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Dose Regimens, Variability, and Complications Associated with Using Repeat-Bolus Dosing to Extend a Surgical Plane of Anesthesia in Laboratory Mice

Samer M Jaber,[1,2] F Claire Hankenson,[1,2] Kathleen Heng,[4] Andrew McKinstry-Wu,[3] Max B Kelz,[3] and James O Marx[1,2,*]

Extending a surgical plane of anesthesia in mice by using injectable anesthetics typically is accomplished by repeat-bolus dosing. We compared the safety and efficacy of redosing protocols administered either during an anesthetic surgical plane (maintaining a continuous surgical plane, CSP), or immediately after leaving this plane (interrupted surgical plane, ISP) in C57BL/6J mice. Anesthesia was induced with ketamine, xylazine, and acepromazine (80, 8, and 1 mg/kg IP, respectively), and redosing protocols included 25% (0.25K), 50% (0.5K), or 100% (1.0K) of the initial ketamine dose or 25% (0.25KX) or 50% (0.5KX) of the initial ketamine–xylazine dose. In the ISP group, the surgical plane was extended by 13.8 ± 2.1 min (mean ± SEM) after redosing for the 0.25K redose with 50% returning to a surgical plane, 42.7 ± 4.5 min for the 0.5K redose with 88% returning to a surgical plane, and 44.3 ± 15.4 min for the 1.0K redose, 52.8 ± 7.2 min for the 0.25KX redose, and 45.9 ± 2.9 min for the 0.5KX redose, with 100% of mice returning to a surgical plane of anesthesia in these 3 groups. Mortality rates for ISP groups were 0%, 12%, 33%, 12%, and 18%, respectively. Mice in CSP groups had 50% mortality, independent of the repeat-dosing protocol. We recommend redosing mice with either 50% of the initial ketamine dose or 25% of the initial ketamine–xylazine dose immediately upon return of the pedal withdrawal reflex to extend the surgical plane of anesthesia in mice, optimize the extension of the surgical plane, and minimize mortality.

Abbreviations: CSP, continuous surgical plane; ISP, interrupted surgical plane; KXA, ketamine–xylazine–acepromazine; PWR, pedal withdrawal reflex; RR, righting reflex.

The goals of achieving a surgical plane of anesthesia are to provide immobility, unconsciousness, analgesia, amnesia, and attenuation of autonomic responses to noxious stimuli[5] without adversely compromising physiologic variables such as heart rate, respiratory rate, body temperature, and blood pressure.[1,5] For prolonged surgical procedures, inhaled anesthetics generally are recommended because of rapid onset of and recovery from anesthesia, efficacy in attaining and maintaining a surgical plane of anesthesia, and relative safety.[15] In laboratory animal practice, there are situations when inhaled anesthetics cannot be used due to technical or experimental limitations.[6,13,15,43] In these cases, an intraperitoneal injectable anesthetic protocol, typically including the combination of ketamine–xylazine–acepromazine (KXA), is used in mice to achieve a surgical plane of anesthesia.[3,8,22,47] If the procedure requires a duration of anesthesia beyond what a single dose of these drugs can provide, repeat-bolus dosing is the default method to extend the duration of anesthesia in mice. Although previous studies have evaluated the safety and efficacy of a single bolus of injectable anesthetics in rodents, no study has evaluated these parameters in the repeated dosing of injectable anesthetics to extend the duration of anesthesia, resulting in minimal information and guidance for researchers and veterinarians beyond anecdotal experience.[3,8-11,14,16,17,22,25,30,39]

When considering repeat-bolus dosing, it is essential to factor in the dose as well as the timing of administration to the animal. Knowledge of pharmacokinetics of each drug, as well as of drug interactions with other combined agents, is imperative for deciding how much to administer and when to provide the repeat dose to avoid either overdosing or underdosing the animal. The elimination half-life of ketamine in various strains of mice is approximately 13 min when administered by the intraperitoneal route,[28] making it a preferred drug for redosing, because systemic concentrations are likely to be diminished at the time of dissipation of the surgical plane of anesthesia. Potential negative effects of ketamine include respiratory depression, arrhythmias, seizures, tremors, muscle hypertonicity, and erratic or prolonged anesthetic recovery.[32,42] Xylazine has a half-life of 1.2 to 1.6 h in rats, and although it has not been evaluated in mice,[44,45,48] the half-life of xylazine in mice is presumably longer than that of ketamine. Due to the lack of information on the half-life of xylazine in mice, caution should be taken with any redosing scenario to prevent overdosing the drug. Adverse effects of xylazine in multiple species include hypotension, bradycardia, decreased gastrointestinal motility, and respiratory and cardiovascular depression.[32,42] The metabolism of xylazine is likely unaffected by concurrent ketamine administration, according to in vitro data.[26] Acepromazine has a relatively long half-life in nonrodent species, for example 7.1 h in dogs and 50 to 183 min in horses,[21,37] and is not routinely redosed in mice.

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[1]University Laboratory Animal Resources, [2]Department of Pathobiology, School of Veterinary Medicine, and [3]Department of Anesthesiology and Critical Care, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania; [4]School of Veterinary Medicine, University of California, Davis, California.
[*]Corresponding author. Email: marx@upenn.edu

The timing of drug administration is another critical factor to consider when addressing anesthetic redosing. Ideally, an effective repeat-dosing regimen achieves an uninterrupted, continuous surgical plane of anesthesia with a predictable anesthetic recovery. The advantage of redosing prior to the animal leaving the surgical plane of anesthesia is that a continuous surgical plane of anesthesia is maintained, eliminating concerns over patient awareness and perception of pain. The main disadvantage of this redosing approach is that adding more drug to the system while there is potentially still a considerable amount affecting the animal increases the risk of adverse side effects. Conversely, dosing at the time of emergence from a surgical plane of anesthesia decreases the risk of anesthetic overdose but may increase the possibility of a patient's transient conscious awareness of the procedure.

The aim of the present study was to identify a safe and effective dosing regimen to extend the surgical plane of anesthesia in mice by using an injectable anesthetic combination of KXA. We hypothesized that the ideal anesthetic regimen would permit the administration of a second dose of anesthetic before the mouse emerged from a surgical plane of anesthesia, thus facilitating an uninterrupted extension of that plane of anesthesia while minimizing mortality.

Materials and Methods

Animals and facility. Male SPF C57BL/6J mice (*Mus musculus*, $n = 56$; age, 8 to 14 wk; weight, 27.3 ± 0.2 g [range, 23 to 31 g]) were obtained from an approved vendor (Jackson Laboratories, Bar Harbor, ME). Male mice were selected to eliminate the potential influence of hormonal variations of the estrus cycle on anesthetic responses. To adhere to the principle of reduction, mice were used for as many as 2 separate anesthetic experiments, with a minimum of 10 d as a washout period between anesthetic exposures. All mice were housed in an AAALAC-accredited rodent facility at a 12:12-h light:dark cycle in static polycarbonate microisolation cages (Max 75, Alternative Design, Siloam Springs, AR) on disposable bedding (0.12-in. Bed-O-Cobs, The Andersons, Maumee, OH). Mice were fed standard pelleted laboratory rodent chow (no. 5010, LabDiet, St Louis, MO) and provided municipal water supplied by bottle. All procedures described were approved by the University of Pennsylvania's IACUC.

A subset of mice ($n = 20$) was used to determine the baseline heart rate for unanesthetized, unrestrained mice. Heart rate was determined on a recording platform by ECG after a 3-min acclimation period (ECGenie, Mouse Specifics, Quincy, MA; eMouse 11 Analysis Software, Mouse Specifics, Quincy, MA). The baseline heart rate of the mice was 734 ± 8 bpm (range, 654 to 788 bpm).

Anesthetic dosing and monitoring. All procedures were performed during the last 6 h of the light cycle to reduce environmental and circadian rhythm variability. Mice were weighed individually on a digital scale (US-ACE, US Balance, Vincennes, IN) prior to dosing. The anesthetic drugs were diluted to 10% of their original concentration by using sterile 0.9% NaCl and combined at the designated doses into a single syringe immediately prior to injection. Doses for each drug were rounded to the nearest 0.01 mL. Each mouse was manually restrained for intraperitoneal injection in the right lower quadrant of the abdomen by using a 27-gauge needle (5/8 in. length) on a 1-mL syringe. Injection volumes ranged from 0.31 to 0.40 mL for initial doses based on weight. Mice received ketamine (100 mg/mL, Ketaset, Fort Dodge Industries, Fort Dodge, IA,) xylazine (20 mg/mL, AnaSed, Lloyd Laboratories, Shenandoah, IA), and acepromazine (10 mg/mL, Acepromazine maleate, Phoenix Pharmaceuticals, St Joseph, MO) at doses of 80, 8, and 1 mg/kg IP, respectively. Artificial tears ointment (Akwa Tears, Akorn, Lake Forest, IL) was applied to the eyes at the beginning of each trial. The initial dose of KXA was based on published reports in rodents[3,8,22,47] and a pilot experiment to test the response of C57BL/6J mice ($n = 14$ mice; 22 anesthetic events) to different anesthetic doses (Table 1).

Mice were monitored every 30 s from the time of injection for the loss of righting reflex (RR). The RR was defined as the ability to return to sternal recumbency or normal standing posture with all 4 limbs on the ground within 60 s after being placed in dorsal recumbency on a flat surface. Once the RR was lost, the mice were placed in dorsal recumbency on a circulating-water heating pad (Gaymar Industries, Orchard Park, NY) set to maintain a surface temperature of 37 °C and instrumented for physiologic monitoring. Temperature was measured by using a temperature probe placed rectally (length of the probe; RET-3, Thermoworks, Lindon, UT) connected to a thermometer (MicroTherma TW2-193, Thermoworks). Starting temperatures and lowest temperatures during anesthesia were 36.5 ± 0.1 °C and 35.6 ± 0.1 °C, respectively. Electrocardiogram leads were placed on immobilized mice on the right forelimb and the base of the tail with coupling gel and held in place by using standard medical adhesive tape. Respiratory rate was measured visually by counting thoracic excursions. Heart rate, respiratory rate, and rectal temperature were recorded every 5 min under anesthesia. These parameters were measured prior to assessment of the pedal withdrawal reflex (PWR) to reflect resting physiologic parameters under anesthesia.

A surgical plane of anesthesia was defined by the absence of any motor response to a noxious stimulus. The PWR was assessed every 5 min immediately after evaluating the physiologic parameters as previously described (300-g, 6.65-gauge Touch-Test Sensory Evaluator, North Coast Medical, Gilroy, CA),.[23,27] The point of compression was on the dorsal aspect of the metatarsal region and alternated between hindlimbs. A positive response was defined as any flexion of the stifle, tarsus, hip, or combination of joints in response to this stimulus or any other spontaneous motion of the mouse not associated with the stimulated limb. Once loss of the PWR was confirmed, this stimulus was reapplied every 5 min until return of the PWR or until the mouse exhibited spontaneous movement. For mice that had spontaneous motion between the 5-min intervals of PWR assessment, the time until return of the PWR was recorded, and the repeat dose was administered immediately, but the assessment interval was not reset and instead continued every 5 min from the previous assessment. The duration of the surgical plane of anesthesia was defined as the time between the loss of the PWR and its return.

Mice were divided randomly into 2 groups according to the timing of the repeat dose. One group was dosed prior to the anticipated return of the PWR, at 30 min after the initial injection, providing a continuous surgical plane (CSP group). This administration time point was approximately 10 min prior to the anticipated return of the PWR, on the basis of the shortest duration of a surgical plane of anesthesia in the pilot study (Table 1). The mice received 25%, 50%, or 100% of their initial dose of ketamine (0.25K, 0.5K, and 1.0K, respectively) or 50% of both the initial doses of ketamine and xylazine combined (0.5KX). The second group of mice, which had an interrupted surgical plane (ISP group), was dosed immediately after return of the PWR or when any spontaneous motion was observed. These mice were redosed with either 0.25K, 0.5K, 1.0K, or 0.5KX or 25% of the initial dose of both ketamine and xylazine

Table 1. Results of the pilot study to determine initial injectable anesthetic dose

Dose group (mg/kg)	No. of mice that reached a surgical plane of anesthesia/ total no. of mice	Duration (min) of surgical plane of anesthesia (mean ± SE [range])	No. of deaths/ total no. of mice
Ketamine 50 Xylazine 5 Acepromazine 1	0/4	not applicable	0/4
Ketamine 80 Xylazine 8 Acepromazine 1	6/7	45.1 ± 9.4 (40–73; *n* = 6)	0/7
Ketamine 100 Xylazine 10 Acepromazine 1–2	7/7	67.7 ± 2.9 (64–72; *n* = 3)	4/7
Ketamine 100 Xylazine 10	3/4	23.3 ± 16.7 (5–50; *n* = 3)	0/4

Methods for monitoring and support of mice in pilot study (*n* = 14 mice; 22 anesthetic events) were identical to those used for the experimental animals.

combined (0.25KX). Two mice initially assigned to the CSP group, one each in the 0.5K and 1.0K groups, demonstrated return of the PWR at 25 min after the initial injection and therefore were included in the ISP group. Acepromazine was not redosed in light of its long duration of action.[32]

After redosing, physiologic parameters and PWR were monitored every 5 min for loss of the PWR, until return of the PWR or spontaneous motion was noted. Monitoring instrumentation then was removed, and the mice remained in dorsal recumbency until the return of the RR. The time to the return of the RR was established as the time until the mouse spontaneously righted itself from dorsal recumbency 3 consecutive times. Mice were monitored until they were ambulatory and subsequently were monitored once daily for 2 d after the experiments for any adverse clinical signs induced due to use of the sensory evaluator, including redness or swelling of the foot or gait abnormalities.

During the course of the experiments, several mice (*n* = 19) developed an agonal pattern of breathing. No mice that developed agonal breathing recovered from anesthesia without intervention. The animals were considered to have reached the point of death when there was complete respiratory arrest for 1 min in conjunction with cardiac arrest noted via ECG. A subset of the mice (7 of 19) that developed agonal breathing while under anesthesia after their repeated dose were give atipamezole (0.1 mg IP per mouse; Antisedan, Zoetis, Florham Park, NJ) to attempt to reverse the injectable anesthesia. For these animals, the time of dosing of atipamezole was considered the time of death for purposes of analysis of response to the redosing protocol. These mice were not reused in subsequent experiments.

Effect of ketamine on heart rate. To evaluate the effect of ketamine independently, heart rate was recorded (ECGenie, Mouse Specifics) both before and after mice were given either ketamine (*n* = 6) or saline (control, *n* = 4). Mice were placed on the platform and allowed to acclimate before recording the baseline ECG. Mice were injected as previously described for the anesthetic protocols. Total administration volumes were diluted with 0.9% NaCl to match drug volumes used in the KXA protocol. The ECG was recorded continuously until the mouse exhibited grooming behavior or the heart rate returned to resting heart rate. Heart rate was measured at 2.5 and 5 min after dosing and at 5-min intervals thereafter. A single mouse was dosed with ketamine to confirm that the drugs had minimal effect on the body temperature of the sedated mice, because thermal support of the sedated mice could not be provided on the ECGenie platform.

Statistical analysis. Means ± SE were calculated for all data. The extension of the surgical plane in surviving mice was analyzed by 2-way ANOVA, with main effects of the timing of the redosing and the dose of anesthetics administered. A repeated-measures, 2-way ANOVA was performed to compare the heart and respiratory rates of survivors and nonsurvivors over time. Two-way repeated-measures ANOVA also was used to analyze the effects of ketamine or saline injection on heart rate. When significant differences were detected, Tukey post hoc analysis was performed. A *t*-test was performed to compare the body weights of mice that died unexpectedly with those of mice that survived. χ^2 tests were performed to determine whether there was a difference in survival between the first and second anesthetic event and for the 4 different ISP and CSP redosing protocols. For all analyses, a *P* value less than 0.05 was considered to be statistically significant. All statistical analysis was done by using SigmaPlot 12.3 (Systat Software, San Jose, CA).

Results

Initial dose. The initial dose of KXA (80, 8, and 1 mg/kg, respectively) was successful in inducing a surgical plane of anesthesia without excessive mortality in 88.4% (76 of 86) of the anesthetic events. The initial dose resulted in death in 3 of the 86 mice; these mice were excluded from further evaluation. In addition, 7 of the 86 mice did not reach a surgical plane of anesthesia after the initial dose. The average time to loss of the RR was 2.5 ± 0.1 min (range, 1.40 to 4.98 min). The average time to loss of the PWR was 17.0 ± 0.8 min (7.9 to 30.8 min). The average time of repeat dosing in ISP group animals was 39.7 ± 1.8 min (range, 25.0 to 90.0 min). Although some mice had mild swelling and erythema of the paw after PWR testing, these were self-resolving, and no lameness could be appreciated on inspection the day after testing.

ISP groups. The duration of surgical plane of anesthesia after redosing, the time to return of the RR, and mortality data for the ISP group are summarized in Table 2. The extension of a surgical plane of anesthesia was significantly ($P < 0.05$) less for the 0.25K dose than for the 0.25KX and 0.5KX doses (Table 3). Mortality in

Table 2. Anesthetic effect, recovery, and complication rate for mice in ISP group.

Dose group	Time (min) at surgical plane of anesthesia after redose (mean ± SE [range])	Time (min) to return of the RR after redose (mean ± SE [range])	No. of deaths/ total no.	Time (min) to death after redose (mean ± SE [range])
0.25K	13.8 ± 2.1 (0-45; *n* = 8)	49.4 ± 5.0 (14-137; *n* = 8)	0/8	not applicable
0.5K	42.7 ± 4.5 (0-84; *n* = 7)	82.7 ± 4.2 (36-129; *n* = 7)	1/8	35.0 (*n* = 1)
1K	44.3 ± 15.4 (20-60; *n* = 6)	83.5 ± 2.7 (64-105; *n* = 6)	3/9	58.3 ± 4.4 (50-65; *n* = 3)
0.25KX	52.8 ± 7.2[a] (35-85; *n* = 7)	140.3 ± 9.0 (114-162; *n* = 7)	1/8	30.0 (*n* = 1)
0.5KX	45.9 ± 2.9[a] (10-79; *n* = 9)	81.3 ± 2.9 (41-109; *n* = 9)	2/11	15.0 ± 5 (10-20; *n* = 2)

[a]Value significantly ($P < 0.05$) different from that for the 0.25K group.

Table 3. Anesthetic effect, recovery, and complication rate for mice in CSP group

Dose group	Time (min) at surgical plane of anesthesia after redose (mean ± SE [range])	Time (min) to return of the RR after redose (mean ± SE [range])	No. of deaths/ total no.	Time (min) to death after redose (mean ± SE [range])
0.25K	21.3 ± 5.0 (0-45; *n* = 4)	57.3 ± 5.1 (38-82; *n* = 4)	3/7[a]	77.5 ± 32.5 (45-110; *n* = 2)
0.5K	25.0 ± 0 (25; *n* = 3)	43.7 ± 0.5 (42-45; *n* = 3)	3/6	81.7 ± 21.7 (45-120; *n* = 3)
1K	61.7 ± 10.5 (35-95; *n* = 3)	105.0 ± 11.9 (80-146; *n* = 3)	4/7	67.5 ± 13.6 (50-100; *n* = 4)
0.5KX	55.0 ± 10.4 (35-70; *n* = 3)	61.7 ± 2.6 (55-70; *n* = 3)	3/6	53.3 ± 12.7 (35-70; *n* = 3)

[a]One of 3 mice died after the experiment; the data from this mouse are not included in the means and ranges in this table.

the ISP groups increased with increasing anesthetic dose, resulting in 0%, 12%, 33%, 12%, and 18% mortality rates in the 0.25K, 0.5K, 1K, 0.25KX, and 0.5KX groups respectively. χ^2 analysis did not reveal significant differences between the mortality rates of the 5 protocols, but this analysis lacks sensitivity at this group size. ISP mice required 5.2 ± 0.5 min (range, 1.1 to 12.1 min) to return to a surgical plane of anesthesia after redosing, and 50% of the 0.25K mice and 12.5% of the 0.5K group failed to return to a surgical plane of anesthesia after repeat dosing.

CSP groups. The duration of the surgical plane of anesthesia after redosing, the time to return of the RR, and mortality data for the CSP group are summarized in Table 3. Mortality in CSP groups was unacceptably high and ranged from 43% to 57% among the redosing protocols. All redosing paradigms extended the surgical plane of anesthesia, except for a single mouse in the 0.25K group, which displayed a positive PWR at 5 min after redosing. The high rate of mortality prevented detection of differences between doses in the CSP groups in regard to extension of the surgical plane of anesthesia. There was no significant difference in the mortality rates between the various protocols.

Heart and respiratory rates in mice with anesthetic death compared with recovery. One of the animals in the CSP 0.25K group died the day after a successful anesthetic recovery and was excluded from the analysis comparing animals that died with the surviving mice. All other deaths occurred during the anesthetic event itself. All the mice that died displayed agonal breathing. None of the mice that displayed this critical sign recovered without pharmacologic intervention with atipamezole. The duration of agonal breathing prior to death was approximately 1 to 2 min in the 12 of 19 mice that did not receive atipamezole. No significant differences were detected between the weights of surviving mice and mice that died or between those that died during the first compared with second anesthetic events. Mice that survived without atipamezole reversal had a progressive increase in heart rate for several minutes prior to recovery, whereas the heart rates of mice that died remained constant during the procedure prior to arrest (Figure 1). In contrast to this pattern, the respiratory rates of the mice that died had a slowly progressive drop in frequency, whereas surviving mice had a nearly constant respiratory rate prior to recovery (Figure 1). No lethal abnormalities, such as ventricular tachycardia or fibrillation, were noted in the ECG tracing of any animals prior to the onset of agonal breathing. ECG tracings at various time points for a mouse that did not survive are shown in Figure 2.

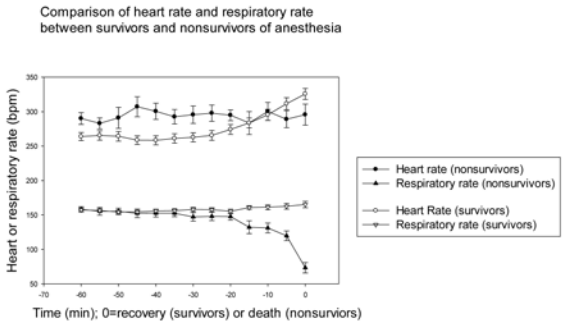

Figure 1. Heart rate and respiratory rate (mean ± SE) of mice in all redosing groups. Time 0 is the time to return of the RR for survivors or the last recordable time point (that is, 5 min) prior to the onset of agonal breathing in mice that died. *, Significant ($P \leq 0.05$) difference between time points in the nonsurviving mice when compared with all time points from –60 min through –35 min. +, Significant ($P \leq 0.05$) difference between time points in the surviving mice when compared with all time points from –60 min through –25 min.

Anesthetic reversal. Of the 7 mice that received atipamezole reversal after exhibiting agonal respiration, 43% (*n* = 3) went on to recover from anesthesia, whereas the remaining 57% (*n* = 4) progressed to death. In the mice that did not recover, death occurred within 10 min after administration of the reversal agent. The mice that recovered displayed a rapid increase in heart rate (658 ± 38 bpm) compared with the animals that did not successfully recover (331 ± 23 bpm) at 5 min after reversal. The same trend was seen for respiratory rate, with survivors having higher rates (212 ± 25 breaths per minute) compared with nonsurvivors (32 ± 13 breaths per minute) at 5 min after reversal. For the 3 animals that recovered, average time to return of the RR from the time of reversal administration was 27.3 ± 9.9 min (range, 13 to 41 min). The PWR was not evaluated after the administration of atipamezole.

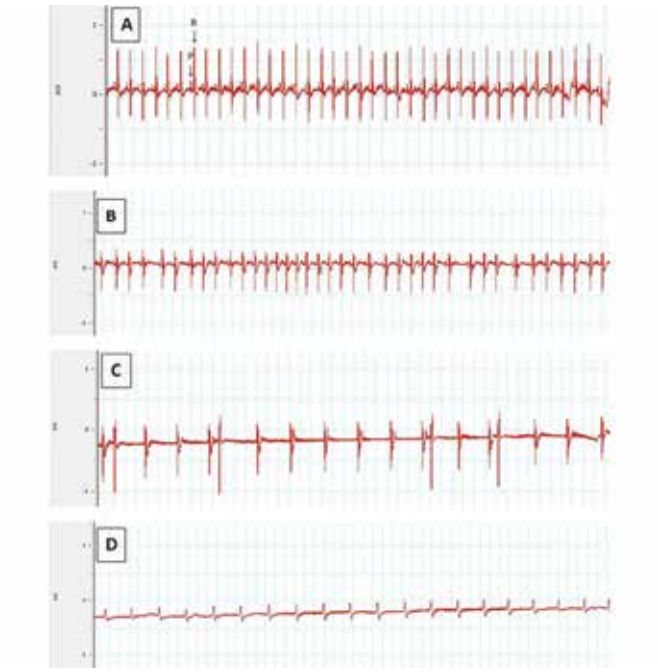

Figure 2. Electrocardiographic tracings from a mouse in the CSP group that did not survive anesthesia. (A) Sinus bradycardia (296 bpm) associated with KXA anesthesia. (B) Decrease in heart rate (253 bpm) and R wave amplitude after 0.25K redose at the onset of agonal breathing. (C) Continued progression of bradycardia (172 bpm) and an absence of P waves after the onset of agonal breathing. (D) After clinical declaration of death on the basis of respiratory arrest. The normal P and R wave morphology and their typical temporal relationship are illustrated in tracing A.

Effect of ketamine administration on heart rate. In the 0.5K and 1.0K dosing groups, 25 of the 30 mice in the main redosing study had a decrease in heart rate after the redosing with ketamine as a sole agent. On the basis of this observation, we tested the effect of ketamine administration on the heart rates of mice. In the mouse tested to determine the effect of the ketamine injection on the body temperature after ketamine injection, the rectal temperature decreased 2.2 °C within 25 min after ketamine injection. The average heart rate was significantly ($P < 0.05$) lower in ketamine group than the control group at both 5 and 10 min after the injection (Figure 3). Thereafter, the mean heart rate approached the baseline and did not differ significantly between the 2 groups. Ketamine-treated mice exhibited decreased alertness and sustained episodes of clonic muscle activity. All mice recovered to consciousness without incident in these experiments.

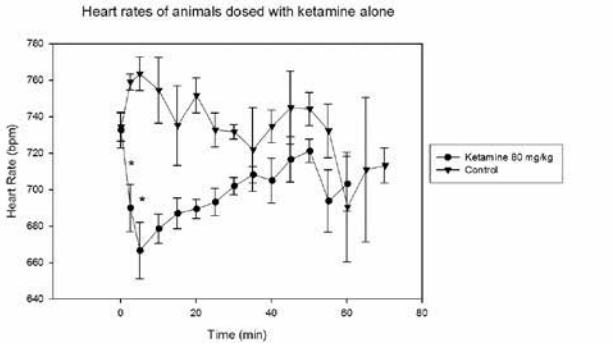

Figure 3. Heart rate (mean ± SE) over time after the administration of a single dose of ketamine only ($n = 6$) or of saline (control, $n = 4$) to awake mice. Time 0, time of injection. *, Value significantly ($P \leq 0.05$) different from that for control (saline-injected) mice.

Discussion

In the current investigation, we assessed the common practice of intraperitoneal administration of injectable anesthetics to extend the surgical plane of anesthesia in C57BL/6J mice. We aimed to determine an ideal repeat dosing regimen that maximized the duration of the extension of the surgical plane of anesthesia yet minimized negative anesthetic side effects. We hypothesized that both the amount and time of administration of the drugs would affect the response to the anesthetics. Through the analysis, we determined that the protocol of administering either 50% of the initial dose of ketamine (0.5K regimen) or 25% of the initial dose of ketamine and xylazine combined (0.25KX regimen) after the return of the PWR (for example, for the ISP groups) optimized the extension of the surgical plane with minimal mortality. We also found that administration of additional bolus anesthesia while the mouse was still at a surgical plane of anesthesia resulted in approximately 50% mortality, independent of the dose administered. These results are important because they are the first to provide empirical evidence on which to base clinical guidance for the extension of a surgical plane of anesthesia using KXA in mice.

The 0.25KX ISP redose regimen, in which no mice failed to return to a surgical plane of anesthesia and only 1 of the 8 mice died after the second bolus of anesthetic, was the most successful redosing protocol. In addition, the 0.5K protocol was comparably safe, and only 1 of the 7 surviving animals failed to return to a surgical plane of anesthesia. This group also had a shorter recovery time to return of the RR than did the 0.25KX animals. The 0.25K ISP protocol resulted in only 4 of 8 mice returning to a surgical plane of anesthesia, demonstrating that this is an inadequate regimen for this purpose. Redosing with either 1.0K or 0.5KX in the ISP strategy returned 100% of mice to a surgical plane, but these protocols were associated with 27% and 18% mortality rates, respectively. The total failure rate (deaths combined with failures to reach a surgical plane of anesthesia) of these 2 protocols is similar to the failure rate of the 0.5K ISP protocol, but the increased deaths among the 1.0K and 0.5KX groups makes this more problematic than failing to return to a surgical plane of anesthesia after redosing. The recommendation of redosing with either 0.5K or 0.25KX at the time of the return of the PWR must be considered a starting point for the biomedical community to test in their specific experimental designs. Experimental factors to consider when selecting an anesthetic regimen include the strain of mice; their sex, age, and health status; and the amount of surgical stimulation anticipated. We believe that the surgical stimulus was likely lower in intensity in our study compared with many surgical procedures, and this intensity will affect anesthetic responses, potentially increasing or decreasing the efficacy of various anesthetic protocols.[19,40,50] When using an ISP protocol, it is important to consider the time required for the mouse to return to the surgical plane of anesthesia after the redosing injection; continued surgical manipulation should not occur until loss of the PWR is noted in a redosed mouse. In the current study, the average time required to return to the surgical plane was approximately 5 min, but this time frame may be highly variable between animals and experimental protocols.

The advantage of administering additional anesthetics prior to return of the PWR is an uninterrupted surgical plane that eliminates the likelihood of patient awareness or perception of pain during the procedure. In the current investigation, approximately 50% mortality occurred when mice were redosed while under a surgical plane of anesthesia, independent of the redose of drug administered. This outcome demonstrates that the plane of anesthesia at the time of drug redosing is critical in predicting the animal's response to the anesthetics and that a more profound effect, consistent with the narrow therapeutic index of anesthetic drugs, occurs when drugs are readministered during a surgical plane of anesthesia. It is very challenging to assess subtle differences in the depth of anesthesia in mice due to the lack of a direct way to clinically detect the fraction of the initial drug that an individual animal has metabolized, redistributed, or eliminated, ultimately resulting in considerable risk of anesthetic overdose. A helpful adjunct for evaluation of depth of anesthesia may be parameters derived from the EEG. This monitoring technique allows for direct assessment of activity with the CNS. The use of EEG in anesthetic monitoring and its limitations are reviewed elsewhere.[38] For EEG to be considered a true reflection of depth of anesthesia in mice, additional investigation is necessary for this species with different types of anesthetic drugs, doses, and combinations. If validated, these measures could prove useful in determining effective anesthetic regimens. However, it should be noted that EEG reflects cortical evidence of anesthetic drug effects, whereas our definition of the surgical plane as loss of the PWR more closely reflects anesthetic drug effects on the spinal cord. The measures of movement may or may not be congruent with processed EEG indices.

Lack of movement in response to noxious stimuli is the veterinary standard for detection of a surgical plane of anesthesia, due to its ease of evaluation and confidence that it provides that the animal is not experiencing any pain or distress. The PWR measured by movement away from a consistent 300-g stimulus provided an objective measure for our studies, but this stimulus is likely less severe than is that incurred by other procedures, particularly invasive surgery. An important factor for the biomedical community to consider when using these guidelines is that the amount of surgical stimulation is a critical factor in determining the anesthetic requirement.[31,49]

Consistent with previous work,[3] the mice in our study demonstrated profound variability in response to the different drug regimens in the current investigation, despite controlling for the strain of mouse, sex, age, health status, circadian rhythm, and stimulation while under anesthesia. The mice had a wide array of responses, which ranged from mice not reaching a surgical plane of anesthesia to mice remaining at a surgical plane of anesthesia for 25 to 90 min and further to mice dying from this initial administration. Another example of the variable response to the anesthetics is demonstrated in the 0.25K CSP group, in which 1 mouse failed to maintain a surgical plane of anesthesia and half of the group had fatal anesthetic outcomes. Demonstrating similar variability, another study[3] tested 8 different anesthesia protocols. Three protocols had some percentage of mice fail to reach a surgical plane of anesthesia, whereas some mice reached a surgical plane of anesthesia and recovered, and some died.[3] This documented variability, along with our findings herein, verifies that there is no specific and ideal regimen for injectable anesthetics that can be applied universally to laboratory mice. Laboratory animal specialists and biomedical scientists must be flexible and, using the current recommendations as a guide, plan to adapt anesthesia protocols to specific procedures and the responses of individual animals.

The cause of the deaths in the current study was not definitively determined, but respiratory depression undoubtedly was a major contributing factor based on anesthetic monitoring prior to cardiac arrest. The mice that died had a significant drop in respiratory rate 10 to 15 min before arrest (Figure 1), whereas surviving mice maintained a constant respiratory rate before regaining consciousness. Both ketamine and xylazine are known to decrease tidal volume and respiratory rate;[4,7,12,18,20,24,29,36,41] however, we cannot rule out that the respiratory changes are a secondary marker of the actual cause of death. A trend toward higher heart rates in nonsurvivors may be indicative of a compensatory change in response to hypotension, and although no definitive statement can be made to this end given that no blood pressure measurements were obtained from these animals, these data are suggestive of a link between changes in the cardiovascular system and death.

The period of respiratory depression before cardiac arrest presents a potential window, based on an easily measured parameter, when intervention to attempt to save animals for which death is imminent may be possible. In fact, the administration of atipamezole alone, which reverses only the effects of the α-agonist xylazine, led to the recovery of 43% of the mice that displayed agonal breathing, an advanced state of respiratory depression. Earlier and more aggressive intervention may lead to a higher success rate in avoiding mortality in these mice. Other possible pharmacologic interventions that may address the underlying cause of death can be evaluated in future studies, including assessments of parasympatholytic agents such as atropine, sympathomimetic agents such as ephedrine, and the respiratory stimulant doxapram. In light of these observations, monitoring respiratory rate may prove to be a valuable predictor of anesthetic complications in mice receiving injectable anesthetics.

Work in other species has shown that ketamine administration in normal animals increases the heart rate due to the stimulation of the sympathetic nervous system.[32] We were surprised to see that the heart rate frequently decreased after the administration of ketamine alone in the redosing protocol. We evaluated the effects of ketamine alone on heart rate in mice; unexpectedly, there was a significant decrease in heart rate after ketamine injection compared with a saline injection. Ketamine is known to depress myocardial function, but this effect is usually overcome by sympathetic stimulation.[42] In addition, some of the cardiovascular depression seen in critically ill patients has been attributed to the depletion of catecholamine stores after release induced by ketamine.[46] If mice in the current experiment had complete depletion of catecholamine stores prior to redosing with ketamine, the direct myocardial depressive effects may have contributed to anesthetic complications. Because of their intact catecholamine stores, healthy mice that received only ketamine should not have demonstrated this effect, yet a decrease was noted. Although the decrease in heart rate in the ketamine-only group was statistically significant compared with controls, what contribution this transient decrease in heart rate has on the adverse events associated with repeat-bolus dosing is unknown.

When evaluating the surgical plane of anesthesia in animals, it is important to remember that the motor response to a noxious stimulus is mediated at the level of the spinal cord and may not necessarily reflect perception or awareness.[2,33-35] The ultimate goal of anesthetic use in experimental animals is to prevent awareness and any pain perception. It is therefore important to acknowledge that even though the surgical plane may have dissipated as evidenced by return of the PWR, it does not nec-

essarily imply that the animals actually emerged sufficiently to regain consciousness or form memories. We caution that the administration of additional anesthetics while animals are still at a surgical plane of anesthesia may lead to unacceptable mortality rates.

In summary, when repeat-bolus dosing is used to extend the surgical plane of anesthesia in mice, we recommend frequent assessment of the PWR and redosing only once the reflex has returned. We recommend providing either 50% of the initial ketamine dose or 25% of the initial combined ketamine–xylazine as an effective and relatively safe protocol to extend the surgical plane of anesthesia in C57BL/6 mice.

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