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Intrahepatic Injection of Sodium Pentobarbital as an Alternative to Intraperitoneal Injection for the Euthanasia of Rats (*Rattus norvegicus*)

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The most commonly accepted method of rat euthanasia in North America is intraperitoneal injection of sodium pentobarbital (PB). However, misinjection can occur, and intraperitoneal PB may cause pain and distress. The objective of this study was to test an alternative method of euthanasia: intrahepatic injection of PB. A pilot study was conducted to develop a method of intrahepatic injections (evaluated using CT scans and test injections), followed by a full study comparing intraperitoneal (*n* = 14) and intrahepatic PB injections (*n* = 66) in adult rats. Full study outcomes were: 1) time from injection to loss of righting reflex (LORR), 2) time from injection to cessation of heartbeat (CHB), 3) number of failed euthanasia attempts, and 4) confirmation of successful intrahepatic injection or misinjection via necropsy. All injections were performed by a veterinary student. CT revealed that intrahepatic injections were feasible. Times (median [range]) to LORR and CHB were faster after successful intrahepatic injections (LORR, 3 s [1 to 5 s]; CHB, 8 s [2 to 242 s]) than after intraperitoneal injections (LORR, 89.5 s [73 to 110 s], CHB: 284.5 s [237 to 423 s]). The misinjection rate was higher with intrahepatic injections (59%) than with intraperitoneal injections (29%), but intrahepatic misinjection still resulted in fast and successful euthanasia (LORR, 29 s [1 to 96 s]; CHB, 216 s [12 to 330 s]), with the injectate distributed between the intraperitoneal and intrahepatic locations. The number of failed euthanasia attempts with intrahepatic injections was low (*n* = 2). Intrahepatic injections show potential as an alternative to intraperitoneal injections for rat euthanasia.

Abbreviations: CCAC, Canadian Council on Animal Care; CHB, cessation of heartbeat; LORR, loss of righting reflex; PB, sodium pentobarbital

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Large numbers of laboratory rats are killed after the completion of research projects worldwide. Currently, the only method that is classified as acceptable by both the AVMA and Canadian Council on Animal Care (CCAC) is an overdose of sodium pentobarbital (PB) via either intraperitoneal or intravenous injection.[3,7] Of these, the intraperitoneal route is more commonly used in rats as it is easier and faster to perform. However, intraperitoneal injection has 2 important disadvantages that interfere with whether 'euthanasia' ("a good death") is always achieved. The first disadvantage is an inherent misinjection rate that varies between 6% to 20% for rats.[5,9,11,12,21,22,27,28,33] The consequences of a misinjection is a delay or failure to achieve loss of consciousness or death.[8,30,32,33] In these cases, the injection may need to be repeated or an alternative killing method applied.[20]

The second disadvantage is the potential for pain and distress. Converging evidence for this drawback includes elevated plasma corticosterone, tachycardia, hyperthermia, expression of immediate early response genes, electroencephalographic changes, visible signs of inflammation and behavioral changes, which have all been associated with intraperitoneal injection of PB.[4,10,17,19,23,25,26,29] The source of nociception and potential pain is mostly due to alkaline pH (typically 10 to 12) of PB solution.[25]

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The CCAC and AVMA euthanasia guidelines acknowledge that intraperitoneal injections may be painful and suggest the addition of a local anesthetic, such as lidocaine. However, neuronal and behavioral studies suggest that the addition of lidocaine does not eliminate nociception and pain.[2,18,29] Furthermore, the amount of lidocaine (or another buffer) that can be added is limited. As the pH descends below approximately 10, the PB precipitates.[32] In addition, when misinjections occur, the potential for pain increases as a result of potential delay until loss of consciousness or pain resulting from the site of misinjection.[25]

These concerns indicate an ongoing need to refine the use of intraperitoneal PB or identify alternative methods to achieve euthanasia. A relatively unexplored injection route is intrahepatic injection. The current AVMA euthanasia guidelines describe intrahepatic injections as acceptable only in unconscious or anesthetized animals (with the exception of cats).[3] This method has successfully been used in conscious cats,[16] in which successful intrahepatic injection resulted in almost immediate recumbency, but intrahepatic injection of PB for euthanasia remains untested in rats. By contrast, intrahepatic injection in mice recently was shown to be unsuccessful.[21]

We first performed a pilot study, with the objectives of: 1) identifying the appropriate injection site and angle and 2) testing the feasibility of the intrahepatic injection methods in rats. These results were applied in the main study, with the objective of investigating whether the intrahepatic injection technique could be an alternative to intraperitoneal injection for euthanasia

in rats. We hypothesized that intrahepatic injection would result in a shorter time to loss of consciousness and death as compared with intraperitoneal injection, and would have fewer instances of failure to achieve death.

Materials and Methods

Ethical statement. All experiments were approved by the Université de Montréal (18 RECH-1892) and the Charles River Laboratories Montreal ULC IACUC. The study of a novel euthanasia procedure that differed from the CCAC guidelines was approved by the animal care and use committees. All rats used in the experiments had been scheduled for euthanasia for other reasons. Rats had not previously undergone other procedures.

Animals. Animals in both the pilot and main study were housed in a conventional facility with environmentally controlled conditions of 12:12-h light:dark cycle (lights on, 0700), 22 ±3 °C, and humidity between 30% and 70%. Animals were group-housed in polycarbonate flat-bottom cages with corncob bedding, nesting material, and a hiding tube. Feed (Certified Rodent Chow No. 5CR4, PMI, St Louis, MO) and municipal tap water that had been softened, purified by reverse osmosis, and exposed to UV light were freely available. Sentinel rats in the housing room tested negative for rat parvoviruses, Toolan H1 virus, Kilham rat virus, rat minute virus, protoparvovirus NS1, rat sialodacryoadenitis virus, rat theilovirus, *Pneumocystis carnii*, Sendai virus, reovirus, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, adenovirus, hantavirus, *Encephalitozoon cuniculi*, cilia-associated respritaory bacillus, rat rotavirus, *Bordetella bornchiseptica*, *Corynebacterium kutscheri*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Rodentibacter pneumotropicus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, β hemolytic *Streptococcus spp.*, *Streptococcus pneumoniae*, *Proteus mirabilis*, *Salmonella* and other bacteria and endo- and ectoparasites.

Pilot study methods. A 2-part pilot study was performed to develop a method of intrahepatic injection by identifying a technique for intrahepatic injection (part 1) and testing injection feasibility (part 2).

Part 1: Injection approach. In a terminal procedure, 4 adult Sprague–Dawley rats (3 male, 1 female; 260 to 390 g) were anesthetized with dexmedetomidine (30 µg/kg IP) and ketamine (100 mg/kg IP), followed by isoflurane (nose cone) for CT scanning. The scanned area included the thoracic and abdominal cavities, from the base of the neck to the base of the tail. Slice thickness was 1 mm, with each rat scanned in both vertical (head up) and dorsal recumbency positions; these positions were selected because they are common positions for performing intraperitoneal injection. Organ location and measurements were taken from reconstructed images. A potential needle insertion site and trajectory were determined based on these measurements, with the goal of minimizing the risk of misinjection into other organs or the thorax.

The xiphoid process was identified as a needle insertion site. The stomach and right kidney were near this site, such that these 2 organs and the thoracic cavity were identified as potential sites for misinjection. The relationships of these sites to the liver was measured (Figure 1, distance a–c, and Figure 2). To determine a potential angle of insertion and needle trajectory, distances from the xiphoid process to the cranial margin of the liver were estimated (Figure 1, distance a–b). In addition, the thickness of the liver at the injection insertion site (xiphoid process) was measured by using digital Vernier calipers at necropsy.

To determine the optimal body position (vertical or dorsal recumbency), distances between the xiphoid process and closest border of the right kidney, stomach and diaphragm (Figure 2, distance d-e, illustrating distance to right kidney) were measured. All measurements were taken in triplicate and median and range reported.

Part 2: Injection protocol. Intrahepatic injection of PB (Euthanyl 240 mg/mL, Bimeda-MTC, Cambridge, Ontario, Canada) into male (n = 8) and female (n = 11) Sprague–Dawley rats (weight: median, 515 g; range, 343 to 980 g) was performed by using the insertion site and needle angle described earlier. For all injections, the dose of PB was 800 mg/kg. Blue food dye (0.05 mL, Club House, Burlington, Ontario, Canada) was added to the PB to facilitate necropsy evaluation of injectate distribution. The order of injections was randomized by using a list randomizer (random.org). All solutions used for euthanasia were placed in 3-mL syringes, and a new hypodermic needle (25 gauge, 5/8-in., 16 mm) was used for each injection.

Injections were performed by using a 2-person technique. The holder restrained the rat using the 'backpack hold,' with one hand supporting the hindlimbs and the other hand cradling the thorax, with the index and middle finger on either side of head and the thumb and remaining fingers beneath the forelimbs). Rats were held vertically (head up; Figure 3). A single person performed all injections (veterinary student [CL]) and another individual held all the rats. The injector identified the xyphoid process by gentle digital palpation and inserted the needle immediately caudal to this point, at an angle of insertion of approximately 45° to the body wall and the needle directed toward the head. The needle was fully inserted in all cases, and the injection was given over 2 to 3 s.

Immediately after injection, loss of righting reflex (LORR) was determined by placing the rat on its back. LORR was considered to have occurred when the rat remained on its back for at least 15 s. If LORR did not occur, the rat was observed continuously until ataxia or sedation (head lowered toward floor) occurred, at which time LORR was reassessed. LORR was reassessed every 30 s until 3 min had elapsed. After LORR occurred, no further testing was performed, and the holder continuously ausculted the thorax to identify cessation of heartbeat (CHB, used as confirmation of death). Both experimenters continuously observed rats for apnea. Two outcomes were required to designate an injection as a failure: 1) the LORR did not occur within 3 min after injection and 2) the heartbeat continued beyond 5 min after injection. After these 2 conditions were met, a secondary killing

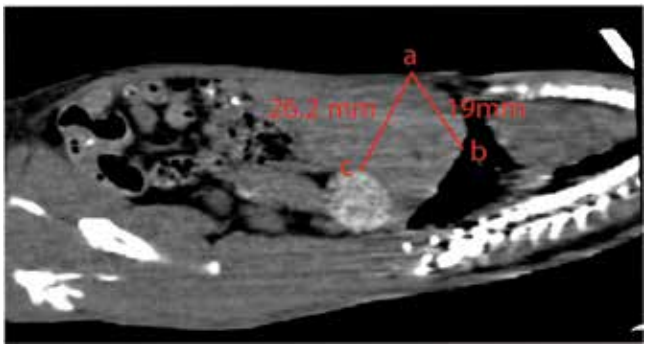

Figure 1. Sagittal CT image of the abdomen of a rat in dorsal recumbency (cranial is to the right, caudal is to the left and ventral toward the top). Shown are measurements used to determine intrahepatic injection protocol and misinjection risks. Distance a–b is an example of thickness of the liver between the injection location and diaphragm. Distance a–c is the distance between the injection location and closest border of the stomach.

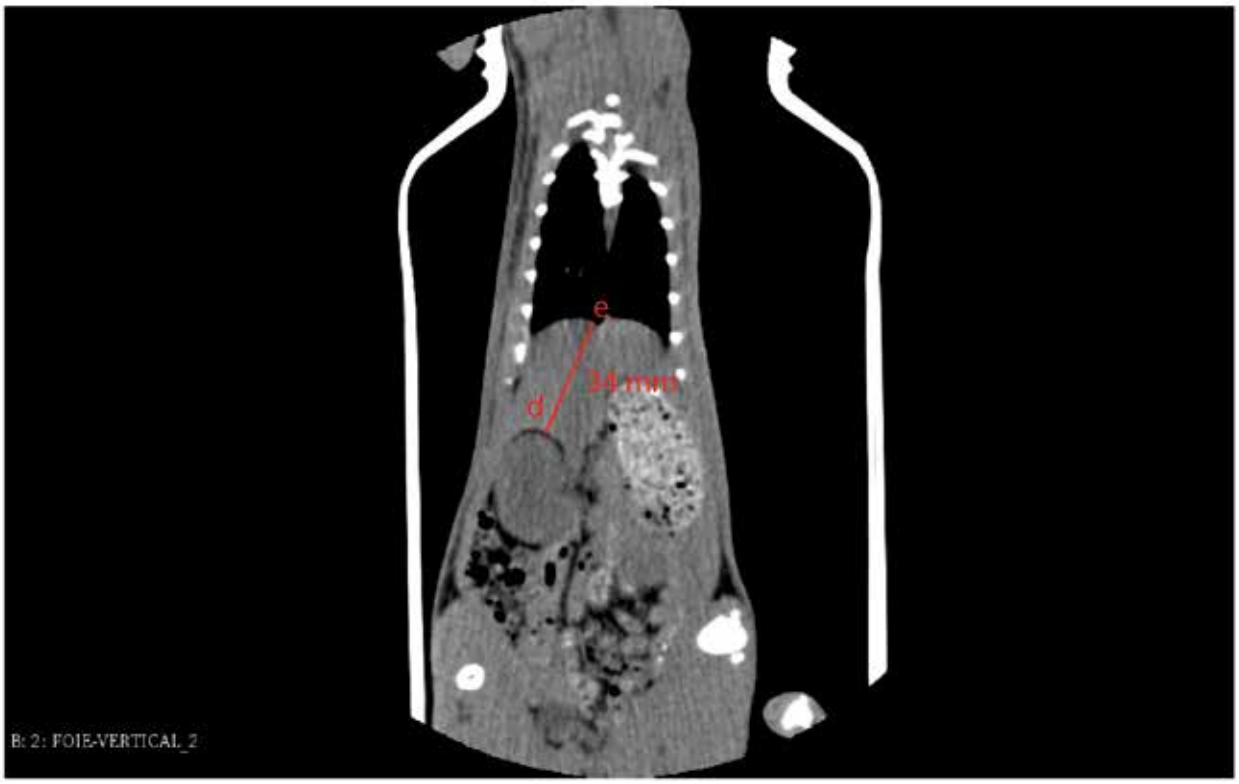

Figure 2. Frontal CT image of a rat thorax and abdomen (cranial is toward the top). The rat was in the vertical position to simulate injection position. Line d–e indicates the distance between the diaphragm and the closest border of the right kidney. This distance was greater in rats held vertically than in dorsal recumbency.

Figure 3. Illustration of the intrahepatic injection technique, showing needle insertion at midline, caudal to the xiphoid process and at 45° to perpendicular. The animal is positioned vertically (head up).

method was used (general anesthesia with isoflurane, followed by overdose with inhaled carbon dioxide).

Necropsy examination. After confirmation of death, a single observer performed a necropsy using standard procedure. With each rat in dorsal recumbency, the abdomen was incised at midline, and the interior was examined to establish injectate distribution. The liver and intestines were removed, incised, and examined for evidence of injectate (blue coloration), followed by examination of the interior abdominal wall and subcutaneous tissue. Lastly, the thorax was opened to confirm absence of injectate in the thoracic cavity.

Injectate location of intended intrahepatic injections was classified as either 'confirmed intrahepatic' (presence of injectate restricted to the liver, no sign of dye elsewhere in the abdomen) or intrahepatic misinjection (injectate was identified at any site outside the liver, e.g., in the abdominal cavity, subcutaneously, intramuscularly, inside an abdominal organ other than the liver, within the thoracic cavity). Misinjections were further classified as 'incomplete intrahepatic' when injectate was present intraperitoneally, with the possibility of some intrahepatic injectate (confirming the presence of dye within hepatic tissue with absolute confidence was difficult) but with no evidence of dye outside the abdominal cavity or within other abdominal organs.

Main study methods. The main study used 80 (66 intrahepatic and 14 intraperitoneal injections) adult male and female Sprague–Dawley rats (*Rattus norvegicus*; weight: median, 455 g; range, 160 to 894 g). Rats that weighed less than 500 g were block-randomized to receive either an intrahepatic or intraperitoneal injection. Rats weighing more than 500 g were all assigned to the intrahepatic group, with a modification of the injection technique (described later). All rats had been identified for euthanasia at a private research facility, and all originated from Charles River Laboratories. All rats had been habituated to handling, with at least once daily handling over a period of 5 to 7 d.

Sample size estimate. A sample size estimate was calculated to determine differences in failed euthanasia rate in groups defined by body mass less or greater than 500 g. The estimated sample size per group, including a potential 20% misinjection rate, was 28 for detection of a 40% difference in failure rates with a power of 0.9 and α of 0.01.[14] Our estimated sample size was greater

than that needed to detect a difference in CHB of 100 s between intraperitoneal and intrahepatic injections (calculated at approximately 9 rats per treatment group, including a potential 20% misinjection rate, with power of 0.9 and α of 0.05)[33]. Therefore, the estimated necessary number of rats given intraperitoneal injections was 9, whereas the estimated number of intrahepatic injections was 56 (28 over 500 g, and 28 under 500 g). During the study, 15 additional rats scheduled for euthanasia became available and were randomly allocated to the intrahepatic and intraperitoneal groups. The number of rats assigned to each injection group was: intrahepatic over 500 g, $n = 36$; intrahepatic under 500 g, $n = 30$; and intraperitoneal (all under 500 g), $n = 14$.

Injection protocol and necropsy examination. For intrahepatic injection, the same protocols were used as described in the pilot study, except that for rats heavier than 500 g, the angle of needle insertion was approximately perpendicular (0°) to the body wall. This modification was based on observations during the pilot study of an increased risk of intrafat injections in animals weighing more than 500 g.

For intraperitoneal injection, each rat was restrained as described earlier, except that body position was dorsal recumbency with the head angled slightly downward (approximately 20 to 25°). The injector performed both restraint of the right pelvic limb of the rat and administration of an intraperitoneal injection in the right caudal quadrant. The needle was inserted at the level of the coxofemoral joint, approximately 5 mm to the right of midline, with the needle tip directed cranially and at a 45° angle to the body wall.[31] At completion of injection, the same steps as described earlier were performed to assess LORR, time to apnea, and CHB. A necropsy was performed to identify injectate location (dye added to the injectate as described earlier), with outcomes classified as 'confirmed intraperitoneal' (presence of injectate in the abdomen) or 'misinjection' (injectate was identified in any unintended location, e.g., within an abdominal organ, intramuscularly, subcutaneously). The person performing the necropsies was blind to treatment group.

Statistical methods. Statistical analyses were performed by using a commercial statistical software package (Prism version 8.2.0, GraphPad Software, La Jolla, CA). Normality was assessed according to the Shapiro–Wilks test. Data did not approximate normal distribution. The Wilcoxon signed-rank test was used to compare the distance between the injection site (xiphoid process) to the right kidney or stomach when rats were held in the vertical or dorsal recumbency position. The Mann–Whitney test was used to compare differences between intrahepatic and intraperitoneal treatment groups for time to LORR, time to CHB, and the effect of rat weight (less than 500 g compared with greater than 500 g). The effects of injectate location (confirmed intrahepatic compared with incomplete intrahepatic compared with intraperitoneal) on time to LORR and CHB were assessed by using the Kruskal–Wallis and Dunn posthoc tests (groups compared with one another). P values less than 0.05 were considered statistically significant for all comparisons. Data are presented in the text as median (range) and in figures as median (10th–90th percentiles).The 95% CI for median differences between comparison are presented where available. Data are available in an electronic repository: https://doi.org/10.7910/DVN/XFG7YH

Results

Pilot study. The optimal intrahepatic injection site and approach was identified as midline, immediately caudal to the xiphoid process, with an angle of insertion of approximately 45° (relative to the sternum), and the needle directed cranially (Figure 3). With this approach, the thicknesses of the adjacent liver lobes were: left lateral lobe, 4.24 mm (range, 3.98 to 5.49 mm); and right medial lobe, 5.95 mm (5.53 to 7.64 mm).

The distance from the needle insertion site to the diaphragm ranged from 16 to 28 mm, with the shortest distance to the ventral border of the diaphragm. Therefore, an injection angle of less than 45° increases the risk of entering the thoracic cavity.

The distances from the needle insertion site (xiphoid process) to the right kidney and stomach were consistently greater when rats were suspended vertically than in dorsal recumbency: kidney vertical, 32.6 mm (19.3 to 34.2 mm); kidney dorsal, 18.7 mm (11.3 to 21.1 mm); $P = 0.03$, Mann–Whitney test); stomach vertical, 21.4 mm (13.3 to 26.2 mm); stomach dorsal, 5.9 mm (3.4 to 10.8 mm); $P = 0.03$, Mann–Whitney test). Therefore, vertical positioning was selected to reduce the risk of misinjection.

Of the 19 intrahepatic trials, 16 resulted in successful euthanasia. The 3 unsuccessful injections entered the falciform fat pad in larger rats (558 g, 910 g, and 980 g). For the 16 successful injections, the time to LORR was 5 s (1 to 114 s), and time to CHB was 135.5 s (8 to 360 s). Necropsy revealed 3 (16%) intrahepatic injections and 16 (84%) misinjections. Among the misinjections, 13 were incomplete intrahepatic (68%), and 3 were in the falciform fat pad (16%).

Time to LORR, grouped according to necropsy results, was: confirmed intrahepatic, 3.5 s (2 to 5 s); incomplete intrahepatic, 5 s (2 to 114 s). Time to CHB was: confirmed intrahepatic, 12 s (8 to 120 s); incomplete intrahepatic, 178 s (86 to 360 s).

Given these preliminary data, intrahepatic injection of PB into rats was determined to be feasible. Because the 3 failed euthanasia attempts occurred in larger rats, body mass may limit use of the intrahepatic route as described. Therefore, we hypothesized that increasing the angle of insertion (closer to 0° and thus perpendicular to the skin) would reduce the failure rate in larger rats.

Main study. ***Misinjections.*** In the intrahepatic group, 64 of the 66 injections performed resulted in death (misinjection rate, 3%). The 2 misinjections resulted from injection into the falciform fat pad (rat weight, 570 g and 660 g). Both of these animals achieved LORR within 3 min (43 and 73 s); however, CHB did not occur within 5 min in either case, and a secondary killing method was applied. Data from these rats were removed from further analysis (i.e., comparisons between treatment groups of time to LORR and time to CHB). In the intraperitoneal group, 10 of the 14 injections performed were confirmed intraperitoneal injections. Three of the misinjections were subcutaneous, and the remaining one was intraintestinal (misinjection rate, 29%). The body weights of these 4 rats were 311, 312, 375, and 402 g. Among the misinjections, one rat died within 5-min, 2 rats achieved LORR within 3 min, and one rat did achieve LORR. None of these last 3 rats achieved CHB within 5 min, and the secondary killing method was used. Data from these 4 rats were not included in the analysis.

Intrahepatic and intraperitoneal injections. The body weights of the intrahepatic injection groups were: intrahepatic <500 g, $n = 30$, median 337.5 g (160 to 490 g); intrahepatic >500 g, $n = 34$, median 644 g (510 to 894 g). Times to LORR and CHB for all intrahepatic attempts were 4 s (1 to 96 s) and 142.5 s (2 to 330 s), respectively (Figure 4). There were 27 (41%) confirmed intrahepatic injections and 39 (59%) misinjections. Of the misinjections, 37 (56%) were incomplete intrahepatic, and the remaining 2 (3%) were into the falciform fat pad. For confirmed intraperitoneal injections ($n = 10$; median, 292.5 g [275 to 445 g]), the times to LORR and CHB were 89.5 s (73 to 110 s) and 284.5 s (237 to 423 s), respectively (Figure 4).

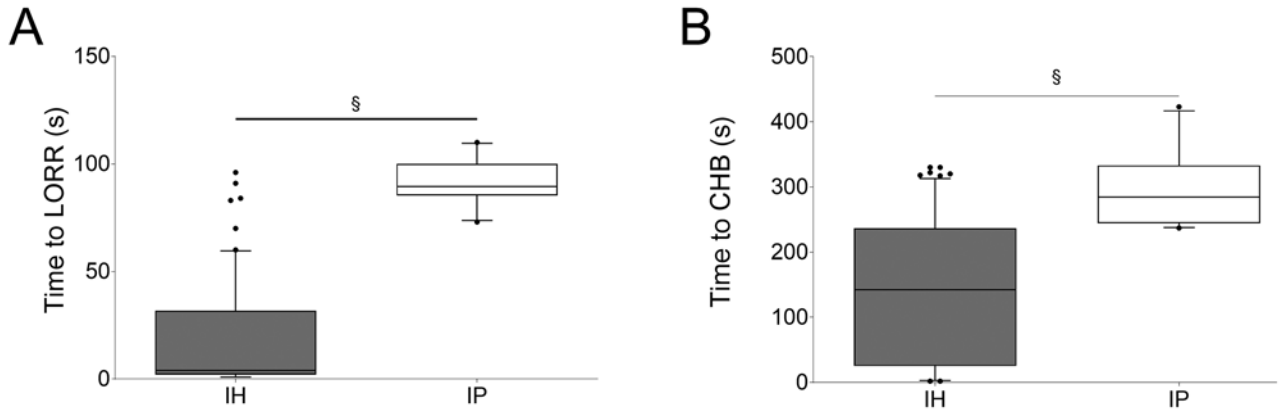

Figure 4. Box and whisker plot of (A) time to loss of righting reflex (LORR) and (B) time to cessation of heartbeat (CHB) for all confirmed intrahepatic (injectate restricted to the liver), incomplete intrahepatic (injectate present intraperitoneally, with the possibility of some injectate in the liver, $n = 64$), and confirmed intraperitoneal (injectate restricted to the abdomen, $n = 10$) injections. The horizontal line within each box represents the median; the lower and upper box limits indicate the interquartile range; and the whiskers denote the 10th through 90th percentiles. Solid circles are data points outside the 10th to 90th percentiles. IH, intrahepatic injection; IP, intraperitoneal injection. §, $P < 0.0001$.

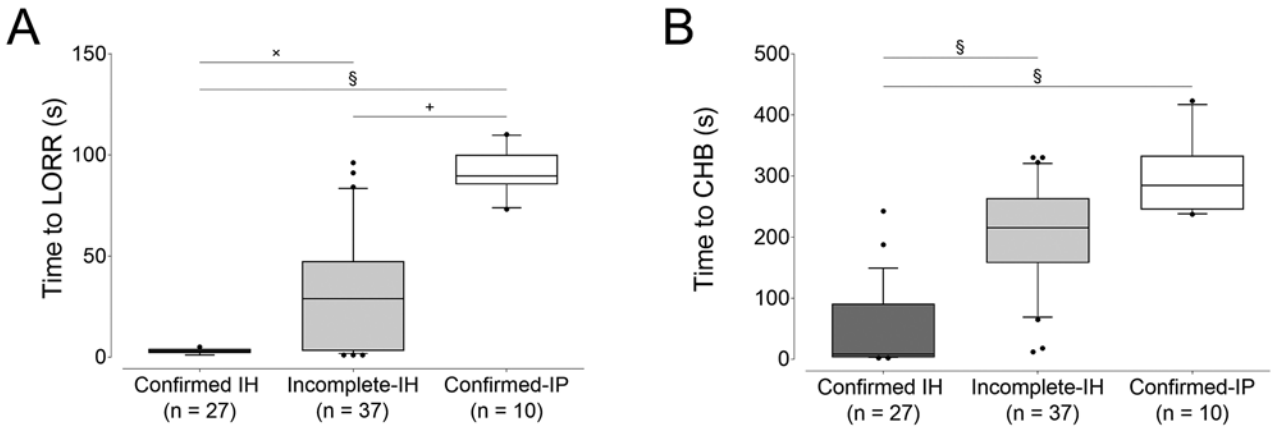

Figure 5. Box and whisker plot of (A) time to loss of righting reflex (LORR) and (B) time to cessation of heartbeat (CHB). Data are presented for each of 3 outcomes: confirmed intrahepatic injection (injectate restricted to the liver), incomplete intrahepatic injection (injectate present intraperitoneally, with the possibility of some injectate in the liver), and confirmed intraperitoneal injection (injectate restricted to the abdomen). The horizontal line within each box represents the median; the lower and upper box limits indicate the interquartile range; and the whiskers denote the 10th through 90th percentiles. Solid circles are data points outside the 10th to 90th percentiles. IH, intrahepatic injection; IP, intraperitoneal injection. +, $P = 0.0017$; ×, $P = 0.0004$; §, $P < 0.0001$.

The difference in time to LORR between all intrahepatic injections (confirmed intrahepatic and incomplete intrahepatic) and confirmed intraperitoneal injections was significant ($P < 0.0001$; 95% CI, 68 to 88 s, Figure 4 A). In addition, time to CHB was significantly longer for confirmed intraperitoneal injections ($P < 0.0001$; 95% CI, 82 to 234 s, Figure 4 B).

Time to LORR was significantly shorter ($P = 0.0004$; 95% CI, 18 to 38 s) for confirmed intrahepatic (3 s [1 to 5 s]) as compared with incomplete intrahepatic (29 s [1 to 96 s]) injections (Figure 5). Similarly, time to CHB was significantly shorter ($P < 0.0001$; 95% CI, 97 to 206 s) for confirmed intrahepatic (8 s [2 to 242 s]) as compared with incomplete intrahepatic (216 s [12 to 330 s]) injections. As compared with the intraperitoneal treatment group (LORR, 89.5 s [73 to 110 s]; CHB, 284.5 s [237 to 423 s]), the time to LORR was shorter for both incomplete intrahepatic ($P = 0.002$; 95% CI, 43 to 78 s) and confirmed intrahepatic ($P < 0.0001$; 95% CI, 80 to 97 s) injections (Figure 5A). For time to CHB, confirmed intrahepatic injections were faster than intraperitoneal injections ($P < 0.0001$; 95% CI, 1712 to 287 s), with no significant difference between incomplete intrahepatic and intraperitoneal injections ($P = 0.10$; 95% CI, 17 to 138 s; Figure 5B).

Within the intrahepatic group, time to LORR ($P = 0.82$; 95% CI, –2 to 2 s) and time to CHB ($P = 0.30$; 95% CI, –31 to 83s) was not different between rats weighing less than 500 g ($n = 30$) and those heavier than 500 g ($n = 34$).

Discussion

This study describes a novel intrahepatic injection technique and shows that 1) intrahepatic injections resulted in a shorter time to LORR than did intraperitoneal injections, 2) the relatively high proportion of incomplete intrahepatic misinjections in the intrahepatic group still resulted in death more rapidly than the intraperitoneal route, and 3) fewer failures to achieve death occurred after intrahepatic than intraperitoneal injection. Overall, the intrahepatic injection technique is efficient, simple to perform, and has a low risk of failure to result in death of rats. These findings support intrahepatic injections as a viable and potentially preferable option to intraperitoneal injection as a euthanasia method in rats.

The pilot study showed the feasibility of the intrahepatic injection technique. Performing a pilot study was a necessary step to evaluate the feasibility of intrahepatic injections before embarking on a full study. This strategy minimized

animal use had the method been impractical or unsuccessful (e.g., inadvertent intrathoracic injection). Once feasibility was confirmed, the full study enabled a formal assessment of the intrahepatic technique. During the pilot study, we noted that inadvertent injection into the falciform fat pad could occur in larger animals. The risk of intrathoracic injection is important to consider, because it is associated with pain and distress in awake animals and has been reported to occur after attempted intrahepatic injection in mice.[3,21] This risk was minimized by avoiding angles of needle insertion that were less than 45° relative to the sternum.

The optimal rat body position was vertical, because it created more distance between the liver and stomach and right kidney. This position differs from that for intraperitoneal injections, in which dorsal recumbency is often preferred.[24]

Intrahepatic injection was more efficient than intraperitoneal injections because of the rapid LORR. Although the misinjection rate was high, the majority of misinjections were incomplete intrahepatic injections, and the time to LORR for incomplete intrahepatic injection was still approximately 3 times faster than for intraperitoneal injections.[33] This outcome suggests that incomplete intrahepatic injection actually deposited some PB into the liver, resulting in a rapid effect. A potential complication of the intrahepatic technique in larger rats is the presence of a prominent falciform fat pad, which may impede the injection. Using a longer needle and maintaining an insertion angle that is approximately perpendicular to the skin may increase the likelihood of a successful intrahepatic injection without an increase in the risk of an intrathoracic injection. Further work is needed to determine whether this risk can be reduced or eliminated.

To our knowledge, reports of intrahepatic injections for killing are limited to cats and mice.[16,21] In the cat study, intrahepatic injections produced a significantly shorter time to recumbency, time to loss of pedal reflex, and time to cardiac standstill (measured by inserting a 3-mL syringe in the heart through the 5th intercostal space and monitoring syringe movement) than intraperitoneal injections.[16] Similar to our study, necropsy evaluations (macroscopic evaluation of dye distribution) helped to determine accuracy of the technique in cats: of all intrahepatic injections, 24% were categorized as intrahepatic, 27% as intrahepatic and intraperitoneal (signs of hepatic and peritoneal absorption), 32% were intraperitoneal only, and the remaining 17% were intrathoracic or intramuscular.[16] These proportions differed from our study in 2 ways: 1) our rate of confirmed intrahepatic injection was much higher (41%) and 2) misinjection into the thorax or intramuscularly did not occur. The other notable difference is the designation of 2 distinct groups ('IH and IP' and 'IP only') in the cat study,[16] whereas we combined both groups (incomplete intrahepatic). This combination was due to our lack of ability to confirm the presence or absence of hepatic absorption when injectate was present in the intraperitoneal space. Regardless of these differences, the proportion of necropsy assignments was similar between our current study and the previous study in cats:[16] 59% for 'IH and IP' and 'IP only' combined, and 56% for incomplete intrahepatic. In both the current study and the one in cats,[16] the outcome of these misinjections was successful LORR and death. By contrast, the intrahepatic technique in mice has been associated with a risk of intrathoracic drug delivery.[21]

The intraperitoneal injections had similar times to LORR and CHB as reported by a group, previously using the same protocol in rats (PB dose and volume, injection technique, LORR and CHB assessment methods).[33] The higher misinjection rate in our study (29%; 16% in the previous study)[33] and 6% to 20% elsewhere[5,9,11,12,21,22,27,28,33] may be due to the inexperience of the injector (CL). Indeed, these were the first intraperitoneal and intrahepatic injection attempts performed by the injector. Assuming that a learning curve exists for performing intraperitoneal and intrahepatic injections, as has been documented for other technical skills,[6,13] the intrahepatic technique was successful (97% of intrahepatic injections resulted in death) even when performed by an inexperienced individual. However, training and experience could nonetheless further improve outcomes. A further potential contribution to the rate of injectate deposition outside the liver in planned intrahepatic injections could be the injectate volume. For example, the injectate volume for a 300-g rat would be 1 mL (240 mg/mL solution), and this volume might exceed the absorptive capacity of the liver for the speed of injection.

Comparing intraperitoneal and intrahepatic misinjections reveals an important contrast: an intrahepatic misinjection is likely to still lead to a rapid LORR and death, whereas an intraperitoneal misinjection often leads to a failed killing attempt.[8,30,32,33] One of the principal sites of intraperitoneal misinjections is the cecum.[8,21,22] Although intraperitoneal injections traditionally are given in the right caudal abdominal quadrant to avoid the predicted position of the cecum in the left caudal quadrant, 2 studies[11,31] reported that cecum position is highly variable: it can be located in the right caudal quadrant or in the middle of the abdomen in approximately 20% to 30% of rats. In contrast, intrahepatic misinjections often lead to incomplete intrahepatic deposition, resulting in rapid LORR and CHB. However, an important caveat to this generalization is the potential for injection into the falciform fat pad in larger rats. The change in needle angle in rats heavier than 500 g did not eliminate this problem, and other technique refinements should be explored. The difference in the ultimate outcome of misinjection is an important advantage of the intrahepatic injection route over intraperitoneal delivery.

The rapid LORR after intrahepatic injection is advantageous when considering the duration of potential distress and pain, in that the shorter time to LORR, which is a proxy index of unconsciousness,[15] would minimize these adverse effects. In contrast to low variability in the time to LORR data, times to CHB varied considerably. A likely contribution to this greater variability is the ease of measuring these 2 outcomes. Measurement of LORR uses a clear and rapid visual indicator, whereas CHB measurement includes the inherent difficulty in hearing increasingly faint heart sounds as the heart slows in order to identify the last audible beat. Although the extent of pain associated with PB in the abdomen has not yet been fully elucidated, current evidence suggests that some degree of pain is possible.[1,2,17,18,25,29] Therefore any novel PB delivery technique should be assessed against this possibility. The rapidity and consistency of effect with intrahepatic delivery makes it an appealing alternative to intraperitoneal injection. The failure rates reported for both intraperitoneal and intrahepatic methods should be considered when selecting a euthanasia method.

A limitation of this study was the lack of pain assessment, an important outcome measure when evaluating killing methods. Therefore, we do not know whether intrahepatic injection of PB is more or less painful than intraperitoneal injection of PB. In an intrahepatic injection study involving cats,[16] behavioral responses associated with pain (vocalization and turning the head toward the injection at the time of injection) were slightly more frequent after intrahepatic injection (8 of 85 animals [9%]) than intraperitoneal injection (4 of 77 animals [4%]). Assessing pain in the presence of drugs that induce muscle relaxation,

sedation, or general anesthesia is challenging, because standard measures of pain require a motor response as part of a behavioral expression. One approach is to use an appropriate control injection, although doing so assumes that the control contains the constituent that may cause pain.[25]

Another limitation of the study was that quantification of the injectate volume in either the liver or intraperitoneal space was not possible. That information would help to clarify the disposition of injectate after misinjection and indicate the extent to which intrahepatic injection occurred. Furthermore, we did not perform aspiration before either intraperitoneal or intrahepatic injection. Although limited evidence indicates that aspirating before intraperitoneal injection is useful,[20] the aspiration of blood could confirm accurate needle placement during intrahepatic injection. Finally, the method described for intrahepatic injection of rats required 2 people. We recognize that this may be a limiting factor in some settings, and we speculate that the technique is amenable to being performed by a single person. Further work is required to confirm this possibility.

In summary, intrahepatic injections of PB are an effective and consistent euthanasia method that should be considered as an alternative to intraperitoneal injections for the euthanasia of rats. When performed according to the protocol outlined in the current study, intrahepatic injections yield a rapid LORR and time to CHB and present little risk of failed euthanasia attempts.

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