Title: IACUC Policy on the Euthanasia of Rodents, Rodent Fetuses and Neonates, P-008-01	
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I. PURPOSE

Animals being euthanized should not experience pain, fear, or other significant stress prior to their death. Outlined below are guidelines for the various acceptable methods of euthanasia for rodents. The PHS Policy requires that euthanasia be conducted according to the American Veterinarian Medical Association (AVMA) Guidelines for Euthanasia (2013 Edition S2. Laboratory Animals – S2.2.4.2.2) (OLAW). The AVMA guidelines set criteria for euthanasia and specify appropriate euthanasia methods and agents based upon the best literature and empirical evidence that minimizes pain and distress to animals during euthanasia. Central University Institutional Animal Care and Use Committee (IACUC) has adopted the following guidelines to:

(1) The euthanasia method must be appropriate to the research goals, species and age of the animal, approved in the animal study proposal and must conform to the most recent AVMA guidelines on euthanasia.

(2) Assist the research community by clarifying the specific procedures relating to the euthanasia of rodents.

(3) Promote best practices and ensure that pain and distress are minimized during laboratory rodent euthanasia. Animals must never be left unattended while succumbing to any euthanasia method.

The IACUC committee must grant an exemption to any deviation from this policy.

This guideline discusses the following topics regarding appropriate euthanasia methods for rodents:

- CO2 Euthanasia
- Barbiturate overdose
- Dissociative agent overdose
- Other inhaled agents
- Physical methods
- Confirmation of death

II. PROCEDURES

A. CARBON DIOXIDE EUTHANASIA OF RODENTS

Carbon dioxide (CO_2) is a frequently used euthanasia agent for small laboratory animals due to its rapid onset of action, safety, and availability.

This portion of the guideline concentrates on the following topics regarding the use of CO_2 euthanasia:

- Mechanism of action (CO_2)
- Species
- Administering CO_2
- Confirmation of death with CO_2

1. Mechanism of Action

Inhalation of high concentrations of CO_2 result in a rapid decrease of intracellular pH, rapidly resulting in decreased function in the CNS and death.

2. Species

Any rodent (mice, rats) used for research may be euthanized by CO_2 by following the guidance described below. The AVMA has determined this to be an "acceptable method with conditions".

3. Administering CO_2

A gradual fill rate of 10-30% chamber volume per minute displacement is expected at all rodent euthanasia locations at CMU (AVMA), (NIH).

Calculating Rodent Euthanasia Chamber Size and Flow Rate

The flow rate of gas for 10%, 20%, 30% displacement per minute:

I. $\frac{\text{Chamber Volume (in L)} = \text{(height in cm)} \times \text{(width in cm)} \times \text{(length in cm)}}{1000}$

Acceptable flow rate (in L/min) = (Chamber Volume in Liters) X 0.20 / min.

or

II.

Calculating Rodent Euthanasia Chamber Size & Flow Rate	
Enter the euthanasia chamber measurements (inches) into the yellow boxes.	
Chamber Height	
Chamber Width	
Chamber Length	
CO2 Flow Rate Calculations (L/min) will appear below in blue boxes.	
Chamber Volume (Cubic Inches)	0.0
Chamber Volume (Liters)	0.0
10% Displacement Rate (minimum)*	0.0
20% Displacement Rate	0.0
30% Displacement Rate (maximum)*	0.0
*See NIH ARAC Guidelines for Euthanasia of Rodents Using Carbon Dioxide for additional details.	

a. All personnel administering CO_2 to rodents must be properly trained (Guide and AVMA). All Principal Investigators must assure that their research staff are duly trained and adhere to animal care and use protocols, policies, and guidelines. Training on the use of the equipment and appropriate methods of euthanasia is required.

b. Compressed gas is the only acceptable source of CO_2 for euthanizing rodents. Dry ice, fire extinguishers, and other sources of CO_2 may not be used.

c. Euthanasia chambers should be constructed of a clear material (e.g. Plexiglas®) to facilitate observation of the animals continuously during the euthanasia procedure.

Animals must not be left alone at any point during the procedure. The chamber must be cleaned between uses.

d. Prefilled chambers are unacceptable. Gas must be delivered in a predictable and controllable fashion, at a low-flow rate of 10-30% volume displacement per minute (as described above).

e. All facilities must post individual signage at the site of the euthanasia station with clear instructions on how to operate the equipment and ensure death of animals (see page 8 for CO_2 Euthanasia Procedure Template).

f. Euthanasia should occur in a procedure room or laboratory, away from other rodent housing.

g. Satellite housing facilities may not euthanize animals in close proximity to the housing area. When possible, rodents should be euthanized in their home cages. Animals from different cages and/or different species cannot to be comingled.

h. CO_2 is denser than room air and will remain at the bottom of the chamber, thus the chamber will need to be emptied between groups of cages.

- **i.** Within CMU facilities, all mice must be kept within the CO_2 chamber until unconscious usually within 2-3 minutes. Maintain CO_2 flow for a minimum of 1 minute after respiration ceases. Observe each rodent to ascertain for lack of respiration, heartbeat and noting fixed, dilated pupils, and faded eye color. If all these signs are observed, than remove the rodents from the chamber otherwise continue exposing them to CO_2. If unconsciousness does not occur, check the chamber fill rate.
- **ii.** It is important to verify death after CO_2 exposure. If an animal is not dead, CO_2 narcosis must be followed by a secondary method of euthanasia, such as bilateral pneumothorax or cervical dislocation.
- **iii.** Examine the system for: a defective flow meter, absence of CO_2 supply, and /or leaks. Appropriate CO_2 concentrations and exposure times will prevent unintended recovery.

j. When euthanizing in laboratory space or a satellite facility outside of the vivarium areas, with IACUC approval, and scientific justification, an adult mouse may be euthanized with CO_2 and kept in the euthanasia chamber with CO_2 flowing for 2 minutes beyond the cessation of respiration. This is only in an effort to harvest tissues in a timely manner rather than completing the full 10 minute cycle. If euthanizing within vivarium, all mice must be kept within the CO_2 chamber for the entire 10 minute cycle, including euthanasia followed by tissue harvest.

k. Confirmation of euthanasia must be performed by an approved secondary physical method. Please see below for approved physical methods.

l. For euthanasia of mouse and rat fetuses and neonates, please refer to "NIH Euthanasia of Mouse and Rat Fetuses and Neonates".

B. INJECTABLE AGENTS

1. Barbiturates

Intraperitoneal injection of a barbiturate, such as pentobarbital, is an acceptable method of euthanasia for rodents.

a. The recommended dosage of sodium pentobarbital is 150 mg/kg for larger rodents and 250 mg/kg for mice or 3x the anesthetic dose. Commercial barbiturate euthanasia formulations are also appropriate.

b. Pentobarbital and pharmaceutical formulations containing pentobarbital are controlled substances and are regulated by the Drug Enforcement Agency (DEA).

c. Confirmation of euthanasia must be performed by an approved physical method. Please see below for instructions on confirmation of euthanasia.

2. Dissociative Agent Combinations

Ketamine and other dissociate agents, in combination with an α-adrenergic receptor agonist such as xylazine or a benzodiazepine such as diazepam, can be administered as a means of euthanizing rodents under certain conditions.

a. Ketamine/xylazine combinations may be administered intraperitoneally or intravenously for the purpose of euthanasia.

b. Doses and volumes of drugs may vary, but at least 4 times the anesthetic doses of ketamine/xylazine combinations should be used.

c. Confirmation of euthanasia must be performed by an approved secondary physical method. Please see below for approved physical methods.

C. INHALANT ANESTHETICS (Halogenated Gaseous Agents)

Agents such as isoflurane, sevoflurane and other halogenated gases may be used as a means to euthanize rodents when delivered by either an anesthetic vaporizer or bell jar set-up.

1. Use of an Anesthetic Vaporizer with Inhalant Anesthetics

Anesthetic vaporizers can be used to rapidly and reliably induce anesthesia followed by euthanasia in rodents when used appropriately. The following guidelines should be followed when using an anesthetic vaporizer to euthanize rodents.

a. The chamber used to expose animals to the volatile gas must be sealed, and have the appropriate waste gas scavenging system in place.

b. The euthanasia chamber should not be overcrowded and only animals originating from the same home cage should be anesthetized and euthanized together.

c. Isoflurane is the preferred inhalant anesthetic agent and the concentration setting on the vaporizer should be at 3-4% in order to induce anesthesia. Once the appropriate anesthetic depth is achieved, the vaporizer setting may be increased to 5% in order to induce death.

d. The animal should remain in the chamber for an additional 2 minutes after breathing has ceased.

e. Rodents should be monitored continuously during the inhalant anesthetic delivery and should never be left alone.

f. Confirmation of euthanasia must be performed by an approved secondary physical method. Please see below for approved physical methods.

g. The euthanasia chamber must be cleaned with a disinfectant after each use. Please refer to the IACUC guideline on "Anesthetic Vaporizers and Gas Scavenging" for more detailed information on vaporizer and scavenging equipment as well as certification.

2. Use of Bell Jars with Inhalant Anesthetics
(Must be preapproved by CMU's Chemical Safety Officer)

Bell jars refer to any small, transparent, sealable container that is filled with a volatile anesthetic via a soaked absorbent material. A bell jar may only be used with small rodents for **euthanasia procedures**. If used, the description of the bell jar must be included in the "Euthanasia" procedure as part of an IACUC-approved protocol. In the procedure's drug chart, the dose on the drug chart should be listed as "bell jar".

The following practices must be followed, and described in your protocol:

a. This equipment must be used in a fume hood.

b. A physical barrier must be used to separate the rodent from the anesthesia soaked cotton or gauze material. The animal should only be exposed to vapors and should never come in contact with the liquid state of the anesthetic as this can be irritating. This separation should be accomplished by using a pre-fabricated container with a "shelf" or other durable screening in the container dedicated to this purpose.

c. A bell jar should not be pre-charged with the volatile anesthetics as this can lead to a sudden hypoxemia in rodents, which can cause distress. Placement of anesthetic soaked materials into the bell jar must occur immediately prior to placement of separator and rodents into the bell jar.

d. The bell jar should not be overcrowded. Only animals originating from the same home cage should be anesthetized and euthanized together. If using the conical tube technique, only one animal should be anesthetized and euthanized at a time.

e. Animals should remain in the bell jar until breathing has ceased or until anesthetic depth is achieved. Immediately after removing animals from the bell jar euthanasia or confirmation of euthanasia must be performed by an approved physical method. See below for methods of confirmation of euthanasia.

f. Rodents should be monitored continuously during the inhalant anesthetic delivery and must not be left alone.

g. The bell jar's interior will be cleaned with disinfectant and wiped clean after each use.

h. If any procedure(s), such as blood collection or terminal surgery, are to be performed, a bell jar **must not be used**. A more refined, controlled method must then be used, i.e., the use of a vaporizer to control the amount of inhalant anesthesia to be delivered.

The following procedure description can be cut-and-pasted into the euthanasia procedure, with the "blanks" completed with lab-specific information (use [] for guidance):

The bell jar used for euthanasia is _____ [describe the "jar"; a commercial product designed for this purpose for a "homemade" system made from other equipment] and will be used in a fume hood located in the laboratory. The animal will be separated from the isoflurane (or other agent)-soaked absorbent material by _____ [described the grid or mesh used]. Only animals originating from the same home cage will be anesthetized and euthanized together. Bell jar will not be pre-charged with the anesthetic prior to introduction of animals. Animals will remain in the jar until breathing has slowed or ceased, then one animal at a time will be removed, and assurance of deep anesthesia confirmed by a firm toe pinch. If there is a pedal reflex, the animal will be returned to the chamber and the above attempted again after at least 30 seconds. When breathing has stopped or the animal is anesthetized, the animal will be immediately euthanized or have death confirmed by _____ [physical method: cervical dislocation,

decapitation, exsanguination, bilateral pneumothorax]. Bell jars containing animals will never be left unattended or used as a transport device.

D. PHYSICAL METHODS WITHOUT ANESTHESIA

1. Cervical Dislocation Without Anesthesia

Manual cervical dislocation can be a humane technique for euthanasia of mice, and rats weighing < 200 g, when it is performed by well-trained individuals that have undergone the appropriate training. This method of euthanasia should only be used when scientifically justified and approved by the IACUC. Confirmation of euthanasia must be performed by an approved secondary physical method. Please see below for approved physical methods.

2. Decapitation Without Anesthesia in Adult Mice and Rats

This technique may be used only when required by the experimental design and approved by the IACUC. Specialized rodent guillotines are available and must be kept clean and in good condition with sharp blades. The use of a species appropriate restrainer (e.g., DecapiCone) will reduce stress from handling, minimize the chance of injury to personnel, and improve the positioning of the animal in the guillotine. Both of these physical methods are acceptable forms of euthanasia for these animals when the animals are fully anesthetized.

E. METHODS OF PRIMARY EUTHANASIA FOR FETAL AND NEONATAL RODENTS

Please refer to the "NIH Guidelines for the Euthanasia of Rodent Fetuses and Neonates".

F. CONFIRMING DEATH

Many of the aforementioned methods of euthanasia can produce a reversible anesthetic state. Therefore, **all animals must also receive a confirmatory method of euthanasia to ensure death**.

These confirmatory methods include exsanguination, decapitation, cervical dislocation (adult mice or rats under 200 grams only), bilateral thoracotomy, or at least 50% additional time in a euthanasia chamber filled with 100% CO_2 (in adult rodents only). The latter may be achieved with a total CO_2 exposure time of 10 minutes at a 20% flow rate, so that animals are euthanized by 7 minutes and then maintained for an additional 3 minutes in the closed chamber. **Death of the animal must be ensured prior to disposal of the rodent carcass. Failure to confirm death of a euthanized rodent is a significant non-compliance, reportable to the appropriate regulatory and accrediting agencies.**

REFERENCES

American Veterinary Medical Association Guidelines for Euthanasia (2013) (**AVMA**).

American College of Laboratory Animal Medicine Report of the ACLAM Task Force on Rodent Euthanasia (**ACLAM**)

Guide for the Care and Use of Laboratory Animals (**Guide**).

Animal Research Advisory Committee Guidelines for the Euthanasia of Mouse and Rat Rodent Fetuses and Neonates (2013) (**ARAC**).

Animal Research Advisory Committee Guidelines for the Use of Non-pharmaceutical Grade Compounds in Laboratory Animals (2013) (**ARAC**).

NIH Guidelines for Euthanasia of Rodent Fetuses and Neonates (last revised 6/22/16)

NIH Guidelines for Euthanasia of Rodents Using Carbon Dioxide (last revised 1/25/17)

Makowska, I.J., et al. Evaluating methods of gas euthanasia for laboratory mice. *Appl Anim Behav Sci*. 2009. 121(3–4):230-235.

Office of Laboratory Animal Welfare IACUC Guidebook (**OLAW**).

Public Health Service Policy: Clarification Regarding Use of Carbon Dioxide for Euthanasia of Small Laboratory Animals (**PHS**).

Penn State Animal Welfare IACUC Guideline EUTHANASIA OF RODENTS (2016)

Pritchett, K., D. Corrow, J. Stockwell, and A. Smith. Euthanasia of Neonatal Mice with Carbon Dioxide. *Comp Med*. 2005. 55(3):275-281.

Pritchett-Corning, K. Euthanasia of Neonatal Rats with Carbon Dioxide. *J Am Assoc Lab Anim Sci*. 2009. 48(1):23-27.

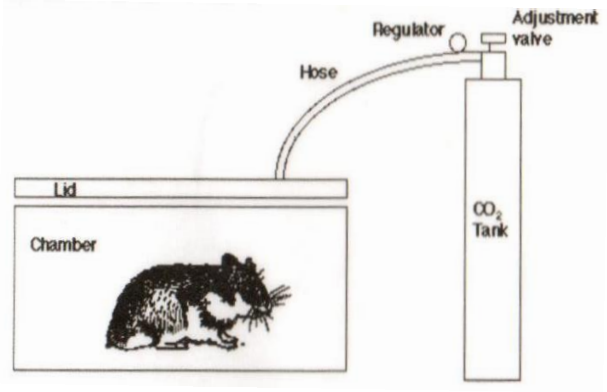
Schoell AR, Heyde BR, Weir DE, Chiang P-C, Hu Y, Tung DK. Euthanasia Method for Mice in Rapid Time-Course Pulmonary Pharmacokinetic Studies. *J Am Assoc Lab Anim Sci*. 2009;48(5):506-511.

Valentine H, Williams WO, Maurer KJ. Sedation or Inhalant Anesthesia before Euthanasia with CO2 Does Not Reduce Behavioral or Physiologic Signs of Pain and Stress in Mice. *J Am Assoc Lab Anim Sci*. 2012. 51(1):50-57.

CO2 Euthanasia Procedure

1. Place the animal(s) in an empty chamber lined with paper towel, or their home cage can be placed in the CO2 chamber. Animals place togerther in the chamber should be of the same species. Chambers must not be overcrowded. In this regard, it is important to also consider that mixing unfamiliar or incompatible animals in the same container may be distresstful. NOTE: If euthansaia is NOT being conducted in the home cage, chambers must be emptied and cleaned/disinfected between uses (each group of animals).
2. Fasten the chamber lid and place the hose into the top port of the chamber securely. Compressed CO2 gas in cylinders is the only recommended source of carbon dioxide. Other methods are unacceppable. Use the regulator key to open the CO2 value. **(Left to loosen, right to tighten).**

3. **Do not pre-fill the chamber.**
4. **Use a gradual flow method:** method is less likely to cause pain due to nociceptor activation by carbonic acid prior to onset of unconsciousness.
5. Start the flow of CO2 from the gas cylinder at a rate that will displace 20-30% of the cage or chamber volume per minute.
   - Small chamber: 20% displacement = 1.3 L/min 30% displacement = 2L/min
   - Large chamber: 20% displacement = 7.3L/min 30% displacement = 11 L/min



This calculated rate will allow a slow increase in the concentration of CO2 to develop. Without wind/hissing type noise in the chamber. **You should NOT observe animals jumping, scratching at the sides of the chamber or rubbing their feet over their nose/mouth/eyes; if you do the volume is incorrect.**

Unconsciousness will occur as indicated by a loss of movement and righting reflex (reflex that corrects the orientation of the body when taken out of a sternal position). The animal's movement will reduce and then stop with evidence of unconsciousness. Maintain CO2 flow rate for at least one minute after respiratory arrest.

6. Turn the gas down to 0%-OFF
7. After turning the gas off, observe the animal(s) until all muscle activity and signs of life have been absent for at least 30 seconds. Eyes generally dilated, and mucous membranes will no longer be pink. Remove the animal from the chamber and confirm respiratory arrest along with absence of heartbeat.
8. Return conscious animals to the chamber and restart gas flow.
9. When an animal is unconscious but still alive, place them back in the chamber or euthanize them utilizing a physical method; i.e. cervical dislocation. "Consistent with the AVMA Guidelines on Euthanasia 2013, cervical dislocation may not be performed on rodents weighing greater than 200 grams".
10. Once death is confirmed, place the carcass (es) in an appropriate plastic bag, label it appropriately with either Raptor or Biohazard, P.I. and date. NOTE: Raptor indicates that the animals have not received any drugs or agents and are non-transgenic.
11. Use the wrench/key to close the CO2 value all the way to the right. Remove the paper towel/excrements from the chamber and disinfect the chamber thoroughly. Do not re-lid the chamber so it can properly dry.

Acceptable Methods in Mouse and Rat Fetuses and Neonates.

- **CO2 acceptable methods:**

Mice and rat fetuses are unconscious in utero and hypoxia does not evoke a response. Thus, it is unnecessary to remove fetuses for euthanasia after the dam is euthanized.

- **Acceptable with conditions method:**

  - Neonatal mice may take up to 50 minutes to die from CO2 exposure. (An adjunctive method should be performed to ensure death cervical dislocation or decapitation) after a neonate is nonresponsive to painful stimuli.)
  - **Decapitation:** Use of scissors or sharp blade is acceptable for mice and rat neonates less than 7 days old (AVMA Guidelines)
  - Cervical Dislocation acceptable for mice and rat neonates greater than 15 days old (NIH Guidelines)