



Title	Euthanasia of Rodents, Rodent Fetuses and Neonates
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I. PURPOSE

To define a set of approved procedures for performing euthanasia on rodents, rodent fetuses and neonates. This standard operating procedure provides additional methods and procedural details in accordance with the IACUC Policy on Euthanasia.

The PHS Policy requires that euthanasia be conducted according to the American Veterinarian Medical Association (AVMA) Guidelines for Euthanasia of Animals (2020 Edition). 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.

II. POLICY

Per IACUC Policy on Euthanasia, “Methods not covered in approved IACUC standard operating procedures need to be approved by the IACUC. The Principal Investigator should confer with the attending or clinical veterinarian prior to submitting the protocol to the IACUC. The IACUC committee must grant an exemption to any deviation from approved policies and procedures.”

This SOP discusses the following approved euthanasia methods for rodents:

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

III. DEFINITIONS

Euthanasia – In the context of this policy, euthanasia is the act of inducing humane death in an animal with rapid unconsciousness and death with a minimum of pain, discomfort, or distress.

IV. PROCEDURES

A. General:

1. All individuals responsible for euthanasia of research animals must be qualified and trained to perform euthanasia humanely, per the IACUC Policy on Training for Research and Animal Care Personnel (P-003).
 - a. The Principal Investigator is responsible for ensuring appropriate documentation.

- b. Documentation of personnel training may be reviewed by the IACUC.
2. These procedures must be posted near the routine area of use – e.g., procedure or laboratory spaces, necropsy rooms. An abbreviated version may be posted at carbon dioxide euthanasia stations. Refer to Appendix A for an example template.
3. Euthanasia should occur in a procedure room or laboratory, away from other rodent housing. Satellite facilities may not euthanize animals in close proximity to the housing area. Similarly, investigators should avoid bringing more animals into the euthanasia space than can be euthanized in one batch.
4. Euthanasia procedures should be documented. See Appendix B for an example.
5. Death must be confirmed by a second physical form of euthanasia for narcotic or inhalant euthanasia processes. Examples:
 - a. Bilateral thoracotomy
 - b. Collections of vital tissues to assure the animal will not recover
 - c. Exsanguination
 - d. Decapitation
 - e. Cervical dislocation
6. Chambers for delivery of inhalant euthanasia agents must not be overcrowded and unfamiliar animals must not be combined to avoid anxiety and/or aggression shortly before euthanasia.
7. Animals must not be left alone at any point during the euthanasia processes.
8. Pentobarbital and pharmaceutical formulations containing pentobarbital are controlled substances and are regulated by the Drug Enforcement Agency and the State of Michigan. Refer to university policy on Oversight of Controlled Substances in Research (3-44) for more information.
9. The methods described in sections IV.B – IV.F are specific for rodents over 10-days-old. Section IV.G describes euthanasia of fetal and neonatal rodents.

B. Hazards and Safety Precautions:

1. Wear appropriate PPE as required per facility standards (e.g., lab coat, gloves)
2. Ensure appropriate ventilation when working with CO₂ (e.g., HVAC is operational, fume hood, down draft table). Build-up of CO₂ gases in the area can be a safety hazard negatively impacting research and facility staff.

C. Carbon Dioxide Euthanasia:

1. Carbon dioxide (CO₂) is a frequently used euthanasia agent for small laboratory animals due to its rapid onset of action, safety, and availability.
2. Mechanism of Action
Inhalation of high concentrations of CO₂ result in a rapid decrease of intracellular pH, rapidly resulting in decreased function in the CNS and death.
3. Administering CO₂
 - a. When possible, rodents should be euthanized in their home cages.
 - b. For euthanasia of mouse and rat fetuses and neonates, please refer to section IV.G.**
 - c. A gradual fill rate of 30-70% chamber volume per minute displacement is required.
Refer to Appendix A for information using the NIH ARAC Guidelines for calculating rodent euthanasia chamber size and flow rate instructions.
 - d. Compressed gas is the only acceptable source of CO₂ for euthanizing rodents.
The following are not permitted: dry ice, fire extinguishers, and other sources of CO₂.
 - e. Euthanasia chambers should be constructed of a clear material (e.g., Plexiglas®) to facilitate observation of the animals during the euthanasia procedure.
 - f. CO₂ 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. The chamber must

- be cleaned between uses.
- g. Prefilled chambers are unacceptable.
 - h. Maintain CO₂ flow for a minimum of 1 minute after respiration ceases.
 - i. 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, then remove the rodents from the chamber otherwise continue exposing them to CO₂.
 - j. If unconsciousness does not occur, check the chamber fill rate. Examine the system for a defective flow meter, regulator, absence of CO₂ gas supply, and leaks.
 - k. Confirmation of euthanasia must be performed by an approved secondary method. Please see Section IV.A.5 and H for approved methods.

D. Injectable Agents

1. Barbiturates

- a. The advantage of using barbiturates for euthanasia far outweighs the disadvantages, and barbiturate overdose is a common euthanasia method for mammals. Intraperitoneal injection of a barbiturate, such as pentobarbital, is an acceptable method of euthanasia for rodents
- b. The recommended intraperitoneal dosage of sodium pentobarbital is 150 mg/kg for rats and 255 mg/kg for mice or 3x the anesthetic dose. Commercial barbiturate euthanasia formulations are also appropriate.
- c. Confirmation of euthanasia must be performed by an approved secondary method. Please see Section IV.A.5 and H for approved methods

2. Dissociative Agent Combinations

- a. 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.
- b. Ketamine/xylazine combinations may be administered intraperitoneally or intravenously for the purpose of euthanasia.
- c. Doses and volumes of drugs may vary, but at least 4 times the anesthetic doses of ketamine/xylazine combinations should be used. Reliable euthanasia dosages have not been established for all species and therefore should not be used as a sole agent. Accordingly, confirmation of euthanasia must be performed by an approved secondary method. Please see Section IV.A.5 and H for approved methods.

E. Inhalent Anesthetics (Halogenated Gaseous Agents)

1. Agents such as isoflurane, sevoflurane and other halogenated gases may be used as a means to euthanize rodents when delivered by an anesthetic vaporizer.
2. **Use of an Anesthetic Vaporizer with Inhalant Anesthetics:**
 - a. 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.
 - b. Anesthetic vaporizers are specific to the agent or gases being used – ensure appropriateness prior to initiating use.
 - c. The chamber used to expose animals to the volatile gas must be sealed and have the appropriate waste gas scavenging system in place.
 - d. The euthanasia chamber must not be overcrowded and only animals originating from the same home cage are to be anesthetized and euthanized together.
 - e. Isoflurane is the preferred inhalant anesthetic agent and the concentration setting on the vaporizer should be at 3-4% in order to induce anesthesia. The oxygen flow rate should be at 1 liter / min.

Once the appropriate anesthetic depth is achieved, the vaporizer setting may be increased to 5% in order to induce death.

- f. The animal should remain in the chamber for an additional 2 minutes after breathing has ceased.
- g. The chamber must be flushed with oxygen and the anesthetic must be scavenged using the waste gas system. 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.
- h. Confirmation of euthanasia must be performed by an approved secondary method. Please see Section IV.A.5 and H for approved methods.
- i. The chamber must be cleaned with a disinfectant after each use.

F. Physical Methods

1. The methods indicated below are the same for anesthetized and anesthetized animals. When the research goals permit, animals should be anesthetized prior to use of physical methods of euthanasia.
2. **Cervical Dislocation**
 - a. 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.
 - b. For mice and rats, the thumb and index finger are placed on either side of the neck at the base of the skull or, alternatively, a rod (e.g., pencil, pen, closed scissor blades) is pressed at the base of the skull. With the other hand, the base of the tail or the hind limbs are quickly pulled, causing separation of the cervical vertebrae from the skull. A commercial device may also be used.
 - c. Alternatively, position the thumb and index finger on either side of the neck at the base of the skull (behind the occipital bone). With the other hand, holding the base of the tail, quickly and solidly pull causing separation of the cervical vertebrae from the skull.
 - d. Confirmation of euthanasia must be performed by an approved secondary method. Please see Section IV.A.5 and H for approved methods
3. **Decapitation in Adult Mice and Rats**
 - a. This technique may be used only when required by the experimental design and approved by the IACUC.
 - b. In unanesthetized animals, 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.
 - c. Specialized rodent guillotines are available.
 - i. The equipment used to perform decapitation must be cleaned and sanitized after each use, maintained in good working order, tested before each use (refer to IACUC SOP Maintenance of Blades used for Euthanasia P-0023-01), and serviced on a regular basis to ensure sharpness of the blades.
 - ii. PIs must have their guillotines and other similar equipment maintained or replaced at least annually or more frequently as needed based on use.
 - d. PIs are required to maintain either a service log or another method of documenting maintenance. Documentation must include (at a minimum):
 - i. Service date and/or next date of service
 - ii. Initials of individual verifying serviceability

G. Methods of Primary Euthanasia for Fetal and Neonatal Rodents

1. 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.
2. If pups are over 10 days old, the euthanasia process is the same as an adult.
3. Neonatal mice, less than 10 days old, may take up to 50 minutes to die from CO₂ exposure. An adjunctive method must be performed to ensure death such as decapitation after a neonate is nonresponsive to painful stimuli.

4. If neonates are anesthetized with a halogenated agent, an adjunctive method must be performed to prevent the possibility of recovery.

H. Confirmation of Death

1. Many of the aforementioned methods of euthanasia can produce a reversible anesthetic state. Therefore, animals must also receive a confirmatory method of euthanasia to ensure death, with the exception of decapitation without anesthesia (IV.F.2).
2. Potential confirmatory methods include exsanguination, decapitation, cervical dislocation (adult mice or rats under 200 grams only), bilateral thoracotomy, and collections of vital tissues to assure the animal will not recover.
3. Death of the animal must be ensured prior to disposal of the rodent carcass.

I. Disposal

1. Death must be confirmed prior to bagging carcasses for disposal.
2. Secure carcass(es) in a plastic bag.
3. Place bag in the appropriate freezer.
4. Vivarium staff will dispose of carcasses as biohazardous waste on a periodic schedule.

V. REFERENCES

American Veterinary Medical Association, Panel on Euthanasia. (2020). *AVMA guidelines for the euthanasia of animals: 2020 edition*. American Veterinary Medical Association.

<https://www.avma.org/sites/default/files/2020-01/2020-Euthanasia-Final-1-17-20.pdf>

Hawk, C. T., Leary, S. L., & Morris, T. H. (2005). *Formulary for laboratory animals* (3rd ed.). Blackwell Publishing.

National Institutes of Health, Office of Intramural Research, Office of Animal Care and Use. (2020, December 9). *Guidelines for euthanasia of rodents using carbon dioxide*. U.S. Department of Health and Human Services. https://oacu.oir.nih.gov/sites/default/files/uploads/arac-guidelines/b5_euthanasia_of_rodents_using_carbon_dioxide.pdf

Appendix A: Example Template - Procedures for CO₂ Euthanasia

1. Determine the necessary flow rate, see the two options for how to determine the minimum and maximum flow rates below.

- a. Double click the table below to use the Flow Rate Calculator:

Flow Rate Calculator		
Instructions:		
<ul style="list-style-type: none"> If chamber volume is already known, simply enter the volume in the first row, below. Otherwise, enter length, width and height/depth measurements into the table to obtain chamber volume and required flow rate. 		
Volume (in L), if known		
	Inches	Centimeters
Length		
Width		
Height/Depth		
Volume (L):		
0.00		
Required Flow Rate*:		
From 0 to 0 L/min		
<small>*Current OLAW and AVMA Guidance requires displacement of air with CO₂ at a rate of 30% -70% of chamber volume per minute.</small>		

- b. Perform the calculations manually:

- i. Determine chamber volume – If known, use the volume of euthanasia chamber. Otherwise calculate the interior volume of the euthanasia chamber as Length X Width X Depth (all dimensions are inside measurements, in centimeters).

Example: A euthanasia chamber measuring 20 cm X 25 cm X 20 cm has a volume of 10,000 cm³, or 10 L.

- ii. Calculate minimum flow rate as 0.3/minute X chamber volume (in L, from preceding). Example:

For a 10 L chamber, the minimum flow rate should be 0.3/minute X 10 L = 3 L/minute.

- iii. Calculate maximum flow rate as 0.7/min X chamber volume (in L, from preceding).

Example: For a 10 L chamber, the maximum flow rate would be 0.7/minute X 10 L = 7 L/minute.

- Place the animal(s) in the chamber. If not leaving animals in their original cage, line the chamber with paper towel. If animals are not euthanized in their home cage, the chamber must be cleaned to remove debris and pheromones between animals.
- Fasten the chamber lid and securely attach the hose to the top port of the chamber.
- Fill the chamber completely with CO₂ using a fill rate of 30-70% of the chamber volume per minute.
- CO₂ 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.
- Unconsciousness typically occurs within two to three minutes. Observe each rodent for lack of respiration. Maintain CO₂ flow for one minute after respiration ceases. If all signs of death (lack of respiration, fixed, dilated pupils and faded eye color) are observed at this point, then remove the rodents from the chamber. Otherwise, continue exposing them to CO₂. If unconsciousness does not occur, check the chamber fill rate. The system should also be checked for a defective flow meter, an empty CO₂ cylinder and/or system leaks.
- It is important to verify death after CO₂ exposure. This process must be followed by a secondary method of euthanasia, such as bilateral pneumothorax, decapitation, or cervical dislocation.

Appendix B: Example - Euthanasia Log

Euthanasia Record

Date	Subject ID*	Species	Time Start	Time End	Gauge Level (if CO2) or Method	Name

*In the event of euthanizing entire cages of animals without identification, indicate the protocol number and number of animals.