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Review of Rodent Euthanasia Methods

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The optimal choice of euthanasia method for laboratory rodents depends on a number of factors, including the scientific goals of the study, the need to minimize animal pain and/or distress, applicable guidelines and laws, the training and proficiency of personnel, and the safety and emotional needs of the personnel performing the euthanasia. This manuscript aims to provide guidance to researchers so they may select the method of euthanasia that results in minimal experimental confounds, such as the creation of artifact and alteration of tissues and analytes. Specific situations addressed include euthanasia of large numbers of rodents and euthanasia of neonates. Recent literature supports the notion of significant strain-dependent differences in response to euthanasia methods such as CO_2 inhalation. To assist researchers in selecting a strain-appropriate method of euthanasia, the authors present a summary of methodologies for assessing the effectiveness of euthanasia techniques, including elements and parameters for a scoring rubric to assess them.

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The goals of this review were to evaluate recent literature related to rodent euthanasia and provide guidance and references to help laboratory animal veterinarians make informed decisions and recommendations related to humane and scientifically appropriate techniques for rodent euthanasia.

The AVMA Guidelines for the Euthanasia of Animals[63] (AVMA Guidelines) is the primary reference in use at most US institutions. We used this document as a primary reference, and in addition, reviewed guidelines from other countries and regulatory bodies, and literature published since the last report. Based on this new literature, veterinarians and institutions may find it advisable to adopt methods that differ from the current AVMA Guidelines. However, readers are reminded that if an institution is required to comply with the Public Health Service Policy, funded institutions must comply with the most recent published AVMA Guidelines. Protocol-specific, but not program-wide, exemptions to this requirement are permissible, but must be scientifically justified and approved by the Institutional Animal Care and Use Committee (IACUC).

Decisions on methods of euthanasia are complicated and should be based on consultation with a laboratory animal veterinarian. Veterinarians with advanced training or expertise in Laboratory Animal Medicine (ACLAM or ECLAM or similar background and expertise) may be best suited to assist with the choice and validation of an optimal technique. Recent literature[1,33,34,40,43,46,79] affirms that animals of different age, sex, disease state, and genetic background may respond differently to euthanasia techniques. To ensure the method selected is appropriate for the experimental animals and the aims of the experimental protocol, a pilot study might be the best way to ascertain the most appropriate euthanasia method for specific cohorts of rodents. This overview will discuss evaluation of the effectiveness of euthanasia techniques, including a scoring rubric to assess euthanasia techniques.

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Considerations for Choice of Euthanasia Method

Compatibility with intended animal use. When euthanizing unwanted rodents (for example, retired breeders or pups of unwanted genotype) or study animals from whom terminal tissue collection is not needed, any of the approved methods in the AVMA Guidelines may be suitable. The choice of method may be based on considerations such as minimizing emotional impact on personnel, efficient workflow, occupational health and safety considerations and logistics of convenience. However, if terminal tissue collection is needed for diagnosis of clinical cases or to collect data or tissues for a study, the veterinarian should recommend a method that minimizes tissue artifact while still providing a humane, painless death. Indeed, the AVMA Guidelines specify that "compatibility with intended animal use and purpose and subsequent evaluation, examination, or use of tissue" should be a consideration in choosing a method.

Tables 1 and 2 summarize literature citations comparing methods of euthanasia and their effects on a variety of measurements. This is not an exhaustive list; many factors can affect the magnitude and even directionality of differences between methods, including sex and age of the animals and handling techniques. The references in these tables provide a starting point for a more detailed literature search and for the design of pilot studies, as warranted. The 2001 ANZCAART publication Euthanasia of Animals Used for Scientific Purposes[3] also contains an exhaustive review. The handling and restraint associated with euthanasia can also result in sympathoadrenal activation, which can have profound effects on certain analytes, tissue quality, and data reproducibility. Methods of euthanasia demonstrated in the literature to minimize sympathoadrenal activation (for example, anesthesia) will not be effective if euthanasia is preceded by prolonged or rough handling or exposure of the animals to stressful stimuli.

Effects on tissues. Euthanasia methods may affect tissues in a variety of ways. Physical methods such as decapitation and cervical dislocation offer the potential for a very quick death with no artifacts from chemical agents, but they also

Table 1. Effects of euthanasia methods on cells and tissues (organized by system)

System	Method	Compared with	Affected parameters
Blood	CD[49]	CD + SP, CO_2, Isoflurane, or halothane	lymphocyte proliferation, cytotoxic lymphocyte response, lymphocyte parameters, granulocyte, leukocyte, and platelet count
Blood	CD[49,105]	Antemortem/anesthetized	murine bone marrow culture
Brain	CD[58]	CD + Isoflurane	plasticity changes in brain slices. CD causes mechanical disruption/anatomic/morphologic changes
Brain	Decapitation[9,37]	Decapitation+ CO_2	GABA receptor function
Cell viability and function	CD[49,105,113]	Antemortem/anesthetized	Normal lymphocyte proliferation
Reproduction	CD[83]	Isoflurane	Isoflurane yielded fewer intact oocytes due to microhemorrhage that hindered collection
Reproduction	CO_2[20]	Isoflurane	Isoflurane reduced motile sperm counts in Sprague–Dawley rats, due to inhibition of vas deferens contractions and decrease in expelled sperm
Reproduction	CO_2[44]	CD	CO_2 decreased fertilization rate of mouse oocytes
Reproduction	Decapitation[92,97]	*multiple – see references	sperm motility, straight-line, average path, curvilinear velocities, linear index, and linearity
Pulmonary	Argon[19]	CO_2	No difference in pulmonary lesions. Note: Argon was aversive.
Pulmonary	CO_2[2,39,40,51,101]	CO_2 + O_2	Lung: congestion, hemorrhage, emphysema, atelectasis; Cardiac muscle: variable degenerative changes (influenced by time of exposure to CO_2 causing acidosis, hypoxia)
Pulmonary	Decapitation[2,39,51,95]	N/A	Lung: emphysema, hemorrhage, blood in alveolar spaces
Miscellaneous	SP[10,39,77,85]	N/A	Splenic enlargement due to smooth muscle relaxation which lets spleen engorge with blood
Miscellaneous	SP[1,33]	Ethanol IP	IP but not IV pentobarbital damages serosal cells; Pentobarbital and ethanol cause loss of tinctorial properties
Metabolic	Decapitation[10]	decapitation + anesthesia	No significant differences in insulin and glucagon receptors from liver plasma membranes
Heart muscle function	CD[85]	SP	Decreased coronary flow; decreased contractile function in isolated perfused heart preparations
Heart muscle function	Decapitation[36]	Antemortem/anesthesia	rat heart mitochondrial function
Smooth muscle function	SP[85]	CD	Decreased contractility in isolated muscle preps; Decreased GI smooth muscle contractility (PI or IV, not IP) Decreased spontaneous and drug induced vascular smooth muscle contractility; IP only increased colonic contractility in response to acetylcholine
Respiratory	Decapitation[39]	Normal tissues	marked focal accumulation of blood in bronchioles/alveolar spaces due to reflex inspiration
Respiratory	SP[39]	Normal tissues	mild congestion of alveolar capillaries

CD, Cervical Dislocation
CO_2, Carbon Dioxide
Decapitation, Decapitation alone
FBMI, Focused Microwave Beam Irradiation
SP, Sodium Pentobarbital
*, overdose with CO_2, euthanasia solutions, enflurane, halothane, isoflurane, or sevoflurane, or decapitation after halothane, pentobarbital, or CO_2
N/A, Not applicable

cause tissue damage that may render certain samples unusable. For example, in addition to the disruption of tissues in the cervical area, the blood collected by decapitation is subject to hemolysis. Euthanasia methods that cause hypoxia can adversely affect viability of the tissues and cells being harvested (for example sperm, oocytes, cells for culture). Certain anesthetic agents can directly affect tissue viability or parameters. Chemical agents may directly damage tissues (for example intraperitoneal alcohol and intraperitoneal pentobarbital both diminish tinctorial qualities in histologic sections). The dyes in some euthanasia solutions (for example rhodamine) may interfere with assays or tissue staining.

Table 1 summarizes the literature on the effects of euthanasia methodology on tissues, organized by system.

Effects on analytes. Euthanasia methods can have a profound direct or indirect effect on analytes in blood or tissues. Some analytes are very labile and are best preserved by physical methods that allow rapid sample collection. In other cases, a method such as focused microwave beam irradiation can be used to preserve labile analytes in tissues. Levels of many analytes may also

Table 2. Effects of euthanasia methods on analytes (organized by system)

System	Method	Compared with	Effect/analytes studied
Blood	CD[49]	SP, CO_2, halothane, CD + methoxyflurane	granulocyte, leukocyte, and platelet count
Blood	CO_2[76,106]	CO_2 + O_2	hematocrit, mean corpuscular volume, hemoglobin
Blood	CO_2[99]	ketamine	respiratory acidosis from CO_2 causes artifactual hyperkalemia
Blood	Decapitation[29,84]	antemortem blood collection	Decapitation increased Ca^{2+}, Mg^{2+}, K^+, Na^+
Brain	CO_2[53]	CD	Brain amines
Brain	CO_2[114]	FBMI	less RNA from FBMI compared with CO_2 inhalation
Brain	Decapitation[9,37]	Decapitation + CO_2	cholinergic parameters in rat brain
Brain	Decapitation[66,68,98]	FBMI	brain vasoactive peptide, adenosine, glutathione, glutamate, alanine, GABA, ethanolamine, NH3, valine, leucine, isoleucine, tyrosine, phenylalanine, glycine, aspartate, prostaglandin and Thromboxane, substance P, neurokinin A, and neurotensin
Brain	Decapitation[9]	Decapitation + SP	acetylcholine release in the brain, Activity of cholinergic markers
Metabolic-stress	CD[75]	anesthesia (CO_2, Isoflurane, ketamine)	metabolomic parameters in mice
Metabolic-stress	CO_2[76,106]	100% CO_2	decreased mean corpuscular hemoglobin
Metabolic-stress	CO_2[18,76,106]	antemortem	Increased serum glucose; Decreased liver glycogen, pyruvate, ATP, serum creatine kinase, aspartate aminotransferase
Metabolic-stress	Decapitation[8]	Decapitation + Isoflurane	Isoflurane: plasma corticosterone but not gene expression of stress markers increased in female but not male rats
Metabolic-stress	Decapitation[8]	Decapitation + anesthesia	anesthesia significantly increased plasma levels of glucose, triglyceride, and insulin but not levels of cholesterol or glucagon
Metabolic-stress	Decapitation[7]	antemortem	plasma ascorbic acid
Metabolic-stress	Decapitation[10,29]	Antemortem/anesthesia	Increase in blood catecholamine levels due to postmortem neurochemical activity
Metabolic-stress	Decapitation[78]	CO_2, SP	No significant difference in serum corticosterone or insulin
Metabolic-stress	Decapitation[18]	CO_2, CO_2/O_2, Isoflurane	liver glycogen levels
Metabolic-stress	Decapitation[54]	Antemortem/anesthesia	Anesthesia alters brain, heart, skeletal muscle concentrations of Fructose -2-6-biphosphate
Metabolic-stress	SP[10]	Multiple agents- see references	plasma glucose, insulin, triglycerides, liver glycogen
Metabolic-stress	SP[111]	Decapitation, Isoflurane	injection of either pentobarbital or saline increased plasma corticosterone, thereby interfering with ability to measure changes in corticosterone associated with footshock
Metabolic-stress	SP[78]	CO_2, Decapitation	increased glucose and decreased cholesterol, stearic, and arachidonic acid
Metabolic-stress	SP[52]	CO_2 or Decapitation	Pentobarbital decreased free fatty acids compared with CO_2 or decapitation.
mRNA expression	CO_2[96,114]	Multiple agents- see references	mRNA expression varies with anesthesia protocol
Pulmonary	CO_2[19]	Argon	No difference in pulmonary lesions. Note- Argon is aversive
Pulmonary	CD[113]	FBMI, antermortem/anesthesia	CD increases platelet serotonin in lungs
Pulmonary	SP[100]	Isoflurane, medetomidine-butorphanol-midazolam	Bronchoalveolar lavage fluid quality- isoflurane was preferred.
Renal	SP[77]	Multiple agents- see reference	partial pressure of CO_2 in arterial blood; serum renin and plasma aldosterone
Reproductive	Decapitation[101]	Decapitation + CO_2	LH, FSH, prolactin
Reproductive	Decapitation[71,112]	Decapitation + anesthesia	hormones in mature, immature, castrated, and intact male rats

CD, Cervical Dislocation
CO_2, Carbon Dioxide
Decapitation, Decapitation alone
FBMI, Focused Microwave Beam Irradiation
SP, Sodium Pentobarbital
(*) Pentobarbital, ketamine hydrochloride, chloral hydrate, chloralose and halothane in combination

change rapidly in the response to stress-related hormones such as corticosterone, so consistent handling, techniques, and timing of procedures is an important way of controlling experimental variability. Chemical euthanasia agents can directly affect serum and plasma analytes, for example, CO_2 causes acidosis and potassium chloride prevents analysis of serum potassium ion levels). Anesthetic agents are frequently given prior to euthanasia (for example, as an adjunct to a physical method) but they too can change the levels of analytes in blood or tissues. For some methods, of euthanasia the literature contains conflicting reports about the degree and direction of analyte alterations, a discrepancy that may be the result of different stress levels in the experimental subjects.

Table 2 summarizes the literature on the effects of euthanasia methodology on blood and tissue analytes, organized by system.

Considerations for Euthanasia of Large Groups of Rodents

In cases of disaster or for containment of an infectious disease, depopulation may be required,[82] and provisions for mass euthanasia should be included in every facility's disaster plan. Depopulation of animals is a distinct topic from euthanasia that is covered in the 2019 AVMA Guideline on depopulation.[4] Some facilities, especially those with large breeding programs, routinely euthanize large numbers of surplus rodents. In the United States, this is typically accomplished using inhaled CO_2 or cervical dislocation. In Canada, the CCAC guidelines on euthanasia of animals used in science[22] recommend the use of inhalant anesthetics prior to CO_2 where practical. However, the 2019 CCAC Guideline on Mice acknowledges that "There is currently a substantial amount of research being conducted in the area of inhalant techniques for euthanasia and it is important to evaluate any new evidence that becomes available."[23] The AVMA Guidelines recommends that animals be euthanized in their home cages, when possible, and that "If animals need to be combined, they should be of the same species and compatible cohorts, and, if needed, restrained or separated so that they will not hurt themselves or others." Recent studies have suggested a calming effect may occur when conspecifics undergo stressful events in concert.[87,88] Despite substantial guidance from oversight and specialty working groups on various aspects of euthanasia, specific guidance for group euthanasia of laboratory rodents is minimal.[4,50,63,81] When animals cannot be euthanized in their home cages or as stable social groups, professional judgment must be used to minimize the social stress caused by mixing unknown conspecifics, and to avoid overcrowding inhalant chambers. Factors to consider are the timing of mixing, the likelihood of immediate aggression, and the risk of trampling conspecifics. Although the number of animals on the floor of a chamber does not influence the CO_2 concentration in the chamber, factors such as primary containment structures and the animals themselves can modify the CO_2 flow dynamics in the chamber.[35] When calculating CO_2 gas exposure time, the number of animals in the chamber should be considered, as it has been reported that a higher density of rats and mice required longer exposure times than single animals.[12] If euthanizing animals of different ages, such as a mix of neonates and adults, the CO_2 exposure time must be sufficient for the least-susceptible animals.

Euthanasia of Fetal and Neonatal Rodents

When euthanizing pregnant females, euthanasia of the dam is considered sufficient for euthanasia of the fetuses if they remain in the uterus. Scientific data cited in the NIH Guidelines for Euthanasia of Rodent Fetuses and Neonates[72] indicate that mammalian embryos and fetuses are in a state of unconsciousness throughout pregnancy and birth and that hypoxia does not evoke a response. Even though fetal heartbeats may continue for an average of 30 to 46 min after euthanasia of the dam, the fetuses remain unconscious and therefore are unable to experience pain or distress.[57,70] Therefore, it is not necessary to remove fetuses for euthanasia after the dam is euthanized. However, if the fetuses are removed from the amniotic sac after euthanizing the dam and are able to breathe (mouse, rat and hamster greater than E15; guinea pigs greater than E35), they should be euthanized by an AVMA-approved method. Such methods may include decapitation, cervical dislocation, hypothermia (avoiding direct contact with ice/cold surface), rapid freezing in liquid nitrogen, or chemical anesthetic overdose, as discussed in the AVMA Guidelines.

According to the AVMA Guidelines, rodents with altricial young, such as mice and rats, must be differentiated from rodents with precocial young, such as guinea pigs. Precocial neonates should be euthanized in the same manner as adult rodents. Caution should be used when using inhaled methods with altricial rodents, who are resistant to hypoxia and hypercarbia via multiple mechanisms including fetal hemoglobin, reduced metabolic rate, enhanced tissue retention of oxygen, and diminished cerebral susceptibility.[31,57,79,80,91,103] While the AVMA Guidelines specifies that inhalant anesthetics are acceptable with conditions for euthanasia of altricial neonatal rodents, a longer exposure time or a secondary method is required. Genetic background also influences the susceptibility of neonatal mice to CO_2; one study showed that 0 to 2 d old mice from a variety of inbred strains took longer to be killed by exposure to 100% CO_2 than a common outbred stock.[80] Age has the greatest effect on time to death after CO_2 exposure, with the youngest animals requiring the longest exposure time, up to 50 min for inbred mice and 35 min for rats on the day of birth.[79,80]

While the AVMA Guidelines stipulate that adequate exposure time should be provided, or an adjunctive method performed after the neonate is nonresponsive to painful stimuli, adequate exposure time is difficult to determine. Animals that appear dead (cold, cyanotic, unmoving) in the anesthetic/euthanasia chamber may revive after removal from the CO_2 or isoflurane and exposure to room air. Neonatal mice have been reported to recover as long as after 30 min of exposure to CO_2[80] or isoflurane.[86] Therefore, a secondary AVMA-accepted physical method of euthanasia (such as decapitation) should be performed to prevent revival.

Physical/Visual Separation of Animals Undergoing Euthanasia

Euthanasia of rodents is frequently performed in an area separate from housing and breeding, although some situations, like biocontainment, may require that all procedures, including euthanasia, take place in the housing room. The AVMA Guidelines recommend that "…for sensitive species, it is desirable that other animals not be present when individual animal euthanasia is performed." However, these guidelines do not define which species are considered sensitive. Research into the question of whether rats or mice are sensitive to euthanasia of conspecifics in the same room by carbon dioxide or decapitation has demonstrated that observation of euthanasia or other procedures does not result in stress as measured by cardiovascular and activity response in animals.[13,88] Rats and mice have poor distance vision and have limited ability to clearly discern euthanasia or other procedures being conducted several feet away.[6,11,73] Further research is warranted to study the potential impact of being present for euthanasia of conspecifics (including visual, auditory, or pheromone exposure) when

rodents are housed in facilities employing individually ventilated cages (IVC) and laminar flow cabinets. Based on the current literature cited above, rats and mice are not sensitive species.[6,11] Visual separation is not required for rats and mice, and IVC and laminar flow cabinets typically provide sufficient auditory and olfactory separation. Professional judgment should be used to determine whether facilities offer sufficient separation to allow euthanasia to be performed in the same room with conspecifics.

Considerations for the use of Carbon Dioxide

Carbon dioxide narcosis and asphyxiation have long been used for euthanasia of rodents and other laboratory species. At present, the method is considered "acceptable with conditions" by the AVMA. The conditions require the use of compressed 100% CO_2 gas in cylinders delivered at a specified displacement rate (currently 10% to 30% of the chamber volume/min,[63] but with a new proposed rate of 30% to 70% in the *Proposed 2019 Updates to the AVMA Guidelines for the Euthanasia of Animals)*.[5] Prefilled chambers are unacceptable. If euthanasia cannot be conducted in the home cage, chambers should be emptied and cleaned between uses, and the user must verify that an animal is dead after exposure to CO_2 as with other inhalant euthanasia techniques. CO_2 has the advantages of being cost-effective, relatively safe for users and the environment, and suitable for euthanasia of multiple rodents at the same time.

Several recent reviews provide in-depth analyses of the controversy about CO_2 euthanasia.[16,104] To summarize briefly, carbon dioxide is an anesthetic at high concentrations (30% to 40% in rats),[109,110] and it renders animals unconscious before they die of respiratory arrest and hypoxia. Other inert gases (for example, nitrogen, argon) can asphyxiate animals, but they are not considered humane methods of euthanasia as a sole agent for rodents because the animals may experience distress when they are conscious during asphyxiation. Similarly, time to death is prolonged when CO_2 is supplemented with oxygen, so the practice of euthanizing animals with a CO_2-O_2 mixture [32,101] is no longer recommended.[63] Carbon dioxide has the disadvantage of reacting with the fluid in mucous membranes to form carbonic acid, which can produce a stinging sensation in the eyes and throat in some humans.[24,32,107] It can also produce anxiety responses in rodents at concentrations above 20%.[46] In the past, CO_2 was administered by putting animals in a prefilled chamber or delivering CO_2 at a very high rate (approximately 70%) of volume displacement. This resulted in rapid loss of consciousness (loss of cortical brain activity within 30 s in mice)[21] but the animals could experience significant distress from the nociceptive effect of the CO_2. Under the current AVMA Guidelines, when using CO_2 as the sole agent, the objective is to achieve loss of consciousness before a noxious dose is delivered. Although the displacement rate of 10% to 30% is supported by literature,[45,48] and many institutions have invested in engineered systems that can reliably deliver CO_2 at this rate, more recent studies show that this rate, especially at the low end of the range, is not optimal or effective for every species or strain.[13,30,32,42,46,56,61,62,69,73] CO_2 exposure with suboptimal flow rates can result in animals remaining conscious for a prolonged period while being exposed to an atmosphere that, at least for humans, causes a distressing sensation of breathlessness.[46] Also, the prolonged induction time (3 to 10 min to achieve 100% fill) is prohibitively long for some experimental studies, for either logistical or experimental reasons. At the time of writing this review, the AVMA's new proposed rate is 30% to 70%.[5]

In recognition of the difficulty in delivering a CO_2 exposure paradigm that uniformly produces loss of consciousness before producing distress, Canadian Council on Animal Care has issued guidelines that require anesthesia or sedation (for example with isoflurane) prior to CO_2 exposure when practical[22] (Table 3). However, assessment of the wellbeing of rodents exposed to isoflurane, especially with repeated exposures, is not well-characterized and may also cause distress prior to causing loss of consciousness.[17,41,46,60-62,64,65,69,108]

CO_2 is recommended as a sole method of euthanasia under the conditions outlined in the AVMA Guidelines when it can be delivered at a rate that rapidly induces loss of consciousness before inducing distress from the nociceptive and dyspneic effects of the gas. For small rodents, such as mice, the AVMA-recommended range of 10% to 30% fill rate may produce this effect, but IACUCs and laboratory animal veterinarians should remain alert to the possibility that for some rodent models or strains, either preanesthesia or a faster CO_2 delivery rate may be required to achieve humane euthanasia. Recent studies[13,46,69] support the humane use of fill rates of 30% to70% to achieve faster loss of consciousness. The proposed 2019 Updates to the Guidelines for euthanasia states that "… as there is no clear evidence of a flow rate that optimally minimizes both pain and distress for all species, sexes, and genetic backgrounds, veterinarians should use their professional judgment to determine which flow rate is appropriate for their circumstances."[5] In cases that require a higher rate than the current AVMA Guidelines, pilot studies should be conducted to establish a more effective rate of CO_2 delivery, and this performance standard used as scientific justification for deviation from the AVMA Guidelines for specific IACUC protocols. When implementing results of local or published studies regarding the use of CO_2, the reader is again reminded that per Public Health Service Policy, institutions with PHS Animal Welfare Assurances must comply with the AVMA Guidelines.

Technical Considerations for CO_2 Euthanasia

The euthanasia chamber should allow easy visibility of the animals. With animals present, the chamber or home cage must be slowly filled with CO_2 at a displacement rate that causes rapid unconsciousness and that avoids exposing conscious animals to aversive high CO_2 concentrations. The CO_2 gas displacement rate is critical to the humane application of CO_2; an appropriate pressure-reducing regulator, flow meter, or restriction valve must be used. A 2-stage regulator typically gives the greatest control over flow rates; an initial regulator steps the pressure from the tank down to a predetermined setting, and then a flowmeter, flow gauge, or restriction valve delivers a precise CO_2 flow to the euthanasia chamber. Commercial vendor claims regarding flow rates of commercially available euthanasia systems should be verified by the end user.

After the animals are unconscious (defined as the point where the righting reflex is lost or achievement of lateral recumbency), the flow rate can be increased to minimize the time to death.

The euthanasia chamber volume is determined by the following equation:

$$\textit{Euthanasia chamber volume in liters} = (\textit{Height in cm}) \times (\textit{Width in cm}) \times (\textit{Length in cm}) / 1000$$

Setting Calculations. Flow meters are typically marked in liters/minute (LPM). The flow meter setting is determined by multiplying the euthanasia chamber volume in liters by the desired chamber displacement rate. For example, the CO_2 flow meter rate for a 30% chamber volume/minute displacement rate is determined by the following equation:

$$\textit{Flow meter setting}\ (LPM) = \textit{Euthanasia chamber volume in liters} \times 0.3$$

Flow Gauge Setting. Flow gauges are typically marked in cubic feet/hour (CFH). (Note: This is different than a *pressure*

Table 3. Published guidelines on euthanasia of laboratory rodents

Agent or Method	2013 AVMA Guidelines[63]	2016 Society of Mammologists Guidelines[89]	2010 CCAC Euthanasia Guidelines[22]	2010 EU Directive[28]	2001 ANZCCART[3]
Barbiturate	A[a]	A[b]	A[a]	A[c]	A[a]
Dissociative Agent Combination	A	A[b]	—	A[c]	—
Ethanol	C[d]	—	—	—	A[d]
T-6131	—	—	C[e,f]	A[c]	—
Carbon Dioxide	C[g,k]	—	C[g,h]	C[g,i]	A[j]
Carbon Monoxide	C[l]	—	—	—	U
Cervical Dislocation	C[n,o]	C[p]	C[n,o,q,r]	C[s]	C[n,s]
Decapitation	C[t]	—	C[t,u]	C[v]	C[t,u]
Inhalant Anesthetic	C[w,x]	A[b]	A[w,y]	A[c]	C[z]
Focused Beam Microwave Irradiation	C[a1]	—	—	—	U
Nitrogen, Argon	U	—	C[b1]	A	U
Nitrous oxide	U	—			U
Exsanguination	U[m]	—	U[m]	U[m]	U[m]
Thoracic Compression	U	C[c1]	—	—	U
Blunt Force Trauma to the Head	C[f,d1]	—	—	C[d1]	C[f]

A = Acceptable; C = Acceptable with Conditions; U = Unacceptable when used as sole agent on conscious animals; - = not addressed.
a, IV preferred over IP; concentrated solutions may cause pain when given IP.
b, Drug use in field can present additional risks to investigators and stress to animals, risk of secondary toxicity if carcass left in field to be eaten.
c, Anesthetic overdose should, where appropriate, be used with prior sedation
d, 0.5 mL of 70% IP for mice; unacceptable for larger species
e, Only IV, slowly
f, Personnel must be well-trained
g, Gradual fill only, displacing 10% to 30% chamber volume per minute; source of gas should be compressed gas cylinder; euthanize in home cage or euthanasia chamber should be emptied and cleaned between uses; verify death has occurred
h, Must have written SOP, written records, regular postapproval monitoring, animals should be anesthetized prior to CO_2 delivery
i, Not to be used on fetuses/neonates
j, Prefilled chamber recommended for guinea pigs to minimize the experience of breathlessness
k, Prolonged exposure required for neonates
l, Requires properly maintained equipment; hazardous to personnel, acceptable only when conditions for safe use can be met
m, Acceptable under deep anesthesia
n, Personnel must be trained and their proficiency validated; availability of secondary method if initial attempt unsuccessful
o, For rodents < 200 g
p, Animals of small body size, performed by experienced personnel
q, Anesthetize or sedate first; scientific justification required for use on conscious animals
r, For rats > 200g use commercial dislocator
s, Mice, rats < 150 g
t, Properly maintained equipment: blades sharp, clean, in good condition; operator skilled in handling/restraint of animals; personnel should be trained on dead/anesthetized animals to demonstrate proficiency
u, Recommend anesthetizing first
v, Use only if other methods are not possible
w, Time to death may be prolonged, consider adjunctive method once animals are deeply anesthetized
x, Maintain compatible groups, clean and maintain induction/euthanasia chamber, adhere to recommended flow rates, it is important to verify death when inhalant method used
y, Not for use in species that breath-hold
z, Occupational health and safety issue for personnel exposed to waste anesthetic gas
a1, Purpose-built equipment, mouse and rat only
b1, When scientifically justified and approved by ACUC; Argon is aversive to rats; O_2 concentration must be <2%, only appropriate for use if O_2 concentration is known/measured; mixtures of argon and nitrogen should only be used if animal is already anesthetized
c1, Personnel skilled in technique and animal small enough to allow thoracic cavity to be collapsed and prevent inspiration
d1, Small laboratory rodents <1kg
e1, T-61 not available in the US

gauge that measures gas pressure in pounds per square inch (PSI).)

To calculate the flow gauge setting, in CFH determine the flow meter setting in LPM as described above then convert the value from LPM to CFH by the following equation:

$$LPM \times 2.12 = CFH$$

Restriction valve settings. Restriction valves are selected and set by the manufacturer to deliver a specific flow rate in response to CO_2 supplied at a specific pressure. These valves typically do not have adjustable settings and must be used with the original euthanasia chambers provided with the system.

Comparison of International Guidelines. Selecting the best method of euthanasia for a given study can be a complicated

Table 4. Parameters for assessing euthanasia efficacy

Assessment	Pros	Cons
Behavioral Assessments		
Time to recumbency or loss of righting reflex (LORR)[16,17,45,46,69]	LORR is defined by anesthesiologists as the point at which veterinary patients experience loss of consciousness	Not consistently defined between laboratories or consistently articulated within articles. Referenced articles are examples that provide definitions
Ultrasonic vocalizations[19,25,104]	Characterized for rats (low frequencies associated with pain/distress; high frequencies associated with play)	Difficult to record. Some evidence 50 KHz associated with distress; not well characterized in species other than rat.
Behavior: Mouse Grimace Scale (MGS)[1,59,67]	Noninvasive, validated for acute abdominal pain.	Blinded observers and high-definition recording equipment are needed. Must be properly powered and statistically evaluated correctly
Light/dark aversion[108]		
Aversion behavior[17,41,60-62,64,69,73,108]	Allows evaluation of animals' choices	Preference test; does not measure distress associated with the euthanasia process
Behavior: Respiratory Distress (dyspnea)[14,19,42,46,69,94,104]	May be indicative of pain and/or distress	Only relevant as an assessment of wellbeing in periods of time when the animal is conscious
Behavior: Agitation (flipping, spinning, abnormal alteration in activity)[14,15,19,42,94]	May be indicative of pain and/or distress	Only relevant as an assessment of wellbeing in periods of time when the animal is conscious
Behavior: pawing at the face)[14,15,104]	May be indicative of pain and/or distress	Only relevant as an assessment of wellbeing in periods of time when the animal is conscious
Telemetry data, including locomotor activity[13,14,46,87]	May be indicative of pain and/or distress	Requires specialized equipment and invasive procedure to measure
Respiration, color, movement (neonates)[57,70,79,80,86]	Subjective assessment of oxygenation and vitality	
Cardiovascular and Respiratory Assessments		
Heart rate – telemetry: Time to cardiac arrest[1,13,15,19,46,87]	Increased heart rate can indicate arousal and stress	In the absence of concurrent behavioral evaluation, it is difficult to interpret increases in heart rate; the heart can continue to beat after loss of CNS control due to the internal pacemaker; requires specialized and/or invasive equipment to measure
Heart rate – ultrasound: absence fetal / maternal heart beat and aorta blood flow[70]	Increases in heart rate can be indicative of a stress state	Ultrasound Equipment and expertise
Blood pressure – telemetry[13,14,26,46,87,94]	Increases in blood pressure can be indicative of a stress state	In the absence of concurrent behavioral evaluation, difficult to interpret increases in heart rate; requires specialized and invasive equipment to measure
Electrocardiography (ECG)[26,27]	Provides information about cessation of heart beat	In the absence of concurrent behavioral evaluation, difficult to interpret increases in heart rate; the heart can continue to beat after loss of CNS control due to the internal pacemaker; Requires specialized and/or invasive equipment to measure
Histology: Respiratory tract[13,15,19,31,40]	Can suggest evidence of pain and/or distress with the presence of inflammatory response	Nonspecific marker of pain or distress
Autonomic Nervous System Assessment		
ACTH[14,45,105]	Measure of acute stress; more accurate representation of the sympathetic autonomic response than markers such as corticosterone	May not be reflective of physiologic responses to slow methods of euthanasia
Serum corticosterone[18,45,105]	Measure of acute stress	Varies significantly dependent upon sex, time of day, degree of satiation and other variables; takes minutes after stressful response to achieve significant increases in the circulation
Blood glucose[45]	Measure of acute stress	Varies significantly dependent upon sex, time of day, degree of satiation and other variables; takes minutes after stressful response to achieve significant increases in the circulation
Central Nervous System Assessment		
c-fos expression[105]	May indicate pain	Changes in c-fos expression take several minutes to manifest
pERK activation [Newsome and colleagues in press]	Changes in phosphorylation state occur very rapidly	Phosphatases in tissue may interfere with measurement unless tissues are collected rapidly, and phosphatase inhibitors used

Table 4. Continued

Assessment	Pros	Cons
Histopathology: Brain[50]		Requires Blinded Pathology expertise
Electroenchephalograms (EEG)[25,31]	Can be used to determine cessation of brain activity	Interpretation of when unconsciousness occurs may be difficult; unclear how this correlates with pain and/or distress
Visual evoked potentials (VEP)[21]	To measure focal cortical responses to a specific stimulus	
Electromyograph (EMG)[30]	Provides information about loss of CNS activity	Requires specialized and invasive equipment to measure; unclear how this correlates to loss of consciousness or the perception of pain and/or distress
Electrocorticograph (ECoG)[30]	Provides information about loss of righting reflex	Requires specialized and invasive equipment to measure.

task. Not only must investigators consider the effects that a given method may have on their data, but they must also consider the impact of the method on the animal and on the ability of other laboratories to reproduce the results of their study. Investigators, veterinarians and institutional animal care and use committee members should be aware of the various published guidelines on euthanasia. Such documents may help investigators to choose, or choose between, euthanasia methods. These guidelines can represent the opinion of various professional societies regarding best practices for euthanasia[90] or like the EU Directive,[38] they may carry the weight of the law. Further complicating the picture, some guidelines describing best practices may be interpreted by federal agencies, accreditation bodies, or the public as though they were binding standards. Examples of this may include the AVMA Guidelines,[63] NIH Guide for the Care and Use of Laboratory Animals,[50] OLAW FAQs,[74] and USDA Animal Care Policy Manual.[102] Table 3 summarizes the recommendations of several euthanasia guidelines to help researchers and veterinarians make informed decisions, especially if international collaborations are part of the study. This table is not meant to be comprehensive, and in all cases, local laws and the most recent guidelines should be consulted. The terminology regarding whether a method of euthanasia is considered humane varies across different guidelines, but generally, methods fall into one of 3 categories: acceptable, conditionally acceptable, or unacceptable. In the context of Table 3, acceptable indicates that the method most often produces a humane death with a low risk of operator error or personnel injury. Conditionally acceptable indicates that the method is considered humane when the conditions for its appropriate use are met, operator error is minimized through training or engineering controls, and personnel safety concerns are mitigated. Unacceptable indicates that the method is not considered humane when used as a sole agent on conscious animals. Some unacceptable methods can be rendered acceptable if the animal is deeply anesthetized or otherwise unconscious prior to the application of such a method. Readers are advised that some methods still carry risks to the operator, bystanders, or the environment; these risks must be addressed to use such methods even on an unconscious animal. The literature is not complete regarding euthanasia methods for all species, strains, and all stages of development. For example, the time at which a fetus or neonate acquires the ability to consciously perceive pain varies between species and may not be known for some species. Similarly, aversion or susceptibility to chemical agents, hypoxia, and stress associated with handling or restraint may vary considerably between species and developmental stages, making it difficult to provide general recommendations.[1,33,34] Until more conclusive research is available, due consideration should be given to minimizing pain or distress for the animal in question.

Designing or Evaluating Studies on Euthanasia Methods

When evaluating or contributing to the literature on different rodent euthanasia methods, a clear understanding of the process of euthanasia, and how to assess the wellbeing of animals is critical.

One of the most important criteria for acceptance of a method of euthanasia is that it has a rapid initial depressive action on the central nervous system (CNS) to ensure immediate insensitivity to pain. It is also important to take steps to minimize distress in the animal prior to the procedure.[19] The evaluator must understand anesthesia and anesthesia overdose to aid in selecting the appropriate period to perform the welfare evaluation. Expression of pain and distress is limited or very subtle for many rodent species, and assessment of these states can be imprecise. Therefore, design of studies should include a combination of physiologic and behavioral assessments to improve the accuracy of the interpretation of the results.

Scientific information on euthanasia is available for certain species, strains, physiologic states (for example, neonatal or pregnant) and scientific situations; however, conclusive information is not available for all species, many strains, and research manipulations. When evaluating methodologies, it is imperative to:

- use professional judgment and technical competence to make an assessment based on both the scientific requirements of the study and the welfare of the animals;
- understand the animal, its normative behavior, and physiology;

Individual animal responses may differ. Therefore, adequate statistical planning and power and use of proper controls is required to detect subtle behavioral or physiologic expressions of pain/distress. Proper reporting of how animal pain and distress is evaluated, especially when using behavioral measures correlated to physiologic measure, enhances reproducibility and provides validation of a chosen technique.

Key to such investigations are well-defined parameters related to:

- Recognition, timing, and assessment of unconsciousness;
- Assessment of adverse effects prior to unconsciousness;
- Methods of ensuring the death of the animal; and
- Recognition and confirmation of death.

Animals should be euthanized during research, teaching, testing or production in a way that ethically ensures the death is as painless and free of distress as possible. The literature includes many methods for determining the level of pain and distress, or the time during the procedure at which pain and distress are perceived. Table 4 attempts to categorically group these assessment techniques and provide information related to strengths and weakness of each.

Conclusion

Euthanasia methods are under constant study, and guidelines often change as new information is gained. As new knowledge expands understanding of practices best suited for humane euthanasia, revised and new guidelines can be expected. At the time of writing, examples include a new version of the CCAC Guidelines on mice with revised guidance on CO_2,[23] a new proposed draft of the AVMA Guidelines for the Euthanasia of Animals[5] and the International Association of Colleges of Laboratory Animal Medicine (IACLAM) Task Force on carbon dioxide; and the recent publication of AVMA Guidelines for the Depopulation of Animals.[4] Those conducting studies on euthanasia with plans for publication are advised to follow the PREPARE[93] and ARRIVE[55] Guidelines for planning and reporting these studies. Following these publication guidelines aids authors in designing reproducible studies and in reporting their experimental conditions in sufficient detail to permit reproducibility between institutions. The literature on the following areas, in particular, is conflicting or lacking: strain-related variation in optimal CO_2 flow rates; flow rates and exposure paradigms for alternative gases; thoracic compression of small mammals.

Veterinarians trained in laboratory animal medicine should use professional judgment to assess whether a proposed euthanasia method is aligned with the goals of the research and will provide valid data. When necessary, a veterinarian should participate in the assessment and validation of euthanasia methods on a case-by-case basis.

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