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Effects of Midazolam/Dexmedetomidine with Buprenorphine or Extended-release Buprenorphine Anesthesia in C57BL/6 Mice

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The effects of commonly used injectable combinations of anesthetics such as ketamine and xylazine, with or without acepromazine, vary widely across individuals, have a shallow-dose response curve, and do not provide long-term analgesia. These drawbacks indicate the importance of continuing efforts to develop safe and effective injectable anesthetic combinations for mice. In this study, a series of experiments was designed to validate the use of dexmedetomidine and midazolam to provide chemical restraint for nonpainful procedures and the addition of buprenorphine or extended-release buprenorphine to reliably provide a surgical plane of anesthesia in C57BL/6J mice. Loss of consciousness was defined as the loss of the righting reflex (LORR); a surgical plane of anesthesia was defined as the LORR and loss of pedal withdrawal after application of a 300 g noxious stimulus to a hind paw. The combination of intraperitoneal 0.25 mg/kg dexmedetomidine and 6 mg/kg midazolam produced LORR, sufficient for nonpainful or noninvasive procedures, without achieving a surgical plane in 19 of 20 mice tested. With the addition of subcutaneous 0.1 mg/kg buprenorphine or 1 mg/kg buprenorphine-ER, 29 of 30 mice achieved a surgical plane of anesthesia. The safety and efficacy of the regimen was then tested by successfully performing a laparotomy in 6 mice. No deaths occurred in any trial, and, when administered 1 mg/kg atipamezole IP, all mice recovered their righting reflex within 11 min. The anesthetic regimen developed in this study is safe, is reversible, and includes analgesics that previous studies have shown provide analgesia beyond the immediate postsurgical period. Buprenorphine-ER can be safely substituted for buprenorphine for longer-lasting analgesia.

Abbreviations and Acronyms: Bup, standard-formulation buprenorphine; BupER, extended-release buprenorphine; Dexmed, dexmedetomidine; HR, heart rate; LORR, loss of righting reflex; Midaz, midazolam; RR, respiratory rate; SpO_2, peripheral oxygen saturation; TT, Touch Test

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Introduction

Mouse anesthesia is a common practice in biomedical research that facilitates many experimental procedures and can be achieved by using inhalant or injectable anesthetics. The combination of the injectable drugs ketamine and xylazine, with or without acepromazine, was the most popular injectable anesthetic regimen at the turn of the century.[37] However, the response to the ketamine-xylazine-acepromazine combination is highly variable, with single doses producing results ranging from light sedation to death.[1,5,11,19] This wide range in response, as well as the minimal postoperative analgesia provided by this combination, supports the importance of continued efforts to validate safe and reliable options for injectable anesthesia in mice.

Researchers have evaluated a novel injectable anesthetic combination of medetomidine, midazolam, and butorphanol for use in mice.[24,28,29] The anesthesia resulting from this combination appears comparable to ketamine and xylazine anesthesia in male ICR mice[22] and reliably induces anesthesia that is suitable for a variety of noninvasive or minimally invasive procedures (e.g., electroretinograms and blood collection) in hamsters,[30] rabbits,[25] and inbred BALB/c and C57BL/6J mice.[24]

To identify an anesthetic combination which provides long-term analgesia for mice, we evaluated a combination of midazolam (Midaz), dexmedetomidine (Dexmed), and buprenorphine (Bup) or buprenorphine-ER LAB (BupER) under nonsurgical and surgical conditions. Midaz is an injectable benzodiazepine used in veterinary medicine primarily as a coinduction anesthetic agent. Dexmed is an α-2 adrenergic agonist used in animals for sedation and analgesia; it has greater specificity for the α-2 receptor than does xylazine. Bup is a synthetic opioid with potent analgesic efficacy via its partial mu agonist actions and weak antagonist effects on the κ receptor. It is a commonly used analgesic in mice and a more potent analgesic as compared with butorphanol, with a duration of action of 3 to 6 h.[13,15] BupER, a polymeric extended-release formulation of the synthetic opioid, maintains therapeutic plasma concentrations for up to 24 h.[13]

All of the anesthetic agents used in this study are reversible. Flumazenil can be used to antagonize the effects of Midaz,[32] Dexmed can be reversed with atipamezole,[6,17,18,40] and naloxone may reduce or reverse both the sedative and analgesic effects of Bup.[32] Because naloxone reverses the analgesic effects of the opiate, consideration should be given to maintaining ongoing analgesia if the Bup is reversed.

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A final benefit of the Midaz/Dexmed/Bup regimen is the lack of ketamine. Combinations that do not contain ketamine may be preferable for patients with high sympathetic tone, coronary artery disease, uncontrolled arterial hypertension, cardiomyopathy, heart failure, and dysfunction of catecholamine systems.[2,26,34] Ketamine also alters cerebral perfusion pressure, which may confound neurologic studies (e.g., studies of traumatic brain injury).[9,16]

We performed 4 experiments to test the ability of Midaz/Dexmed/Bup to provide a surgical plane of anesthesia to mice undergoing invasive procedures. We first tested 2 doses of the combination for their ability to safely and effectively achieve a surgical plane of anesthesia. We then tested the ability of Midaz/Dexmed to produce loss of consciousness while still retaining the pedal withdrawal reflex, indicating that the combination would be appropriate for nonpainful procedures. In the third experiment, we determined whether BupER could replace the shorter acting Bup formulation, the ability of atipamezole to reverse the anesthesia, and the effects of oxygen supplementation on the anesthetized mice. In the last experiment, we tested the anesthetic regimen under actual surgical conditions by performing a laparotomy.

The challenges of monitoring numerous physiologic values that are easily measured in larger species (e.g., blood pressure, ECG, end tidal CO_2) make accurate assessment of the depth of anesthesia challenging in mice. In the current study, our tests focused on the use of techniques that are commonly used in research that uses mice to provide relevant information that can help to guide the decision-making process during anesthesia. The physical responses of the LORR to assess loss of consciousness and lack of a motor response to a noxious stimulus are practical tests of whether a mouse is adequately anesthetized for various procedures.[7,14] The use of pulse oximetry to assess oxygenation and heart rate (HR) further refine these assessments to ensure that the mouse is not becoming too deeply anesthetized and is at risk of death.

The hypothesis of this study was that the combination of Midaz/Dexmed/Bup or BupER would provide adequate anesthesia for laparotomy without causing death and that anesthesia would be reliably reversed with atipamezole. We also hypothesized that the combination of Midaz/Dexmed would produce an anesthetic plane sufficient for nonpainful procedures, as indicated by LORR without the loss of pedal withdrawal.

Materials and Methods

Animals. Experiments were performed using adult (aged 8 to 16 wk) male (weight range, 22 to 33 g) and female (weight range, 17 to 24 g) C57BL/6 mice (*Mus musculus*, $n = 38$, Jackson Laboratories, Bar Harbor, ME). An additional 4 mice were used to train personnel on this project. Mice were housed in static polycarbonate microisolation cages with 75 square inches of floor space (Max 75, Alternative Design, Siloam Springs, AR). Cages contained corncob bedding (0.12-in. BedO-Cobs, The Andersons, Maumee, OH) and cotton squares (cotton squares, Ancare, Bellmore, NY). Mice were maintained under a 12:12 light/dark cycle with fluorescent lighting at 325 lx in an AAALAC accredited facility. Room temperature and humidity ranged from 21.1 to 23.3 °C and 30 to 70%, respectively. Mice were fed standard laboratory rodent chow (5001, LabDiet, St. Louis, MO) and provided with municipal water by bottle.

Sentinel mice that were exposed to dirty bedding were used for routine health monitoring. Mice were free of fur mites, pinworms, 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. All mice were acclimated for at least 7 d after arrival before being used in the study. Each mouse was anesthetized no more than twice with at least a 10-d washout period between anesthetic events. All experimental procedures were approved by the IACUC at the University of Pennsylvania.

Experimental design. This study consisted of 4 experiments. In the first experiment, we evaluated the combination of a low-dose of Midaz/Dexmed/Bup and a high-dose of Midaz/Dexmed/Bup to determine the dosages required to achieve a surgical plane of anesthesia. A surgical plane of anesthesia was defined as a LORR when placed into dorsal recumbency and loss of pedal withdrawal after application of a 300 g noxious stimulus to a hind paw. In the second experiment, a Midaz/Dexmed combination was tested to see whether loss of consciousness could be achieved without Bup. A loss of consciousness was defined as having the mouse placed on its back and to remain in that position while maintaining a withdrawal response to a noxious stimulus, indicating that the degree of sedation was sufficient for noninvasive procedures. The third experiment compared Bup and BupER to determine whether both groups would reach a surgical plane of anesthesia. In addition, atipamezole was administered to confirm the reversibility of the anesthetic combination while preserving the analgesic effects of the opiate. The fourth experiment tested the combination of Midaz/Dexmed/Bup under surgical conditions.

Drugs and dosing. Mice were weighed on a gram scale (Tanita Digital Scale, KD-160, Arlington Heights, IL) before dosing. Dexmed (Dexmedesed, Dechra, Overland Park, KS, 0.5 mg/mL) and Midaz (Midazolam Injection USP, Westward, Eatontown, NJ, 5 mg/mL) were diluted together with sterile saline to provide the desired concentration at 0.1 mL per 10 g of body weight. Drugs were diluted with 0.9% sodium chloride, used within 48 h, and dosed as described in each experimental section. The combination of Dexmed and Midaz was administered intraperitoneally as a single injection in the lower left or right quadrants of the abdomen using a 1-mL syringe and 25-gauge needle. Bup (0.1 mg/kg SC; Buprenex, C III, Patterson Vet Generics; 0.3 mg/mL diluted to 0.02 mg/mL with 0.9% NaCl) (experiments 1, 3, and 4) or BupER (1 mg/kg SC; Buprenorphine ER-LAB ZooPharm, Windsor, CO) (experiment 3) was administered subcutaneously between the scapulae using a 1-mL syringe and 25-gauge needle immediately after the intraperitoneal injection of Dexmed/Midaz. Bup and BupER were not combined with the intraperitoneal injection so that the each could be evaluated based on the current recommended doses. Because of differences in viscosity of the drugs and injection amounts, the experimenter could not be blind to the experimental group.

Anesthetic depth and physiologic monitoring. After induction of anesthesia, mice were monitored for LORR, which was defined as the mouse lying in dorsal recumbency, without returning to standing or sternal recumbency, and was interpreted as an estimate of the time until loss of consciousness.[14] The time of loss of consciousness (i.e., LORR) was defined as $T = 0$. Once this occurred, mice were transferred to a circulating warm water blanket (Stryker T/Pump, Kalamazoo, MI), and eye lubrication was applied (Akorn, Lake Forest, IL). Mice were instrumented with a rectal temperature probe (ThermoWorks, Alpine, UT), and body temperature was maintained between 35 to 37 °C by the use of the warm water blanket and a heat lamp. HR and peripheral oxygen saturation of hemoglobin (SpO_2) were measured using a mouse-specific pulse oximeter (MouseSTAT

Jr., Kent Scientific Corporation, Torrington, CT). Pulse oximetry measurements were taken from the left or right hind foot at each time point. A continuous electrocardiogram monitoring system (ECGenie and eMouse 11 Analysis Software, Mouse Specifics) was used to monitor HR and rhythm. Respiratory rate (RR) was measured by counting thoracic excursions. Supplemental 100% oxygen was provided via nose cone at 0.6 L/min for mice in experiments 2, 3, and 4. For all experiments, temperature, HR, RR, and SpO_2 were recorded every 5 min. In experiments 1, 2 and 3, the presence of the paw withdrawal reflex was recorded every 5 min for the duration of anesthesia until the return of spontaneous movement. In experiment 4, the paw withdrawal reflex was confirmed to be negative before making an initial incision, and mice were closely monitored for movement, changes in HR, or changes in SpO_2 for detection of signs of a response to the surgical stimulus. A brisk application of a 300-g noxious stimulus (Touch Test [TT]; North Coast Medical, Gilroy, CA) was applied to the hind foot, alternating the left and right foot at each 5-min time point to simulate a surgical stimulus. The 300-g TT device (Figure 1) was selected for its consistent deliverance of a full 300-g stimulus when applied at a 90-degree angle to the foot, as recommended by the manufacturer. This approach was used in lieu of toe pinching, which is prone to differences in user pressure. A positive response was characterized as any movement by the mouse in response to the noxious stimulus. The 300-g TT is a robust stimulus for a mouse, but past experiments[3,10,18,26] have not resulted in pain or lameness after recovery.[3,11,19,27] In experiment 1, pulse oximeter measurements were recorded immediately before the application of the noxious stimulus and for up to 10 s after the stimulus. The peak SpO_2 values were recorded before and after application of the TT. The return of the righting reflex was defined as the time when the mouse would immediately right itself from its back on 3 consecutive trials. The return of the righting reflex defined the return of consciousness, as the concentrations of injectable and inhalant anesthetics that cause LORR in mice are highly correlated in the concentrations at which humans lose consciousness.[14] After the return of the righting reflex, mice were monitored continuously until able to stand and fully ambulatory.

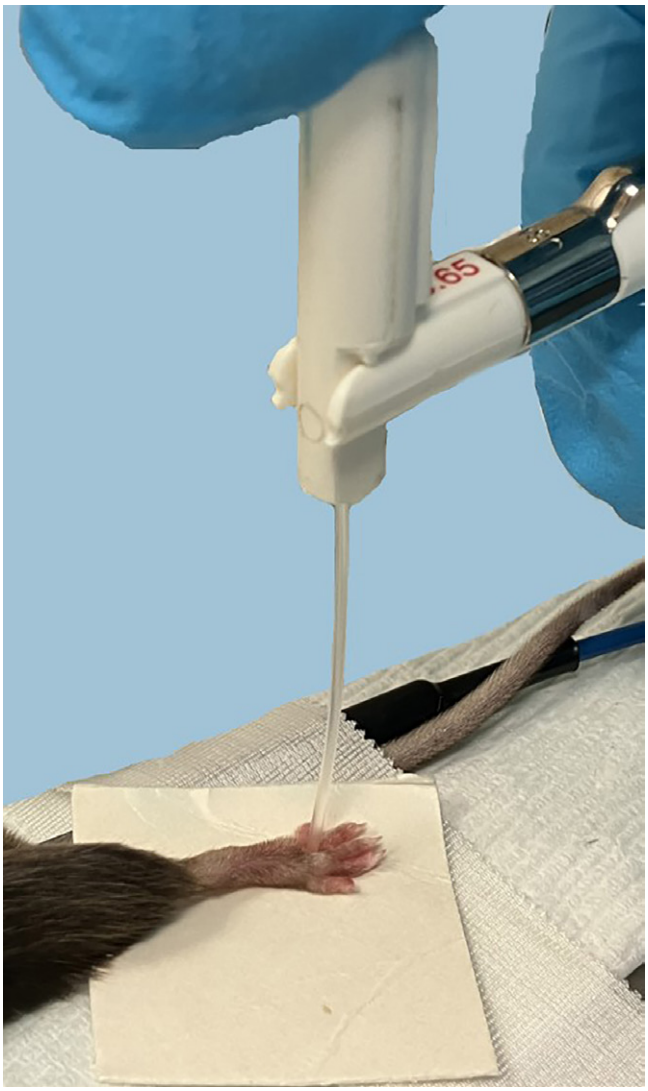

Figure 1. An anesthetized mouse during the application of the 300-g TT device noxious stimulus. The mouse had no motor response to the stimulus, which is considered a negative result.

Experiment 1: Establish dosing regimen to achieve a surgical plane of anesthesia. Mice (n = 8; male mice, 3; female mice, 5) were injected with 0.15 mg/kg Dexmed and 4 mg/kg Midaz IP and with 0.1 mg/kg Bup SC. The doses of Midaz and Dexmed were based on previous publications.[24,28,29] Bup dosing was based on current recommendations for the use of the drug for analgesia in mice.[13] Mice were induced and monitored as described above. Based upon the results of this experiment, a second trial of mice (n = 10; 5 males and 5 females) was injected with a higher dose consisting of 0.25 mg/kg Dexmed and 6 mg/kg Midaz IP, and 0.1 mg/kg Bup SC. The Bup dose was not altered so that the expected analgesia from the opiate would not change from previous work. For both cohorts, SpO_2 readings were obtained before and after the TT.

Sample size and statistical analysis. **Sample size.** The sample size was based on previous work[1] that reported an 85% success rate for a ketamine, xylazine, and acepromazine combination. We tested 10 mice in each dosing group, with an acceptable success rate of higher than the 85% standard achieved in the previous paper. If 15% of mice failed to achieve a surgical plane of anesthesia, no additional mice were tested with that combination. Ultimately, the high-dose combination proved effective at achieving a surgical plane of anesthesia in more than 85% of mice tested.

Analysis of HR, RR, and SpO_2. A block design was used for the statistical analysis 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 a t test. A 2-way, repeated measure ANOVA was then performed using time and anesthetic regimen as main effects for the 3 dependent variables. When significant differences were detected, a Tukey post hoc analysis was performed. A P value < 0.05 was considered statistically significant.

Responsiveness of autonomic nervous system. To test the responsiveness of the autonomic nervous system to the TT, a repeated measures ANOVA was performed comparing the SpO_2 before and after the TT.

Experiment 2: Evaluation of dexmedetomidine and midazolam for sedation for noninvasive procedures. This experiment was designed to identify a combination of Dexmed and Midaz that would result in LORR but would not eliminate the spinal reflexes, therefore not achieving a surgical plane of anesthesia. Mice (n = 10; male mice, 5; female mice, 5) were anesthetized twice with Dexmed (0.25 mg/kg) and Midaz (6 mg/kg) IP, receiving supplemental oxygen in one trial (100% oxygen at a flow rate of 0.6 L/min provided via a nose cone), and no supplemental oxygen in the other. The order of the 2 trials was randomized for each mouse, so half of the mice received oxygen

supplementation in the first trial and half received oxygen during the second trial. The mice were induced and monitored as described above.

Statistical analysis and sample size. The sample size was based on the same criteria as experiment 1.

Effects of oxygen and sex on the LORR A 2-way repeated measures ANOVA was performed to test the effects of the independent variables sex and oxygen supplementation on the length of the time necessary for LORR or to achieve a surgical plane of anesthesia.

Analysis of HR, RR, and SpO_2 and responsiveness of the autonomic nervous system. These analyses were performed the same as in experiment 1, with testing of the autonomic nervous system being done for both mice receiving oxygen supplementation and not receiving oxygen supplementation.

Experiment 3: Evaluation of buprenorphine and extended-release buprenorphine with reversal. In this experiment, mice (n = 10; 5 males and 5 females) received 0.25 mg/kg Dexmed and 6 mg/kg Midaz, and then were randomly assigned to receive either 0.1 mg/kg Bup or 1 mg/kg BupER. Mice were induced, instrumented, and monitored as described above. At 45 min after LORR, mice received an intraperitoneal injection of 1 mg/kg atipamezole and were then monitored until the return of a righting reflex.[17,18] In contrast to experiment 1, experiment 2 included the provision of supplemental oxygen (100% O_2 at a flow rate of 0.6 L/min) via a nose cone. The response of the SpO_2 to TT was not measured in this experiment because of the high baseline values created by the oxygen supplementation. Two weeks later, mice were again anesthetized by using the other Bup formulation. The order of the 2 anesthetic regimens was randomized for each mouse, so half of the mice received Bup, and the remaining half received BupER in the first event.

Sample size and statistical analysis. **Sample size.** The basis for the sample size was the same as was used in experiment 1.

Analysis of HR, RR, and SpO_2. Differences in HR, RR, and SpO_2 were analyzed for 45 min after LORR. The analysis of these dependent variables was performed the same as in experiment 1.

Experiment 4: Testing dexmedetomidine/midazolam/buprenorphine under surgical conditions. A total of 6 laparotomy procedures were performed. Mice (n = 6; 3 males and 3 females) were injected intraperitoneally with 0.25 mg/kg Dexmed and 6 mg/kg Midaz and with 0.1 mg/kg Bup SC. Mice were induced and monitored as described above. As in experiment 2, supplemental oxygen (100% O_2 at a flow rate of 0.6 L/min) was provided via a nose cone.

Mice were shaved from xiphoid to pubis, and the abdomen was aseptically scrubbed with 3 alternating rounds of diluted chlorhexidine and alcohol after the induction of anesthesia. Before making the incision, the mice were confirmed to be at a surgical plane of anesthesia indicated by a negative response to the TT. The laparotomy procedure was designed to provide extensive manipulation of the abdominal viscera with low risk of perforation of an abdominal organ. A 2.0-cm midline incision was made, and the cranial mesenteric artery was identified and exposed for 5 min. The abdominal viscera were then shifted to the right side of the abdomen and the left kidney was then identified and exposed for 5 min. A 2-layer closure was performed with the body wall sutured with 4-0 monofilament suture in a simple interrupted fashion. The skin was sutured with 3-0 monofilament suture in a simple continuous fashion. After closure of the skin, mice received an intraperitoneal injection of atipamezole. After a return of righting reflex was observed, mice were deeply anesthetized with isoflurane until a deep surgical plane of anesthesia was reached, as defined by no response to firm toe pinch on both hind limbs, and the mouse was then euthanized by cervical dislocation. All 6 mice used for experiment 4 were euthanized in this manner. Mice in the other experiments were either used for training or euthanized by exposure to carbon dioxide.

Sample size and statistical analysis. Six mice were used for terminal laparotomy based on the success rate of achieving a surgical plane in the 20 trials performed using Bup in experiments 1 and 3. SpO_2, HR, and RR were analyzed by 2-way ANOVA, with sex and time as the main effects. When significant differences were detected, a Tukey post hoc analysis was performed. A P value < 0.05 was considered statistically significant.

Results

Experiment 1: Establish dosing protocol to achieve a surgical plane of anesthesia. The low-dose regimen of 0.15 mg/kg Dexmed, 4 mg/kg Midaz, and 0.1 mg/kg Bup produced highly variable results ranging from no LORR (n = 2) to a surgical plane of anesthesia (n = 6). One of the mice that did reach a surgical plane was a statistical outlier with regard to the amount of time it remained at a nonsurgical plane and surgical plane of anesthesia, 248 and 205 min, respectively. Because the combination did not achieve the 85% success rate within 10 mice as discussed above, no further mice were tested using this combination. The combination also failed to reliably achieve an anesthetic plane appropriate for nonpainful imaging procedures, as 2 of the 8 mice never lost the righting reflex.

The high-dose regimen of 0.25 mg/kg Dexmed, 6 mg/kg Midaz, and 0.1 mg/kg Bup provided sufficient anesthesia to reliably keep all mice (n = 10) at a surgical plane of anesthesia (Table 1). Nine of the 10 mice lost the righting reflex within 2 min of the injection, with one mouse requiring 11 min to lose the righting reflex. Nine of the 10 mice reached a surgical plane within 15 min of the injection, with one requiring 25 min from the time of injection. The HR, RR, and SpO_2 recordings are presented in Figure 2. Sex was used as a blocking factor for all 3. The sex differences in HR and RR were not significant (P = 0.198 and P = 0.835, respectively). The sex difference for SpO_2 was statistically significant (P = 0.026) with the males being slightly more hypoxic (67% ± 10%) than the females (70% ± 8%). Despite the statistical significance, we do not believe the difference is physiologically significant as both indicate severe, but not fatal, hypoxia. Differences in HR, SpO_2, and RR were not significantly different as a function of dose, but HR and SpO_2 varied significantly over time (Figure 2). Supplemental oxygen was not provided in this experiment. All the mice were profoundly hypoxic, although no morbidity was observed after the procedure or mortality. Because

Table 1. Duration of LORR and surgical plane anesthesia of mice in experiment 1 receiving either the low-dose drug regimen or the high-dose drug regimen

	Mean ± SD (min)	Range (min)	95% CI
Low dose, LORR (n = 5)	66 ± 32	25–91	26–106
Low dose, Sx plane (n = 5)	44 ± 36	10–95	0–89
High dose, LORR (n = 10)	112 ± 36	67–192	86–138
High dose, Sx plane (n = 10)	86 ± 31	25–135	63–108

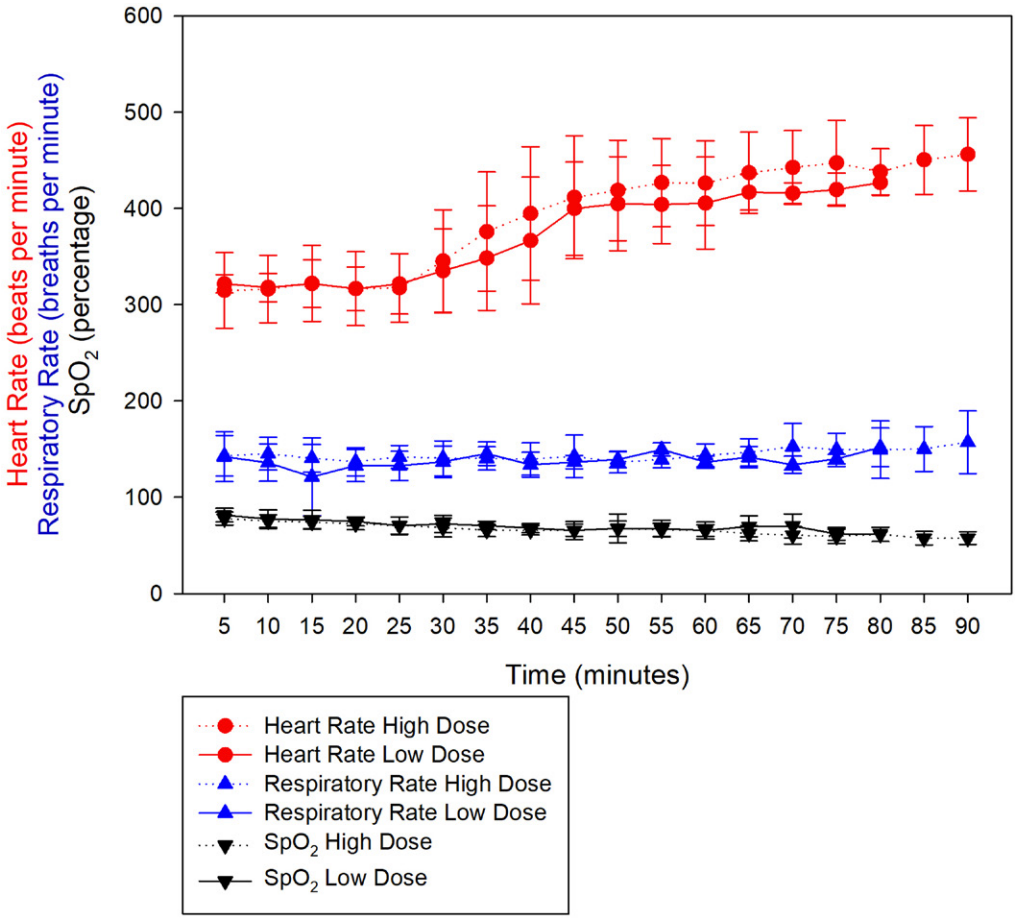

Figure 2. HR, RR, and SpO_2 of male and female C57BL/6J mice without oxygen supplementation under 2 doses of injectable anesthesia: (A) Low-dose Dexmed/Mizdaz/Bup (0.15/4/0.1 mg/kg); (B) high-dose Dexmed/Midaz/Bup (0.25/6/0.1 mg/kg). No statistically significant differences were detected for any of the variables as a function of dose, but HR and SpO_2 did significantly change over time.

no mice died during this or any of the other procedures, no necropsies were performed.

Because of an observed increase in peripheral oxygen saturation after TT, we monitored the SpO_2 before and after TT. Data were recorded from mice that were either in or not in a surgical plane of anesthesia. In mice that were not in a surgical plane (n = 23 time points), the SpO_2 before the TT was 73% ± 8% and peaked at 76% ± 6% within 10 s after the TT. In mice that were in a surgical plane of anesthesia (n = 178 time points), the SpO_2 was 65% ± 8% before TT and peaked at 68% ± 9% within 10 s after the TT. Values before and after the TT differed significantly for mice in either anesthetic plane (nonsurgical, $P = 0.008$; surgical, $P < 0.001$). The statistical analysis information is presented as Supplemental Data S1.

Experiment 2: Evaluation of dexmedetomidine and midazolam for sedation for noninvasive procedures. Mice in 19 of the 20 trials lost the righting reflex but did not lose the withdrawal reflex to the TT and thus were not considered to reach a surgical plane of anesthesia. The mouse that did not lose the righting reflex was in the oxygen supplementation group, and data from that mouse were not included in the subsequent data analysis. The mice receiving and not receiving oxygen supplementation lost the righting reflex for 86 ± 30 and 79 ± 22 min, respectively. This difference was not statistically significant ($P = 0.61$), nor was the effect of sex on the loss of the righting reflex ($P = 0.76$).

HR, RR, and SpO_2 for both oxygen supplementation conditions are shown as a function of time in Figure 3. Sex was not associated with significant differences in HR and SpO_2 ($P = 0.080$ and $P = 0.949$, respectively), whereas sex was associated with a significant difference in RR ($P = 0.001$). Oxygen supplementation had a significant effect only on the SpO_2 ($P < 0.001$). HR changed significantly with time ($P < 0.001$). A significant difference was detected in the SpO_2 after the noxious stimulus in mice that either received oxygen supplementation (before and after stimulus: 95% ± 3% and 96% ± 2%, respectively) or did not (before and after stimulus: 70% ± 5% and 81% ± 5%, respectively) ($P < 0.001$ for both groups). The results of the statistical analysis are presented in the Supplemental Data S1.

Experiment 3: Evaluation of buprenorphine and extended-release buprenorphine with reversal. Mice that received the high-dose combination all reached a surgical plane of anesthesia without mortality. However, one mouse receiving BupER was considered an anesthetic failure because its surgical plane of anesthesia lasted less than 5 min. The other 19 mice maintained a surgical plane of anesthesia for at least 25 min by the 45-min time period after the LORR, at which time the reversal agent was given. Two mice, both within the Bup group, had not lost the righting reflex until 22 and 28 min after injection and became hypothermic, with rectal temperatures of 32.9 and 32.4 °C at the time of LORR; the hypothermia was restored to normothermia within 20 min of placement on the circulating warm water blanket and a heat lamp. The more profoundly hypothermic of the 2 mice also displayed numerous premature atrial contractions while hypothermic; this problem resolved when the mouse

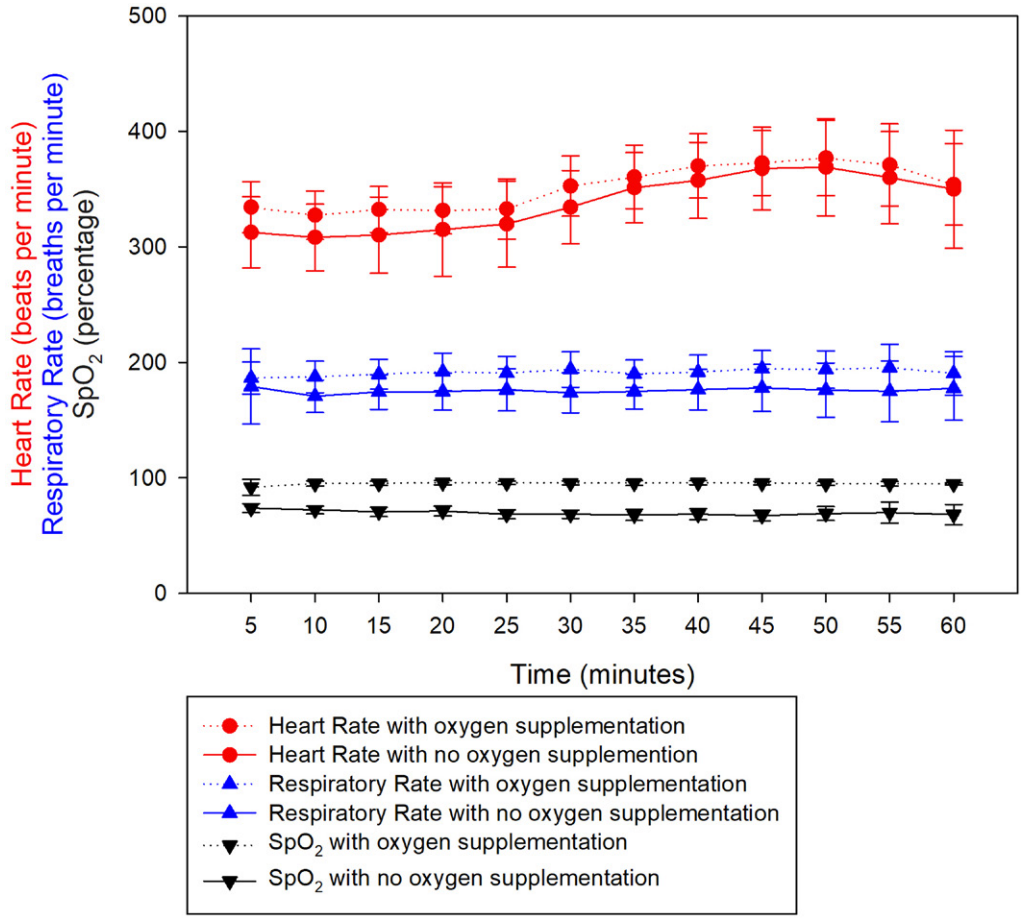

Figure 3. HR, RR, and SpO_2 of male and female C57BL/6J mice that are or are not receiving supplemental 100% O_2, using an injected drug combination (Dexmed/Midaz, 0.25/6 mg/kg) designed to achieve immobility, but not a surgical plane of anesthesia. HR was the only dependent variable to change significantly change over time. SpO_2 was the only variable that changed significantly changed.

regained normothermia, and no morbidity was observed after recovery. Of mice that successfully reached a surgical plane, the surgical plane of anesthesia was maintained for 25 to 45 min. After atipamezole administration, all mice regained righting reflex within 11 min. The administration of Bup compared with BupER had no significant impact on the time to recovery after atipamezole administration (Bup, 4.5 ± 2.9 min; BupER, 3.5 ± 2.3 min; $P = 0.40$). These mice received supplemental 100% oxygen via nose cone and maintained a peripheral oxygen saturation above 90% for the duration of anesthesia. One mouse was below 90% for approximately 20 min but returned to values above 90% with no intervention. No obvious cause was identified for this transient decrease.

Sex was not a significant factor for HR or RR but was a statistically significant factor for SpO_2 (females, 95.7% ± 1.8%; males, 96.8% ± 2.1%). This difference was not considered to be physiologically significant because all the mice on supplemental oxygen maintained normal oxygen saturation. HR, RR, and SpO_2 were analyzed by 2-way repeated measures ANOVA, with time and drug as main effects. Time, but not drug, was a significant factor in HR ($P < 0.001$). For RR, both time and drug were significant factors ($P = 0.001$ and $P = 0.047$, respectively). For SpO_2, drug was not significant, and time nearly reached significance ($P = 0.067$); however, this difference had no apparent physiologic significance (Figure 4). The statistical analysis is presented in the Supplemental Data S1.

Experiment 4: Testing dexmedetomidine/midazolam/buprenorphine under surgical conditions. Mice ($n = 6$) received 0.25 mg/kg Dexmed, 6 mg/kg Midaz, and 0.1 mg/kg Bup; this combination provided sufficient anesthesia to perform a laparotomy with recovery of righting reflexes after atipamezole administration. Total surgical time ranged from 35 to 45 min before the administration of atipamezole. As in experiment 3, mice that received supplemental 100% oxygen mice maintained normal peripheral oxygen saturation. Five of these 6 mice maintained a SpO_2 at or above 90% at all time points. One mouse had a gradual decrease in peripheral oxygen saturation, reaching a low of 76%, at which point the surgery had been completed and the mouse was given atipamezole. Within 5 min of atipamezole administration, the SpO_2 returned to over 90%.

Time and sex were not significant factors for HR or SpO_2 (HR: $P = 0.740$ and $P = 0.737$; SpO_2: $P = 0.470$ and $P = 0.131$, respectively). For RR, time was significant ($P = 0.038$), with the RR decreasing over the course of the surgery, but sex was not significant ($P = 0.133$) (Figure 5).

Discussion

Anesthetic drug combinations for mice must be tested for safety and efficacy to provide options for safe injectable anesthesia in mice other than ketamine-based combinations. The current study demonstrated that a dose of 0.25 mg/kg Dexmed and 6 mg/kg Midaz is a viable option for injectable anesthesia for minimally invasive, nonpainful procedures as demonstrated by a total of 19 of 20 mice that reliably achieved a LORR without mortality or observable morbidity. The addition of 0.1 mg/kg Bup or 1 mg/kg BupER results in a reliable

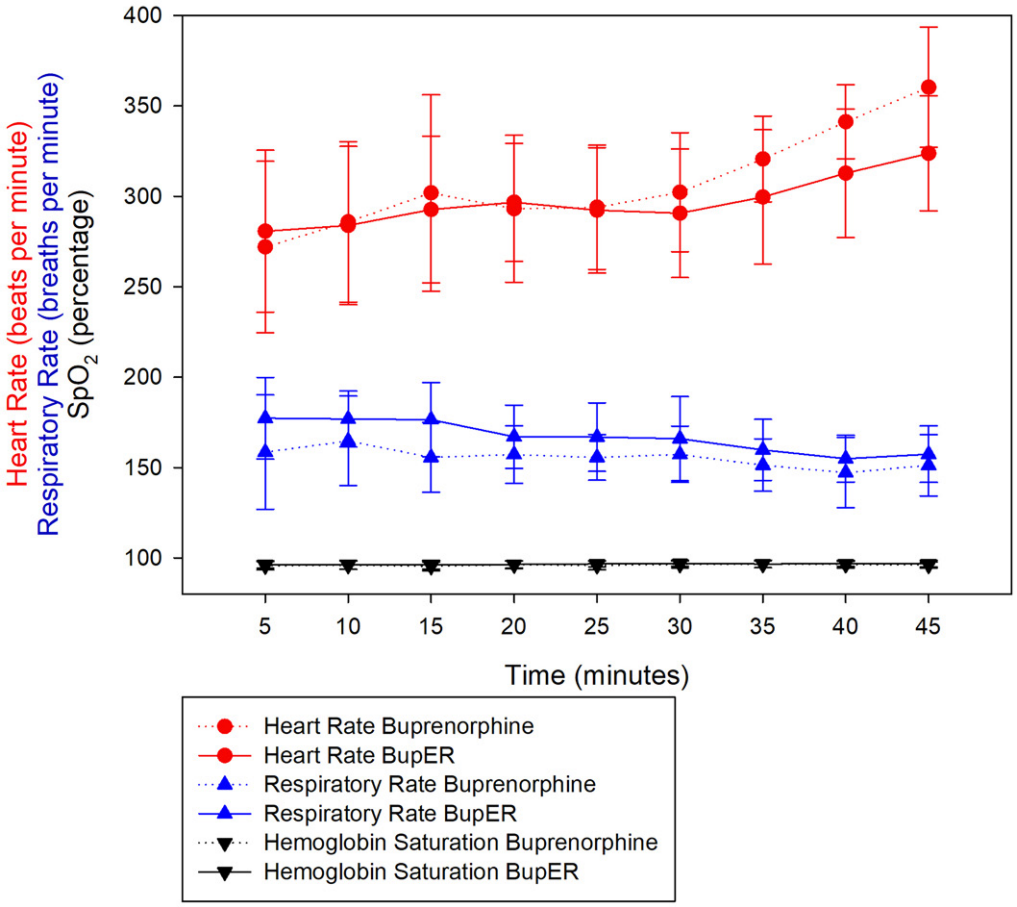

Figure 4. HR, RR, and SpO_2 of male and female C57BL/6J mice supplemented with 100% O_2 under 2 injected anesthetic combinations designed to achieve a surgical plane of anesthesia: (A) Dexmed/Mizdaz/Bup (0.25/6/0.1 mg/kg); (B) Dexmed/Midaz/BupER (0.25/6/1 mg/kg). HR and RR both changed significantly over time. RR differed significantly between the 2 forms of buprenorphine.

option for surgical procedures, as demonstrated by 35 of 36 mice successfully achieving a surgical plane across 3 of our 4 experiments and includes buprenorphine, which has been shown in previous studies to provide analgesia to mice beyond the perioperative period.[13,15] All anesthetic agents used in our injectable combination are reversible, enhancing its safety, but reversal was consistently achieved with use of atipamezole as a single reversal agent. We also showed that mice at a surgical plane of anesthesia retained autonomic nervous system activity by demonstrating increases in peripheral blood oxygen saturation after application of a noxious stimulus.

Variability in the response to injectable anesthesia is a significant challenge in mouse anesthesia. This variability extends to the depth of anesthesia, the time required for induction and duration of anesthesia, and the impact on the cardiovascular and respiratory responses to the drugs. This variability is overcome in most large animal species by administering the drugs intravenously, which results in a rapid distribution of the drugs and finer minute-to-minute control of these variables than intraperitoneal administration, which is the route commonly used in mice. The current regimens in this study were not exempt to this inherent variability, which should be a consideration when using our novel combination. One mouse that received Dexmed/Midaz in experiment 2 never developed LORR. In experiment 3, one mouse that had received the Dexmed/Midaz/BupER combination did not maintain consistent negative pedal withdrawal and thus was not considered to have reliably reached a surgical plane of anesthesia. In experiment 3, 2 mice receiving Dexmed/Midaz/Bup combination required over 20 min to achieve LORR.

The delayed LORR experienced by 2 mice receiving Dexmed/Midaz/Bup combinations raises a concern that researchers should be aware of when using this combination. One male and one female, both in the Dexmed/Midaz/Bup group, did not show LORR until 28 and 22 min after injection, respectively. Both mice were hypothermic (32.4 and 32.9 °C, respectively) at the initial body temperature reading. The more profoundly hypothermic mouse showed an arrhythmia on the ECG; this has been described in human literature as a consequence of hypothermia.[10,35] Hypothermia is a known side effect of Dexmed and has been reported with injectable anesthetic regimens in mice even despite external warming.[1] A previous study of injected medetomidine/midazolam/butorphanol anesthesia found that mice remained hypothermic for 5 h after the injection.[38] This documented risk of hypothermia indicates that mice should receive supplemental heat while anesthetized and have their body temperature monitored to confirm the efficacy of the warming. Continued thermal support may be necessary during the recovery period until the mouse can fully thermoregulate.

The variability observed in this study has several possible explanations. In species that include humans, dogs, and cats, benzodiazepines like Midaz reportedly trigger various responses that range from excitement to sedation.[12,34,36] Theories for the paradoxical excitement phenomenon include a loss of cortical restraint similar to what occurs after alcohol consumption or a

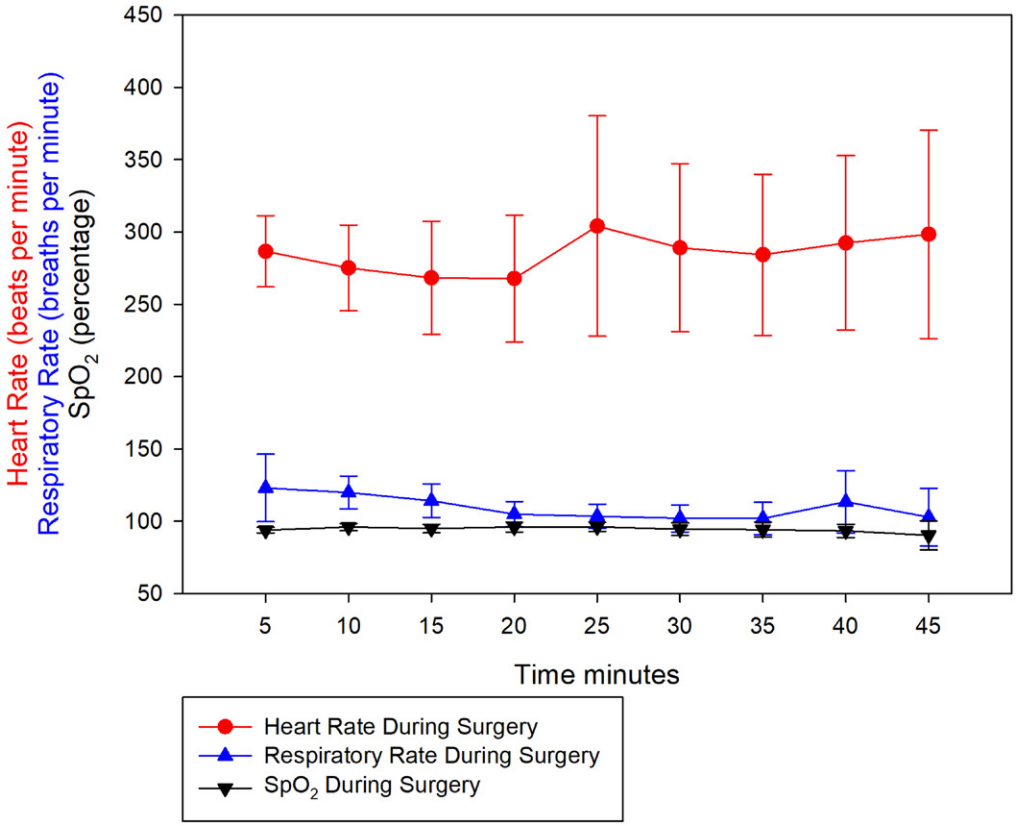

Figure 5. HR, RR, and SpO_2 of male and female C57BL/6J mice with oxygen supplementation and injected with the high-dose formulation of Dexmed/Mizdaz/Bup (0.25/6/0.1 mg/kg) before surgery. RR was the only variable to change significantly over time.

reduction in serotonin neurotransmission, which may precipitate aggressive behavior.[33] While paradoxical excitement has not been reported in mice given Midaz, some variability seen may be similar to the variable response reported in other species.[32] No current pharmacokinetic studies have evaluated Bup or BupER during the first 30 min after administration; experiments 1, 3, and 4 indicate that this early time period is critical to achieving a surgical plane of anesthesia with this drug combination. Variability of onset has been documented for sustained-release formulations because of their nonlinear absorption rates after subcutaneous administration, which has been previously shown to affect the onset of anesthesia.[27] A final factor contributing to variability is that the drugs are administered intraperitoneally, which means their absorption and distribution will be much slower and more variable than what occurs after intravenous administration.[39]

The ideal plane of anesthesia is achieved when there is loss of consciousness so that the animal cannot experience pain, the spinal reflexes have been anesthetized to achieve immobility, but the animals still have a functioning autonomic nervous system and are able to respond to physiologic disturbances. We consistently observed a rise in peripheral oxygen saturation after applying a painful stimulus when supplemental oxygen was not provided. We speculate that this increase in SpO_2 indicates preservation of autonomic nervous system function despite respiratory depression caused by the anesthetic agents. Some anesthetics (e.g., ketamine and isoflurane) impair normal physiologic responses to hypoxemia and hypercapnia by depressing the function of chemoreceptors that respond to those conditions by increasing RR and tidal volume.[3,21,32] Although no literature is currently available to directly link the increase in peripheral oxygen saturation with a lack of pedal withdrawal as a means of confirming the preservation of autonomic reflexes, future studies could consider measuring tidal volume or instantaneous RR changes to confirm this relationship.

One factor that should be considered when measuring SpO_2 after administration of Dexmed is α-2 adrenoceptor-mediated vasoconstriction, which can affect pulse oximetry readings.[31] Similar to xylazine, Dexmed causes α-2 adrenoceptor-mediated vasoconstriction, which, in conjunction with room air, may explain low pulse oximetry readings. Additional factors that affect measurements are sensor placement and tissue pigmentation and thickness, which were controlled in our study by consistent pedal placement of the oximeter across experimental groups. Although no deaths occurred in experiment 1, a previous study reported that oxygen supplementation can reduce mortality when using injected anesthetics and thus increases the safety of mouse anesthesia.[3]

Supplemental oxygen may affect RR, as was observed in experiment 2 in which the mice receiving supplemental oxygen had a higher RR than those not receiving oxygen. This finding is not consistent with the effects of ketamine/xylazine anesthesia and warrants further exploration.[3] In experiment 3, the mice receiving BupER had a higher RR than did mice receiving Bup; however, both groups maintained a normal peripheral oxygen saturation with oxygen supplementation. Researchers using these drug combinations should be aware of these effects with regard to monitoring mice and should also understand that without the use of supplemental oxygen, mice in experiments 1 and 2 developed profound hypoxia.

Validation and recommendation of a novel anesthetic combination should provide researchers with normal monitoring data that can provide a basis for identification of abnormalities that indicate a need for intervention. Increases in HR and RR

above expected levels could indicate that anesthesia is wearing off and that additional anesthetics may be required to extend the anesthetic plane. Conversely, large decreases in HR, RR, or SpO_2 could signal impending death and require reversal of anesthesia. In experiments 1, 2, and 3, HR increased significantly over time. Although we did not monitor blood pressure in this study, we speculate that the gradual rise in HR beginning at approximately 30 min after injection in first 3 experiments is compensating for hypotension triggered by Dexmed.[29,34] Further studies using this drug combination could monitor blood pressure to test this idea. Mice in our experiment maintained a consistent plane of anesthesia during period of increasing HR. Mice should be routinely monitored for their pedal withdrawal with changes in HR and possibly in blood pressure changes to confirm an adequate plane of anesthesia.

Because the recovery period is the most common time of death associated with anesthesia in animals,[4] supporting the mice through this period is critical to a successful outcome. A previous study has shown in female C57BL/6J mice that atipamezole reverses the anesthesia produced by ketamine/Dexmed without reversing the antinociceptive effects of Bup.[18] In experiment 3, atipamezole administration reliably hastened the termination of anesthesia without requiring the reversal of midazolam or either buprenorphine formulation. Anesthetic reversal has been recommended to decrease the anesthetic morbidities such as hypothermia and bradycardia and is recommended for short procedures that do not require prolonged recovery.[20,38] Furthermore, the use of a reversal agent likely reduces the time demand on research staff by reducing the time needed to monitor anesthetized mice.

An important benefit of our combination was its use of preemptive analgesia, which avoids a gap between anesthesia and analgesia after a painful procedure. Bup is important in achieving the surgical plane of anesthesia because of its sedation and analgesic effects, as demonstrated by our finding that when it is not part of the anesthetic combination, none of the 20 mice in experiment 2 reached a surgical plane of anesthesia. Pharmacokinetic analyses have demonstrated that plasma concentrations of standard formulation buprenorphine maintain a therapeutic threshold for 4 to 6 h, helping to ensure that analgesia is present during the immediate postoperative period. The long acting BupER produced was equally effective at achieving a surgical plane of anesthesia and also provides analgesia for up to 24 h after administration; this is an improvement as compared with the standard formulation buprenorphine because if a longer duration of analgesia is required, the need for frequent redosing and associated stress from handling is eliminated.[8,23] Postoperative analgesia was not evaluated in this series of experiments, but the Bup dosing was consistent with existing pharmacokinetic literature.[8,23] Future users of this anesthetic regimen should be mindful of monitoring mice for pain in the immediate postoperative period considering the lack of long-term evaluation performed in this series.

Future studies to refine this anesthetic combination could include evaluating the use of repeat-bolus dosing to extend the duration of the surgical plane of anesthesia. Given what we know from previous studies of injectable anesthetics, finding a single dose that will produce a surgical plane of anesthesia in 100% of mice without any deaths will be difficult.[11,19] With some injected drug combinations (e.g., ketamine/xylazine/acepromazine and alfaxalone/xylazine), repeat-bolus dosing is the default method to extend the duration of anesthesia.[11,19] Repeated injections of Dexmed/Midaz, with or without Bup or BupER, might be an option if individual mice do not reach a surgical plane of anesthesia at the initial dose. The use of blood pressure monitoring would better evaluate time-dependent changes in HR and provide information about the function of the autonomic nervous system during surgical and nonsurgical procedures. Studying the pharmacokinetics of Bup after simultaneous administration of an α-2 agonist (e.g., Dexmed) could confirm that a therapeutic plasma level was present at the time of LORR. Recently, a pharmacokinetic study of BupER demonstrated that a therapeutic plasma level was achieved within 30 min in male C57BL/6 mice; the same study showed this BupER did not affect the safety of either isoflurane or ketamine/xylazine anesthesia.[8] A pharmacokinetic study of Bup or BupER could test whether the addition of α-2 adrenergic agonists alters absorption of Bup and BupER.

In conclusion, this work demonstrated the effective use of Dexmed and Midaz as an effective alternative to ketamine and xylazine for nonpainful procedures requiring chemical restraint. With the addition of Bup or BupER, this combination represents a refinement to ketamine, xylazine, and acepromazine for procedures that require postprocedural analgesia. The use of a single reversal agent, atipamezole, reliably and effectively reversed anesthesia due to Dexmed. Our study findings provide an alternative injectable anesthetic regimen for mice and is a refinement in mouse anesthesia due to its reliability, reversibility, and inclusion of drugs to provide analgesia beyond the perioperative period.

Supplemental Material

Data S1. Statistical data for Experiments 1 to 4.

Conflict of Interest

The authors have no conflicts of interest to declare.

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