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Evaluating Intrahepatic and Intraperitoneal Sodium Pentobarbital or Ethanol for Mouse Euthanasia

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Intraperitoneal (IP) injection of sodium pentobarbital (PB) is an accepted method of euthanasia for mice. However, this method has important drawbacks, including the potential for pain or misinjection. The objective of this prospective, randomized, blinded study was to determine whether intrahepatic (IH) injection of PB is more effective than IP delivery for mouse euthanasia. Secondary objectives were to: 1) determine whether IP ethanol (ET) is a suitable alternative to PB and 2) study the effect of isoflurane anesthesia on euthanasia with either PB or ET. Eighty adult CD1 mice were randomly assigned to 6 different treatment groups, were euthanized by using IP or IH injections of either PB or ET, and were either anesthetized or conscious before injection. Variables of interest were: 1) misinjection rates (based on necropsy evaluation), 2) time from injection to apnea and 3) time to cessation of heartbeat (CHB). The misinjection rate for IH injections was 93% (28/30). Two successful IH injections resulted in death within 4 s, but this method cannot be recommended due to the possibility for intrathoracic injection (n = 4). In nonanesthetized mice, time to apnea and CHB was significantly shorter with IP ET (apnea: 72.5 s [median], CHB: 115 s) than with IP PB (apnea: 136 s, CHB: 176 s). Anesthesia at time of injection was associated with a shorter CHB time for IP PB. These data show the difficulty in achieving successful IH injections in mice, but confirm that IP ET is a viable and potentially superior alternative to IP PB. Lastly, anesthesia can shorten time to death after IP injection of PB.

Abbreviations: CHB, Cessation of heartbeat; IH, Intrahepatic; ET, Ethanol; IP, Intraperitoneal; PB, Sodium Pentobarbital

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Euthanasia is a commonly performed laboratory procedure, with many different techniques available for mice. Intraperitoneal (IP) injection of sodium pentobarbital (PB) is a method accepted by both the American Veterinary Medical Association (AVMA) and Canadian Council on Animal Care.[3,5] IP injection is a relatively simple procedure that allows for substances to be administered and absorbed rapidly.[20] However, this technique has some disadvantages.

First, the IP misinjection rate varies from 10% to 20% in mice.[1,6,10,22,23] Misinjections occur when the injectate is delivered into an abdominal organ or into the subcutaneous space instead of into the peritoneal cavity. In mice, most misinjections are administered into the stomach, intestine, uterine horn, and the skin.[19,22] The result of a misinjection is often a failure to achieve death or a significant delay in the cessation of breathing and heartbeat, requiring either an additional injection or an alternative method of euthanasia.[1,2,25,27]

Second, IP injections have the potential for pain and distress. IP injections of saline have been associated with increases in plasma corticosterone levels,[4,21] tachycardia and hyperthermia,[7,14,18] and the expression of immediate early response genes, such as cfos.[21] Nociception has also been associated with IP injections, as a result of needle entry and potentially from the alkalinity (pH 10 to 12) of PB, which may be irritating.[20,25] Pain-related behaviors, such as vocalizations, writhing, hunched posture, flinching and increased locomotion, have all been observed after IP injections of PB in rats and mice.[1,9,26] Electrical activity in the brain[12] and neuronal markers[24] have also been used to infer the existence of pain and nociception after IP injections of PB. Although adding a local anesthetic, such as lidocaine, can alleviate some of these responses in rats, it does not remove them entirely.[13,24] While the temporal relationship between nociception, pain, and loss of consciousness is not always apparent, a significant number of animals nonetheless risk experiencing pain given the inherent variability in the onset of anesthetic effect, time to death and the frequency of misinjections.

Therefore, continued evaluation of alternative methods of chemical euthanasia in mice is warranted. This can be done by evaluating alternative injection techniques and/or alternative injectable euthanasia agents. Intrahepatic (IH) injection has been described as effective for the euthanasia of shelter cats.[11] IH injections resulted in a significantly shorter time to recumbency, loss of pedal reflex, and cardiac arrest. Cats with successful IH injections confirmed at necropsy showed immediate recumbency postinjection. Therefore, IH is a method that may be useful in other species, given the potential for rapid induction of unconsciousness and death.

Few alternative injectable agents for PB have been extensively studied, but one that shows promise is ethanol (ET). ET is already described as 'acceptable with conditions' for mice in the AVMA guidelines on euthanasia; however, few studies have investigated its use.[1,8,15-17] ET has an important advantage over PB in that it is more readily available than barbiturates.[3]

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One study found no significant difference in times to respiratory and cardiac arrest (estimated by time to asystole using ECG) with either IP ET (100%, approximately 15.3 g/kg) or IP PB (approximately 5.4 g/kg) in mice.[1] Furthermore, this study found no differences in pain-related behaviors (vocalizations, writhing, and hunched posture) between mice injected with ET or PB, and concluded that it was an acceptable alternative to PB.[1] In contrast, another group reported that ET should be limited to mice over 35 d of age because the time to death in younger mice (estimated as 2 min post apnea) and time to loss of consciousness (measured using the righting reflex) exceeded the times obtained with PB.[8]

The objective of the current study was to test IH injection as an alternative to IP injection in mice. We hypothesized that IH PB or IH ET would result in a shorter time to respiratory and cardiac arrest death than IP-injected PB or ET. Two secondary objectives were to: 1) confirm the earlier findings[1] that IP ET is a viable alternative to PB by using a more specific method (auscultation) to assess time to death, and 2) evaluate the effect of performing IP overdose with PB or ET in animals anesthetized with isoflurane. We hypothesized that the efficacy of IP ET would be similar to that of IP PB and that anesthesia would prolong the time to apnea and death.

Materials and Methods

Study Design. This was a prospective, randomized, blinded study. The animal use protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Medicine (Université de Montréal protocol ID: 17-Rech-1892).

Eighty adult, SPF, CD1 male and female mice (mean weight, 26.8g; range, 23 to 34g) were used. Mice were purchased from a commercial vendor (Charles River Laboratories, Senneville, Quebec) for an unrelated project and scheduled for euthanasia. For the primary objective and outcome measures of determining time to apnea and death, a sample size estimate of 60 animals (n = 15 per treatment group, (α of 0.05, 80% power, effect size 0.6) was determined using pilot data and data from the literature, including a potential 20% misinjection rate.[1,27] For the secondary objectives, with the same outcome measures, a sample size estimate of 10 animals (α of 0.05, 80% power, effect size 0.6), including a potential 20% misinjection rate, was determined based on data from the literature.[1]

Animals were housed in groups of 5 and were not habituated to handling before the experiment. Cages were ventilated with HEPA filters and contained wood chip bedding (Betachip, Charles River Laboratories, Senneville, Quebec) and cage enrichment (Cotton squares). The housing environment was controlled: 12h light-12h dark cycle (lights on at 0700 h), room temperature 22 °C, humidity from 30% to 35%. All mice had access to tap water and food ad libitum (rodent food 5075, Charles River Laboratories, Senneville, Quebec) and had a visual health inspection twice daily. All procedures were performed between 1600 and 1900.

Treatment groups. In the first part of the experiment, anesthetized mice were block randomized to one of 4 treatment groups (n = 15 per group): 1) IH injection of PB (IH PB), 2) IH injection of ET (IH ET), 3) IP injection of PB (IP PB) and 4) IP injection of ET (IP ET). Immediately before injection, all mice were anesthetized (isoflurane, vaporizer setting of 5% in 2 L/min of oxygen) in an induction chamber (27 (L) × 12.5 (W) × 12.5 cm (H)). Appropriate depth of anesthesia was confirmed by loss of the withdrawal reflex (toe pinch with tissue forceps) before the treatment injection was administered.

In the second part of the experiment, mice were not anesthetized prior to injection. Mice were block randomized to one of 2 treatment groups (n = 10 per group): 1) IP injection of ET (IP ET_{awake}) and 2) IP injection of PB (IP PB_{awake}).

Injection protocol. For all injections, the dose of PB used was 5.4 g/kg (Dorminal, Rafter 8 Products, Calgary, Alberta, 240 mg/mL) and the concentration of ET was 96%. The PB dose was selected to provide a similar injectate volume for both treatments (approximately 0.55 mL). Blue food dye (0.01 mL, Club House, Burlington, Ontario) was added to each volume of injectate to facilitate necropsy evaluations of injectate distribution.

The injectate was prepared in a 1 mL syringe and a new hypodermic needle used for each injection (25g, 5/8inch). Injections were performed using a 2-person technique, an injector and a holder. For IP injections, the holder maintained the mice in horizontal dorsal recumbency by gently gripping the scruff (loose skin over shoulders) and stabilizing the tail against the palm (with the little finger). The injector inserted the needle into the right caudal quadrant of the abdomen at the approximate level of the coxofemoral joint, midway between midline and the lateral abdominal wall. The needle tip was directed cranially at a 20° angle to the body wall.

IH injections were performed using a small reusable stop that was fashioned out of a needle cap by piercing it with a 20g needle. The cap was then threaded over the needle to shorten its usable length to 3/8 in. This was done to reduce the risk of needle insertion and injection into the thorax, with the length selected based on multiple necropsy evaluations of liver location and thickness in the pilot study. The holder held the animal vertically (head upward) using the technique described IP injection. The injector located the xyphoid process by digital palpation immediately before introducing the needle in the midline, with the needle tip directed cranially at an angle (approximately 45 to 75° relative to the long axis of the body wall).

Both the holder and the injector were blind to treatment allocation. After the injection, an observer started a timer, and the holder monitored the presence of a heartbeat (thoracic auscultation) and respiratory movement (visual assessment). The times from injection to apnea and injection to cessation of heartbeat (CHB) were recorded.

For the procedures in awake animals (IP ET_{awake} and IP PB_{awake}), additional monitoring was performed with an ECG to assess the time to asystole. Atraumatic skin electrodes were placed immediately before injection on each of the thoracic limbs and the left pelvic limb.

For all animals, if death did not occur within 5 min, the attempt was classified as a failure and the animal was euthanized with an overdose of CO_2 gas according to the AVMA Guidelines for Euthanasia.[3]

Necropsy examination. After death, each animal was necropsied and evaluated by a single observer who was blind to treatment. A midline abdominal and thoracic incision was made and the body cavity examined for the distribution of injectate. Examination included removal of the liver and intestines. The intestines were opened to determine injectate distribution. The inner surface of the abdominal wall and ventral abdominal subcutaneous tissues were also examined. Based on necropsy, injectate location was classified as: intraperitoneal (no evidence of injectate within an organ or the skin), intrahepatic (injectate distribution restricted to the liver) or misinjection (injectate present at unintended site). The latter classification differed depending on the treatment group: for IP groups, misinjections included subcutaneous, intramuscular, intraorgan, and

intrathoracic locations, whereas misinjections in the IH groups also included the peritoneal cavity.

Statistical methods. Statistical analyses was performed using commercial software (GraphPad Prism v.8.02, GraphPad Software La Jolla, CA). A Shapiro–Wilks test determined that data were not normally distributed. Comparisons of time to apnea, to CHB, to asystole between IP ET and IP PB groups (anesthetized and awake) were analyzed with a Mann–Whitney test. The effect of anesthesia was compared with a Kruskal–Wallis test with Dunn post hoc test (IP ET compared with IP ET_{awake}, IP PB compared with IP PB_{awake}). Agreement between CHB (assessed with a stethoscope) and asystole (assessed with an ECG) was assessed with Bland–Altman analysis (time to CHB [criterion method] subtracted from time to asystole) with data pooled for treatment (IP ET_{awake} and IP PB_{awake}) and time to achieve each outcome (CHB or asystole) compared with a Wilcoxon test. Data presented as median and 10 to 90 percentile in figures and median (range) in the text. Values of $P < 0.05$ were considered significant. Data supporting the results are available in an electronic repository: https://doi.org/10.7910/DVN/DLWLOI.

Results

Misinjections. For the anesthetized mice, misinjections were as follows: IP ET ($n = 0$), IP PB ($n = 3$, subcutaneous), IH ET ($n = 2$, intrathoracic; $n = 1$, intramuscular; $n = 12$, intraperitoneal), IH PB ($n = 1$, intrathoracic; $n = 1$, intrapulmonary; $n = 11$, intraperitoneal). For awake mice, misinjections was as follows: IP PB_{awake} ($n = 1$, subcutaneous), IP ET_{awake} ($n = 0$). Therefore, the incidence of misinjections reported as percentages were: IP ET 0%, IP PB 20%, IH ET 100%, IH PB 86.7%, IP PB_{awake} 10%, IP ET_{awake} 0%.

Of the 2 successful IH injections (both from the IH PB group), time to CHB occurred almost instantaneously (within the time to place the stethoscope on the thorax). The 4 intrathoracic misinjections resulted in death (time to CHB of 2, 22, 27 and 28 s). The intramuscular injection failed to achieve CHB within 5 min, so the animal was killed with CO_2. IH misinjections, where IP placement occurred when IH was intended, resulted in apnea in 44.5s (12 to 91) and 72s (27 to 105) for IH ET and IH PB groups, respectively. CHB was achieved in 123.5s (63 to 264) and 124s (88 to 222) for IH ET and IH PB groups, respectively.

Of the 4 misinjections from the IP treatment groups, 3 resulted in death (CHB in 146, 154 and 265s). One misinjection failed to result in death within 5 min and overdose of CO_2 was performed. The IP misinjections were excluded from further analysis, leaving the following group sizes for analysis: IP ET ($n = 15$), IP PB ($n = 12$), IP ET_{awake} ($n = 9$) and IP PB_{awake} ($n = 10$). Statistical analysis of the IH treatment groups could not be performed because of the high misinjection rates and these data were therefore excluded from analysis.

Comparison of IP injections of ethanol and pentobarbital. IP ET was superior to IP PB in quickly achieving apnea and CHB. Time to apnea was longer in the IP PB group than the IP ET group for both anesthetized ($P = 0.009$) and awake ($P = 0.023$) states (Figure 1). Similarly, time to CHB was also longer with IP PB when anesthetized ($P = 0.045$) or awake ($P = 0.010$, Figure 2). The time to asystole was longer in the IP PB_{awake} than the IP ET_{awake} group ($P = 0.047$, Figure 3).

Effect of using ECG compared with thoracic auscultation to confirm death. One mouse was excluded from the IP PB_{awake} group due to technical failure to record asystole. The time to achieve CHB was significantly shorter than the time to reach asystole in both IP ET_{awake} and IP PB_{awake} groups ($P = 0.004$ both comparisons, Figure 4). Time to CHB underestimated time to asystole (bias: 170s, limits of agreement -3.1 to 344s, Figure 5).

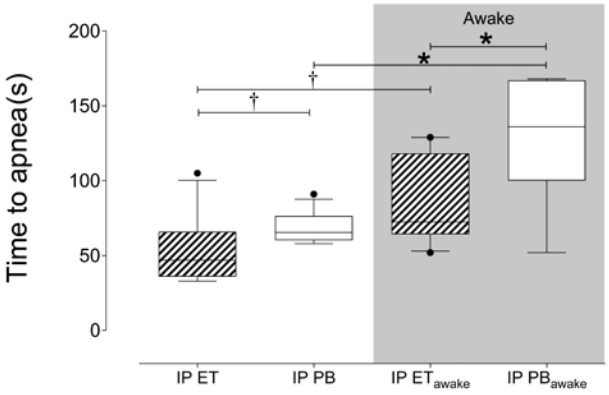

Figure 1. Box and whisker plot of the time to apnea (seconds). The horizontal line within each box represents the median, the lower and upper box limits represent the interquartile range and the whiskers represent 10-90 percentile. Treatment groups are: intraperitoneal (IP) injection, with sodium pentobarbital (PB) or ethanol (ET). Anesthetized groups are located in the nonshaded area, awake groups are located in the gray shaded box. Significant differences are indicated by * ($P < 0.05$) or † ($P < 0.01$).

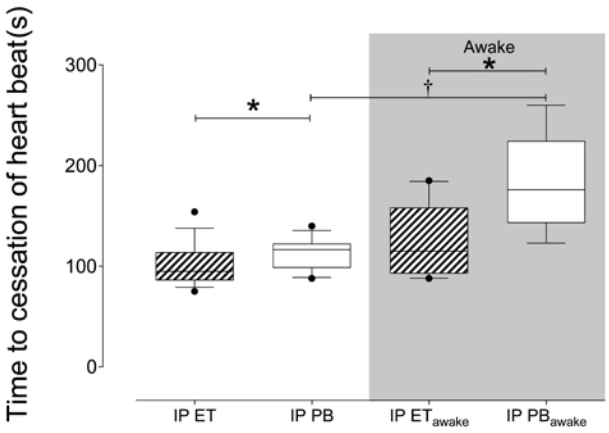

Figure 2. Box and whisker plot of the time to cessation of heartbeat (CHB, seconds). The horizontal line within each box represents the median, the lower and upper box limits represent the interquartile range and the whiskers represent the 10-90 percentile. Treatment groups are: intraperitoneal injections (IP), with sodium pentobarbital (PB) or ethanol (ET). Anesthetized groups are located in the nonshaded area, awake groups are located in the gray shaded box. Significant differences are indicated by * ($P < 0.05$) or † ($P < 0.01$).

Effect of anesthesia. Time to apnea was achieved more quickly in anesthetized animals in comparison to awake animals (IP ET *compared with* IP ET_{awake}: $P = 0.007$, IP PB *compared with IP* PB_{awake}: $P = 0.019$, Figure 1). Time to CHB was achieved more quickly in the IP PB than IP PB_{awake} group ($P = 0.008$, Figure 2). Time to CHB was not significantly different between anesthetized and awake groups given IP ET injections ($P = 0.182$, Figure 2).

Discussion

The main findings of this study are: 1) IH injection, with the technique employed, cannot be recommended due to the risk of inadvertent intrathoracic drug delivery, 2) confirmation that IP ET is a valuable alternative to IP PB and results in a shorter time to death than previously identified and 3) IP injection of PB and ET resulted in a faster death in animals anesthetized with isoflurane than awake animals.

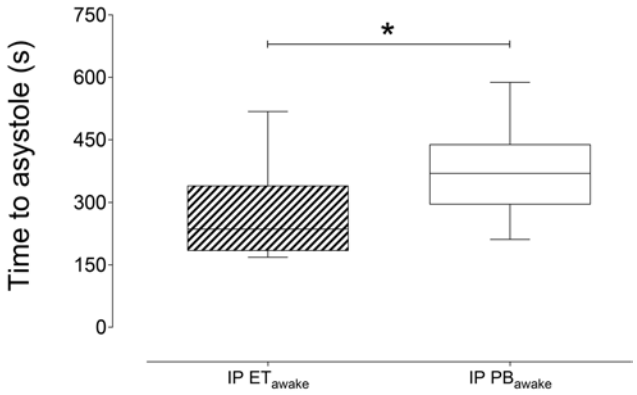

Figure 3. Box and whisker plot of the time to asystole (seconds). The horizontal line within each box represents the median, the lower and upper box limits represent the interquartile range and the whiskers represent the 10-90 percentile. Treatment groups are: intraperitoneal injections (IP), with sodium pentobarbital (PB) or ethanol (ET). Mice were not anesthetized prior to injections. Significant differences are indicated by * ($P < 0.05$).

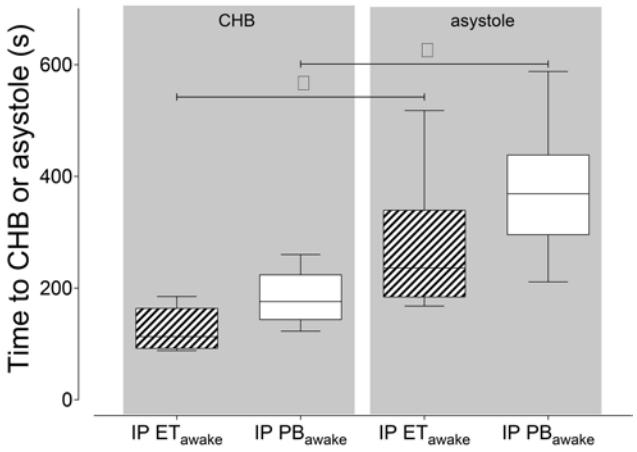

Figure 4. Box and whisker plot of the time to cessation of heartbeat (CHB) or asystole (seconds). The horizontal line within each box represents the median, the lower and upper box limits represent the interquartile range and the whiskers represent the 10-90 percentile. Treatment groups are: intraperitoneal injections (IP), with sodium pentobarbital (PB) or ethanol (ET). Mice were not anesthetized prior to injections. Assessments of CHB are located in the left shaded area and assessments of asystole are located in the right shaded area. Significant differences are indicated by † ($P < 0.01$).

IH injections have been used with success in cats.[11] One study evaluated and compared efficacy, accuracy (via assessment of misinjections in necropsy) and response to pain (defined as vocalization or turning head toward the injection at the moment of injection) of IH injections and IP injections in adult cats.[11] IH injections (n = 85) produced a significantly quicker onset of effects (recumbency, loss of pedal reflex and cardiac standstill) than IP injections (n = 77). Moreover, it was reported that successful IH injections caused immediate recumbency. Successful IH injections represented approximately 24% of the 85 intended IH injections. A further 27% were categorized as IH and IP (signs of hepatic and peritoneal delivery were both present), 32% were categorized as only IP (with no signs of hepatic delivery), and the remainder were in the thoracic cavity or intra muscular. Lastly, pain-responses were observed in 8/85 (9%) animals injected IH, compared with 4/77 (4%) of animals injected IP.

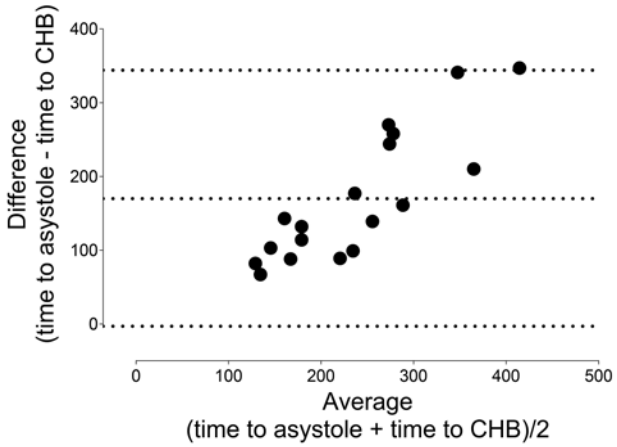

Figure 5. Bland-Altman plot comparing time to cessation of heartbeat (CHB) and time to asystole. Time to CHB underestimated time to asystole by 170s with limits of agreement ranging from -3.1 to 344s. Data were pooled from awake animals administered intraperitoneal (IP) pentobarbital (PB) or ethanol (ET).

The IH misinjection rate observed in the current study is markedly higher than the misinjection rate typically reported for IP injection in rodents (6% to 20%).[1,6,10,22,23] Although the majority of IH misinjections still yielded successful and rapid euthanasia, reflecting the IP placement of injectate in many cases, 4 were intrathoracic injections. Intrathoracic injections are limited to use in anesthetized animals due to the possibility of pain associated with this technique.[3] As a result, we cannot recommend the IH route of injection in conscious mice due to the potential for intrathoracic delivery. Furthermore, given the low proportion of successful IH injections, there are no apparent benefits over IP ET. Perhaps a change in IH injection technique, such as using a shorter needle or changing the insertion angle or site, could yield better results.

The second objective of this study was to evaluate the efficacy of ethanol as an alternative agent to PB. One group found ethanol to be as efficacious as PB in terms of the onset of anesthetic effect (loss of righting reflex), apnea and time to death (as indicated by asystole).[1] Our results differ in that time to apnea and time to CHB were both significantly shorter for ET than for PB. For the time to apnea, the difference in awake animals was small and unlikely to be clinically important. The difference between groups in time to CHB was also small, but the technique used to identify cardiac arrest was more accurate and precise. This is shown by the reduced variability observed in the CHB data compared with the asystole data. In addition, relying on the ECG alone to diagnose CHB is misleading as pulseless electrical activity gives the impression of continued cardiac function in the absence of myocardial contraction. This difference was highlighted by the 2-fold difference in time when identifying CHB with auscultation compared with the presence of asystole. Together, these results confirm and build on the previous findings, further supporting for IP ET over PB for mouse euthanasia.[1]

The shorter time to achieve CHB and apnea in the anesthetized groups was an unexpected result. Our initial hypothesis was that anesthesia would prolong the time to death, based on well-established effects of isoflurane to depress cardiovascular function and consequently, injectable drug absorption and circulation. However, despite the presence of cardiovascular depression, the central nervous system depression associated with general anesthesia appear to have made it easier to achieve apnea and CHB. Further work to standardize the depth

of anesthesia and quantify cardiovascular depression, would be required to elucidate the mechanism of these observations.

Because we did not measure loss of consciousness, we cannot comment on the length of time animals may be experiencing pain, which is an important factor when discussing optimal euthanasia methods. A further limitation of our study was the absence of pain assessment. Our primary goal in evaluating a novel route of delivery was to examine feasibility as an initial step. Many difficulties are associated with measuring behavioral pain responses in the presence of a drug that depresses motor function as sedation and general anesthesia occur. Others have measured certain pain-related behaviors, including vocalization, writhing (abdominal contraction), and hunched posture and found no statistical differences in these behaviors between the mice treated with ethanol or the mice treated with PB.[1] Moreover, in that study group displaying the most signs of pain was the saline-injected group.[1] This outcome probably reflects the difficulties in assessing behavioral motor outcomes (such as those associated with pain) in the presence of sedation, as occurs after PB or ET injection.

Based on the technique employed and results, we conclude that IH injection cannot be recommended for euthanasia of unanesthetized mice. In contrast, IP ET is a viable and appealing alternative to IP PB due to its rapid action. We further found that the speed of death induced by IP PB or ET in anesthetized mice was faster as compared with awake mice.

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