Reference Resources

Caveats from AAALAC's Council on Accreditation regarding this resource:

Report of the ACLAM Task Force on Rodent Euthanasia*.
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JAALAS. 45(1):98-105, 2006.

*This reference was adopted by the Council on Accreditation with the following caveats:

Hypothermia is identified as an accepted method for inducing anesthesia in rodent fetuses (B. Euthanasia of Fetuses 2.d.) or pups 6 days of age or less (C. Euthanasia of Neonates 1.f.). This practice is common in the United States, but may not be accepted internationally. Suitability of this technique should be determined on a case-by-case basis with consideration of input from the Attending Veterinarian and the IACUC or other oversight body.

The science surrounding the use of carbon dioxide for rodent euthanasia is evolving. At present, there is no universally accepted procedure for euthanizing rodents using carbon dioxide. Participating institutions should be aware that updates to the science may result in refined methodology and may alter the Council on Accreditation's view of the methods described in this report.

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Public Statement

Report of the ACLAM Task Force on Rodent Euthanasia

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The ACLAM Task Force on Rodent Euthanasia was appointed by President Lynn Anderson in 2002 in response to growing concerns and controversy regarding techniques that were commonly used for rodent euthanasia. Three issues were targeted as the focus of the report: euthanasia of fetal and neonatal rodents, the use of carbon dioxide for rodent euthanasia, and the impact of euthanasia techniques on data. The charge to the Task Force was to create a document that summarized in a scholarly and comprehensive manner all available data-based literature relevant to these topics, to assess the scientific merit of the design and conclusions of those studies, and to compile valid information into a concise and cohesive document that could serve as a resource for diplomates, other veterinarians, IACUC members, regulatory bodies, and research scientists.

The Task Force has fulfilled this charge in an exemplary manner. During 2004-2005, the ACLAM officers and Board of Directors (BOD) reviewed and critiqued 2 draft versions of the report, and suggestions for change were incorporated into the document presented here. In July 2005, the BOD voted to forego the usual process of distributing the document to the ACLAM membership for comment before release based on 2 considerations. First, the literature relevant to rodent euthanasia is continually expanding. As such, at each revision, the Task Force was compelled to incorporate new data and citations. Their consensus view was that new data would continue to emerge, and the document would require continual revision as the review process continued. Related to that, the 2ndconsideration of the BOD was that information already accumulated would be of immediate utility to the stake-holders listed above.

In lieu of a pre-publication comment period, the BOD and the Task Force instead invite all diplomates, as well as other parties, to comment via email or mail to the BOD liaison for this project, who will compile and maintain all remarks. After an interval deemed appropriate by the ACLAM President, a 2nd Task Force will be appointed to update and modify the Report. Comments will be considered at that time.

I want to personally thank all members of the Task Force for their conscientious and comprehensive efforts in compiling this information. They have created a valuable and informative synthesis that should serve as a resource to the community for years to come.

—Linda A Toth, DVM, PhD
ACLAM BOD Liaison to Task Force on Rodent Euthanasia

Introduction

The guidelines below were prepared by the American College of Laboratory Animal Medicine (ACLAM) to expand upon the information provided by the Report of the AVMA Panel on Euthanasia with regard to euthanasia of rodents in biomedical settings. Database searches were designed with assistance from a library scientist with advanced degrees in public health, education and research, training by AWIC and responsibility for veterinary reference materials. Keywords were selected to include all ages of rodents and all categories of rodent euthanasia identified by the 2000 AVMA Panel on Euthanasia.[2] Peer reviewed publications from 1912 to 2005 were identified and evaluated prior to inclusion as references.

Professional consultation with the attending veterinarian is essential when developing plans for euthanasia and when applying these guidelines. The intent of this document is to provide guidance on rodent euthanasia performed at biomedical research facilities. Selection of the optimal euthanasia methods must be assessed on an individual basis; however, there should be consistency in the goals of all methods employed. The euthanasia of rodents should be humane, minimizing pain and fear, delivered in accordance with current regulations, ensure rapid onset of unconsciousness followed by death, and avoid risk and aversion for animals and personnel. Specific information on fetal and neonatal euthanasia, use of CO_2 as an euthanasia agent, and the influence of euthanasia methods on frequently measured scientific parameters should be incorporated into the institution's educational program for investigators.

Euthanasia of Fetal and Neonatal Rodents

A. Background

The Report of the AVMA Panel on Euthanasia[2] provides limited recommendations for the euthanasia of prenatal or neonatal animals and no specific recommendations on altricial or precocial rodents. The report states: "When ovarian hysterectomies are performed, euthanasia of feti should be accomplished as soon as possible after removal from the dam." It also states "Neonatal animals appear to be resistant to hypoxia, and because all inhalant agents ultimately cause hypoxia, neonatal animals take longer to die than adults."[2] The Panel recommends "inhalant agents not be used alone in animals less than 16 weeks old except to induce loss of consciousness, followed by the use of some other method to kill the animal."[2]

The current scientific literature provides limited evidence for the effectiveness of any of the recommended rodent euthanasia

methods when performed on fetuses or neonates or the outcome on the fetuses when performed on the pregnant mother.

B. Euthanasia of Fetuses

By the 3rd trimester of gestation, the neural tube has developed into a functional brain, and the likelihood that a fetus may perceive pain should be considered.[12,30] No definitive evidence indicates that prenatal rodents perceive pain, but reflexive behavior observed in fetal animals correlates with adult responses to painful stimuli.[15,55] However, low arterial oxygen concentrations may limit higher cortical processing that would mediate fetal arousal and awareness.[43]

1. Mouse, Rat, and Hamster Fetuses up to 15 Days' and Guinea Pig Fetuses up to 35 Days' Gestation
   a. Neural development during this developmental stage is minimal and pain perception is considered unlikely.[36,73]
   b. Euthanasia of the mother or removal of the fetus should ensure rapid death of the fetus due to loss of blood supply and non-viability of fetuses at this stage of development.[37]
2. Mouse, Rat, and Hamster Fetuses over 15 Days' and Guinea Pig Fetuses over 35 Days' Gestation through Birth
   a. The neural development during this developmental period supports the likelihood that pain may be perceived.[30,36,73] Observations of near-term mouse and rat fetuses in vivo indicate behavioral responses to sensory stimulation.[17,65]
   b. Methods of euthanasia of fetuses
      i. Skillful injection of chemical anesthetics in sufficient quantities to ensure death.
      ii. Decapitation with sharp surgical scissors or cervical dislocation.
   c. Rapid freezing in liquid nitrogen without prior anesthesia is not considered to be humane.[2]
   d. When chemical fixation of the whole fetus is required, fetuses should be anesthetized prior to immersion in, or perfusion with, fixative solutions. Anesthesia may be induced by hypothermia,[19,54] or by injection with a chemical anesthetic.[67]
   e. Rodent fetuses are resistant to hypoxia.[63] Near-term rat fetuses experiencing umbilical cord occlusion exhibited respiratory movements for up to 40 min after occlusion.[58] Fetuses require extended exposure to inhalant anesthetics, including CO_2.[37]
   f. When fetuses are not required for study, the method chosen for euthanasia of a pregnant mother should ensure cerebral anoxia to the fetus and minimally disturb the uterine milieu to minimize fetal arousal.[37,43] A recommended method for euthanasia of the mother is CO_2 exposure followed by cervical dislocation.[47]

C. Euthanasia of Neonates

1. Mouse, Rat, and Hamster Neonates up to 10 Days of Age
   a. Maturation of nociceptors and the development of excitatory and inhibitory receptor systems occur during the period just prior to birth and extend into the 2 wk of postnatal life.[26,28,57,70]
   b. Resistance to hypoxia results in a prolonged time to unconsciousness when CO_2 inhalation is used as a euthanasia agent.[2,37,47] The duration of exposure to carbon dioxide varies with the age of the neonate. Inbred and outbred neonatal mice less than 7 d of age may differ in susceptibility to CO_2, requiring exposures as long as 50 min to ensure euthanasia.[55]
   c. When using CO_2 for euthanasia, death must be verified prior to disposal of the carcass.[51]
   d. Other methods for the euthanasia of neonatal mice and rats
      i. Injection of chemical anesthetics in sufficient quantities to ensure death.
      ii. Decapitation.
      iii. Cervical dislocation.
   e. Immersion in liquid nitrogen should be performed only if preceded by anesthesia. Anesthesia should precede immersion in, or perfusion with, chemical fixatives.
   f. Anesthesia in neonatal rodents may be induced by inhalant or injectable anesthetics. Prolonged exposure to inhalant anesthetics (e.g., halothane or isoflurane) may be necessary. Alternatively, hypothermia may be used to induce anesthesia in pups 6 d of age or less.[19,54] The attending veterinarian should be consulted for appropriate techniques and drug dosages.
2. Guinea Pig Neonates
   Follow guidelines for adults.[2]
3. Mouse, Rat, and Hamster Neonates over 10 Days of Age
   Follow guidelines for adults.[2]

The Use of CO_2 for Euthanasia of Rodents

A. Background

Carbon dioxide (CO_2) is a frequently used euthanasia agent for small laboratory animals due to its rapid onset of action, safety, low cost, and ready availability. It most commonly is used to euthanize rats and mice, which constitute the majority of animals used in biomedical research and are the focus of most studies on the use of CO_2 euthanasia. The same delivery system and equipment can be used to euthanize either single animals or groups of animals with CO_2. Despite its widespread use, euthanasia methods using CO_2 are not standardized. The current peer reviewed literature does not establish consistent requirements for CO_2 euthanasia and or even provide a clear definition of what constitutes a humane death. The acceptability of CO_2 for euthanasia under various conditions, and for various species and ages of animals, must continue to be re-evaluated as new data become available.

B. General Considerations

Changes in the animal's environment or novel conditions should be minimized to the degree that is practical. Rodents are sensitive to their environment and to handling.[42,59,62] Removal from the home cage,[14] regrouping with other animals,[44] introduction to new sites and odors,[11] and transport and placement into the euthanasia chamber can alter physiologic and metabolic parameters and possibly cause stress. Researchers and animal care staff should seek methods that minimize the stress experienced by rodents that undergo CO_2 euthanasia. Transporting animals and performing euthanasia in the home cages, using carts that are quiet, roll freely, and do not jostle cages or occupants, and minimizing regrouping to prevent social aggression are simple approaches to lessening potentially stressful conditions.

C. Euthanasia Chambers

1. Euthanasia chambers should be kept clean and free of debris and excreta.
2. The euthanasia chamber should be large enough to permit each animal to stand on the floor of the chamber with all 4 feet and have sufficient space to turn around and perform normal postural adjustments.

D. CO_2 Gas Delivery Systems

1. Sufficient carbon dioxide must be introduced into the chamber to totally displace the residual air by both mixing and dilution. Ideally, the inlet for delivery of CO_2 and any diffusion devices in the euthanasia chamber should provide a predictable and controllable elevation in CO_2 concentration.
2. Excess gas must be allowed to escape from the chamber in a way that allows a gradual increase in the concentration of CO_2 at the floor of the container that holds the animal. Escape of the gas mixture through a port, or other opening at the top of the chamber, must occur in a controlled manner that neither pressurizes the chamber nor permits reflux of room air into the chamber.
3. Carbon dioxide should be delivered using a 2-stage regulator, with the 2nd stage capable of adjustable fixed flow rates.
4. Large chambers designed for euthanasia of groups of animals may require multiple inlets, or diffusion devices, to facilitate different configurations for CO_2 introduction.
5. The use of heated valves assures constant delivery of gas to the chamber by avoiding the formation of dry ice within valves and regulating systems when units are used for prolonged or repeated periods.
6. The filling rate of the chamber should be based upon the time required to rapidly and successfully render the animals unconscious. This may differ from the amount of time required to achieve a lethal concentration of CO_2. Each type of chamber will have a different CO_2 filling profile based upon gas flow rate, gas dispersion characteristics, gas inlet locations and chamber dimensions.
7. Chambers should be filled with CO_2 at a flow rate that balances the time to unconsciousness with such associated aversive stimuli as noise or high velocity air movement.
8. A fill rate of 20% of the chamber volume per minute has been recommended as an appropriate means to achieve a lethal concentration.[31] However, animals should be closely observed during the filling process, as individual systems may require adjustment to achieve the desired effect.[2]

E. Pre-filling vs Not Pre-filling the Euthanasia Chamber

1. Because inspiration of high concentrations of CO_2 is both aversive and painful,[9,19,40] a recommended procedure is to place animals into a chamber that contains room air and then to gradually introduce CO_2.
2. The use of CO_2/oxygen gas mixtures and slow fill rates prolong the time to unconsciousness and death and may increase distress for the animals. There is no conclusive evidence that adding pure oxygen to carbon dioxide makes this procedure less stressful to animals.[13,20,29,39] A fill rate of 20% of the chamber volume per minute with carbon dioxide, added to existing room air in the chamber should be appropriate to achieve a balanced gas mixture to fulfill the objective of rapid unconsciousness with minimal distress to the animals.

F. Cautionary Information

1. Animal carcasses should not be exposed to room air until death has ensued with high certainty, as the anesthetic effects of CO_2 can be quickly reversed in the presence of oxygen.
2. Individual rodents may become apneic at certain concentrations of CO_2, giving the false impression that death has occurred.[9]
3. Confirmation of death should be based not on a single sign, such as cessation of breathing, but on multiple signs, such as physical examination, exposure to room air (under observation), or adjunctive methods of euthanasia (decapitation, cervical dislocation, pneumothorax).
4. Euthanasia apparatus should be regularly evaluated to ensure proper functionality sufficient to achieve 100% euthanasia of all animals. Failure to function correctly may result in the need to re-expose an animal to carbon dioxide to achieve euthanasia. Re-exposure should take place before the animal regains consciousness.

The Influence of Euthanasia Method on Frequently Measured Scientific Parameters

A. Background

The method of euthanasia can influence the validity of scientific results. *The Guide for the Care and Use of Laboratory Animals* indicates the appropriate euthanasia method depends on many criteria, including compatibility with research objectives.[48] It further states, "The selection of specific agents and methods for euthanasia will depend on the species involved and the objectives of the protocol."[48] Euthanasia, as a process, separates the presentation of new variables, treatments or environmental changes to the living system from the terminal collection of tissues and blood for additional study or analysis. In itself, the euthanasia method can alter physiologic parameters and responses.

The effects of handling during the euthanasia process, proficiency of personnel performing euthanasia, and mechanical efficiency of equipment can introduce variables that influence the welfare of the animal and the interpretation of the data. Such factors as the species and age of animal, measurements to be assessed, sampling sites, and time of tissue collection, additionally influence sample analysis and histology.

The existing literature should be assessed for general information. However, the specific impact of any euthanasia method on scientific results may require case-by-case validation.

The euthanasia technique should minimally impact the welfare of the animal and the handler and must support collection of reproducible scientific data.

The researcher must evaluate the scientific consequences of the chosen method of euthanasia.

B. Biological Effects of Euthanasia Techniques

Table 1. Biologic effects of decapitation[3,5,16,49,56,60,66]

Effect	Mechanism
Increase in plasma sodium Increase in plasma potassium Increase in GABA concentrations (brain) Increase in Alanine (brain) Increase in plasma ascorbic acid (30-40% > resting state)	Hemolysis
Increase in blood catecholamine levels	Continued postmortem neurochemical alterations
Increased plasma calcium, magnesium No change in vasoactive intestinal peptides (brain) No change in neuropeptide Y (brain) Alteration in rat heart mitochondria function	
Increase in serum corticosterone	Stress stimulus → mobilization from tissues to blood; generalized metabolic response secondary to sympathoadrenal response some handling related stimulation. Possible handling stress

Table 2. Effects of physical and pharmacological euthanasia methods

Method	Physiologic effect
Methoxyflurane and decapitation[10]	Increase in prostacyclin (vasodilator that inhibits platelet aggregation) Vascular contractility suppressed Decreased vascular contractility
Ether and decapitation, or decapitation alone[50]	No statistical difference in prolactin levels or LH/FSH secretory properties of cultured anterior pituitary cells
Ether and decapitation[74]	No change in estrogen receptors/progesterone receptors in rat uteri
Ketamine and decapitation[50,74]	No change in estrogen receptors/progesterone receptors in rat uteri
Pentobarbital and decapitation[4]	Increase in acetylcholine release in the brain
Halothane and decapitation[21]	Increase in plasma ascorbic acid Increase in plasma catecholamines

Table 3. Effects on reproductive hormones: The following combinations may be unsuitable for studies of serum androgens

Decapitation in combination with agents listed below[49, 71]	Male rats								Mechanism: direct effect on testes Circulating Androstenedione	
	Immature				Mature					
	LH	FSH	Prolactin	Testosterone	LH	FSH	Prolactin	Testosterone	Castrated	Intact
Xylazine	–	–		↓	–	–	↑	↓		↓ or –
Biotal	–	–			–	–		↓		↓ or –
Thiopental	–	–			–	–		↓		↓ or –
Pentobarbital	–	–		↓	–	–	↑	↓		↓ or –
Ketamine	↓	↓		↓	–	–		↓	↑	↓ or –
Halothane	↓	↓		↓	–	–		↓		↓ or –
Ether (tested on castrated rats)	↑	↑	↑	↓	–	–		↓		↓ or –

↓ = decreased ↑ = increased – = no change.

Table 4. Biologic effects of euthanasia induced by pharmacologic and/or physical methods

Method of euthanasia	Effect	Mechanism
Injectable Pentobarbital[5,53,61] [a,b]	Decreased muscular contractility in isolated muscle preps Decreased GI smooth muscle contractility when given orally or intravenously; not seen in intraperitoneal route Intraperitoneal administration causes increased colonic contractility in response to acetylcholine Decreased spontaneous and drug induced vascular smooth muscle contractility Decreased catecholamine levels	Decreased calcium transport
	Increased partial pressure of CO_2 in arterial blood Increased serum activity renin Increased plasma aldosterone Splenic enlargement	
	Increased plasma glucose and insulin Increased liver glycogen	Increased CO_2 in arterial blood may change blood pH, which then changes metabolic indices
	Decreased plasma triglycerides	
	Increase in plasma insulin	Increased glucose production or decreased glucose clearance
Cervical dislocation/ cervical fracture[32, 68, 72]	Decreased coronary flow; decreased contractile function in isolated perfused heart preparations	Possible decreased sensitivity of B-adrenergic receptors secondary to cervical fracture
	Normal lymphocyte proliferation	
	High levels of serotonin in lung	Entrapment of platelets in pulmonary capillaries
	Increase in granulocyte and macrophage colony forming cell counts in murine bone marrow cultures	Apparent alteration of marrow stem cell pool
Cervical dislocation and methoxyflurane[32]	Increased mitogen induced lymphocyte proliferation Normal cytolytic T lymphocytes (CTL) response	
Cervical dislocation and pentobarbital[32]	Increased mitogen induced lymphocyte proliferation Decreased CTL response	
Cervical dislocation and halothane[32]	Normal mitogen induced lymphocyte proliferation Decreased CTL response	
CO_2 and cervical dislocation[32]	Normal mitogen induced lymphocyte proliferation Decreased CTL response	
CO_2 and decapitation[4,23,66]	Normal LH, FSH, prolactin, corticosterone Activity of cholinergic markers identical to decapitation only Altered $GABA_A$ receptor function	
Focused beam microwave irradiation (FBMI)[41,45] [c]	Best technique for measuring adenosine levels Decreased brain amino acids: alanine, GABA, ethanolamine, NH_3, valine, leucine, isoleucine, tyrosine, phenylalanine, glycine, aspartate Increased levels of reduced glutathione, glutamate	Due to rapid inactivation of metabolizing enzyme
	5 fold decrease in d prostaglandin and Thromboxane B_2 (mouse brain)	
	Twice the concentration of substance P, neurokinin A, and neurotensin in brain tissue compared to decapitation	Possible enzyme inactivation by microwave irradiation causing increased recovery of peptides Possible disintegration of neuropeptide containing tissue compartments, or decreased binding of carrier proteins, releasing more peptides
CO_2[8,52,69]	100% CO_2: decreased mean corpuscular hemoglobin (NP)[d] Increased total leukocytes and granulocytes (P)[e] Decreased liver glycogen, pyruvate, ATP No change in platelet counts	CO_2 causes acidosis that affects RBC parameters
CO_2 or CO_2/O_2[8,27,34,46,52,69]	Increased hematocrit, mean corpuscular volume No change in serum norephinephrine, dopamine, serotonin, corticosterone[f] Decreased serum creatine kinase, aspartate aminotransferase	
	Significant decreased liver glycogen stores Increased serum glucose	CO_2 causes acidosis that produces stimulation of enzymes of the glycolytic pathway
	Decreased activity of enzymes regulating branched chain amino acid degradation Decreased mean erythrocyte hemoglobin, mean corpuscular hemoglobin concentration	
70% CO_2/30% O_2 vs 100% (Pre-charged)[52]	Decreased number of circulating $CD3^+$ and $CD8^+$ T cells Increase in $CD10^+$ B cells in circulation	
70% CO_2/30% O_2 vs 100% (Not pre-charged)[52]	Increased number of circulating $CD3^+$, $CD4^+$, and $CD8^+$ T cells	NOTE: 100% CO_2 (Non-precharged and pre-charged) had overall greater T-cell counts than 70% CO_2/30% O_2 euthanized animals
Isoflurane[8]	No change in liver glycogen	

[a]preferred for isolated beating heart preparations.
[b]preferred method (by IV route) for collection of tissues, including liver, for cyclic AMP assay.
[c]FBMI minimizes post-mortem neurochemical changes. Leaves brain landmarks intact.
[d](NP) = Not precharged.
[e](P) = Precharged.
[f]exposure to CO_2 prior to decapitation.

Table 5. Anesthetics – ketamine hydrochloride, pentobarbital, chloral hydrate, chloralose and halothane in combination

Fructose -2-6-biphosphate[35]	Significant increase in brain, heart, skeletal muscle concentrations

reported to release massive sympathetic response with ↑ catecholamines from adrenal gland.

Table 6. Gross/histopathology changes[1,24,25,33,64]

Ether	Decapitation	CO_2[a]	Methoxyflurane	Pentobarbital	Physical Methods (DC, CD)	Methods Listed in this Chart
Lung: interstitial edema, marked alveolar emphysema	Lung: emphysema, hemorrhage, blood in alveolar spaces	Lung: congestion, hemorrhage, emphysema, atelectasis; Cardiac muscle: variable degenerative changes (influenced by time of exposure to CO_2 causing acidosis, hypoxia) $CO_2 + O_2$ Lung: severe edema and hemorrhage, extravasation to alveoli Cardiac muscle: variable degenerative changes (influenced by time of exposure to CO_2 causing acidosis, hypoxia), capillary bleeding causing marked extravasation of blood	Lung: congestion Spleen: splenomegaly	Lung: emphysema congestion Spleen: emphysema, congestion GI serosa: emphysema, congestion Cardiac muscle: Acute degenerative lesions Kidney cortex: circulatory changes Other: Peritoneal congestion, sanguinous fluid in abdominal cavity	Lung: emphysema, bleeding Neck/Brain: local tissue trauma	No change in sperm motion

NOTE: DC (decapitation), CD (cervical dislocation), CO_2, Intracardiac pentobarbital more suitable for histology of abdominal viscera.
[a]produces changes in hemodynamics—capillary contraction, followed by dilation of capillaries and veins (except lung vessels); depresses cerebral cortex, stimulates chemoreceptors; extravasation to alveoli: Not seen in all rodent species.

Table 7. Additional Factors that Influence the Outcome of Euthanasia[6,7,18,22,38,56]

1. Handling: May cause sympathoadrenal discharge, which affects plasma glucose, progesterone plasma catecholamines. Habituating the animals to handling may mitigate this effect.
2. Environmental stimuli (for example, noise) can increase plasma corticosterone concentrations.
3. Sequence: The order of euthanasia for rats housed in pairs produced significant differences in plasma tryptophan and unesterified fatty acids, plasma corticosterone, plasma protein lactate levels, substance P, cholecystokinin, somatostatin.

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