 3.6; xylazine, 3.8 ± 1.2), where a lower score represented a less stressful induction. Posthoc analysis showed that scores of mice in the xylazine group were significantly lower during induction than were those of either the control (P = 0.033) or ketamine groups (P = 0.002) and that the scores of the ketamine and control groups were not significantly different. One mouse in the ketamine group died immediately after intraperitoneal injection of ketamine and was not included in this statistical analysis; necropsy revealed a mild amount of subcutaneous hemorrhage at the injection site, and histopathology showed no lesions. Further analysis revealed that the control mice had significantly lower stress scores before being moved to the induction chamber than did either of the other groups (control, 0.0 ± 0.0; ketamine, 3.0 ± 0.8; xylazine, 0.7 ± 0.4; P < 0.001 for both groups). After being moved to the induction chamber, xylazine-premedicated mice had lower scores than did either of the other 2 groups (control, 7.3 ± 1.5; ketamine, 6.0 ± 3.0; xylazine, 3.1 ± 1.0; P < 0.003 between xylazine and control groups; P = 0.036 between xylazine and ketamine groups), which did not differ significantly from each other. After intraperitoneal injection, control mice returned to normal behavior very rapidly, whereas some of the mice that received xylazine showed ataxia and those that received ketamine showed both ataxia and tremors. During anesthesia induction with isoflurane, scores of control and ketamine-treated mice were not different, but the 2 groups had qualitative differences. In particular, control mice displayed frantic behavior and ataxia, whereas most of the mice given ketamine had tremors and some ataxia. However, the responses of mice that received ketamine varied markedly during induction, with some showing sedation that appeared to lessen stress in the induction chamber. This sedation resulted in the mice becoming nonambulatory and showing minimal movement during induction. This variable response resulted in the high standard deviation in this group's scores, indicating an unpredictable response to ketamine. Mice that received xylazine were calm and heavily sedated while in the induction chamber and showed few signs of distress during induction. Representative videos of mice during induction with each of the 3 protocols are provided as supplemental material (Videos S1–S3).

Experiment 2: MAC determination. Based on quantal analysis, both ketamine and xylazine caused left-shifting of the isoflurane dose–response curve, indicating a reduction in MAC (Figure 2). The analysis found an isoflurane MAC of 0.96% for the ketamine group, 1.20% for the xylazine group, and 1.89% for the control group. The MAC value for the control group is consistent with previous studies using similar noxious stimuli in C57BL/6 mice.[4,23] The bracketing analysis yielded similar MAC values as the quantal analysis; 1.07% ± 0.20% isoflurane for the ketamine group, 1.32% ± 0.11% isoflurane for xylazine-treated mice, and 1.86% ± 0.05% for control animals. These values were all significantly different from each other (P < 0.001 between the control group and both the ketamine and xylazine groups; P = 0.025 between the ketamine and xylazine groups). The greater variability of the transition point when using the injectable products is consistent with the steep dose–response curve associated with inhalant anesthesia.[8]

Experiment 3: 60-min trials. All mice survived 60 min of anesthesia and remained at a surgical plane of anesthesia for the duration of the experiment. The data analysis showed that sex was not a significant factor in the HR, RR, and SpO_2 associated with each protocol (Figure 3). In particular, both time and drug protocol significantly affected HR (P < 0.001 for both independent variables), such that the control mice had significantly higher HR at all time points as compared with mice that received ketamine or xylazine. In addition, for almost all time points after 15 min, HR was significantly higher in ketamine-treated mice than in the mice receiving xylazine. Finally, the HR of the control mice increased gradually over the course of the experiment, with the HR at first 2 time points being significantly lower than those at the last 2 time points.

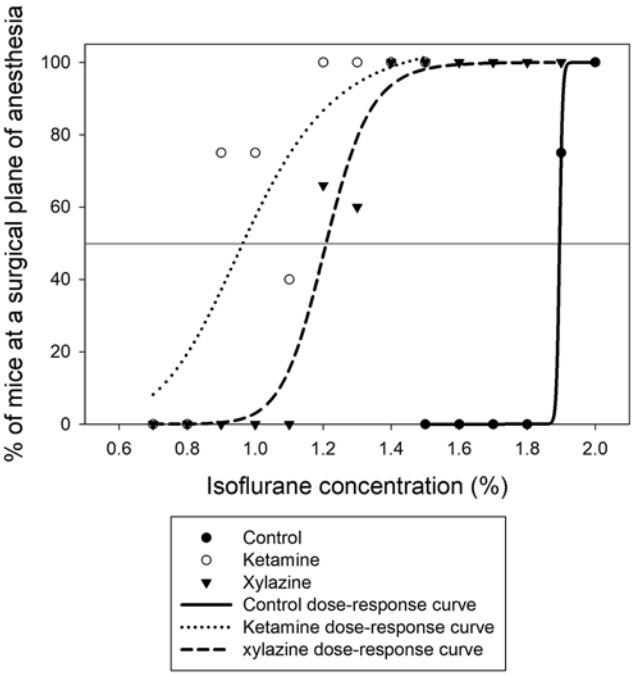

Figure 2. Quantal analysis of xylazine, ketamine, and control groups (data pooled for male and female mice). The horizontal black line bisecting the graph is the 50% line: the isoflurane concentration at which 50% of the mice were not responsive to the noxious stimulus (i.e., the minimum alveolar concentration, MAC). The results show left-shifting of the curves (i.e., decreased isoflurane MAC) for mice that received xylazine or ketamine.

Both time and drug protocol had a significant effect on RR (P < 0.001 for both independent variables). The control mice had the lowest RR at all time points, and RR was lower in mice given xylazine than in those given ketamine at all time points except 35 min. RR in the control mice fell gradually throughout the experiment, achieving statistical significance between the 5-min and the 55- and 60-min time points. Mice given ketamine had no significant changes in RR over time, whereas the xylazine group showed a significant difference only between the 15- and 35-min time points. No significant differences were detected in SpO_2, and none of the mice became hypoxic.

Experiment 4: Physiologic responses to an anesthetic challenge. All mice in all 3 groups survived 60 min of anesthesia. Sex had a significant effect on SpO_2 but not on HR or RR. Posthoc analysis showed that the sex effect was due to the mice given xylazine (P < 0.001) rather than the control and ketamine groups. In the xylazine-treated group, the SpO_2 was 70.5% ± 4.9% in male mice and 79.2% ± 5.0% in female mice.

The data analysis focused on changes in the dependent variables between 20 min and subsequent measurements, because this interval covered the period from immediately before to just after the induced blood loss. Both time (P = 0.006) and drug protocol (P < 0.001) had a significant effect on HR. The HR of the mice given ketamine fell significantly at all time points after blood loss (Figure 4), compared with no significant changes in

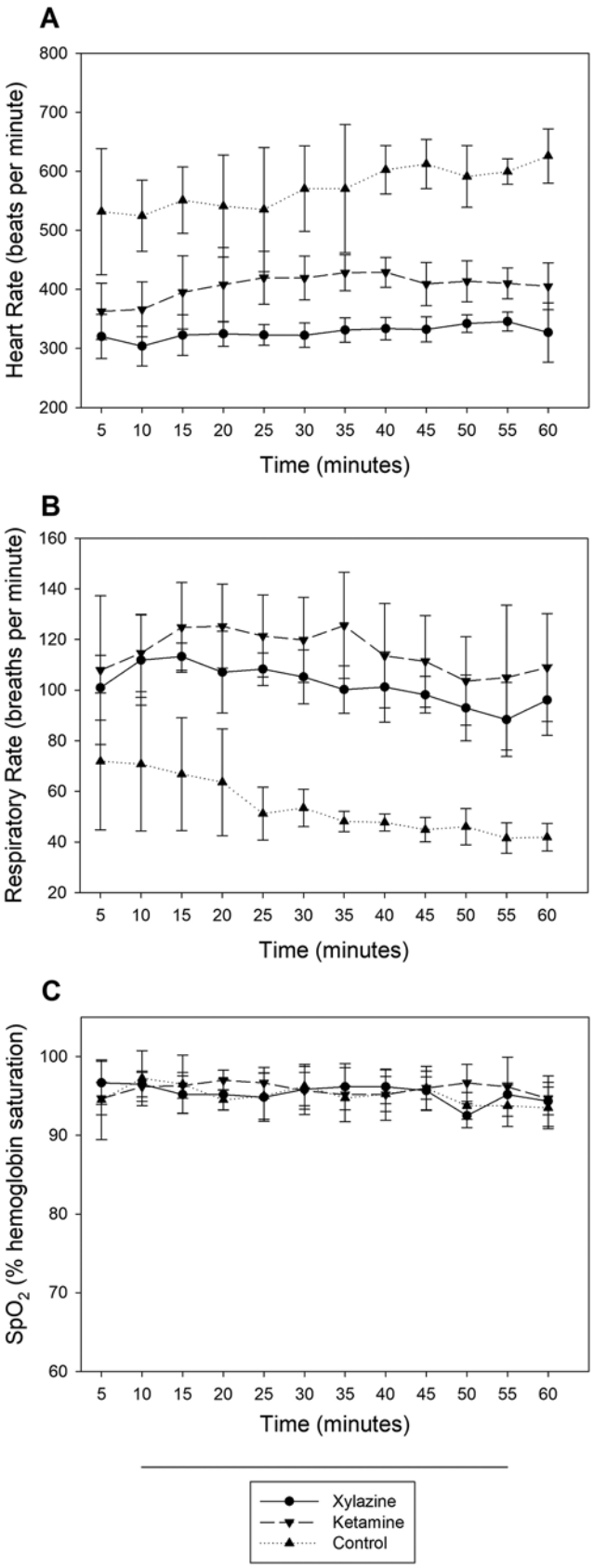

Figure 3. Experiment 3, which used 100% oxygen as the carrier gas. (A) Effect of each anesthetic combination on heart rate over time. (B) Effect of each anesthetic combination on respiratory rate over time. (C) Effect of each anesthetic combination on SpO_2 over time. T = 0 min is the initiation of anesthesia.

the HR of the control or xylazine groups. Even in the context of hypoxia and hypovolemia, the pattern of HR was the same as for experiment 3, with control mice having the highest HR, followed by the mice given ketamine and then those treated with xylazine. In addition, the HR of the control mice gradually increased over the 60 min of anesthesia, as in experiment 3.

Both time ($P < 0.001$) and drug protocol ($P < 0.001$) significantly affected RR, with ketamine and xylazine groups showing significantly lower RR at the 25- and 30-min time points compared with the 20-min time point (before blood loss; Figure 3). The RR in the control group gradually fell over the course of the experiment, similar to the pattern in experiment 3.

All the mice were clinically hypoxic during the 60 min of anesthesia when 21% oxygen was used as the carrier gas (experiment 4). Time had a significant effect on the SpO_2 of the mice ($P = 0.007$), and due to the profound hypoxia seen in the male mice, the xylazine-treated mice tended ($P = 0.057$) toward a lower SpO_2 than those of the other 2 groups. No significant changes were detected in SpO_2 within any of the drug protocols around the time of blood loss.

Neither time nor drug protocol had a significant effect on HR variability. Analysis of the ECG recordings revealed that one of the control mice had 2 premature ventricular contractions (Figure 5A). In addition, mice had infrequent episodes of atrial premature contractions (Figure 5B) under all 3 protocols.

The effect of hypoxia on autonomic functions was analyzed using data from the first 20 min of experiments 3 and 4, before blood collection, when the only difference between the experiments was the carrier gas—either 100% or 21% oxygen. As expected, the mice that received 100% oxygen as the carrier gas had significantly ($P < 0.001$) higher SpO_2 than did the mice given 21% oxygen (Figures 3 and 4). In addition, no difference in SpO_2 was detected among groups when mice received 100% oxygen, but when 21% oxygen was used, SpO_2 was lower in the mice given xylazine as compared with the other 2 protocols. Similarly, both oxygen level ($P < 0.001$ for both) and anesthetic protocol significantly affected HR and RR ($P < 0.001$ and $P = 0.005$, respectively). As compared with 100% oxygen, 21% oxygen yielded higher HR in all 3 anesthetic protocols, achieving statistical significance for the mice receiving ketamine. Mice that received 21% oxygen had significantly higher RR than did those receiving 100% oxygen for both the control and ketamine groups, with no significant difference between the 2 in the mice given xylazine. Time had a significant effect on RR ($P = 0.022$) but not on SpO_2 ($P = 0.356$) or HR ($P = 0.878$).

Discussion

Due to the ability of balanced anesthesia to facilitate smooth induction and recovery and to reduce the dose-dependent adverse effects of the component drugs, this anesthetic technique is widely used in human and veterinary medicine. The hypothesis of the current study was that premedication with ketamine or xylazine prior to isoflurane anesthesia would produce similar benefits in mice. We found that the use of xylazine minimized the stress of induction, and both ketamine and xylazine safely reduced the amount of isoflurane required to maintain a surgical plane of anesthesia in mice. Furthermore, when mice experienced induced hypoxia and hypovolemia under anesthesia, all mice survived these challenges. Both of the tested balanced anesthetic protocols resulted in surprising changes at the time of induced blood loss, including a decrease in HR despite the acute hypovolemia.

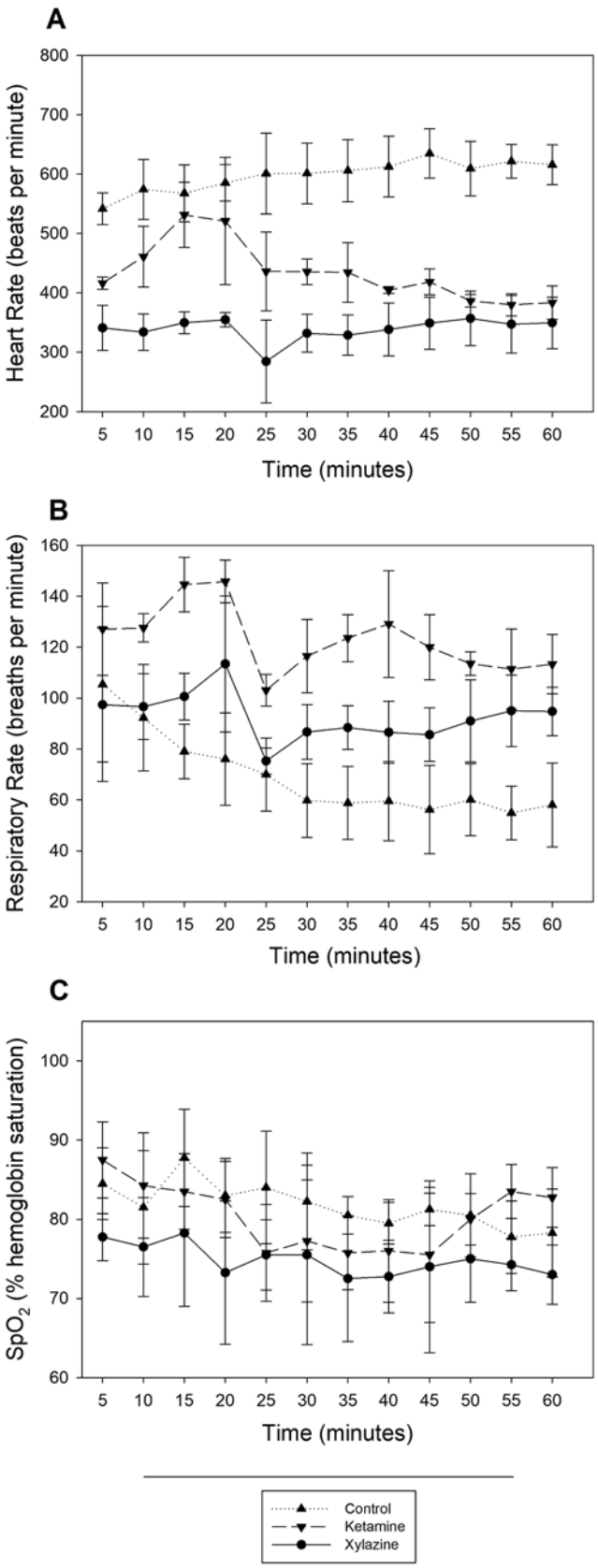

Figure 4. Experiment 4, which used compressed air containing 21% oxygen as the carrier gas. (A) Effect of each anesthetic combination on heart rate over time. (B) Effect of each anesthetic combination on respiratory rate over time. (C) Effect of each anesthetic combination on SpO_2 over time. T = 0 min is the initiation of anesthesia; blood collected at T = 20 min.

Isoflurane induction is not only distressful but is also potentially dangerous in some species, including guinea pigs and rabbits, which have been reported to breath-hold when induced with isoflurane.[14,15,17,38,39] In rabbits, this breath-holding is accompanied by bradycardia and leads to hypercapnia and acidosis.[14] In many species, premedication prior to gas induction provides a quicker induction and milder changes in vital parameters including HR, blood pressure, and RR.[33] Studies in mice and other species have shown that premedication can decrease the length of induction and reduce stressful behavior.[9,26,33]

Experiment 1 showed a significant reduction in the stress of anesthetic induction for mice premedicated with xylazine as compared with control mice. Whereas control mice displayed frantic behaviors and appeared distressed during induction, the mice premedicated with xylazine were calm, with no tremors or frantic behaviors noted. This response was consistent across all mice in the group, with little difference noted between individual mice. The response to premedication with ketamine was more variable than for both xylazine premedication and in the control group. One mouse died after ketamine administration, and 1 of the 4 surviving mice was profoundly sedated, whereas the other 3 mice behaved similar to control mice, showing twitching, tremors, and incoordination both before and during induction. These behaviors are consistent with a previous study that examined the effects of premedication with ketamine on sevoflurane induction in mice.[9] A second study evaluating ketamine premedication prior to sevoflurane anesthesia reported a variable response to ketamine injection, with some mice displaying tremors and ataxia, similar to the observations in the present study.[26] The variability in the response to ketamine premedication not only complicates establishing recommendations for anesthetic regimens but also has the potential to confound experimental results. Intraperitoneal injection clearly is stressful to mice.[13,30] One relevant study found that intraperitoneal injection can increase plasma concentrations of corticosterone and exacerbate the glucocorticoid response in mice, and another showed that mice given intraperitoneal injections show a higher HR than those that receive intramuscular or subcutaneous injections.[13,30] When evaluating the benefit of administering a premedication prior to induction, the benefits of drug administration must be sufficient to justify the stress associated with the injection.

In view of the variability in response and the abnormal behavior of mice given ketamine during induction, we do not recommend this premedication for mice. If ketamine is used in a balanced anesthetic protocol with isoflurane, it should be given after isoflurane induction. Experiment 1 showed that premedication with xylazine at the dose tested safely and reliably reduces the distress associated with inhalant anesthetic induction. Future studies to examine the effect of a ketamine–xylazine combination on induction would be worthwhile, because the muscle relaxation properties of xylazine would potentially reduce the severity of the tremors and ataxia that we observed in ketamine-treated mice.[25,40]

Quantal and bracketing analysis demonstrated that premedication with xylazine or ketamine reduces the MAC of isoflurane. The MAC-reducing effect of ketamine and α2-adrenergic agonists on inhalant anesthetics is consistent with previous studies in dogs.[18,41] However, not all studies have shown a MAC-reducing effect of ketamine in mice, including one that showed no effect of ketamine on the MAC of sevoflurane; in addition, some mice in that study showed apnea, tachypnea, and cardiac arrhythmias, and 2 mice in the ketamine group died.[9]

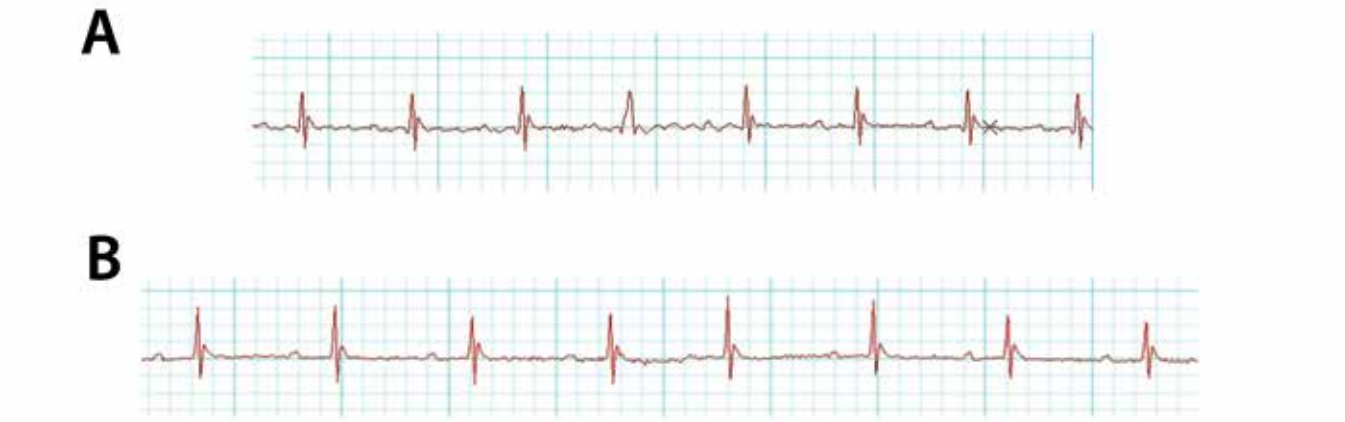

Figure 5. (A) ECG recording from a control mouse showing a premature ventricular contraction. Note the changes in the conformation of the 4th contraction compared with the other, normal contractions. (B) ECG recording showing atrial premature contractions. Note the decreased interval between the 4th and 5th complexes. Mice in all 3 groups had atrial premature contractions noted on ECG.

Similarly, in our present study, one mouse died immediately after intraperitoneal injection of ketamine, whereas none of the mice in the xylazine group died, thus supporting a better safety profile for xylazine than ketamine at the doses tested. No significant gross or histologic abnormalities were noted on examination of the mouse that died after receiving ketamine.

In experiments 3 and 4, all of the mice survived 60 min of anesthesia and maintained a surgical plane of anesthesia. Isoflurane alone has been the 'gold standard' for mouse anesthesia for approximately 2 decades.[17] The results of the current study show that the addition of an injectable anesthetic to inhalant protocols can not only reduce the stress of isoflurane anesthesia and decrease the MAC of isoflurane but also safely prolong the surgical plane of anesthesia at lower isoflurane concentrations, even after the injectable drugs likely have been metabolized. In our study, all mice remained at a surgical plane of anesthesia for 60 min without the need for additional doses of the injectable drug or increased isoflurane administration. This prolonged efficacy was particularly apparent for mice in the ketamine group, given that the half-life of ketamine in mice has been reported as 13 min , whereas the half-life of xylazine is approximately 1 to 2 h.[20,29,48] The concept of neural inertia, which is defined as the brain's resistance to transition between arousal states, perhaps contributes to our observations regarding the prolonged duration of a surgical plane of anesthesia despite relatively short half-lives of the individual drugs.[16] Plasma concentration of either xylazine or ketamine at 60-min time point, although not measured in the current study, combined with isoflurane at the concentration delivered in this experiment would likely not result in a surgical plane of anesthesia in a mouse; however, if a mouse was already at a surgical plane, this anesthetic combination would probably maintain the surgical plane. In the current study, the anesthetic protocols were equalized using MAC testing to determine the lowest anesthetic concentration that would achieve a surgical plane of anesthesia in essentially 100% of the mice in each protocol; consequently, anesthetic depths were similar in all 3 protocols.

The anesthetic challenge that we developed for experiment 4 evaluated the ability of mice to survive acute pathologic conditions that are common during anesthesia. The goal of this test was to assess whether the evaluated anesthetic protocols are safe for procedures with a risk of hypovolemia, hypoxia, or both—for example, surgery with a significant risk of hemorrhage. Pulse oximetry data confirmed that all mice were hypoxic when isoflurane was delivered in 21% oxygen prior to blood loss, consistent with previous studies demonstrating that mice become hypoxic under anesthesia when not supplied with 100% oxygen.[4] Our group has previously shown that even in healthy mice, the acute loss of 10% to 15% of the blood volume significantly alters cardiovascular parameters.[28]

Despite the combined hypoxia and hypovolemia, all mice in experiment 4 survived 60 min of anesthesia, thus indicating the safety of isoflurane, the anesthetic common to all 3 protocols, in mice. This safety is related to the steep dose–response curve of the inhalant anesthetics, such that a single concentration of isoflurane keeps mice at a surgical plane of anesthesia without causing a deeper, unsafe plane, leading to potentially blunted autonomic reflexes or even death.[8,43] The addition of injectable anesthetics, which typically have more shallow dose–response curves than inhalant agents, did not impair the ability of the mice to survive the pathologic challenge.

Monitoring basic vital parameters during anesthesia can be particularly difficult in mice, given the need for specialized equipment and the fact that a single person is often responsible for both surgery and anesthesia. Despite these challenges, when evaluating novel anesthetic protocols, the vital parameters during anesthesia should be monitored and reported. This information prompts researchers to monitor vital parameters when using these protocols by providing reference points that indicate abnormalities and the need to intervene before clinical complications arise.

In experiment 3, HR differed significantly among the 3 groups, with the control mice having the highest HR, followed by the ketamine-treated mice and then the xylazine group. The high HR in the control group, which received the highest concentration of isoflurane, gradually increased over the course of the anesthesia. Isoflurane causes dose–dependent hypotension;[17] thus, the high HR in the control group may be a compensatory response to this hypotension, such that HR rose due to declining blood pressure over the 60 min of anesthesia. One of the most important benefits of balanced anesthesia is that it allows the use of lower doses of the component drugs, consequently reducing adverse side effects. For example, ketamine administration improved ventilation, oxygenation, and hemodynamics in isoflurane-anesthetized dogs.[6] Our group has shown that ketamine causes a small decrease in HR in mice, thus contrasting with most other species, in which ketamine causes elevations in HR, cardiac output, and blood pressure due to increased sympathetic tone.[20,36] The ability of ketamine to preserve blood pressure and lower the amount of isoflurane

required to maintain an equivalent plane of anesthesia may have contributed to the lower HR in the ketamine group compared with the control mice; however, we cannot confirm this hypothesis because we did not measure blood pressure in the current study. Lastly, α2-adrenergic agonists cause profound bradycardia due to both central effects and influences on the peripheral blood pressure, thereby explaining the decreased HR in the xylazine-treated mice.[40]

The hypothesis of experiment 4 was that all mice would retain enough autonomic nervous function to permit increased HR to help compensate for the hypoxia before acute blood loss: this hypothesis was correct. For all 3 protocols, mice had significantly higher HR during the first 20 min of experiment 4 (i.e., before the induced blood loss) than in the first 20 min of experiment 3. This outcome is strong evidence that the mice were at the desired plane of anesthesia: deep enough to prevent a motor response to the noxious stimulus but with sufficient autonomic function to compensate for physiologic challenges.

That said, an unexpected decrease in HR was associated with acute blood loss in experiment 4. We had hypothesized that blood loss would cause a compensatory increase in HR in all 3 protocols due the induced hypovolemia. However, the mice in the xylazine and ketamine groups both showed reductions in HR after the induced blood loss. A potential explanation for this is the oculocardiac reflex, which causes a reduction in HR secondary to direct pressure placed on the eye. Immediately after blood collection via retroorbital bleeding, firm pressure was applied to the globe for approximately 10 s. Recent studies in humans have shown that premedication with the α2 adrenergic agonist dexmedetomidine enhanced the bradycardia associated with the oculocardiac reflex, whereas deeper inhalant anesthetics blocked the oculocardiac reflex.[1,2,5] Further investigation into the oculocardiac reflex in mice is needed to determine whether this reflex might explain the decrease in HR after retroorbital bleeding in these groups.

RR can be monitored in anesthetized mice without expensive equipment. Because anesthesia impairs respiratory function, this parameter is an important component of anesthesia management. Furthermore, changes in respiratory rate can predict anesthetic complications in some situations.[20,31] Anesthetic-related respiratory depression describes the inability of the body to detect or respond to abnormal blood gas concentrations. Although anesthesia is known to cause respiratory depression, we could not study this condition in experiment 3 with our available monitoring measures. When provided with 100% oxygen, mice maintained normal SpO_2; we did not measure either end-tidal CO_2 concentrations or the blood acid–base status. In experiment 4, in which isoflurane was delivered in 21% oxygen, all 3 anesthetic protocols caused respiratory depression—that is, the SpO_2 was between 70% and 85% for substantial time—demonstrating that the anesthetized mice could not compensate for the low oxygen concentrations. This finding demonstrates that providing 100% oxygen as the carrier gas for isoflurane can help to raise peripheral oxygen saturation in mice. The SpO_2 was lower in mice that received xylazine as compared with the other protocols. Arterial hypoxia has been attributed to the use of α2-adrenergic agonists in ruminants, but this effect is typically considered to be species-specific and has not previously been confirmed in mice.[22] A potentially confounding factor in the analysis of SpO_2 is that vasoactive drugs affect the accuracy of pulse oximeters.[34] Xylazine causes α2-adrenoceptor–mediated vasoconstriction, that together with the low concentration of delivered oxygen, could explain the lower pulse oximetry readings in these mice.[44] In addition, the pulse oximeter that we used works by passing 2 wavelengths of low-intensity light, one red and one infrared, through tissue to a photodetector; its outputs can be affected by sensor placement and by tissue pigmentation and thickness. In future studies, arterial PaO_2 values could be confirmed through blood gas analysis. However, due to their small physical size and correspondingly small blood volume, blood gas analysis is a far more invasive procedure in mice than larger in animals.

RR in mice in the control and ketamine protocols were higher when mice received 21% oxygen as compared with 100% oxygen. In contrast, RR of xylazine-treated mice did not differ between the 2 oxygen concentrations, likely contributing to the lower SpO_2 values in the 21% oxygen mice. However, the higher RR did not maintain normal oxygenation in the control and ketamine groups although the SpO_2 concentrations were above those of the mice receiving xylazine. Further analysis revealed that the difference in SpO_2 between protocols was due to a sex-associated difference in response to the xylazine–isoflurane combination, with male mice having a significantly lower SpO_2 than their female counterparts on other protocols. The mice on the other 2 protocols showed no significant sex-associated differences in SpO_2 responses. Sex-associated differences have not previously been reported in response to xylazine anesthesia in mice. Ultimately, determining which physiologic values of isoflurane-anesthetized mice are predictive of death would be valuable but a considerable amount of effort would be necessary to accumulate enough data to be predictive. In addition, tidal volume, end-tidal CO_2 measurements, and arterial blood gas values would be useful to further interpret the data but can be technically challenging to obtain in mice and impractical for researchers in most settings.

All 3 anesthetic protocols studied were safe under the normal and pathologic conditions we tested and all 3 maintained mice at a surgical plane of anesthesia (no motor response to a noxious stimulus during stable physiologic parameters) for 60 min. Premedication with either ketamine or xylazine reduced the MAC of isoflurane. Xylazine facilitated a smooth induction, without the tremors and ataxia noted in the control and ketamine groups. Compared with ketamine, xylazine has additional benefits, including the fact that xylazine is reversible and is not a controlled substance. The findings in experiment 4 demonstrate that although xylazine showed greater blunting of the respiratory response to hypoxia, mice in all groups survived when challenged with hypoxia and hypovolemia. The results of this study support the use of xylazine as a premedication for the induction and maintenance of isoflurane anesthesia in healthy C57BL/6J mice when 100% oxygen is provided and anticipated blood loss is minimal.

Supplementary Materials

Video S1. Mouse after control injection.
Video S2. Mouse induction after ketamine injection.
Video S3. Mouse induction after xylazine injection.

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References

1. **Arnold RW, Biggs RE, Beerle BJ.** 2018. Intravenous dexmedetomidine augments the oculocardiac reflex. J AAPOS **22:**211–213. https://doi.org/10.1016/j.jaapos.2018.01.016.

2. **Arnold RW, Bond AN, McCall M, Lunoe L.** 2019. The oculocardiac reflex and depth of anesthesia measured by brain wave. BMC Anesthesiol **19:**36. https://doi.org/10.1186/s12871-019-0712-z.
3. **Arras M, Autenried P, Rettich A, Spaeni D, Rülicke T.** 2001. Optimization of intraperitoneal injection anesthesia in mice: drugs, dosages, adverse effects, and anesthesia depth. Comp Med **51:**443–456.
4. **Blevins CE, Celeste NA, Marx JO.** 2021. Effects of oxygen supplementation on injectable and inhalant anesthesia in C57BL/6 mice. J Am Assoc Lab Anim Sci **60:**289–297. https://doi.org/10.30802/AALAS-JAALAS-20-000143.
5. **Bond AN, Beerle BJ, Arnold RW.** 2016. Dexmedetomidine nasal sedation produces more oculocardiac reflex during strabismus surgery. J Pediatr Ophthalmol Strabismus **53:**318. https://doi.org/10.3928/01913913-20160629-05.
6. **Boscan P, Pypendop BH, Solano AM, Ilkiw JE.** 2005. Cardiovascular and respiratory effects of ketamine infusions in isoflurane-anesthetized dogs before and during noxious stimulation. Am J Vet Res **66:**2122–2129. https://doi.org/10.2460/ajvr.2005.66.2122.
7. **Buitrago S, Martin TE, Tetens-Woodring J, Belicha-Villanueva A, Wilding GE.** 2008. Safety and efficacy of various combinations of injectable anesthetics in BALB/c mice. J Am Assoc Lab Anim Sci **47:**11–17.
8. **Campagna JA, Miller KW, Forman SA.** 2003. Mechanisms of actions of inhaled anesthetics. N Engl J Med **348:**2110–2124. https://doi.org/10.1056/NEJMra021261.
9. **Cesarovic N, Jirkof P, Rettich A, Nicholls F, Arras M.** 2012. Combining sevoflurane anesthesia with fentanyl–midazolam or s-ketamine in laboratory mice. J Am Assoc Lab Anim Sci **51:**209–218.
10. **Cesarovic N, Nicholls F, Rettich A, Kronen P, Hässig M, Jirkof P, Arras M.** 2010. Isoflurane and sevoflurane provide equally effective anaesthesia in laboratory mice. Lab Anim **44:**329–336. https://doi.org/10.1258/la.2010.009085.
11. **Criado AB, de Segura IAG, Tendillo F, Marsico F.** 2000. Reduction of isoflurane MAC with buprenorphine and morphine in rats. Lab Anim **34:**252–259. https://doi.org/10.1258/002367700780384717.
12. **Criado AB, De Segura IAG.** 2003. Reduction of isoflurane MAC by fentanyl or remifentanil in rats. Vet Anaesth Analg **30:**250–256. https://doi.org/10.1046/j.1467-2995.2003.00123.x.
13. **Drude S, Geissler A, Olfe J, Starke A, Domanska G, Schuett C, Kiank-Nussbaum C.** 2011. Side effects of control treatment can conceal experimental data when studying stress responses to injection and psychological stress in mice. Lab Anim (NY) **40:**119–128. https://doi.org/10.1038/laban0411-119.
14. **Flecknell PA, Cruz I, Liles J, Whelan G.** 1996. Induction of anaesthesia with halothane and isoflurane in the rabbit: a comparison of the use of a face-mask or an anaesthetic chamber. Lab Anim **30:**67–74. https://doi.org/10.1258/002367796780744910.
15. **Flecknell PA, Roughan J, Hedenqvist P.** 1999. Induction of anaesthesia with sevoflurane and isoflurane in the rabbit. Lab Anim **33:**41–46. https://doi.org/10.1258/002367799780578516.
16. **Friedman EB, Sun Y, Moore JT, Hung H-T, Meng QC, Perera P, Joiner WJ, Thomas SA, Eckenhoff RG, Sehgal A.** 2010. A conserved behavioral state barrier impedes transitions between anesthetic-induced unconsciousness and wakefulness: evidence for neural inertia. PLoS One **5:**e11903. https://doi.org/10.1371/journal.pone.0011903.
17. **Gaertner D, Hallman T, Hankenson F, Batchelder M.** 2008. P 239-297.–Anesthesia and Analgesia for Laboratory Rodents. In: Fish RE, Brown MJ, Danneman PJ, Karas AZ, editors. Anesthesia and Analgesia in Laboratory Animals (Second Edition). San Diego (CA): Academic Press
18. **Gutierrez-Blanco E, Victoria-Mora JM, Ibancovichi-Camarillo JA, Sauri-Arceo CH, Bolio-González ME, Acevedo-Arcique CM, Marin-Cano G, Steagall PV.** 2013. Evaluation of the isoflurane-sparing effects of fentanyl, lidocaine, ketamine, dexmedetomidine, or the combination lidocaine-ketamine-dexmedetomidine during ovariohysterectomy in dogs. Vet Anaesth Analg **40:**599–609. https://doi.org/10.1111/vaa.12079.
19. **Ilkiw JE, Pascoe P, Tripp L.** 2002. Effects of morphine, butorphanol, buprenorphine, and U50488H on the minimum alveolar concentration of isoflurane in cats. Am J Vet Res **63:**1198–1202. https://doi.org/10.2460/ajvr.2002.63.1198.
20. **Jaber SM, Hankenson FC, Heng K, McKinstry-Wu A, Kelz MB, Marx JO.** 2014. Dose regimens, variability, and complications associated with using repeat-bolus dosing to extend a surgical plane of anesthesia in laboratory mice. J Am Assoc Lab Anim Sci **53:**684–691.
21. **Janssen BJ, De Celle T, Debets JJ, Brouns AE, Callahan MF, Smith TL.** 2004. Effects of anesthetics on systemic hemodynamics in mice. Am J Physiol Heart Circ Physiol **287:**H1618–H1624. https://doi.org/10.1152/ajpheart.01192.2003.
22. **Kästner SB.** 2006. A2-agonists in sheep: a review. Vet Anaesth Analg **33:**79–96. https://doi.org/10.1111/j.1467-2995.2005.00243.x.
23. **LaTourette PC, David EM, Pacharinsak C, Jampachaisri K, Smith JC, Marx JO.** 2020. Effects of Standard and Sustained-release Buprenorphine on the Minimum Alveolar Concentration of Isoflurane in C57BL/6 Mice. J Am Assoc Lab Anim Sci **59:**298–304. https://doi.org/10.30802/AALAS-JAALAS-19-000106.
24. **Leach MC, Bowell V, Allan T, Morton D.** 2002. Degrees of aversion shown by rats and mice to different concentrations of inhalational anaesthetics. Vet Rec **150:**808–815. https://doi.org/10.1136/vr.150.26.808.
25. **Leece EA.** 2009. Lumb & Jones' Veterinary Anesthesia and Analgesia. Vet Anaesth Analg **36:**98. https://doi.org/10.1111/j.1467-2995.2008.00425.x.
26. **Lipiski M, Arras M, Jirkof P, Cesarovic N.** 2017. Premedication with fentanyl-midazolam improves sevoflurane anesthesia for surgical intervention in laboratory mice. Exp Biol Med (Maywood) **242:**1287–1298. https://doi.org/10.1177/1535370217707730.
27. **Makowska IJ, Weary DM.** 2009. Rat aversion to induction with inhalant anaesthetics. Appl Anim Behav Sci **119:**229–235. https://doi.org/10.1016/j.applanim.2009.04.003.
28. **Marx JO, Jensen JA, Seelye S, Walton RM, Hankenson FC.** 2015. The effects of acute blood loss for diagnostic bloodwork and fluid replacement in clinically ill mice. Comp Med **65:**202–216.
29. **Maxwell CR, Ehrlichman RS, Liang Y, Trief D, Kanes SJ, Karp J, Siegel SJ.** 2006. Ketamine produces lasting disruptions in encoding of sensory stimuli. J Pharmacol Exp Ther **316:**315–324. https://doi.org/10.1124/jpet.105.091199.
30. **Meijer MK, Spruijt B, Van Zutphen L, Baumans V.** 2006. Effect of restraint and injection methods on heart rate and body temperature in mice. Lab Anim **40:**382–391. https://doi.org/10.1258/002367706778476370.
31. **Miller RD, Eriksson LI, Fleisher LA, Wiener-Kronish JP, Cohen NH, Young WL.** 2014. Miller's anesthesia e-book. Elsevier Health Sciences.
32. **Mircioiu C, Atkinson J.** 2017. A comparison of parametric and non-parametric methods applied to a Likert scale. Pharmacy (Basel) **5:**26. https://doi.org/10.3390/pharmacy5020026.
33. **Mutoh T, Nishimura R, Sasaki N.** 2002. Effects of medetomidine-midazolam, midazolambutorphanol, or acepromazine-butorphanol as premedicants for mask induction of anesthesia with sevoflurane in dogs. Am J Vet Res **63:**1022–1028. https://doi.org/10.2460/ajvr.2002.63.1022.
34. **Nixdorff J, Zablotski Y, Hartmann K, Dörfelt R.** 2021. Comparison of Transmittance and Reflectance Pulse Oximetry in Anesthetized Dogs. Front Vet Sci **8:**643966. https://doi.org/10.3389/fvets.2021.643966.
35. **Norman G.** 2010. Likert scales, levels of measurement and the "laws" of statistics. Adv Health Sci Educ Theory Pract **15:**625–632. https://doi.org/10.1007/s10459-010-9222-y.
36. **Plumb DC.** 2018. Plumb's Veterinary Drug Handbook: Desk, 9th edition. Hoboken (NJ): John Wiley & Sons.
37. **Reiter CP, Christy AC, Olsen CH, Bentzel DE.** 2017. Effect of home cage bedding in the induction chamber on serum cortisol and corticosterone levels in response to isoflurane-induced anesthesia in C57BL/6J Mice. J Am Assoc Lab Anim Sci **56:**118–121.

38. **Ross MC, Zoeffel L, McMonagle J, McDonough J.** 2000. Isoflurane anesthesia for guinea pigs (Cavia porcella) in a stereotaxic surgical apparatus. Contemp Top Lab Anim Sci **39:**43–46.
39. **Schmitz S, Tacke S, Guth B, Henke J.** 2016. Comparison of physiological parameters and anaesthesia specific observations during isoflurane, ketamine-xylazine or medetomidine-midazolam-fentanyl anaesthesia in male guinea pigs. PLoS One **11:**e0161258. https://doi.org/10.1371/journal.pone.0161258.
40. **Sinclair MD.** 2003. A review of the physiological effects of α2-agonists related to the clinical use of medetomidine in small animal practice. Can Vet J **44:**885.
41. **Solano AM, Pypendop BH, Boscan PL, Ilkiw JE.** 2006. Effect of intravenous administration of ketamine on the minimum alveolar concentration of isoflurane in anesthetized dogs. Am J Vet Res **67:**21–25. https://doi.org/10.2460/ajvr.67.1.21.
42. **Steffey EP.** 2014. A history of veterinary anesthesia, p 293–302. In: Eger EI II, Saidman LJ, Westhorpe RN, editors. The Wondrous Story of Anesthesia. New York (NY): Springer.
43. **Sun Y, Chen J, Pruckmayr G, Baumgardner JE, Eckmann DM, Eckenhoff RG, Kelz MB.** 2006. High throughput modular chambers for rapid evaluation of anesthetic sensitivity. BMC Anesthesiol **6:**13. https://doi.org/10.1186/1471-2253-6-13.
44. **Talke P, Stapelfeldt C.** 2006. Effect of peripheral vasoconstriction on pulse oximetry. J Clin Monit Comput **20:**305–309. https://doi.org/10.1007/s10877-006-9022-3.
45. **Tao F, Skinner J, Yang Y, Johns RA.** 2010. Effect of PSD-95/SAP90 and/or PSD-93/chapsyn-110 deficiency on the minimum alveolar anesthetic concentration of halothane in mice. Anesthesiology **112:**1444–1451. https://doi.org/10.1097/ALN.0b013e3181dcd3dc.
46. **Tarnal V, Vlisides PE, Mashour GA.** 2016. The neurobiology of anesthetic emergence. J Neurosurg Anesthesiol **28:**250–255. https://doi.org/10.1097/ANA.0000000000000212.
47. **Tsukamoto A, Iimuro M, Sato R, Yamazaki J, Inomata T.** 2015. Effect of midazolam and butorphanol premedication on inhalant isoflurane anesthesia in mice. Exp Anim **64:**139–145. https://doi.org/10.1538/expanim.14-0073.
48. **Xu Q, Ming Z, Dart AM, Du XJ.** 2007. Optimizing dosage of ketamine and xylazine in murine echocardiography. Clin Exp Pharmacol Physiol **34:**499–507. https://doi.org/10.1111/j.1440-1681.2007.04601.x.