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Euthanasia in Laboratory Rodents: Alternatives to Intraperitoneal Injection of Sodium Pentobarbital

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Ce mémoire intitulé

Euthanasia in Laboratory Rodents: Alternatives to Intraperitoneal Injections of Sodium Pentobarbital

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Résumé

L'utilisation du pentobarbital de sodium (PB), injecté par voie intrapéritonéale (IP), est décrite comme une technique acceptable par les directives d'euthanasie de l'AVMA et du CCPA pour tuer les rongeurs. Cependant, de plus en plus de preuves contestent l'acceptabilité de l'IP PB. Celle-ci a été décrite comme inconsistante et il existe des données suggérant que cette technique pourrait induire de la douleur et du stress. L'objectif de cette thèse était donc de développer et d'évaluer des méthodes alternatives d'euthanasie. Au cours de l'étude pilote, nous avons développé un protocole d'injection pour les injections intrahépatiques (IH) de PB. Ensuite, nous avons testé cette injection sur des souris et des rats. Comme objectif secondaire, nous avons évalué l'utilisation de l'éthanol (ET) comme alternative au PB pour l'euthanasie des souris.

Pour les souris, quatre-vingts souris CD1 adultes (mâles et femelles- 26,8 g [23-34 g], moyenne [intervalle]) ont été assignées au hasard à 6 groupes de traitement et ont été tuées par des injections IH ou des injections IP, en utilisant soit ET ou PB. Le taux de mauvaise injection (mauvais placement du contenu de l'injection) pour les essais IH était de 93% (28/30), y compris 14% intrathoracique (4/28), le reste ayant abouti dans la cavité péritonéale telle une injection IP. Ainsi, seulement 7% (2/30) des injections ont donné lieu à une administration hépatique (selon l'évaluation d'autopsie). Les injections IH ayant abouti dans le foie ont entraîné des décès quasi instantanés. Ces données montrent que les injections IH ne sont pas réalisables chez la souris étant donné la difficulté à frapper le foie et le risque d'injections intrathoraciques. D'autre part, l'IP ET a produit des temps significativement ($p = 0.010$; Mann-Whitney) plus courts de l'injection à l'arrêt du rythme cardiaque (CHB) (115s [88-185] médian [intervalle]) par rapport à l'IP PB (176s [123-260]), confirmant que l'ET est une alternative viable et potentiellement supérieure à la PB.

Pour les rats, 66 injections IH et 14 injections IP ont été tentées sur des rats Sprague-Dawley mâles et femelles adultes (poids médian 371g, plage 170-730g), et ont entraîné un délai significativement plus rapide pour la perte du réflexe de redressement (LORR) ($p < 0.0001$, 95%CI 68 to 88s, Mann-Whitney) et temps de CHB ($p < 0.0001$, 95%CI 82 to 234s, Mann-Whitney) par rapport aux injections IP. Le temps médian de LORR et CHB après les injections IH était de 4s [1 to 96] et 142.5s [2 to 330] respectivement; alors que le temps médian de LORR et CHB après les injections IP était de 89.5s [73 to 110] et 275s [237 to 423], respectivement. Le taux de mauvaise injection, basé sur les évaluations d'autopsie, était plus élevé avec les injections IH qu'avec les injections IP (IH: 59%, IP: 29%); cependant, 97% des mauvaises injections IH ont tout de même produit une euthanasie réussie et rapide (LORR: 29s [1 to 96], CHB: 216s [12 to 330]. Les injections IH sont donc une alternative efficace aux injections IP pour l'euthanasie chez le rat, et présentent moins de risques d'échec des tentatives d'euthanasie.

Mots-clés : euthanasie, rongeur, intrapéritonéale, pentobarbitale de sodium, éthanol, intrahépatique.

Abstract

The use of sodium pentobarbital (PB), injected intraperitoneally (IP), for killing rodents is described as an acceptable technique by the American Veterinary Medical Association (AVMA) and Canadian Council on Animal Care (CCAC) euthanasia guidelines. However, there is a growing body of evidence challenging the acceptability of IP PB. It has been described as inconsistent and there is evidence that it may induce pain and stress.

The objective of this thesis was to develop and evaluate alternative methods of euthanasia. During the pilot study, an injection protocol for intrahepatic (IH) injections of PB was developed and then tested on both mice and rats. As a secondary objective, the use of ethanol (ET) was evaluated as an alternative to PB for mice.

For mice, eighty adult (male and female) CD1 mice (26.8g [23-34g], mean [range]) were randomly assigned to 6 treatment groups and were killed by IH injections or IP injections, using either ET or PB. the misinjection rate (misplacement of injectate) for IH injections was 93% (28/30), including 14% intrathoracic (4/28), and the remainder were IP delivery. Only 7% (2/30) of IH attempts resulted in successful IH delivery, per necropsy evaluation. These yielded quasi-instantaneous deaths. These data show that IH injections are not feasible in mice given the difficulty in hitting the liver and the risk of intrathoracic injections. On the other hand, IP ET produced significantly ($p = 0.010$; Mann-Whitney) shorter time from injection to cessation of heartbeat (CHB) (115s [88-185] median [range]) compared with IP PB (176s [123-260]), confirming that ET is a viable and potentially superior alternative to PB.

For rats, 66 IH injections and 14 IP injections were attempted on adult male and female Sprague-Dawley rats (median weight 371g, range 170-730g), and resulted in significantly faster time to loss of righting reflex (LORR) ($p < 0.0001$, 95%CI 68 to 88s, Mann-Whitney) and time to CHB ($p < 0.0001$, 95%CI 82 to 234s, Mann-Whitney) compared with IP injections. Time to LORR and CHB following IH injections were: LORR of 4s [1 to 96], CHB of 142.5s [2 to 330]; compared with IP injections: LORR of 89.5s [73 to 110], CHB of 275s [237 to 423). The misinjection rate was higher with IH injections than with IP injections (IH: 59%, IP: 29%); however, 97 % of IH misinjections resulted in fast and successful euthanasia (LORR: 29s [1 to 96], CHB: 216s [12 to 330]. IH injections are thus an efficacious alternative to IP injections for rat euthanasia and pose less risk of failed euthanasia attempts.

Keywords: euthanasia, rodents, intraperitoneal, sodium pentobarbital, ethanol, intrahepatic

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General Discussion

List of Abbreviations

ACTH: Adrenocorticotropic hormone

AVMA: American Veterinary Medical Association

CCAC: Canadian Council on Animal Care

CHB: Cessation of Heartbeat

ET: Ethanol

FLI: Fos Like Immunoreactive

IH: Intrahepatic

IP: Intraperitoneal

PB: Sodium Pentobarbital

pERK: Phosphorylated Extracellular Signal-Regulated Kinases

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Introduction

The word *euthanasia*, from the Greek terms *eu* (good) and *thanatos* (death), means the induction of a good, gentle, or humane death. It is a seemingly simple concept in form but difficult in application, especially in the diverse field of laboratory animal medicine. Yet it is a subject that should be at the heart of any evaluation of laboratory animal procedures, because euthanasia of mice and rats is one of the most commonly performed procedures in many countries around the world. Indeed, tens of millions of animals are used, and euthanized, every year for various types of experiments (Taylor et al 2005).

Because of the ubiquity and the important ethical issues engendered by the practice of euthanizing laboratory animals, many governments world-wide publish guidelines to which all institutions engaged in animal research must adhere. The literature on rodent euthanasia methods is in constant flux and expansion (Artwhol et al. 2006). Therefore, these guidelines are regularly updated, and the updates depend upon a continual search for better ways to evaluate and improve upon the methods.

This is the overall objective of the present thesis: to review a method of euthanasia that is widely used and currently judged as acceptable, and to attempt to offer more efficacious and ethical alternatives. This method consists of the intraperitoneal injection of sodium pentobarbital (IP PB). This is a method that has been in practice for decades, and, as will be described in Chapter 1, one that is universally accepted as an ethical means of euthanizing rats and mice.

As a first step, a comprehensive review of the literature on IP PB as a method of euthanasia was conducted in Chapter 1. This review has been accepted for publication in the journal JAALAS (Journal of the American Association for Laboratory Animal Science, manuscript ID: JAALAS-19-000081) and is, to the author's knowledge, the first such published review. The conclusions drawn from the literature review serve as the cornerstone for the experiments that followed, in which alternative methods to IP PB were tested. In one paper (Chapter 2), the efficacy of intrahepatic (IH) injections of sodium pentobarbital in mice was tested, as well as that of ethanol as an alternative to PB. This paper has also been published in JAALAS. In a follow-up paper (Chapter 3), a protocol for IH injections in rats was also tested. This paper will be submitted shortly.

Chapter 1 - Literature Review

Laferriere CA, Pang DSJ. 2000. Review of Intraperitoneal Injection of Sodium Pentobarbital as a Method of Euthanasia in Laboratory Rodents. ***JAALAS: 59(3): 254-263***

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CL: literature search, drafting and revision, approval of final version

DP: concept, revision, approval of final version

Intraperitoneal injection of sodium pentobarbital as a method of euthanasia in laboratory rodents: a narrative review

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Running Title: Rodent euthanasia with pentobarbital review

1. Abstract

Euthanasia is one of the most commonly performed procedures in biomedical research, involving tens of millions of animals in North America and Europe every year. The use of sodium pentobarbital (PB), injected intraperitoneally (IP), for killing rodents is described as an acceptable technique by the AVMA and CCAC euthanasia guidelines. It is recommended over inhalant anesthetics, carbon dioxide, and physical methods for ethical and aesthetic reasons, as well as for its efficiency. However, there is a growing body of evidence challenging the acceptability of IP PB. It has been described as inconsistent and there is evidence that it may induce pain and stress. With these considerations in mind, there is a need for a review of the literature in order to assess the scientific knowledge regarding this killing method, and evaluate its current status, codified by the CCAC and AVMA, as an acceptable euthanasia method.

2. Introduction

There are approximately 17 million rodents (rats and mice) used in research annually in the European Union, UK and Canada.[16,38,101] An overwhelming majority of these rodents are euthanized either as part of a study protocol or at the end of a research project, making euthanasia one of the most commonly performed laboratory procedures. Despite the prevalence of euthanasia procedures and its conceptual simplicity, ensuring a good death across a wide variety of laboratory settings and involving such large numbers of animals is difficult.

Killing methods in research consist of multiple, often conflicting objectives: ideally, death must be rapid and painless whilst also being aesthetic and compatible with research protocols.[49] Furthermore, killing methods must be simple to apply and yield consistent results. It may be difficult, even impossible, to completely satisfy all of these objectives in all instances, more so given the challenges in identifying important outcomes (e.g. distress, pain, aesthetic appeal).

In North America, the American Veterinary Medical Association (AVMA) and the Canadian Council on Animal Care (CCAC) provide guidance on euthanasia procedures. Both organisations publish guidelines regularly, reflecting the latest evidence on killing methods in a wide range of species. In rodents, the AVMA classifies the intraperitoneal (IP) or intravenous (IV) injection of barbituric acid derivatives (or barbiturate combinations), as well as injection of dissociative agent

combinations as the only acceptable methods,[4] and the CCAC classifies IP injection of buffered and diluted barbiturate as well as overdose of inhalant anesthetics (followed by another method to ensure death) as acceptable.[15]

While the above methods are categorized as acceptable, there exists a secondary category for methods termed 'acceptable with conditions'[4] or 'conditionally acceptable'.[15] A method is classified as such if one of the following is true: it lacks proper scientific documentation, it is unsuitable on its own and requires a secondary method, it has greater potential for error, it has been shown that the technique may not consistently produce humane death or may require certain conditions to do so.[4,15,23] Euthanasia procedures that are thusly categorized remain common because acceptable methods may interfere with research data collection or are less practical for larger numbers of animals. For example, overdose with carbon dioxide (CO_2) is one of the most commonly used killing methods.[25] It is practical, easy to use, inexpensive, relatively fast in action, can be applied to multiple animals simultaneously and requires little or no direct handling.[3,25,56] However, many studies have demonstrated that CO_2 exposure elicits an aversive response in rodents and may induce stress and pain.[20,25,51,63,79,80,81,82]

Overdose with barbiturate is the only method classified as acceptable by both the AVMA *guidelines* and the CCAC *guidelines*[4,15] Inhalant anesthetics, such as isoflurane induce aversive behaviour in rodents.[19,63,70,71,104,116] Additionally, their use requires specialised equipment, and time to death can be slow.[15] Thus, the CCAC *guidelines* rate inhalant anesthetics as acceptable in rodents only if used with a secondary method to ensure death whereas it is 'acceptable with conditions' in the AVMA guidelines. As for dissociative agents (typically ketamine or a combination of ketamine and an alpha-2 adrenergic receptor agonist, such as xylazine), there is limited discussion of this euthanasia method in the current AVMA guidelines[4] with one supporting study cited in which the primary objective was not a qualitative evaluation of dissociative agents for killing.[106]

Sodium pentobarbital (PB) is the most commonly used barbiturate for killing. It has a narrow safety margin, is potent, can be formulated as a concentrated solution so that relatively small volumes are needed, and has a rapid onset of action when given IV.[4,23] Furthermore, PB has a long shelf life, is stable in solution, and is inexpensive.[4] Access and licensing requirements for purchase vary between countries.[23] When given as an intentional overdose to cause death, general anesthesia

is induced, followed by depression of the respiratory and cardiovascular centres of the brainstem, leading to cardiorespiratory arrest.[25,23]

Although the intravenous (IV) route of injection is preferred for PB, it is often impractical to perform on rodents as achieving consistently successful IV injections requires training, restraint and often a means to induce venodilation.[40,43] As such, PB for killing rats and mice is often administered via IP injections.[23,78,100] Although the onset of anesthetic effect following an IP injection is not as quick as via IV injection, the absorption and distribution of drugs occurs much faster than it does following intra-muscular or subcutaneous injections.[78; 87] Furthermore more, IP injections are relatively simple and quick to perform, require minimal training, allow large volumes to be administered, and repeated injections are possible.

Thus, intraperitoneal injections of sodium pentobarbital (IP PB) is one of the most widely accepted methods of rodent euthanasia that does not require conditions for its use. This is a rating shared not only by the CCAC and AVMA, but also by animal care guidelines in Europe[5], India[55] and Australia[27]. Importantly, despite its many advantages and widespread use, this killing method is not without drawbacks. The aim of this overview is to provide a narrative review of the literature on IP PB as a killing method, evaluate its current designation as "acceptable" (by the AVMA and CCAC), identify gaps in knowledge that may be pertinent to the refinement of this method and to suggest potential alternatives.

3. Methods

A literature search was performed using combinations of four key words ('euthanasia' 'rodents' 'pentobarbital' 'intraperitoneal'; creating 11 search terms) in three databases (PubMed, Cab Abstracts, Web of Science) in English or French from 1950 to the search date (2019-05-15). Document types were set as journal article, book or government report.

Search results were screened for inclusion by reading titles and abstracts. After initial screening, full articles were obtained for review. Data, results and conclusions from these downloaded articles were then analysed and reviewed for relevant content. Additional articles, books, theses and government reports were identified and included from a manual search of the reference list of articles found during the database search.

4. Results

A total of 247 articles, government reports or books were kept past initial screening and read for the purpose of this review, of which 118 were used as cited references in the writing of the review. A full list of all the post-screening articles is available in an online repository.

5. Discussion

5.1. Intraperitoneal Injection Technique

Several techniques for IP injection have been described, the most common variations are described here and available in table format (Table 2). A two person technique with one holder and one injector, rather than one person technique, is considered more efficient when working with mice as it led to a reduction in misinjection rate from 8-12 % to just over 1%.[2,6] The holder grips the upper limbs and head of the animal and maintains the animal in a horizontal position. It is often recommended that the holder tilts the animal slightly downwards so that its head is lower than its abdomen. This supposedly creates more space in the caudal quadrants of the abdominal cavity by moving organs cranially.[109] However there is little evidence that the tilting of the animal has any effect on the success of the injection.[78] Indeed similar misinjection rates have been obtained whilst injecting rats vertically[26] or horizontally with a tilt.[78; 119] The injector, holding the right hind limb of the animal with one hand visualizes the abdomen as if divided into four quadrants and injects into the caudal left quadrant (the animal's right side). [9,26,102,119] Within the caudal left quadrant, the injection should be made at the level of the coxofemoral joint, approximately halfway between midline and the lateral abdominal wall.[102] The goal of the injection is to deposit injectate into the peritoneal cavity without piercing any of the abdominal organs. In order to avoid doing so, the injection angle should be approximately 10-20 degrees relative to the body wall in mice and 20-45 degrees in rats, with the needle directed cranially.[102] Before injection, it is often suggested to aspirate the needle to assure its correct placement in the peritoneal cavity, though there is no evidence supporting the usefulness of this practice.[11]

Various sizes of needles and gauges can be used, but a 3/8 inch needle (9.5mm) is long enough and may be the least likely to puncture the organs in the abdominal cavity.[11, 26] For a successful IP injection, the needle does not need to exceed a depth of 4-5 mm beyond the skin, so a long needle

is unnecessary. The maximum suggested injectable volume is 0.5-1.0 ml in mice and 5-10 ml in rats.[11,78]

5.2. Mechanism of action

Sodium pentobarbital is an oxybarbiturate ligand of the gamma-aminobutyric acid subtype A ($GABA_A$) receptor. It increases chloride conductance through the receptor channel, causing neuronal hyperpolarisation and consequent central nervous system depression. Following a successful lethal dose of PB, animals become ataxic and sedated, followed by loss of consciousness, apnea, cardiac arrest and death.

The aim of an IP injection is to deposit PB into the peritoneal cavity from where it is absorbed into the circulation. The peritoneal cavity is defined as the potential space between the visceral and parietal peritonea of the abdomen. The visceral peritoneum adheres to most of the abdominal organs whereas the parietal peritoneum adheres to the interior of the abdominal wall. The blood vessels of the visceral peritoneum connect to the mesenteric, colic and intestinal veins to converge into the anterior mesenteric vein and then carry blood to the liver via the portal system.[111] Thus IP substances may undergo first pass metabolism if absorbed via the visceral peritoneum.[18,92] After the liver, venous blood flows to the heart via the caudal vena cava and then on to the pulmonary circulation before returning to the left side of the heart for distribution to the systemic circulation.

Although IP administration is considered a parenteral route, the pharmacokinetics of substances administered via IP injections have similarities to substances administered orally because of the potential for hepatic metabolism.[77,100] However, the very large surface area provided by the peritonea and the omentum, as well as the abundant blood supply allows for absorption that is more rapid than oral or intramuscular injection.[78, 87]

Absorption across the visceral peritoneum and omentum is the predominant, but not the only route of absorption following IP injection. Other routes to attain the systemic circulation include absorption via the parietal peritoneum and lymphatic drainage. These different absorption routes contribute variability to the pharmacokinetics and pharmacodynamics of IP injections.[21] Vessels draining the parietal peritoneum do not connect to the portal veins but instead empty directly into systemic veins.[61] This provides a route to bypass first pass metabolism.

In addition to peritoneal absorption, lymphatic absorption across the diaphragm can affect the fate of substances delivered IP and further contribute to variability in IP absorption and distribution.[1] Access to the lymphatic system occurs through stomatas, small openings on the surface of the peritoneal mesothelium, that allow the passage of fluids from the abdominal cavity into sub-peritoneal lacunae that drain directly into the lymphatic system.[99] These stomatas are located on the muscular portion of the diaphragm in rats.

Lymphatic drainage can be rapid and effective in delivering substances within the peritoneal space to the systemic circulation.[64,94] Indeed, a large amount of drained fluid from the abdominal cavity ends up, within minutes, in the subclavian vein which feeds directly into the cranial vena cava.[94] However the speed of lymphatic drainage varies and is affected by the stretching of the diaphragm during respiration.[12,108] Furthermore, the posture of the animal affects the rate of drainage.[10] Indeed, a slower rate of absorption was measured whilst rats were held with their body in a vertical position (head up) compared to a head down position, whilst a sternal posture produced an intermediate rate of absorption.

Although lymphatic drainage of the peritoneal cavity has been widely studied in multiple species, this system is often undescribed in discussions of IP injection of PB.[26,78,102,110] These tend to focus entirely on peritoneal absorption, omitting what may be a major component of IP injections. Many authors have suggested that lymphatic drainage is more important quantitatively than absorption from either the peritoneal or visceral peritoneums.[1,13,28,42,76] Compelling evidence demonstrating the relative importance of lymphatic drainage is the observation that a 59% decrease in absorptive capacity from the peritoneal space resulted following closure of the diaphragmatic stomata with fibrous tissue (induced by abrasion).[66] Additionally, ligation of the principle lymphatic ducts draining the lymphatic vessels emanating from the diaphragm markedly reduced the amount of dye (delivered via IP injection) absorbed from the peritoneal cavity.[29, 30]

These variable routes of absorption alter the pharmacokinetics of substances delivered by IP injection by providing different means of attaining the systemic circulation. These undoubtedly play a role in affecting the variability in timing of physiological responses following successful IP injections. Successful IP injections refer to injections that administer injectate into the peritoneal cavity, as opposed to misinjections which fail to do so. Misinjections can cause significant variability, as discussed in the following chapter. In terms of euthanasia procedures, the variable

routes of absorption following successful injections translate into inconsistent timing of effects.[21,31,119] Table 1, which presents data from various euthanasia studies, illustrates this variability. Additionally, different operational definitions of measured endpoints, and methodologies for their determination are apparent and contribute to variations (Table 1).

Although many different doses, volumes and concentrations of PB have been used for euthanasia, a dose of 800 mg /kg in rats is associated with increased consistency and speed of effect than 200 mg/kg.[119] Doses in the range of 150-200 mg/kg are commonly used and are based on being approximately 5 x the dose required to induce general anesthesia.[23,43] Furthermore, using 800 mg/kg, the decreased variability of effect facilitates identifying misinjections based on the time taken to achieve loss of righting reflex and apnea. At this dose, it has been suggested that the time to loss of righting reflex and apnea should not exceed approximately 2.5 to 4.5 minutes, respectively (calculated from mean + 2 SD of study population).[119] If these times are exceeded, it is likely a misinjection has occurred.

There is little information regarding optimal dose and volume for euthanasia with PB in mice, although 150 mg/kg has long been a suggested dose.[43] The anesthetic dose using PB is 40-50 mg/kg when given IP[11,40] so by applying the same principal as described above, the minimum dose for cause death should be 200-250 mg/kg, but doses described in the literature for mice range from 150 mg/kg to 5400 mg/kg (Table 1). However, a recent study found that increasing the concentration of the PB solution to 390 mg/ml, which resulted in a dose of over 1300mg/kg for males and 1680mg/kg for females, caused a significantly faster time to unconsciousness and time to death than a dose of 250mg/kg (at a concentration of 50mg/ml).[37] Thus, similarly to what was observed in rats, increasing the dose of PB seems to create a more efficacious killing method when using IP PB in mice.

The extent of current scientific knowledge regarding the pharmacokinetics of IP PB is still limited in many areas.[98,100] Indeed, some of the basic principles of this route of administration, such as absorption from the peritoneal cavity, have yet to be fully elucidated. Determining the effect that different placements of an injectate within the abdominal cavity has on absorption rates and quantifying the various absorption routes could lead to greater consistency and predictability as well as improve our ability to detect misinjections.

5.3. Disadvantages of IP injections of Sodium Pentobarbital

5.3.1. Variability in effect

A major disadvantage of IP injections, arguably the most significant, is its variability. This can be divided into two categories: inherent variability and misinjections. Inherent variability refers to the existence of different pathways of absorption and distribution, as described above, which tend to produce a wide range of responses following a successful IP injection and thus create variability in euthanasia procedures.

The second source of major variability is misinjections, or improper placement of administered substances. These have long been described as an issue with IP injections, and occur when the injection fails to be made into the peritoneal cavity.[65,96] The principal consequence of this failure is an important delay in the onset of drug action. Indeed, misinjections caused an increase in the time from IP PB injection (667 mg/kg) to loss of the pedal reflex in rats, from 175 seconds (successful injection) to 588 seconds (misinjection).[107] In one study, approximately 40% of misinjections were classified as failed euthanasia procedures because time to death exceeded 20 minutes.[119] Furthermore, there is the possibility of complications arising out of misinjections which may include local irritation and inflammation, perforation of abdominal organs, hemorrhage and respiratory distress.[100]

The most common sites of misinjection in rats, ranked by frequency of occurrence, are into the cecum, into the small intestine, subcutaneously, retroperitoneally and into the urinary bladder.[65] In mice, the most frequent locations of misinjections are: stomach, small intestine, uterine horn in females, and subcutaneous.[75,96] Reported rates of misinjection based on necropsy findings are variable, ranging from 6 to 20 % in rats, and 10-20 % in mice.[2,9,21,26,31,65,90,96,97,119] Although application of proper technique and training can reduce these rates,[6,21,75] the incidence of misinjection rarely appears to fall below 6%, particularly in rats. A study did manage to diminish the rate of misinjection in mice from around 12% to just above 1% (both values obtained after injecting 250 mice)[6]. This reduction occurred after the injections were performed with a two person technique rather than by a single person.

Misinjections can be difficult to identify given the inherent variability of successful IP injections. Aspirating before injecting is often suggested, but cecum and intestinal content may not easily be

aspirated through a small gauge needle.[31] Therefore, misinjection can only be quantified with any confidence at necropsy, by adding dye to the injectate.

Two studies examined the cecum position in rats and mice and found its positioning to be predominantly in the left caudal abdominal quadrant.[26,102] This finding is consistent with the practice of giving injections in the right caudal quadrant to avoid penetrating the cecum. However, the lateral dominance of the organ never exceeded 80%. Moreover, the percentage varied among strains, sexes and even among different colonies. So the cecum, which is the main site of IP misinjections in rats, is in the middle or on the right (directly in the target of an IP injection) in up to 20% of cases. This unpredictability is unavoidable and is an important factor contributing to the high misinjection rate in rats. Cecal position may be less variable in mice and is indeed less frequently involved during misinjections.[75,96,102]

The peritoneal space is best understood as being a potential rather than actual space.[31] Therefore, the shifting and changeable position of the abdominal organs such as the cecum within this space make it impossible to completely avoid unsuccessful injections. This becomes a concern especially when considering the number of animals killed via this technique each year on a global scale.

5.3.2. Histopathological and physiological changes

PB can damage local tissue in which it comes in contact;[7,39] significant damage to superficial cells of the organs near or at the injection site have been observed,[45] suggesting the agent was causing this damage. The same study also noted splenomegaly following IP PB, most likely caused by relaxation of smooth muscle, causing splenic engorgement with blood. Other histological changes affecting organs farther from the injection site may include focal congestion of intestinal serosa, congestion in pulmonary veins, necrosis in sub-capsular levels of the liver and pancreas, lung emphysema and oedema, and hyperaemic kidneys (see reference 6 for review).

In light of these physiological disruptions, it is wise to consider alternative euthanasia options where blood or tissue samples are required for a research protocol. In such cases decapitation may offer a viable alternative to IP PB. Rapid decapitation without anesthesia has been shown to have the smallest increase in plasma ACTH and corticosterone concentrations, even when compared to anesthesia followed by decapitation.[83,103] Rapid non-anesthetized decapitation is a chemical free and rapid means of killing, with loss of cortical function occurring very rapidly. This procedure is

thus the least likely to interfere with metabolic parameters.[4,53] But important concerns exist over non-anesthetized decapitation. Mikeska and Klemm showed that some degree of electrical brain activity can persist 13-14 seconds after decapitation and concluded that pain and stress might be present post-decapitation. However, similar electrical brain activity is seen in deeply anesthetised rats, decapitation completely severs nociceptive input to the brain, and hypoxia following decapitation causes unconsciousness in less than three seconds.[17,33,53,105] Therefore, the majority of evidence indicates that there is no pain or distress associated with successful, rapid decapitations. An important concern is the aesthetics of this procedure, contributing to its widespread unpopularity among experimenters, animal care staff and the general public.[52] Furthermore, decapitation should be considered a high stakes procedure, in which there is a high risk of distress and pain should there be failure to completely sever the spinal cord. For these reasons, rapid non-anesthetized decapitation is classified as 'acceptable with conditions', requiring justification based on experimental outcomes.[4]

5.3.3. Pain associated with intraperitoneal pentobarbital

The act of IP injection, regardless of the chemical agent used, can induce stress and pain. Behavioural patterns related to pain, such as vocalisations, increased locomotion and flinching, have been observed immediately following IP injections.[2; 37,110] Additionally, PB is highly alkaline, with a pH of 11-12, whereas the range of pH that is said to be non-irritating to local tissue at the site of IP injections is approximately 4.5-8.0 in rats.[78,107] It follows that IP injection of PB may result in irritation to the peritoneum or surfaces of visceral organs as well as pain. Indeed, Wadham[107] noted signs of local redness and swelling following IP PB at a dose of 667 mg/kg with no additives added to the solution. Writhing behaviour was also observed, beginning approximately 11 +/- 2.26 seconds after injection. Writhing was defined as 'an abnormal posture in which the rats contract their abdomen and extend their hind legs backwards'. Writhing has been reported in both rats and mice following IP injections,[2,3,59,119] and it is recognised as a behavioural response to abdominal pain as it is commonly observed following injection of a known irritant into the abdominal cavity,[95] and following abdominal surgeries such as laparotomy and vasectomy.[88,117]

Many of these data however should be interpreted cautiously given the difficulty in properly quantifying behavioural responses dependent on gross movement in the presence of an agent

depressing motor function. Some authors have attempted to circumvent this issue by means of indirect quantification of nociception. One study used the presence of electrical brain activity in anesthetized piglets to infer the presence of nociceptive input following IP PB injection.[58] Yet another injected PB into a different injection site (the hind paw) in mice and quantified the response to varying PB concentrations using a paw-lick test.[37]

Additional evidence for the occurrence of pain following IP injections can be obtained via the study of neuronal markers c-fos and fos. C-fos is an early response proto-oncogene that is rapidly activated and expressed in certain nociceptive neurons of the dorsal horn following noxious or sensory stimulation.[24,48,54] The c-fos gene encodes for the protein fos which acts as an intermediary between extracellular events and long term intracellular adaptations. Although much is still unknown about the physiological role of c-fos and fos including during nociceptive processes, these markers are nonetheless commonly used in research to measure the activity of nociceptive neurones.[48] Indeed, immunohistochemical staining is a practical and reliable way to detect fos activity, hence the use of FLI (fos-like-immunoreactive) neurones in research.[41]

The quantification of FLI neurones has thus been used to infer pain post IP PB. A study of FLI neurones has shown an increase in neuronal activity post IP PB administration in areas of the dorsal horn related to visceral nociception.[98] A 4-fold increase in the number of FLI neurones in rats administered IP PB was observed compared to rats receiving saline IP injections, although this latter group also showed an increase in FLI neurones when compared to basal levels. Also, the addition of lidocaine (10 mg/ml) to IP PB lowered the number of FLI neurones in the spinal cord compared to IP PB administered alone. The FLI neurones were present in laminae I, II, V and X of the spinal dorsal horn. These laminae mainly receive input from visceral nociceptive fibers.[46,73]

The relationship between FLI neurons and writhing behaviour provides further evidence that IP PB produces pain. There exists a positive correlation between the number of FLI neurones in the same laminae mentioned above and the amount of writhing behaviours, or stretches, observed following the IP administration of acetic acid, a known irritant.[46] The administration of analgesics (including morphine) produced a dose-dependent inhibition of the writhing behaviour and concurrently a reduction in the amount of FLI neurones.

However, there remains much debate surrounding the use of FLI neurones in pain studies. The relationship between FLI neurone activity and the conscious, cortical perception of pain is unclear.

FLI neurones did not significantly increase in the ventroposterolateral nucleus in the thalamus following a noxious stimulus;[18] a region implicated in the perception of pain.[114,115] Additionally, rats treated with morphine a up to 10 m/kg (s.c) still showed fos expression in the dorsal horn whilst displaying little to no behavioral signs of pain, although the fos activity did diminish with an increasing dose of morphine (from 1mg/kg to 10 mg/kg, all s.c).[85] Thus, the quantification of FLI neurones to infer pain is probably oversimplistic. Moreover, stress is a confounding factor as it has been reported to increase FLI neurone activity.[84] Given the lack of specificity, converging lines of evidence (pain-related behaviours, biomarkers, gross and histological tissue changes) should be used to draw inferences about the likely presence of pain.

The AVMA and CCAC euthanasia guidelines acknowledge that IP PB may cause pain.[8, 21] The CCAC *guidelines* suggests that a local anaesthetic such as lidocaine be used concurrently with a buffered and diluted barbiturate, and that steps be taken to ensure that the pH is within a non-irritating range.[21] However, the pH of PB cannot be lowered below 10 without risk of precipitation and a pH of 10 remains irritating to tissue.[39,78] The addition of lidocaine to PB (in a 50-50 mixture) reduces pain, as measured by writhing behaviour.[3] This has been confirmed by Khoo *et al.* who also noted a decrease in writhing when either lidocaine (10 mg/ml of PB solution) or bupivacaine (2.5 mg/ml of PB solution) was added to pentobarbital (pH of 10.1 and 10.2 respectively). This suggests that addition of lidocaine represents a refinement of the IP PB technique. While beneficial, the lidocaine and PB mixture still significantly increased the number of FLI neurones 3-fold compared to a control saline injection.[98] Writhing was also still observed following injection IP PB with lidocaine, albeit less so than IP PB alone.[59]

The potential for pain during IP PB is compounded by the time to achieve loss of consciousness with this killing method, especially in rats. In addition to implications for tissue harvesting and sample quality, this is an important concern for animal welfare. Development of a technique that induces a consistently shorter time to unconsciousness and death is desirable.

In light of the limited available evidence, both the AVMA *guidelines* and Svendsen *et al.* raise the need for additional studies. Different local anesthetic agents could be explored. For example, piperocaine, which has a higher pH in solution than lidocaine, could potentially be used at a higher concentration to provide analgesia without causing precipitation of the PB.[3] Additionally, further research using a different neuronal marker than c-fos, such as phosphorylated extracellular signal-

regulated kinases (pERK), could add valuable insight to pain related to IP PB. pERK are expressed much more rapidly than c-fos following a noxious stimulus; these proteins reach peak intensity 2-3 minutes post stimuli,[57] whereas c-fos induction and expression in the spinal cord takes at least 30 minutes.[36] This rapid expression make pERK more suited as neuronal pain markers for timeline and behavioural studies.[41]

5.3.4 Stress

The stressful nature of IP injections has been inferred via the measurement of hormonal markers. In one study, ACTH levels increased up to twofold compared to basal levels in Sprague Dawley rats after IP injections of saline.[34] However, there was substantial variation in the concentrations of ACTH measured. A similar increase was also observed in mice.[8] Plasma corticosterone levels in both mice and rats may also be increased by IP injections of saline. In mice, Baek *et al.* and Ryabinin *et al.* reported a significant increase in corticosterone following IP injection of saline. In rats, results have varied: Wu *et al.* reported an increased level of corticosterone in Sprague-Dawleys following IP saline injections, whereas Deutsch-Feldman *et al.* reported no such increase.

IP injection of saline has been associated with hyperthermia and tachycardia in both rats [35,47,93] and mice.[22,62] Meijer *et al.* found that IP injection, of either saline or sham (needle insertion with no fluid administered), increased the heart rate of mice over basal levels for up to 30 minutes following injection. The authors also showed that changes in heart rate parallel those of plasma corticosterone, and may therefore be another useful indicator of stress.

Stress related to laboratory procedures vary significantly between strains of laboratory rodents. Ryabinin *et al.* reported plasma levels of corticosterone increasing from 3-7 fold across different strains of mice. If ACTH physiology is similar in different rat strains, Lewis rats may be less susceptible to stress; this strain had a minimal increase in plasma ACTH concentrations following handling or injection compared with Sprague Dawleys.[34]

Thus, there is evidence that handling necessary for IP injections can be stressful. Handling stress can be reduced. Repeated exposure, or habituation, can reduce the stress response associated with laboratory procedures such as IP injections. Following habituation to saline IP injections, there was a reduced expression of immediate early response genes and corticosterone levels.[34,89]

Behavioural assessments of pain and stress are equally difficult because they lack consistency and tend to be fairly subjective.[112] However, although quantifying the effects of IP injections in laboratory rodents is difficult, establishing that there is an effect is less so: in the majority of studies, IP injections are a potential source of pain and stress.

5.4 Alternatives to IP PB

There are few, if any, well described alternatives to PB as an injectable killing method for rats and mice. One chemical agent that has shown promising results is ethanol. In many countries, government drug regulations require accounting of barbiturate drugs,[4] which makes ethanol an attractive alternative. It is currently described in the AVMA *guidelines* as 'acceptable with conditions' based on a series of studies that explored its use as a killing method.[67,68,69] Interest in this agent has recently resurged and it has been determined that 100% ethanol at a dose varying between 15.3 g/kg and 15.8 g/kg result in a similar rate of onset of respiratory and cardiac arrest as a similar volume (approximately 0.5ml) of IP PB in mice.[2,32] The injection of ethanol did induce pain-related behaviours such as vocalization,[2] as well as kicking at the needle.[32] In both studies, these behaviours were not more frequent than with IP PB, and vocalisation is not a specific indicator of pain in mice.[112,113] Therefore, ethanol appears at least similarly effective to PB, but with the advantage of not being a controlled agent. A study of the number of FLI neurones post injection could be used to quantify neuronal activity associated with ethanol injections vs. IP PB injections. In contrast to mice, IP injection of ethanol in rats as a killing method was unsuccessful due to the large volumes of ethanol required (over 7.1 ml for a dose of 20.1 g/kg) and the fact that time from injection to respiratory arrest was slow (8 +/- 5 minutes).[2]

Therefore, PB remains the only practical killing method via injection in rats. Alternative injectable techniques that show potential include retro-orbital and intra-hepatic injections. In mice, retro-orbital injection with an overdose of ketamine and xylazine resulted in rapid death (cessation of heartbeat occurring in approximately 5 seconds) as the pharmacokinetics of retro-orbital injection closely resembles that of intravenous injection.[50,86,91] However, the potential risks and prevalence of misinjections with this route of administration have not been studied, nor has the potential for pain. This technique has not been evaluated in rats.

Another potential route of administration is intrahepatic. One article has reported use of this injection technique for euthanasia of shelter cats.[44] The technique proved more accurate and faster

than IP injections as most cats became recumbent almost immediately after injection of PB. A small number of cats did respond negatively to the injection. A negative response was defined as vocalization or turning towards the injection site; however, these behaviours occurred with similar frequency during intrahepatic and intramuscular injections. The feasibility and consistency of this technique remains to be evaluated in laboratory rodents.

These alternative methods of drug administration, though incompletely investigated, could compare favourably to IP injection in terms of inherent variability, likelihood of misinjection and speed of action. Speed in inducing unconsciousness and death are important factors to consider for animal welfare because euthanasia procedures should be as rapid as possible. Development of a technique that induces a quicker time to unconsciousness and death than IP PB would greatly reduce the amount of pain and stress to which the animals are potentially subjected.

6. Conclusion

In light of the data cited in this review, it is legitimate to question whether IP PB needs to be refined as a method of rodent euthanasia. Indeed, there are many important gaps in the scientific knowledge related to this procedure. The following remains to be established:

1. The optimal dose of PB in mice and predictability of misinjection.

2. The optimal dilution of PB to reduce pain associated with its alkali pH without compromising efficacy.

3. The refinement of the addition of lidocaine to PB (dose and volume)

4. The potential role of other local anesthetics for mixing with PB

5. The elucidation of the pharmacokinetic parameters following IP administration, most notably the relationship between injection locus and peritoneal absorption

6. The potential for alternative injectable routes of administration to replace IP injection.

7. The importance and role of training in the procedure and its success rate

8. Development of a protocol to follow in cases of IP misinjections

In addition to these important scientific gaps, there is a distinct possibility that IP PB causes stress and pain. Also, according to the AVMA *guidelines* euthanasia procedures should be consistent, easy to perform, reliable and predictable; it is arguable whether IP PB meet these criteria, in large part due to the unavoidable risk of misinjections. These greatly prolong the onset of anesthetic effect in up to 20% of IP attempts.

In conclusion, although IP PB is a relatively simple procedure used extensively, it is clear that numerous aspects of IP PB give cause for concern. Novel approaches, such as the use of IP ethanol or alternative routes of injection, are promising but require further research to establish their strengths and weaknesses before they can be proposed as suitable alternatives for, or improvements upon, IP PB. The important limitations described for IP PB, current lack of suitable alternatives and large number of animals killed underline the importance of further research in this field.

7. Tables

Table 1. Timing of the various effects of IP SP euthanasia with different doses in rats and mice.

AUTHOR	SPECIES	DOSE (MG OF PB/KG OF BODYWEIGHT)	EFFECTS	TIME IN SECONDS (RANGE) OR ± SD
REFERENCE 2	Mice	5400[1]	Loss of Righting Reflex	156
			Respiratory Arrest	45
			Cardiac arrest	276 ± 30
	Rats	200[1]	Loss of Righting Reflex	75
			Respiratory Arrest	270
REFERENCE 3	Mice	330[2]	Ataxia	52 ± 8.08
			Cessation of movement	80.11 ±21.15
			Cessation of breathing	317 ± 150.53
REFERENCE 14	Mice	150 [1]	Ataxia	35.6 ± 11
			Death	343.3 ± 110.3
REFERENCE 20	Rats	200	Recumbency	174.6 ± 125.4
			Loss of righting Reflex	272.1 ± 204.8
			Quiescent EMG	259 ± 201
REFERENCE 37	Mice	250 [3]	Loss of Righting Reflex	98.5 ± 4.7
			Cessation of Heartbeat	619.6 ± 359.6
		250 [4]	Loss of Righting Reflex	74.3 ± 4.9
			Cessation of Heartbeat	508.3 ± 303.4
		1300-1680 [5]	Loss of Righting Reflex	66.4 ± 4.5
			Cessation of Heartbeat	253.8 ± 118.1
REFERENCE 56	Rats	150	Induction of Unconsciousness	152 (105-195)
			Time to Respiratory Arrest	676 (510-815)
	Mice	150	Induction of Unconsciousness	80 (45-120)
			Time to Respiratory Arrest	482 (315-720)
REFERENCE 90	Mice	100	Death	235
REFERENCE 106	Rats	667	Ataxia	40.60 ± 4.35
			Cessation of movement	63 ± 8.15
			Loss of Pedal Reflex	175.00 ± 6.52
			Heart rate < 150 bpm	444

REFERENCE 118	Rats	200	Loss of Righting Reflex	111.6 ±19.7
			Cessation of Heartbeat	485.8 ± 140.7
		200[6]	Loss of Righting Reflex	104.2 ± 19.3
			Cessation of Heartbeat	347.7 ± 72
		800	Loss of Righting Reflex	139.5 ± 29.6
			Cessation of Heartbeat	283.7 ± 38

[1] Pentobarbital/Phenytoin combination product (390 mg/ml sodium pentobarbital and 50 mg sodium phenytoin)

[2] 50:50 Mixture 100mg/ml sodium pentobarbital with 10 mg/ml of lidocaine hydrochloride

[3] Diluted to 5mg/ml with USP sterile water from a 50mg/ml solution

[5] 50mg/ml

[5] 390 mg/ml with no Phenytoin added

[6] Diluted 1:3 with phosphate-buffered saline (PBS)

Table 2.IP PB suggested best practices

	RATS	MICE
AREA OF INJECTION	Left caudal quadrant, at the level of the coxofemoral joint, midway between midline and left abdominal wall[101]	
ANGLE OF INJECTION	20-45 degrees (in relation to the body wall)[101]	10-20 degrees (in relation to the body wall)[101]
NEEDLE LENGTH & GAUGE	3/8 inch[11], 20-25G[60,119]	3/8 inch[26], 25-30G[11]
VOLUME OF INJECTION	Maximum 5-10ml[78]	Maximum 0.5-1 ML[11]
PB DOSE	800mg/kg[119]	1300-1620 mg/kg[37]
LOCAL ANESTHETIC	Lidocaine (10 mg/ml) in a 50:50 mixture[3,59] Bupivacaine (2.5 mg/ml) in a 50:50 mixture[59]	
EXPECTED LORR	139.5 ± 29.6s[119]	283.7 ± 38s[37]
EXPECTED CHB	66.4 ± 4.5s[119]	253.8 ± 118.1s[37]

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Chapter 2 - Alternative Mice Euthanasia Methods

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Mice were the first animals on which IH injections were attempted, and this technique had never been described with this species. Also, as described in the literature review, ethanol has shown promise as an alternative to PB, so it was of interest to evaluate this agent as a potential euthanasia agent.

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Evaluating intrahepatic versus intraperitoneal sodium pentobarbital or ethanol for killing mice

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Running Title: Alternative mice euthanasia techniques

Abbreviations and Acronyms:

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

1. Abstract

Intraperitoneal (IP) injection of sodium pentobarbital (PB) is an accepted method for euthanizing mice. However, this method has important drawbacks, including misinjection and the potential for pain. The objective of this prospective, randomized, blinded study was to test if intrahepatic (IH) injection of PB is more effective than IP delivery. Secondary objectives were to: 1) confirm if IP ethanol (ET) is a suitable alternative to PB and 2) to 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 and killed via IP or IH injections, with 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 93.3% (28/30). Two successful IH injections yielded quasi-instantaneous deaths (within 4s) but this method cannot be recommended due to the possibility for intrathoracic injection (n = 4). In awake animals, time to apnea and CHB was significantly shorter with IP ET (apnea: 72.5 [median], CHB: 115s) than IP PB (apnea: 136s, p = 0.023, CHB: 176s) when animals were conscious (p = 0.01). Anesthesia at time of injection was associated with shorter CHB time for IP PB (p = 0.008). These data show that IH injections are not feasible in mice, but confirm that ET is a viable and potentially superior alternative to PB. Lastly, anesthesia can shorten time to death with IP injection of PB.

2. Introduction

Euthanasia is one of the most commonly performed laboratory procedures and there are many different techniques used to kill 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.[3,6] IP injection is a relatively simple procedure that allows for substances to be administered and absorbed rapidly,[23] but this it is not without its disadvantages.

Firstly, there is an inherent misinjection rate that varies from 10-20% in mice.[2,5,8,10,26,27] Misinjections occur when the injectate is delivered into an abdominal organ or subcutaneously instead of into the peritoneal cavity. In mice, the sites of most misinjections are the stomach, intestine, uterine horn and subcutaneously.[22,26] The result of a misinjection is often a failure to

achieve death or a significant delay, requiring an additional injection or alternative killing method.[2,7,29,32]

Secondly, there is the potential for pain and distress. IP injections of saline have been associated with: increases in plasma corticosterone levels[4,24] tachycardia and hyperthermia[9,16,21] and the expression of immediate early response genes, such as cfos[24]. Nociception has also been associated with IP injections, as a result of needle entry and potentially from the alkalinity (pH 10-12) of PB, which may be irritating.[23,29] Pain-related behaviours, such as vocalisations, writhing, hunched posture, flinching and increased locomotion have all been observed following IP injections of PB in rats and/or mice.[2,12,30] Electrical brain activity[14] and neuronal markers[28] have also been used to infer the existence of pain and nociception following 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 completely.[15,28] While the temporal relationship between nociception, pain and loss of consciousness is not always apparent, it is clear that a significant number of animals are at risk of experiencing pain given inherent variability in the onset of anesthetic effect, time to death and occurrence of misinjections.

Therefore, it remains important to continue to evaluate alternative methods of injectable euthanasia in mice. This can be done by evaluating alternative injection techniques, and/or alternative injection agents. Intrahepatic (IH) injection has been described as effective for the euthanasia of shelter cats.[13] IH injections resulted in a significantly shorter time to recumbency, loss of pedal reflex and cardiac arrest. Cats that had successful IH injections confirmed at necropsy, achieved immediate recumbency post-injection. IH is therefore a method that deserves exploration in other species, given the potential for rapid absorption of injectate.

There are few alternative injectable agents to PB that have been extensively studied, but one that shows promise is ethanol (ET). ET is already described as 'acceptable with conditions' in the AVMA guidelines on euthanasia; however, there are few studies that have investigated its use.[2,11,18,19,20] ET has an important advantage over PB in that it is much easier to access than barbiturates.[3] Allen-Worthington *et al.* (2015) reported no significant difference in times to respiratory and cardiac arrest (estimated by time to asystole via ECG) with either IP PB (100%, approximately 15.3 g/kg) or IP PB (approximately 5.4 g/kg) in mice.[2] Furthermore, this study found no differences in pain-related behaviors (vocalisations, writhing, and hunched posture) between

mice injected with ET or PB, and concluded that it was an acceptable alternative to PB. In contrast, de Souza Dyer *et al.* (2017) reported that ET should be limited to mice over 35 days of age because the time to death in younger mice (estimated as 2 min post apnea) and time to loss of consciousness (measured via the righting reflex) exceeded the times obtained with PB.[11]

The objective of this 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 PB or ET. Two secondary objectives were to: 1. confirm the findings of Allen-Worthington *et al.* (2015), showing that IP ET is a viable alternative to PB, by using a more specific method to assess time to death (via auscultation).[2] We hypothesized that the efficacy of IP ET would be similar to that of IP PB. 2. Evaluate the effect of performing IP overdose with PB or ET in animals anesthetised with isoflurane. We hypothesised that anesthesia would lead to a slower time to apnea and death.

3. Methods

3.1. Study Design

This was a prospective, randomized, blinded study and the study protocol was approved by the institutional animal care and use committee of the Faculty of Veterinary Medicine (Université de Montréal protocol ID: 17-Rech-1892).

80 adult, SPF, CD1 male and female mice (26.8g [23-34g], mean [range]) were used. Mice were purchased from a commercial vendor (Charles River Laboratories, Senneville, Quebec) for an unrelated project and scheduled for euthanasia (surplus animals). 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 (alpha 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.[2,32] For the secondary objectives, with the same outcome measures, a sample size estimate of 10 animals (alpha of 0.05, 80% power, effect size 0.6), including a potential 20% misinjection rate, was determined based on data from the literature.[2]

Animals were housed in groups of five 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 (Nestlets). The housing environment was controlled: 12h light-12h dark cycle (lights on at 0700 hours), room temperature 22°C, humidity from 30-35%. 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 16:00 and 19:00 hours.

3.2. Treatment Groups

There were two parts to the experiment. In the first part, anesthetized mice were block randomized to one of four 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) x 12.5 (W) x 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 two treatment groups (n = 10 per group): 1) IP injection of ET (IP ET_{awake}) and 2) IP injection of PB (IP PB_{awake}).

3.3. 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 two-person technique; an injector and a holder. For IP injections, the holder maintained the mice in horizontal dorsal recumbency by gently gripping the scruff. 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 degree angle to the body wall.

For intrahepatic injections a small stop was fashioned out of a needle cap and threaded over the needle to shorten its usable length to 3/8 inch. This was done to reduce the risk of needle insertion and injection into the thorax. The holder held the animal vertically (head upwards). 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 45-75 degrees relative to the long axis of the body wall).

Both the holder and the injector were blinded to treatment allocation. Following injection, a timer was started by an observer and presence of a heart beat (thoracic auscultation) and respiratory movement (visual assessment) was monitored by the holder. The following times were recorded: injection to apnea and injection to cessation of heartbeat (CHB).

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. 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 minutes, the attempt was classified as a failure and the animal was killed with an overdose of CO_2 gas (gradual fill method).

3.4. Necropsy Examination

Following death, each animal was placed in dorsal recumbency for necropsy evaluation by a single observer, blinded 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 confirm 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 subcutaneously), 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, intra-organ, intra-thoracic locations, whereas misinjections in the IH groups also included injectate located in the peritoneal cavity

3.5. Statistical Methods

Statistical analyses was performed using commercial software (GraphPad Prism v.8.02, GraphPad Software, Inc. La Jolla, California, USA). A Shapiro-Wilks test was used to determine if data were 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's post hoc test (IP ET vs IP ET_{awake}, IP PB vs 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 10-90 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.

4. Results

4.1. 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 were 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 two 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 intra-thoracic misinjections resulted in death (time to CHB of 2, 22, 27 and 28 seconds). The intramuscular injection failed to achieve CHB within 5 minutes, 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 – 91) and 72s (27 – 105) for IH ET and IH PB groups, respectively. CHB was achieved in 123.5s (63 – 264) and 124s (88 – 222) for IH ET and IH PB groups, respectively.

Of the four misinjections from the IP treatment groups, three resulted in death (CHB in 146, 154 and 265s). One misinjection failed to result in death within 5 minutes 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), awake IP ET (n = 9) and awake IP PB (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.

4.2. 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).

4.3. Effect of using ECG versus 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).

4.4. Effect of anesthesia

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

5. 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 resulted in a faster death in animals anesthetized with isoflurane than awake animals.

5.1. Intrahepatic Injections

IH injections have been used with success in cats.[13] Grier (1990) evaluated and compared efficacy, accuracy (via assessment of misinjections in necropsy) and response to pain (defined as vocalization or turning head towards the injection at the moment of injection) of IH injections and IP injections in adult cats.[13] 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, versus 4/77 (4%) of animals injected IP.

The IH misinjection rate observed in the study reported here is markedly higher than the misinjection rate typically reported for IP injection in rodents (6-20%).[2,5,8,10,26,27] Although the majority of IH misinjections still yielded successful and rapid euthanasia, reflecting the IP placement of injectate in many cases, four 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 currently recommend the IH route of injection in awake mice due to the potential for intrathoracic delivery and furthermore, with the low proportion of successful IH injections, there are no apparent benefits over IP ET. It may be that a change in IH injection technique, such as using a shorter needle or changing the insertion angle or site, could yield better results. In contrast to the findings of this study, preliminary data from rats[17] suggest greater success can be achieved in other species, though this requires further investigation.

5.2. Ethanol

The second objective of this study was to evaluate the efficacy of ethanol as an alternative agent to PB. Allen-Worthington *et al.* (2015) found it was 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).[2] 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. Additionally, 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 two fold difference in time when identifying CHB with auscultation versus the presence of asystole. Together, these results confirm and build on the findings of Allen-Worthington *et al.* (2015), adding further support for IP ET over PB for the killing of mice.[2]

5.3. Effect of anesthesia

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. It may be that despite the presence of cardiovascular depression, the central nervous system depression associated with general anesthesia made it easier to achieve apnea and CHB. Further work, standardising the depth of anesthesia and quantifying cardiovascular depression, would be required to elucidate the mechanism of these observations.

5.4. Limitations

We did not measure loss of consciousness, therefore we cannot comment on the time animals may be experiencing pain, if present. This is an important factor when discussing optimal killing methods. A further limitation to 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. There are many difficulties associated with measuring behavioral pain responses in the presence of a drug that depresses motor function as sedation occurs followed by general anesthesia. Allen-Worthington *et al.* (2015) measured certain pain-related behaviours such as vocalization, writhing (abdominal contraction), and hunched posture.[2] There were no statistical differences in these behaviors between the mice treated with ethanol or the mice treated with PB. Interestingly, the group displaying the most signs of pain was the saline injected group. We believe this highlights the

difficulties in assessing behavioral motor outcomes (such as those associated with pain) in the presence of an increasing level of sedation, as occurs following PB or ET injection.

6. Conclusion

IH injection cannot be currently recommended for killing non-anesthetized mice. In contrast, IP ET is a viable and appealing alternative to IP PB due to its rapid action. The speed of death induced by IP PB or ET in anesthetized (versus awake) mice was an unexpected result and requires further investigation.

7. Acknowledgments

The authors thank Dr Frédérik Rousseau-Blass for technical support and insightful discussions.

8. Figures

Figure 1 Comparison of time to apnea per treatment group

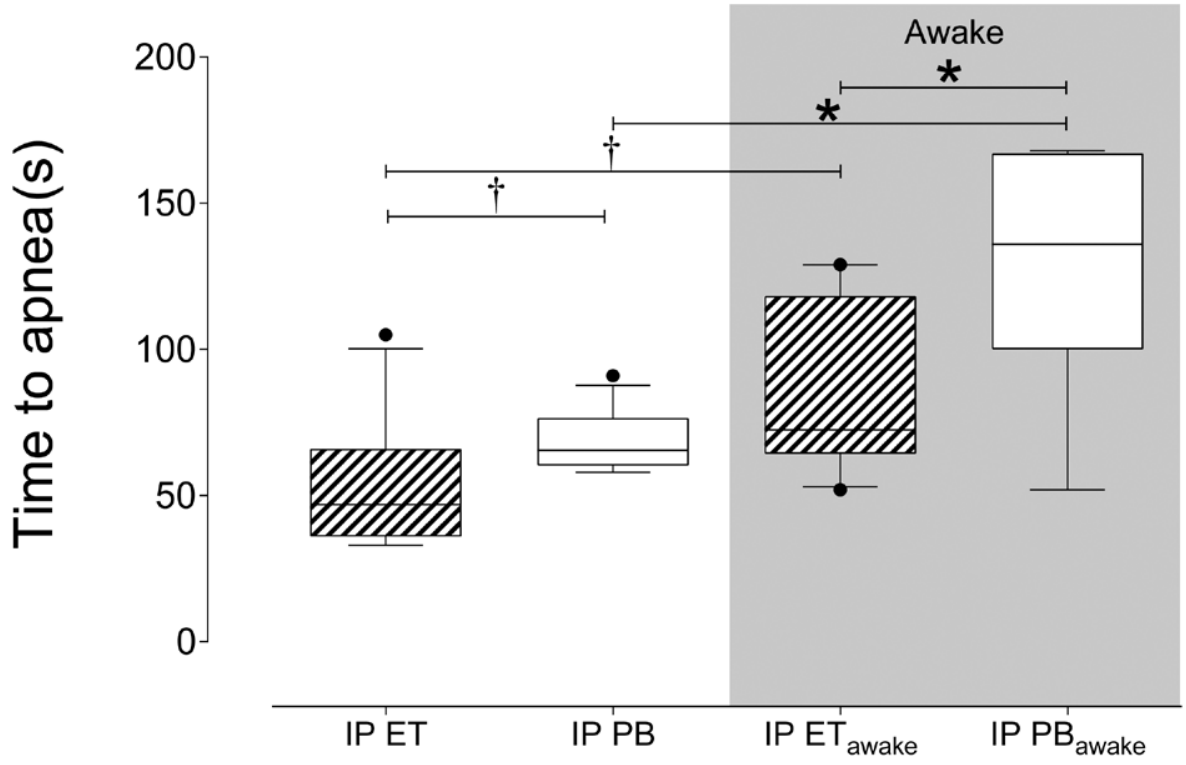

Figure 1. Box and whisker plot of the time to apnea (seconds). The horizontal line within each box represents the median, the lower and upper box limits represent the interquartile range and the whiskers represent 10-90 percentile. Treatment groups are: intraperitoneal (IP) injection, with sodium pentobarbital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

Figure 2. Comparison of Time to Death per treatment group

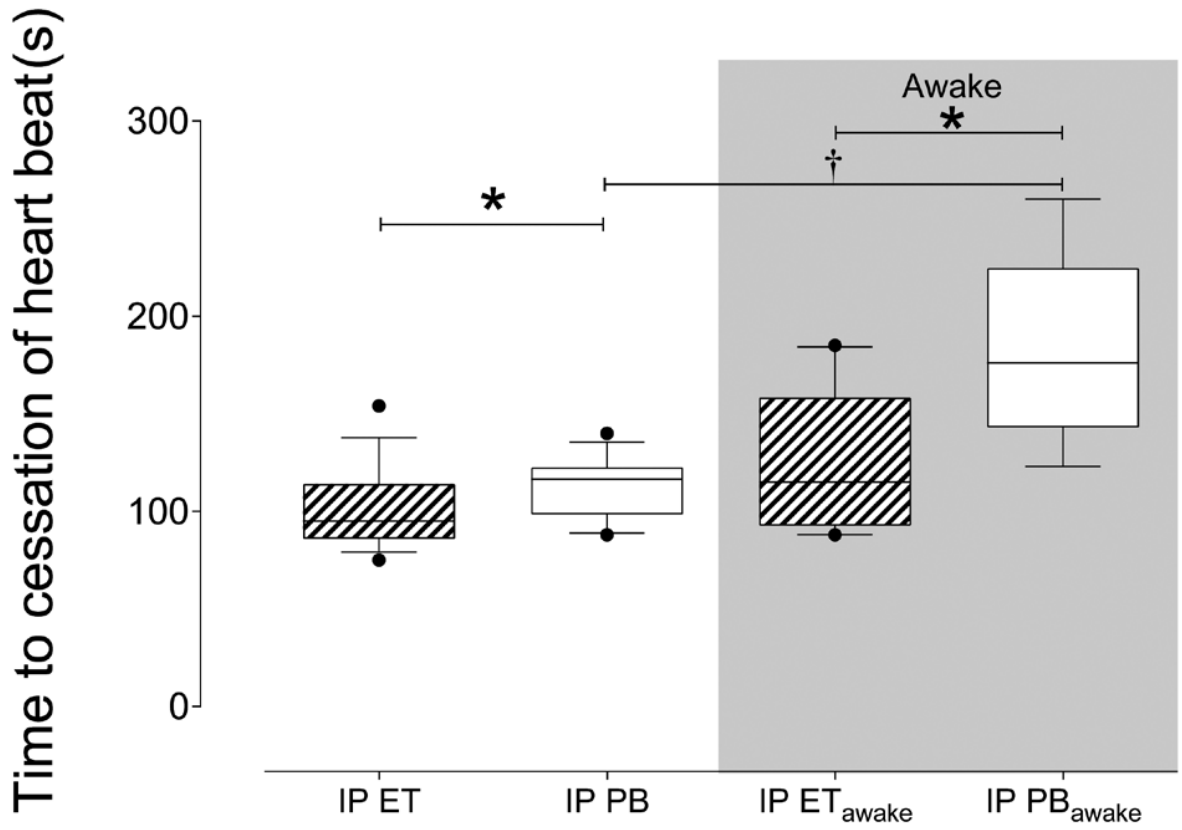

Figure 2. Box and whisker plot of the time to cessation of heartbeat (CHB, seconds). The horizontal line within each box represents the median, the lower and upper box limits represent the interquartile range and the whiskers represent the 10-90 percentile. Treatment groups are intraperitoneal injections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

Figure 3. Comparison of time to asystole for ET and PB

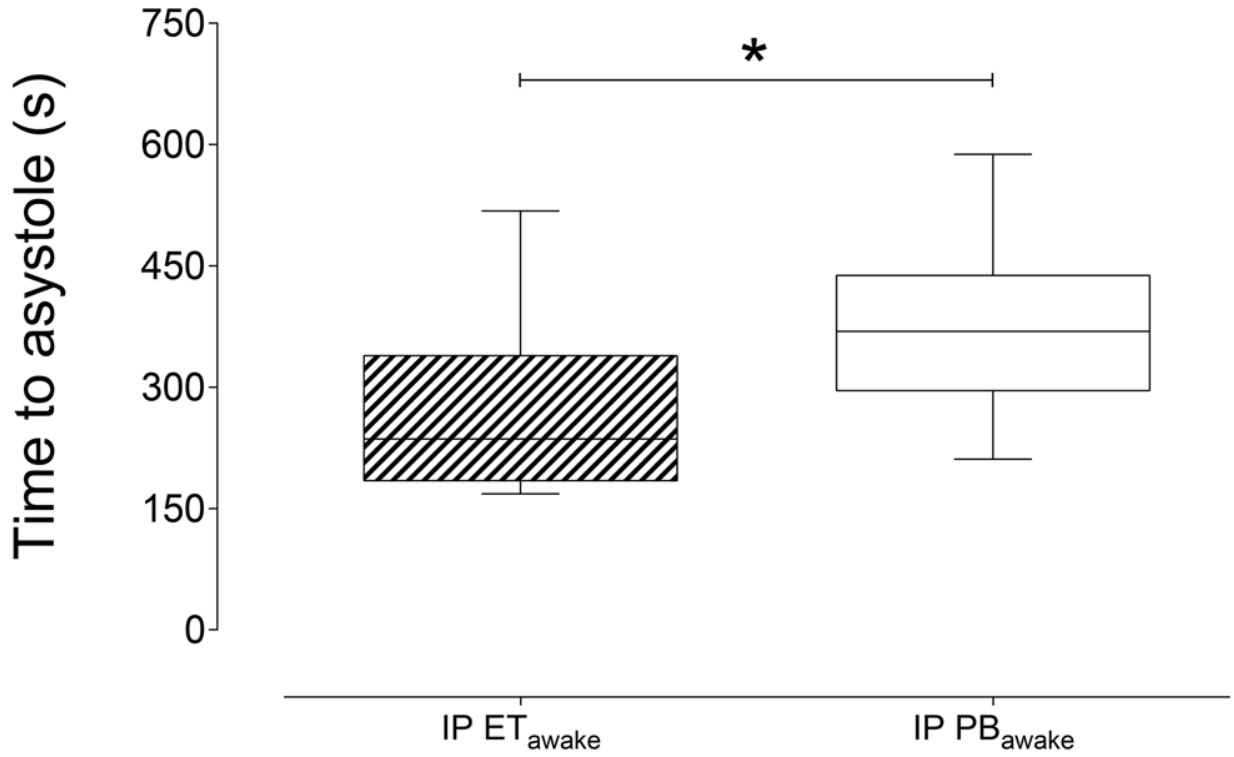

Figure 3. Box and whisker plot of the time to asystole (seconds). The horizontal line within each box represents the median, the lower and upper box limits represent the interquartile range and the whiskers represent the 10-90 percentile. Treatment groups are: intraperitoneal injections (IP), with sodium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

Figure 4. Comparison of time to death for ET and PB

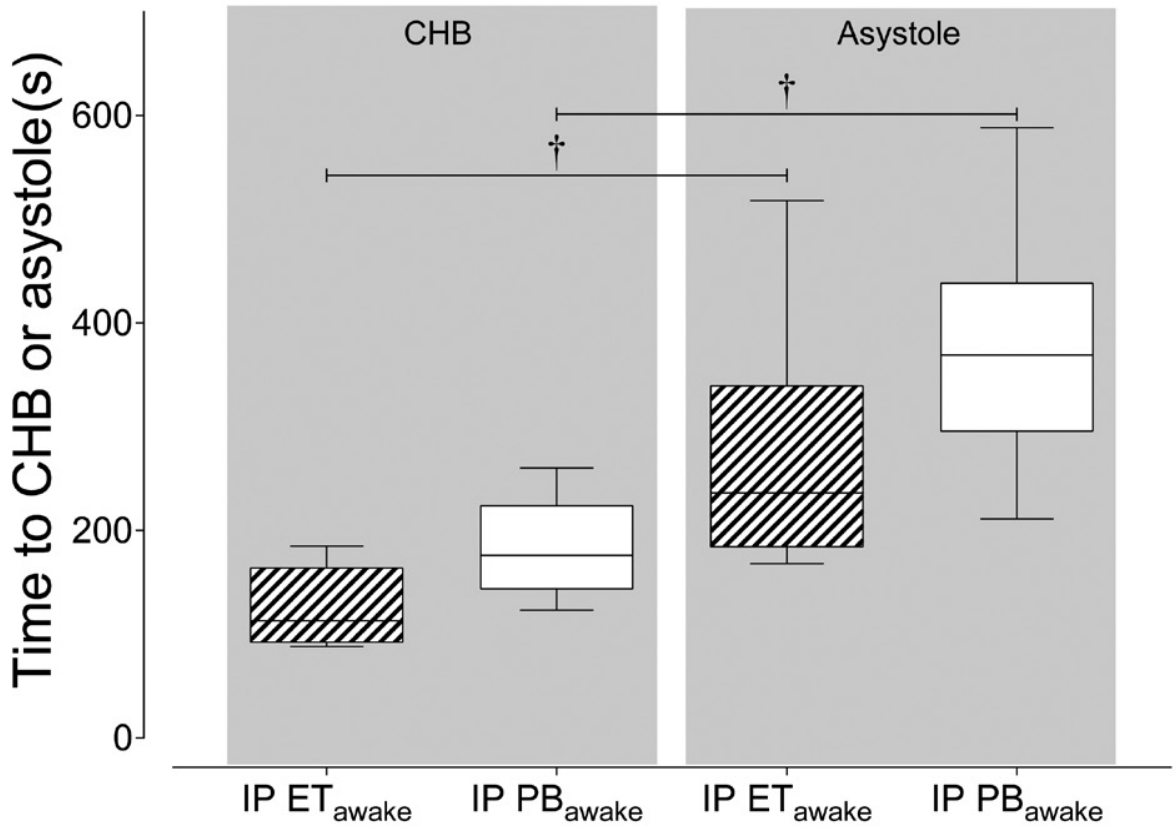

Figure 4. Box and whisker plot of the time to cessation of heartbeat (CHB) or asystole (seconds). The horizontal line within each box represents the median, the lower and upper box limits represent the interquartile range and the whiskers represent the 10-90 percentile. Treatment groups are: intraperitoneal 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 ** ($P < 0.01$).

Figure 5. Relation between time to CHB and time to asystole

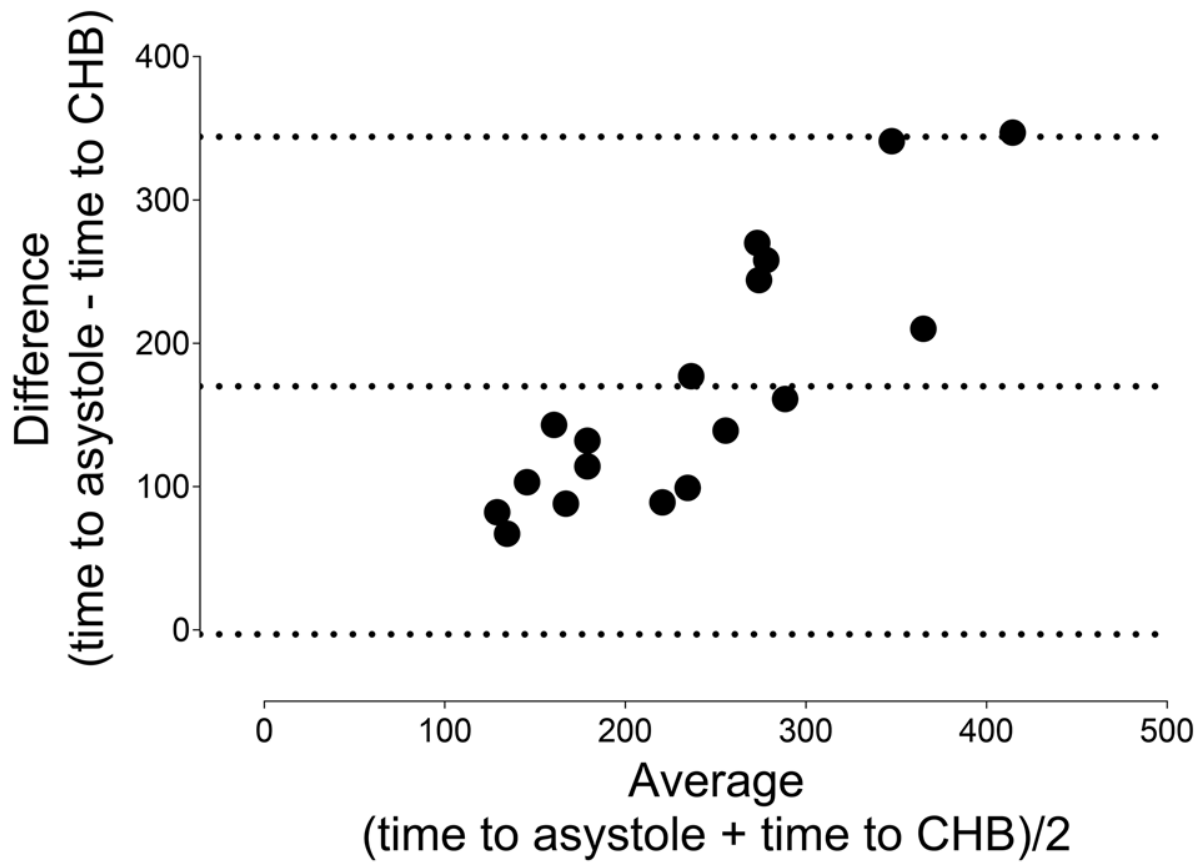

Figure 5. Bland-Altman plot comparing time to cessation of heartbeat (CHB) and time to asystole. Time to CHB underestimated time to asystole by 170s with limits of agreement ranging from -3.1 to 344s. Data were pooled from awake animals administered intraperitoneal (IP) pentobarbital (PB) or ethanol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

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Chapter 3 - Alternative Rat Euthanasia Methods

The following article will be submitted for publication in the journal JAALAS. The focus of the article is on the IH technique, as this technique had never been described as a euthanasia method for rats before. The article also includes a description of the pilot study wherein the objective was to develop a protocol of injection and test its feasibility.

Laferriere CA, Leung VSY, Rousseau-Blass F, Lalonde-Robert V, Pang DSJ. Intrahepatic injections of sodium pentobarbital as an alternative to intraperitoneal injections for the euthanasia of rats. ***Soon to be submitted***

Author contribution:

CL: literature search, concept, drafting and revision, data collection, analysis, interpretation.

approval of final version

VL: data collection, analysis, interpretation, approval of final version

FRB: data collection, approval of final version

VLR: data collection, drafting and revision, approval of final version

DP: data collection, concept, revision, approval of final version

Intrahepatic injections of sodium pentobarbital as an alternative to intraperitoneal injections for the euthanasia of rats

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Running Title: Alternative rat euthanasia technique

Abbreviations and Acronyms:

CHB, Cessation of heartbeat; IH, Intrahepatic; IP, Intraperitoneal; PB, Sodium Pentobarbi

1. Abstract

The most commonly accepted method of rat euthanasia is an intraperitoneal (IP) injection of sodium pentobarbital (PB). However, misinjections are unavoidable (6-20% occurrence) and IP PB may cause pain and stress. The objective of this study was to test an alternative method of euthanasia, intrahepatic (IH) injections of PB. A pilot study was conducted to assess the feasibility of IH injections, followed by a comparison of IP (n = 14) and IH PB injections (n = 66). 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 IH injection or misinjection via necropsy. All injections were performed by a veterinary student. Time to LORR and CHB were significantly faster with IH injections (LORR: 4s (1 to 96) [median (range)], CHB: 142.5s (2 to 330)) than IP injections (LORR: 89.5s (73 to 110), CHB: 275s (237 to

423), $p < 0.0001$). The misinjection rate was higher with IH injections than IP injections (IH: 59%, IP: 29%); however, IH misinjections still resulted in fast and successful euthanasia (LORR: 29s, CHB: 216s) with the injectate distributed between the IP and IH locations. The number of failed euthanasias with IH injections was low (n = 2). IH injections are an alternative to IP injections for rat euthanasia.

2. Introduction

Millions of laboratory rats are killed upon completion of research projects worldwide.[3,7] Currently, the only method that is classified as acceptable by both the American Veterinary Medical Association (AVMA) and the Canadian Council on Animal Care (CCAC) is an overdose of sodium pentobarbital (PB) by intraperitoneal (IP) or intravenous injection.

Of these, the IP route is more common as it is easier and faster to perform. However, it has two important disadvantages that interfere with whether "euthanasia" (a good death) is always achieved. The first disadvantage is an inherent misinjection rate that varies between 6-20% for rats.[5; 9,11,12,18,22,24,29] The consequences of a misinjection is a delay or failure to achieve loss of consciousness or death.[8,26,28,29] In these cases, the injection may need to be repeated or an alternative killing method applied.

The second disadvantage is the potential for pain and distress. Converging evidence for this 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 IP injection of PB.[4,10,15,17,19,21,25] The source of nociception and potential pain is unclear, but the alkaline pH (typically 10-12) of PB solution is a potential factor.

The CCAC and AVMA euthanasia guidelines acknowledge that IP 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.[2,16,25] Furthermore, the amount of lidocaine (or other buffer) that can be added is limited. As the pH descends below approximately 10, the PB will precipitate.[28] Additionally, the potential for pain increases when misinjections occur as a result of the potential delay until loss of consciousness or

pain resulting from the site of misinjection. When IP PB is successful, the mean time to loss of consciousness is approximately 1.5-2 minutes, during which time pain may be experienced.[29]

Therefore, there is an ongoing need to refine IP PB or identify alternative killing methods to achieve euthanasia. A relatively unexplored injection route is intrahepatic (IH) injection. This has been successfully applied in cats,[14] where successful IH injection resulted in almost immediate recumbency but remains untested in rats.

The objectives of a pilot study were to: 1) identify the appropriate injection site and angle and 2) test the feasibility of the IH injection methods in rats. These results were applied to the main study, with the objective of investigating if the IH injection technique could be an alternative killing method to IP injection in rats. We hypothesized that an IH injection would result in a shorter time to loss of consciousness and death than IP injection, with a reduction in the number of failures to achieve death.

Ethical statement

All experiments were approved by the Université de Montréal (18 RECH-1892) and the Charles River Laboratories Montreal ULC Institutional Animal Care and Use Committee. The study of a novel euthanasia procedure, differing from the Canadian Council on Animal Care guidelines, was approved by the animal care and use committees. All rats used in the experiments were scheduled for euthanasia.

3. Pilot Study

A two-part pilot study was performed to identify a potential IH injection approach (part 1) and to test injection feasibility (part 2).

3.1. Part 1

In a terminal procedure, four Adult Sprague Dawley rats (3 males and 1 female; 260-390g) were anesthetized with dexmedetomidine (30 ug/kg IP) and ketamine (100 mg/kg IP), followed by isoflurane for computed tomography 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 vertical (head up) and dorsal recumbency (positions selected based on common

positions for performing IP injection). From reconstructed images, organ location and measurements were taken. A potential needle insertion site and trajectory was determined based on these measurements, with the goal of minimizing risk of misinjection into other organs or the thorax.

A needle insertion site at the xiphoid process was identified. At this site there were two organs in proximity (stomach and right kidney), with these organs and the thoracic cavity as potential sites for misinjection. The relationships of these sites to the liver was measured (Fig. 1, distance a-c and Fig. 2). To determine a potential angle of insertion and needle trajectory, distances from the xiphoid process to the cranial margin of the liver was estimated (Fig 1, distance a-b).

Additionally, the liver thickness of the liver at the injection insertion site (xiphoid process) was measured at necropsy using digital Vernier calipers.

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

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

The locations of the stomach and the right kidney were 19.75 mm (18 to 25; and 22mm (17 to 28.5), respectively, from the insertion site. Distances 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 < 45 degrees increases the risk of entering the thoracic cavity.

Distances from the xiphoid process to the right kidney and stomach were consistently greater with rats suspended vertically than in dorsal recumbency: kidney (vertical; 32.6 [19.3-34.2] dorsal; 18.65 [11.3-21.1] mm, $p = 0.03$, Mann-Whitney test), stomach (vertical; 21.4 [13.3-26.2] dorsal; 5.85 [3.4-10.8], $p = 0.03$, Mann-Whitney test). Therefore, vertical positioning was selected to reduce the risk of misinjection.

3.2. Part 2 – Injection Protocol

IH injection of PB was performed using the identified insertion site and needle angle described above on male (n = 8) and female (n = 11) Sprague Dawley rats (515 [343-980] g). For all injections, the dose of PB was 800 mg/kg (euthanyl, Bimeda-MTC, Cambridge, Ontario, 240 mg/ml). Blue food dye (0.05 ml, Club House, Burlington, Ontario) was added to the PB to facilitate necropsy evaluation of injectate distribution.

The order of injections was randomised with a list randomizer (random.org). All solutions used for euthanasia were placed in 3 ml syringes and a new hypodermic needle used for each injection (25g, 5/8 inch, 16 mm).

Injections were performed using a two-person technique, with an injector and a holder. For the IH group, the holder gently restrained the rat ("backpack hold"; one hand supporting hindlimbs with other hand cradling thorax [index and middle finger on either side of head, thumb and remaining fingers beneath forelimbs]). Rats were held vertically (head up). A single experimenter performed all injections (veterinary student, CL).

The injector identified the xyphoid process by gentle digital palpation and inserted the needle immediately caudal to this point, with an angle of insertion of approximately 45 degrees to the body wall, needle directed towards the head. The needle was fully inserted in all cases and the injection given over 2-3 seconds.

Immediately following injection, an attempt was made to test the LORR by placing the animal on its back. LORR was determined to have occurred if the rat remained on its back for at least 15 seconds. If LORR did not occur, the rat was continuously observed until ataxia or sedation (head lowered towards floor) occurred, at which time LORR was re-assessed. The LORR was re-assessed every 30 seconds until 3 minutes had elapsed. Once LORR occurred, there was no further testing and the thorax was auscultated continuously by the holder using a stethoscope, to identify CHB. Both experimenters continuously observed the rat for apnea. Two outcomes were required in order to designate an injection as a failure: 1) If LORR did not occur within three minutes after injection and 2) if a heartbeat continued beyond 5 minutes after injection. If these two conditions were met, a secondary killing method was used (general anaesthesia with isoflurane, followed by overdose with inhaled carbon dioxide).

3.3 Necropsy Examination Main Study

Following confirmation of death, a single observer (CL), who was blinded to treatment as the rats were opened in necropsy by a secondary person and presented to the observer randomly, performed a necropsy examination, following a standard procedure. With each animal in dorsal recumbency, the abdomen (midline) was incised and the interior examined to establish injectate distribution. The liver and intestines were removed, incised and examined for evidence of injectate (blue coloration), followed by examining 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 IH injections was classified as: 1. “confirmed IH” (presence of injectate restricted to the liver, no sign of dye elsewhere in the abdomen) or 2. IH misinjection, in cases where injectate was identified at any site outside the liver (e.g. in the abdominal cavity, subcutaneously, intramuscularly, inside an abdominal organ, within the thoracic cavity). Misinjections were further classified as “incomplete-IH” if injectate was present intraperitoneally (with the possibility of some injectate IH). regardless of presence/absence of injectate in liver.

For intended IP injections, successful trials were classified as: 1. “confirmed IP” (presence of injectate in the abdomen) or 2. IP misinjection, where injectate was identified in any unintended location (e.g. within an abdominal organ, IM, SC).

Of the 19 IH trials, 16 resulted in successful euthanasia. The three unsuccessful attempts were all misinjections into the falciform fat pad in larger rats (558g, 910g, and 980g). For the 16 successful injections, the time to LORR was 4.75 seconds (1 to 114) and time to CHB was 135.5 (8 to 360). At necropsy, there were 3 (15.8%) confirmed IH injections and 16 (84.2%) misinjections, of which 13 were incomplete-IH (68.4%) and 3 were located in the falciform fat pad (15.8%).

Time to LORR, grouped according to necropsy results, were: confirmed IH 3.5s (2 to 5), incomplete-IH 5s (2 to 114). For times to CHB: confirmed IH 12s (8 to 120), incomplete-IH 178s (86 to 360).

Based on these preliminary data, IH injection of PB was determined to be feasible. The 3 failed euthanasia attempts occurred in larger rats. It may be that body mass imposes a limitation to the IH route described. Therefore, we hypothesized that increasing the angle of insertion (closer to 0 degrees, perpendicular to the skin), would reduce the failure rate for larger rats.

6. Discussion

This study describes a novel IH injection technique and has shown 2) that IH injections resulted in a shorter time to LORR than IP injections, 3) that the relatively high proportion of incomplete-IH misinjections in the IH group still resulted in a faster time to LORR than the IP route and 4) there are fewer failures to achieve death following IH than IP injection. Overall, the IH injection technique is efficient, simple to perform and has a low risk of failure to result in death. These findings support the hypothesis that IH injections are a viable and potentially preferable option to IP injection as a humane killing method in rats.

6.1. Feasibility of IH Injections

The pilot study showed the IH injection technique to be feasible. However, it was observed that inadvertent injection into the falciform fat pad could occur in larger animals. The risk of intra-thoracic injection is important to consider as it is associated with pain and distress in awake animals.[3] This risk was minimised by avoiding angles of needle insertion of less than 45 degrees to the sternum.

The ideal body position was vertical as it created more distance between the liver and stomach and right kidney as measured via the CT-images. This differs from IP injections, in which dorsal recumbency is often preferred.[20]

Two concerns arose from these pilot data. The first was the high misinjection rate; successful IH injections were only confirmed in 15.8% of attempts, with all other attempts classified as misinjections. Importantly, the majority of misinjections (81%), were classified as incomplete-IH and still resulted in a rapid LORR. The second concern was the possibility of an increase in failures to achieve death in rats larger than 500g. These concerns were incorporated into the main study design.

6.2. IH Injections

IH injections were more efficient than IP injections because of the rapid LORR. While the misinjection rate was high, the majority of misinjections were incomplete-IH injections and time to LORR for incomplete-IH injections remained approximately 3 times faster than IP injections.[29] This suggests that in the incomplete-IH injections, some PB was being deposited IH, resulting in a

rapid action. A potential complication of the IH technique in larger rats is the presence of a prominent falciform fat pad, which may impede the injection. Further work is needed to determine if this risk can be reduced or eliminated.

6.3. IP Injections

Comparing IP and IH misinjections reveals an important contrast: an IH misinjection is likely to still lead to a rapid LORR and death, whereas an IP misinjection often leads to a failed killing attempt.[8,26,28,29] One of the principal sites of IP misinjections is the cecum.[18] Though IP injections are traditionally given in the right caudal abdominal quadrant based on the predicted position of the cecum in the left caudal quadrant, two studies[11,27] reported that cecum position is highly variable. It may be located in the right caudal quadrant or in the middle of the abdomen approximately 20-30% of rats. In contrast, IH misinjections most often lead to incomplete-IH deposition, resulting in rapid LORR and CHB. The differences in fates of misinjections is an important advantage of the IH injection route over IP delivery.

The rapid LORR following IH injection is advantageous when considering the duration of potential distress and pain as shorter times to these outcomes would minimise these adverse effects. While the extent of pain associated with PB in the abdomen remains to be fully elucidated, current evidence suggests that some degree of pain is likely.[1; 2,16,25,15,25] Therefore any novel PB delivery technique should also be assessed against this possibility. The rapidity and consistency of effect with IH delivery makes it an appealing alternative to IP injection.

6.4. Limitations

An important limitation of this study was the lack of pain assessment, an important outcome measure when evaluating killing methods. As such, it is impossible to know if IH injections of PB are more or less painful than IP injections of PB. In an IH injection study with cats,[14] it was reported that behavioral responses associated with pain (vocalization and turning the head towards the injection at the time of injection) were slightly greater following IH injections than IP injections: responses were observed in 8/85 (9%) animals injected IH, versus 4/77 (4%) injected IP. A study including appropriate behavioral outcomes, such as the Rat Grimace Scale[23] and writhing, could help determine the presence of pain.

Unfortunately, it was not possible to quantify the injectate volume in either the liver or intraperitoneal space. Doing so would be useful to clarify the disposition of injectate following misinjection and indicate the extent to which IH injection occurred.

7. Conclusion

In conclusion, IH injections of sodium pentobarbital are an efficacious and consistent killing method that should be considered as an alternative to IP injections for the killing of rats. Following the protocol outlined in this study, IH injections yield a rapid LORR and time to CHB, and present little risk of failed euthanasia attempts.

8. Figures

Figure 1 CT-scan image of rat's abdomen - Transverse view

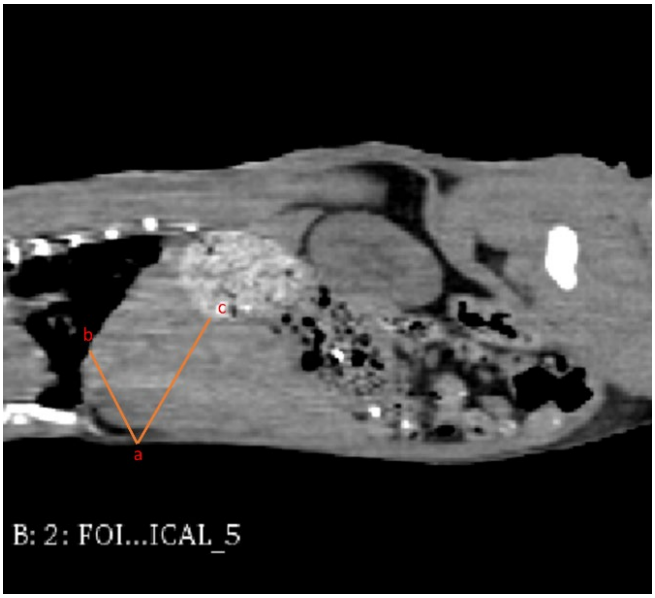

Figure 1. Transverse slide image of computated tomography scan of a rat's abdomen. Cranial is towards the left and dorsal towards the top. Shown are measurements used to determine intrahepatic injection protocol and misinjection risks. Measurement a-b is an example of thickness of liver between injection location and diaphragm. Distance a-c is distance between injection location and nearesr aspect of the stomach.

Figure 2 CT-scan image of a rat's abdomen - Frontal view

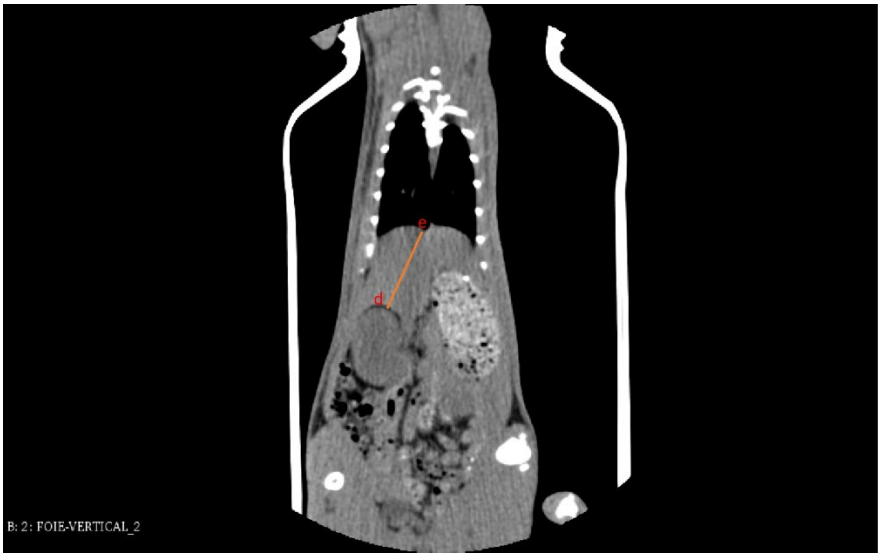

Figure 2. Frontal slide image of computated tomography scan of a rat's abdomen. Cranial is towards the top. The rat was in vertical position for the scan to emulate injection position (using glass jar). Shown are an example of measurements used to determine intrahepatic injection protocol and misinjection risks. Measurement d-e is an example of space between injection location and the nearest aspect of the right kidney.This distance was greater whenr ats were held in vertical position (versus dorsal recumbency)

Figure 3. Comparison of time to LORR and time to CHB, IP versus IH

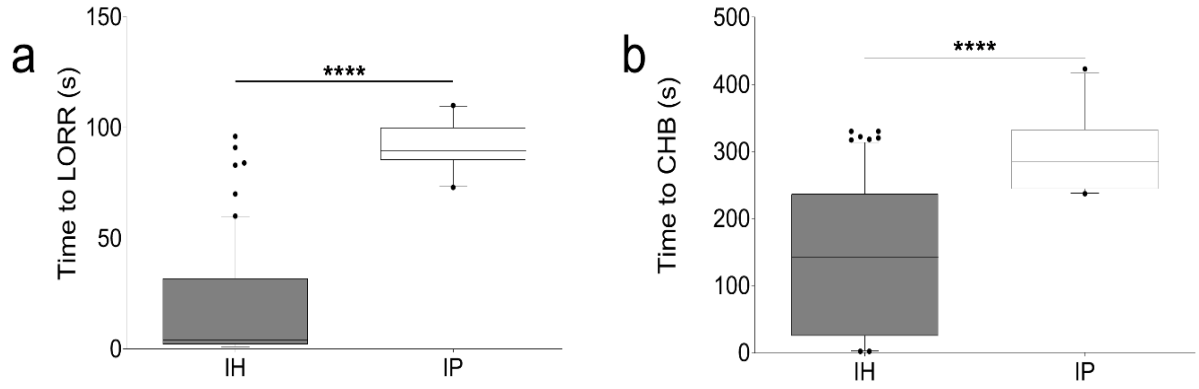

Figure 3. Box and whisker plot of a) time to Loss of Righting Reflex (LORR) and b) time to Cessation of Heartbeat (CHB) for all successful IH and IP injections. The horizontal line within each box represents the median, the lower and upper box limits represent the interquartile range and the whiskers represent the 10-90 percentile. Treatment groups are: intrahepatic injections (IH) or intraperitoneal injections (IP), both with sodium pentobarbital (PB). Significant differences are indicated by **** ($p < 0.0001$)

Figure 4. Comparison of time to CHB and time to LORR per necropsy evaluation groups

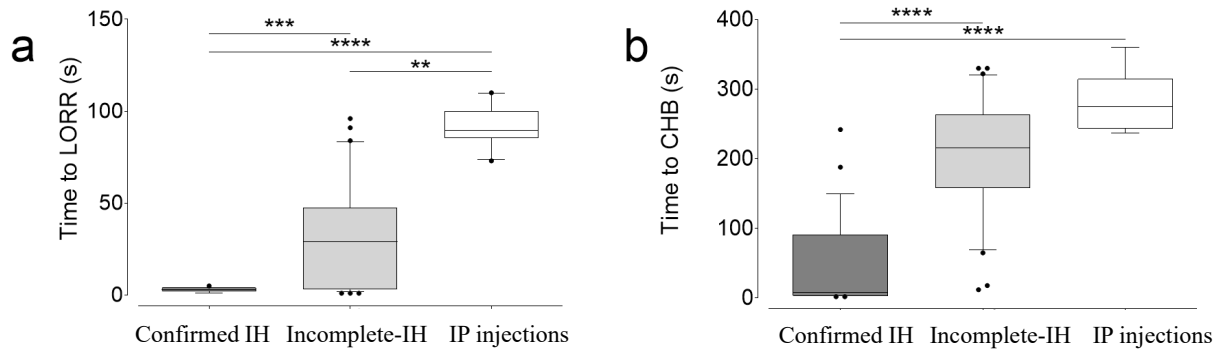

Figure 4. Box and whisker plot of a) time to Loss of Righting Reflex (LORR) and b) time to Cessation of Heartbeat (CHB). The horizontal line within each box represents the median, the lower and upper box limits represent the interquartile range and the whiskers represent the 10-90 percentile. Treatment groups are based on necropsy evaluations: IH and incomplete-IH are outcomes that could occur following IH injection attempts (IH is a success, whereas incomplete-IH is a misinjection), whereas IP is an outcome following IP injection attempts. Significant differences are indicated by ** ($p = 0.0017$) *** ($p = 0.0004$) **** ($p < 0.0001$)

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General Discussion

Chapter 1: 'Literature Review'

In some countries (including Canada and countries of the EU), there is available access to the exact numbers of laboratory animals euthanized on an annual basis. In other countries such as the US, there are no precise data because rats and mice do not fall under the purview of the Animal Welfare Act. Nonetheless, Taylor et al (2005) estimated that, globally, well over 100 million laboratory animals, mostly rats and mice, are euthanized, placing euthanasia squarely in the position of being one of the most commonly-used laboratory procedures. As such, it is essential to assure that all procedures involving euthanasia are done with the utmost confidence in their consistency and reliability. With such large numbers of animals, even minor deviations in efficacy of a procedure can put the wellbeing of millions of animals at risk. For instance, the misinjection rate of IP PB injections has been covered in all chapters of this thesis and is generally accepted to be between 6-20% (Lewis et al. 1966; Miner et al. 1969; Coria-Avila et al. 2007; Ballard 2009; Uysal et al. 2017). If this could be consistently lowered in both rats and mice, then potentially millions of animals every year would not need to suffer from the delays in anesthetic effect caused by misinjections. The widespread impact of even minor improvements, as well as the judicious selection of techniques, make research in this domain essential.

This was the main driving focus of this thesis and, in light of the new techniques and refinements described herein, it is important to revisit the guidelines and rating system as well as other methods of euthanasia to evaluate IH injections in rats and ethanol injections in mice.

The two regulating bodies that publish jurisdictional guidelines on the euthanasia methods used in this thesis are the AVMA (American Veterinary Medical Associations) and the CCAC (Canada Council on Animal Care). Both organisations assess different euthanasia methods and rate them based on various guiding principles. The AVMA explicitly cite the criteria used to assess and evaluate the various euthanasia principles. These criteria are displayed in Table 1 and serve as the main assessment tools with which all euthanasia methods are rated. It is useful, in any assessment of a novel euthanasia procedure, to refer to these criteria during assessment of its usefulness compared with other methods. The CCAC describe general guidelines with which euthanasia procedures should be performed and these are similar in essence to the 14 criteria in Table 1.

For both the AVMA and the CCAC, acceptable methods are those methods that based on the current scientific literature, provide the most consistent and rapid results, yield the least amount of distress/pain and finally do not impede the other outlined principles/criteria (Table 1). If a method is proven to be less efficacious, involve more risk or if it does not provide the same consistent outcomes, then it may be deemed 'conditionally acceptable' or 'acceptable with conditions' by the CCAC or AVMA respectively. The four following methods: injection of barbiturics, injection of a combination of dissociative agents, overdose of inhalant anesthetics, and carbon dioxide, are the accepted methods and/or the most commonly used in North America.

There are no perfect methods, and the paucity of information available on these various methods has, in practice, often led to an idiosyncratic approach being used to determine which method of euthanasia is to be employed in each case. Indeed, despite the ratings, it is not uncommon to see methods that are categorized as "conditionally acceptable" used more often than methods categorized as "acceptable".

The use of carbon dioxide is one such example. CO_2 remains one of the most used methods of euthanasia, despite it being categorized as conditionally rather than universally acceptable by both the AVMA and CCAC. Indeed, gaseous agents such as CO_2 are economical, practical and appealing means of euthanizing animals, especially when many animals need to be euthanized or the animals cannot be easily or safely handled, and some authors report no pain or distress following CO_2 euthanasia (Blackshaw et al. 1988, Smith and Harrap 1997, Hackbarth et al. 2000 However, many more studies have demonstrated that CO_2 is aversive and may induce stress and pain (Leach et al. 2002, Conlee et al. 2003, Niel and Weary 2006, Niel and Weary 2007, Niel et al. 2008a, Niel et al. 2008b, Chisholm and Pang 2016, Hickman et al. 2016). Moreover, studies on humans revealed that relatively low levels of CO_2 (8%) cause dyspnea (Dripps and Comroe 1947, Liotti et al. 2001) whereas higher concentrations (30-54%) can cause pain to the cornea, conjunctiva and nasal mucosa (Anton et al. 1992, Chen et al. 1995, Thurauf et al. 2002, Feng and Simpson 2003). In a review, Conlee et al. (2003) found that the majority of articles (15/21) published on the subject did report either stress, pain or aversion related to the use of CO_2 and suggested that this gas is not an ethical means of euthanasia. It does not meet the objectives of Criterion #1 (Table I) and thus it should not be used unless a particular set of circumstances renders other methods impossible.

Inhalant anesthetics such as isoflurane, when used a pre-sedation to CO_2 or as a sole killing agent, are usually considered a refinement over CO_2 alone because they cause less aversive behavior and induce less signs of stress than does CO_2 (Leach et al. 2002, Makowska and Weary 2009, Makowska et al. 2009, Chisholm et al. 2013, Wong et al. 2013). Yet this is not universally accepted, as Valentine et al. 2012 found an increase in the neuromolecular and behavioral signs of stress when mice were sedated with isoflurane prior to CO_2 euthanasia compared to mice euthanized with CO_2 alone. Therefore the issue remains debatable, even though the most commonly accepted conclusion is that inhalant anesthetics are less aversive than CO_2. Despite their relative appeal, inhalant anesthetic agents such as isoflurane and halothane have still been associated with aversive behaviour in rodents, so it is not clear if their use as pre-sedation or as a sole agent of euthanasia can actually be considered humane (Leach et al. 2002, Makowska and Weary 2009, Makowska et al. 2009, Valentine et al. 2012). In addition to the aversive nature of inhalant anesthetics, their use also requires specialised equipment, and time to death can be quite lengthy (CCAC 2010). So even though gaseous anesthetic agents may be an improvement compared to CO_2, their use in euthanasia is still controversial; the CCAC rate inhalant anesthetics as "acceptable" in rodents if used with a secondary method to ensure death. It is "acceptable with conditions" in the AVMA guidelines, as it is arguable whether it attains the objectives of Criteria 1, 2 & 4 (Table-1).

Dissociative agents, such as ketamine or a combination of ketamine and an alpha-2 adrenergic receptor agonist such as xylazine or diazepam, are poorly studied with respect to their use as euthanasia agents in rodents despite their rating of acceptable. The AVMA only briefly cover their use in rodents, and cite one article (Vaupel et al. 1984) to support the "acceptable" rating. Yet the objective of the Vaupel study was not to determine the efficacy of dissociative agents in euthanasia procedures. Indeed, the authors do not provide any relevant information for the assessment of various drug combinations as stress/pain free and efficacious agents of euthanasia. There are other studies concerned with the anesthetic effects of similar drug combinations (Arras et al. 2001, Wellington et al. 2013), but none were aimed at testing them as agents for euthanasia. Furthermore, the widespread use of dissociative agents as means of euthanasia is hampered by their difficulty of administration; most of the dissociative agents require IV administration, which is not often used in rodents because IV administration takes skill and venodilation prior to injection (Green 1982; Flecknell 2016). So dissociative agents are poorly studied and their administration on a large scale

is problematic given the difficulties of IV injections in rodents. Only the AVMA (and not the CCAC) classify this method as “acceptable”, despite the fact that there is a lack of information.

Physical methods of euthanasia such as decapitation were discussed in Chapter 1. Although a method such as decapitation may be advantageous in certain instances, for example when post-mortem sampling is required for a research protocol, it is a method that requires a steeliness of purpose not always found in even the most experienced lab personnel, and thus is not categorized as acceptable. The emotional impact of laboratory procedures on the lab workers is important in considering euthanasia methods (Criterion #7-Table 1).

The only method that is rated as acceptable by both the CCAC and AVMA is injection of barbituric. Over time, the method of injecting barbiturates intraperitoneally (IP PB) has come to be one of the most widely used methods of euthanasia. However, as the literature review in Chapter 1 made clear, it is far from perfect. Many studies have demonstrated that there may be pain associated with the IP administration of PB (Clement et al. 1989; Ambrose 1998; Ryabinin et al. 1999; Svendsen et al. 2006; Kells et al. 2018; Khoo et al. 2018,). What is more, the technique is inconsistent, as up to 20% of attempts result in misinjections which often require re-injections (Lewis et al. 1966; Miner et al. 1969; Coria-Avila et al. 2007; Ballard 2009; Uysal et al. 2017). This misinjection rate is present in both rats and mice and seems to be an unavoidable limitation of the technique. The time to unconsciousness is also problematic, as animals may be exposed to pain and stress for long periods, especially in cases of misinjections (Wadham 1996; Zatroch et al. 2017). That IP PB remains the method of choice attests in part to the lack of information that exists regarding any alternatives, because one might well wonder whether or not this method meets any of the first three criteria listed in Table 1.

In all, current euthanasia methods, even the only one that is consistently labelled acceptable by the CCAC and AVMA (IP PB), are associated with pain and distress as well as other issues such as lack of consistency. In light of these apparent shortcomings, and because the objective of any ethically based procedure in laboratory animals is to reduce pain, distress and unreliability, it is of interest to explore more efficacious methods of euthanasia. As such, the primary objectives of the rest of this thesis were to develop alternative methods to IP PB for both mice (Chapter 2) and rats (Chapter 3).

Chapter 2: 'Alternative Mice Euthanasia Methods'

In Chapter 2, an alternative method of administration of sodium pentobarbital (PB) in mice was explored: intra hepatic (IH) injections. Our findings indicate this method is untenable in mice given the size of the liver. The small size made it impossible to inject consistently into the organ, and thus made the misinjection rate very high (93%). This might be considered as acceptable, given that most misinjections resulted in successful delivery in the peritoneal cavity, emulating an IP injection. However, a significant portion of the misinjections, approximately 13% of all IH attempts, resulted in intrathoracic delivery. These injections are not normally used on awake animals given the possibility of pain (AVMA 2013). Therefore, it is our recommendation that this technique not be used in mice since this type of injection does not yield more consistent results than IP PB, and yields a high risk of potentially painful misinjections into the thorax. There may be ways to amend the technique that would reduce the risks and increase the reliability, for example using a shorter needle and/or changing the insertion angle of the needle (instead of injecting with a 90-degree angle relative to the body wall). These could be explored further to confirm if the IH technique is potentially useful in mice.

Still in Chapter 2, an alternative injectable agent, ethanol, was explored as a potential alternative to PB. This was work that built on previous studies showing that 100% ethanol had a similar time to death as did PB (Allen-Worthington *et al.* 2015). These previous findings were firstly confirmed, proving that intraperitoneal injections of ethanol (IP ET) are a viable alternative to IP PB. Moreover, these ET injections actually yielded a quicker time to death than did IP PB. This is the first known published paper that has demonstrated the existence of a difference in time to death between ethanol and PB for adult mice and is an argument for the use of ethanol over PB, particularly as ethanol is cheaper and has smaller propensity for abuse or misuse among laboratory personnel (criteria 9 – Table 1).

A third finding in this chapter was the observation of a significant difference in measured time to death when using an ECG versus using auscultation. Allen-Worthington (2015) had used an ECG to compare time to death between IP ET and IP PB and found both to be similar. However, ECG alone may be an unreliable means of determining time to death given the presence of pulseless electrical activity. This can give the impression of continued cardiac function, even though

myocardial contractions have ceased. There was a two-fold difference in time to death when identifying cessation of heartbeat (CHB) with auscultation versus the presence of asystole.

It should be noted here that a quicker time to death is not *per se* an improvement according to any of the AVMA's criteria; a quicker time to unconsciousness is (criteria 1 and 2). Moreover, there has not been any extended study on the potential of ET to induce pain. Allen-Worthington et al. (2015) did compare certain behavioral and vocal signs of pain post injection of ET and PB and found no significant differences. However, behavioural and vocal signs of pain may not be accurate measures in the presence of a drug that depresses motor function and gradually increases the level of sedation. It would therefore be of interest to conduct more extensive pain studies, as described in the section 'future work'. Such data are critical in determining whether or not ethanol should be given precedence over PB in euthanizing mice.

Chapter 3: 'Alternative Rat Euthanasia Methods'

In Chapter 3, a novel method was suggested that can serve as an alternative to IP PB in rats: IH injections of PB. Indeed, to the best of the author's knowledge, the study described in Chapter 3 is the first to evaluate and describe comprehensively the use of intrahepatic injections as a method of euthanasia for rats. In order to evaluate its use as an alternative method of euthanasia, it was first necessary to evaluate its feasibility. To do so, pilot experiments were done that were designed to describe the anatomical disposition of the liver in rats and develop a protocol for successfully injecting the liver (Chapter 3 - Figures 1-2). Through imaging studies, an easily reproducible protocol that allows for successful IH administration of PB in rats was elaborated and was then tested during this pilot phase.

Once the feasibility of IH injections in rats had been established, the injection technique could be tested and compared to IP injections. The variables of interest in this comparison were: time to cessation of heartbeat (CHB), time to loss of righting reflex (LORR), euthanasia success rate (where successful euthanasia was defined as time to LORR and time to CHB under 3 and 5 minutes, respectively) and misinjection rate (evaluated via necropsy).

The results in Chapter 3 were conclusive in demonstrating that IH injections are more efficient than IP injections because of the much faster time to LORR (4 seconds (1 to 96) [median range] vs 89.5 (79 to 110) seconds. As for success rate, IH injections were either classified as successful IH injections if the distribution was found to be exclusively in the liver or misinjection if there was

any evidence of injectate having been distributed anywhere else than the liver (for example in the peritoneal cavity). Interestingly, the misinjection rate was quite high for IH injections (59%), much higher compared to the 6-20% misinjection rate of IP injections (Lewis et al. 1966; Miner et al. 1969; Coria-Avila et al. 2007; Ballard 2009; Uysal et al. 2017). However, 95% of IH misinjections injections were categorized as incomplete-IH injections which were into the peritoneal cavity (many having some evidence of hepatic absorption). Importantly, time to LORR for these incomplete-IH injections remained approximately 3 times faster than IP injections as reported by Zatroch et al. (2017).

Even though a certain amount of injectate may have missed the liver and been dispersed in the peritoneal cavity, some PB was still undoubtedly being deposited into the liver, potentially accounting for the rapid onset of anesthetic effect. The fate of misinjections following IH injections is an important point of contrast with the fate of misinjections following IP injections. For the former, these result in rapid and successful euthanasia, for the latter, most result in significant delays and generally failed euthanasia attempts requiring re-injections (Wadham 1996; Castro Alves et al 2010; Turner et al 2011; Zatroch et al 2017).

Given the successful outcome of these IH misinjections, the failure rate (3%) may be a more useful tool of comparison to the 6-20% misinjection rate of IP injections. These occurred because of injections being made into fat pads. Indeed, this is a potential risk that may be less important for IP injections: the presence of a prominent falciform fat pad in larger rats, which may impede the injection. It is possible that slight amendments to the techniques (longer needle, different injection angle) may reduce these risks.

Given the novelty of our technique of using IH injections in rats, the only comparisons that can be made within the literature necessitate crossing species barriers. There is only one other published study describing IH injections for euthanasia, it was done on cats (Grier 1990). Similar to the article presented in chapter 3, this cat study also performed necropsy evaluations post injections to evaluate the success rate. The salient differences between both articles are: 1) the rate of confirmed IH was much higher in our study (40.9%) compared to theirs (24%) and 2) the misinjections into the thorax and IM did not occur in our study and did in theirs (17%). Despite these differences, 59% of injections in cats yielded ‘IH and IP’ and ‘IP only’ combined, whereas our incomplete-IH rate was 56%. The study in Chapter 3 and Grier’s study used different terms for referring to IH hits

and misses. However, incomplete-IH category (from chapter 3) included any rat that had signs of IP delivery, with or without signs of IH absorption, comparable to a combination of the categories of 'IH and IP' and 'IP only' from the cat study. Significantly, in both studies, the rates of these types of misinjections were similar and the outcome of these misinjections resulted in both cases in rapid LORR and death.

This inter-specific comparisons of the efficacy of IH PB can be extended to mice, based upon the results described in Chapter 2. When comparing outcomes between the mice study of Chapter 2 and the rat study of Chapter 3, the difficulty of giving successful IH injections to mice represents a real obstacle to using this method of euthanasia. The few cases that were successful IH injections did result in rapid LORR and death, similarly to the successful IH injections from the rat study. However, the low success rate, as well as the risk of intrathoracic injections in mice, stood in sharp contrast to the successful IH injections in the rats of Chapter 3.

The success of IH injections in rats strongly suggest that this is a reliable and efficacious means of euthanasia. Indeed, these injections result in very rapid onset of anesthetic effect (loss of righting reflex occurs almost instantaneously) and rapid death that are both significantly faster than after IP injections. There are very few risks associated with this procedure compared to IP PB. Misinjections do occur, but 97% of these misinjections simply fail to hit the liver while still resulting in a successful IP delivery of PB.

In both the rat and mouse studies, there was a serious limitation: the absence of any type of pain assessment. Assessing the feasibility of IH in rats and confirming the viability of ethanol euthanasia in mice being the primary objectives with these experiments, objective evaluations of pain were not included in the protocols. The next section below, will focus more closely on these considerations.

Overall, the present thesis shows that IH injections of PB in rats may be a preferable alternative to IP given the more rapid induction of anesthesia (criteria 2-Table 1) and death, as well as the reduced risk of misinjections (criteria 3- Table 1). In contrast, misinjections following IP injections are essentially unavoidable given the variable positioning of the cecum which is the main site of the misinjections (Lewis et al 1966). The cecum may be positioned in the right caudal quadrant or middle of the abdomen (the principal sites of IP injections) in up to 30% of rats (Coria-Avila et al 2007; Turner et al 2011). IP injections thus involve an inherent risk which affects their reliability

and consistency. These data would seem to provide a compelling case for re-evaluating our preference for IP injections of barbiturates as the method of choice in euthanizing rodent laboratory animals.

IH injections however do involve significant handling and an invasive, potentially painful injection. Rats that are unhabituated to human contact may be stressed during these procedures, and the consistency of the procedure itself can be affected (affecting Criteria 1 and 3 – Table 1). Thus, inhalant anesthetics or CO^2 could be considered in such cases.

Future work

The habituation of rats to handling, alluded to above, represents an important difference between the pilot study rats and the rats of the main study in Chapter 3. The pilot study rats were not habituated to human contact and were thus more combative during manipulation whereas the rats from the main part of the study had been previously habituated to human contact and manipulation. This was an unexpected difference between the two groups, and became an interesting side study: does habituation affect the rate of success of IH injections? We hypothesized that the non-habituated rats, because of their combativeness, would have a greater failure rate than the habituated rats.

In order to evaluate the effects of habituation, a comparison was done between failed euthanasia rate and misinjection rate between the habituated rats (n=66) and the non-habituated (n = 19) rats using a Chi-Square test.

The difference in misinjection rate and failed euthanasia rate following IH injection attempts between the habituated rats and the non-habituated rats from the pilot study was significant (P = 0.0248) (Figure 1). There were proportionally more confirmed IH for the habituated group (27/66 -40.9%) compared to the non-habituated group (3/19 - 15.78%) as well as fewer failed euthanasia attempts (3/19-15.78% for non-habituated and 3.01% - 2/66 for habituated).

Thus, these preliminary data indicate that habituation may have a significant impact on the effectiveness of IH injections. The increased combativeness of the rats during the pilot study may explain the reduced rate of successfully hitting the liver, as well as the increase in failed euthanasia attempts. However, there are possible confounding factors that may have contributed

to this difference. Obviously, the pilot (unhabituated) rats were euthanized before the habituated rats. It would not be surprising to find that the technical expertise gained through practice of the same manoeuvre might have enhanced the success rate of injections in later rats. Such learning curves have been demonstrated for other technical skills (Campbell et al 2014). Second, slight adjustments to the injection angle for large rats were made following the pilot study in an attempt to reduce the misinjections into fat pads; these may have also contributed to the differences seen in figure 1.

These preliminary results are nonetheless compelling and merit more study. Habituation is not a factor that is often or consistently considered in euthanasia procedures, but it may well be a tool to increase success; future studies could further measure the impact of habituation on IH injections as well as other euthanasia procedures (IP, physical methods). It is possible that such studies could lead to euthanasia guidelines that include the habituation of animals as a necessary part of euthanasia protocols.

Another obvious avenue of future research, one that was alluded to in the previous section, is the study of pain. The purpose of a quicker death is to minimize pain and stress. To the extent that no empirically-derived measures of pain were employed in these studies for IH injections (due in no small part to the rapidity of the effects, as unconsciousness usually occurred within 4 seconds post injection), it would be of interest to extend these experiments to include behavioural measures of pain such as the rat grimace scale (Sotocinal et al. 2011) and writhing (Wadham 1996) which could be measured after administration of a vehicle agent. This vehicle agent would have the same physicochemical properties as PB (same pH) but would not have any of the sedative effects.

The following protocol would allow for such a study. Rats could be randomly allocated to two groups, one vehicle group, and one saline control group. Following an IH injection of either a vehicle agent or saline (with blue dye added to the solution), the rats could be placed in a plexiglass observation chamber. The chambers would allow for video-based assessments to measure responses to treatment. The principal outcomes measured would be the Rat Grimace Scale (RGS) and body scoring. Rats would be filmed in these observation chambers for a certain amount of time (representing minimally the amount of time an animal may be theoretically conscious following IH PB). Images could then be taken from the video and placed into a

commercial presentation software, randomized and then scored by a blind observer, as previously described by Sotocinal et al. (2011); for RGS, there are four action units: orbital tightening, nose/cheek flattening, ear changes, and whisker changes scored on a scale of 0 to 2 - a higher score representing pain. An overall score, based on the average of 4 scores from the action units, would thus be assigned to each rat and would allow for a quantitative comparison between both groups (saline and vehicle).

Body scoring could also be measured using the videos (writhing, back arching). Writhing is defined as a 'posture in which the rats contract their abdomen and extend their hind legs backwards', and has been observed following IP injections of PB (Wadham 1996; Ambrose 1998; Khoo et al. 2017). Such behavioural markers, along with the RGS scale would allow for a direct comparison between both groups and would thus help determine if IH injections, of saline and of a vehicle agent, are more or less painful than IP injections.

In addition to this study, alternative methods of quantifying nociception could also add valuable information and help compare different techniques (IH vs IP in rats; ethanol vs PB in mice). As discussed in Chapter 1, neuronal markers such as C-FOS or PERK can quantify nociceptive input following a potentially noxious stimulus such as an IP injection (Svendsen et al. 2006). These markers could be measured for the same groups described above, as well as for IP PB and IP ET, to identify if any difference in nociceptive input exists between the various treatments. These data, combined with the behavioural studies using vehicle injections, would be good indications of the presence of significant pain following IH injections. As long as they are not any more painful, then they would be clearly advantageous due to their increased efficacy and reliability as shown in Chapter 3.

Given the alkaline nature of PB, and the invasive nature of IH or IP injections, some pain may be present, but the question is whether IH injections are significantly more painful than IP injections, albeit for a much-abbreviated period of time. Indeed, any data resulting from such experiments should not be interpreted in a fashion that precludes the obvious contribution of the almost instantaneous sedative and anaesthetic effects of IH PB. In other words, it would be arguable to exclude the use of IH injections even if they were found to be painful given the rapidity of anesthetic and sedative effect: for most animals, there would be approximately four

seconds of pain following injections to the liver, compared to approximately 89 seconds following IP injections.

Nonetheless when thinking over this latter argument, which is considering the possibility that IH PB injections might be more painful but still preferable than traditional IP PB given the shorter time of exposure, an ethical issue comes to the forefront: is a short period of exposure to a more intense pain preferable to a longer exposure to a less intense pain? There is no scientific consensus. Pain is a complex physiological system. It can be qualified and, to some extent, quantified by describing its character (stinging, aching, burning, etc), location, duration and intensity (NRC 2009). However a painful experience involves much more than a simple noxious stimulus; there are significant cognitive, emotional and psychological aspects to pain (NRC 2009). Well-established laws and guidelines such as the US Animal Welfare Act, or the three Rs (Russel & Burch 1959) offer little clarity with regard to this ethical dilemma. There is, however, some evidence in the scientific literature that researchers have a preference for using between-group experimental designs in pain research rather than within-group/repeated dosing designs (Coderre & Laferriere 2019). A between-group experimental design means that more animals are subjected to a painful stimulus than is the case for within-groups designs (which involve the same painful stimulus repeatedly administered to the same subjects). The conclusion that might be drawn is that there is a bias in the research community toward reducing exposure to pain to any single animal, even at the cost of subjecting a greater number of animals to pain. One might liberally interpret this trend as an indication that a lesser degree of pain over a longer period is preferable for any single animal, if one equivocates more animals experiencing a lesser pain to one animal experiencing a lesser pain for a longer period. The counterinterpretation to that is: although a reduction of pain might be accomplished by avoiding re-exposure through the use of additional animals when designing a research protocol, in the case of a necessary procedure such as euthanasia, the best way of reducing exposure to any pain is by reducing time of exposure, because time may utimately be the most quantifiable variable that can be controlled in assessing and attempting to compare two painful experiences.

Unfortunately, there are no clear answers to these questions. But these considerations do make it clear that it would be of interest and importance to expand the current studies. Ethical dilemmas might not be resolved but would be considerably reduced by having access to quick, efficient,

and empirically validated data on various methods of euthanizing laboratory animals and the pain and distress associated with these methods.

Conclusion

Veterinary medicine occupies itself with ensuring the wellbeing of animals. In most fields of veterinary medicine, this involves reducing pain and ensuring the health of animals or of populations of animals. In farm animal medicine, some compromises of wellbeing are accepted with respect to husbandry. However, the objective to ensure that animals are pain- and stress-free remains the same. Furthermore, the goal in farm animal medicine is ultimately quantifiable in terms of the production of a secure and safe source of food for human consumption.

In contrast, the goals of the field of laboratory animal medicine, in which the infliction of some degree of stress and pain is often part of the experimental protocol, are often not as clear. This difficulty is exacerbated by the very nature of scientific procedure, in which the results of any manipulation remain unknown until after the experiments are completed; in other words the reason, or rationale, for inflicting pain or stress on animals can remain somewhat unclear. Furthermore, excess numbers of animals are bred but never used in any research protocol, adding to the total number of animals that require euthanasia.

The veterinarian's objective in the field of laboratory animal medicine is thus different because it is impossible to ensure that all animals are pain and stress free. The objective, therefore, is to reduce the pain and stress by ensuring that common laboratory procedures, such as euthanasia, are as efficient and reliable as possible.

The experiments of the present thesis work towards this objective firstly by challenging the reliability and efficacy of the most universally accepted method of lab animal euthanasia, secondly by evaluating ethanol as an alternative euthanizing agent in mice and lastly, by developing an innovative and effective method of euthanizing rats – intrahepatic injections.

The implications of this work can be viewed at two levels. The first is the practical aspect: IH and ethanol could be suggested in animal care guidelines of euthanasia alongside IP PB. The second is the far broader message that the acceptance of a laboratory procedure should not lead us to complacency. IH injections had not been systematically tested in rodents before the present paper, and there could be other routes or alternatives that could be equally promising, yet

unexplored. This should always be the objective: to push the limits of our knowledge in order to improve the wellbeing of all animals under our care, even for laboratory methods that are considered well-accepted.

Tables

Table 1. AVMA criteria for euthanasia guidelines

	AVMA CRITERA
1	Inducing loss of consciousness/death with a minimum of pain/distress
2	Time to loss of consciousness
3	Reliability
4	Safety of personnel
5	Irreversibility
6	Compatibility with intended animal use/purpose
7	Documented emotional effect on observers
8	Compatibility with subsequent evaluation/examination/use of tissue
9	Drug availability and human abuse potential
10	Compatibility with species/age/health status
11	Ability to maintain equipment to proper working order
12	Safety of predators/scavengers if animal remains are consumed
13	Legal requirements
14	Environmental impacts

Figures

Figure 1. Effects of habituation on IH injection success

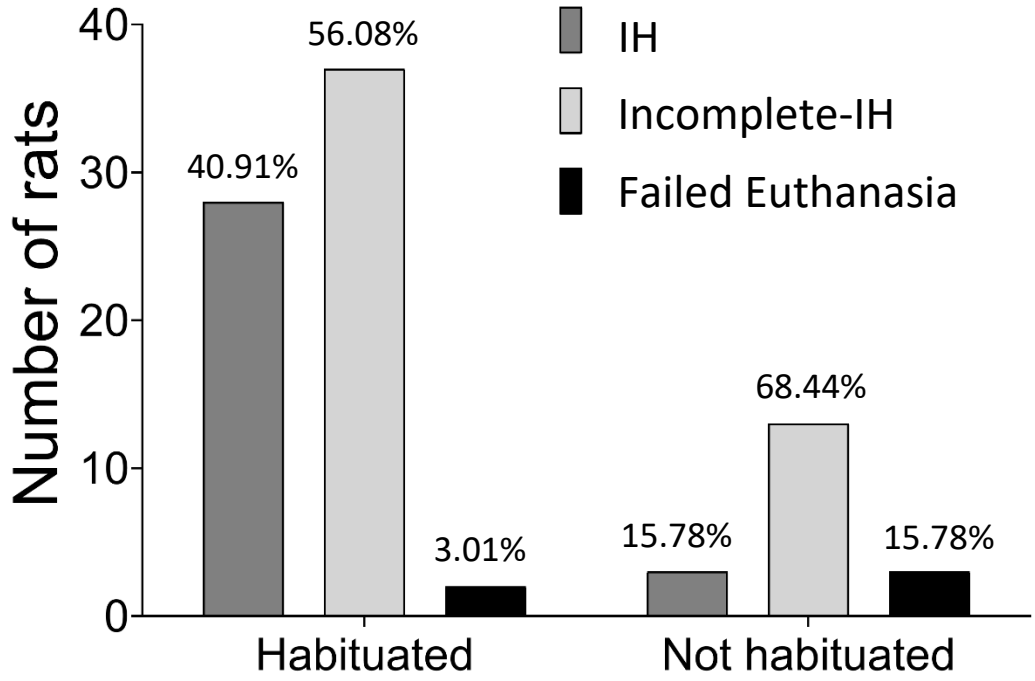

Figure 1. Boxplot representation of necropsy evaluations for all IH injections of habituated rats (n = 66) and non-habituated rats (n = 19) with the percentage indicated over every column. The differences in proportions between the two groups is significant (Chi square test, P = 0.0248), as the percentage of failed euthanasia attempts dropped from 15.78% in not habituated to 3.01% in habituated, and the number of successful IH injections increased from 15.78% in not habituated to 40.91% in habituated.

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