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) (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. The University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) has adopted the following guidelines to: (1) assist the research community by clarifying the specific procedures relating to the euthanasia of rodents and (2) 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.

This guideline discusses on the following topics regarding appropriate euthanasia methods for rodents:
- CO2 Euthanasia
- Barbiturate overdose
- Dissociative agent overdose
- Ethanol administration
- Other inhaled agents
- Physical methods
- Confirmation of death

A. CARBON DIOXIDE EUTHANASIA OF RODENTS

Carbon dioxide (CO2) 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 CO2 euthanasia:
- Mechanism of action (CO2)
- Species
- Administering CO2
- Confirmation of death with CO2

1. 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.

2. Species
Any rodent (mice, rats, hamsters, GPs, gerbils, degus, cotton rats) used for research may be euthanized by CO2 by following the guidance described below. The AVMA has determined this to be an “acceptable method with conditions”.

3. Administering CO2
A gradual fill rate of 10-30% chamber volume per minute displacement is expected at all rodent euthanasia locations across Penn (AVMA).
To calculate the flow rate of gas for 20% displacement per minute:
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Chamber Volume (in L) = (height in cm) x (width in cm) x (length in cm)

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

a. All personnel administering CO₂ 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 from the ULAR Training Division via Knowledge Link.

b. Compressed gas is the only acceptable source of CO₂ for euthanizing rodents. Dry ice, fire extinguishers, and other sources of CO₂ 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 (click here for signage template).

f. Euthanasia should occur in a procedure room or laboratory, away from other rodent housing. Satellite housing facilities may not euthanize animals in close proximity to the housing area.

g. When possible, rodents should be euthanized in their home cages. Animals from different cages and/or different species cannot to be com mingled.

h. 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.

i. Within ULAR facilities, all mice must be kept within the CO₂ chamber for 10 minutes. This serves as both the primary method of euthanasia and confirmation of death.

j. When euthanizing in laboratory space or a satellite facility outside of ULAR areas, with IACUC approval, and scientific justification, an adult mouse may be euthanized with CO₂ and kept in the euthanasia chamber with CO₂ 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 ULAR facilities, all mice must be kept within the CO₂ 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 “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.
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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 formulations containing pentobarbital are controlled substances and are regulated by the Drug Enforcement Agency (DEA).
c. For more information on preparation and storage/handling please refer to “Use of Non-Pharmaceutical-Grade Chemicals and Other Compounds in Research with Animals”.
d. 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 benzodiazepines such as diazepam, can be administered as a means of euthanizing rodents under certain conditions.

a. Ketamine/xylazine combinations may be administered intraperitoneally, intravenously or retro-orbitally 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.

3. Ethanol
Intraperitoneal (IP) injection of 70-100% ethanol is a conditionally acceptable method of euthanasia for adult mice. This method has been shown to result in unconsciousness and ultimately euthanasia in less than 7 minutes in adult mice, however, is suspected to be unreliable in rats and in mice less than 28 days of age (Allen-Worthington). A veterinarian should be consulted when composing a protocol involving IP ethanol injection as a means of euthanasia in mice. Please see below for instructions on confirmation of euthanasia.

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. The animal should remain in the chamber for an additional 2 minutes after breathing has ceased.
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d. Rodents should be monitored continuously during the inhalant anesthetic delivery and should never be left alone.
e. Confirmation of euthanasia must be performed by an approved secondary physical method. Please see below for approved physical methods.
f. 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
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
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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 confirm 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 ULAR 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 “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% CO₂ (in adult rodents only). The latter may be achieved with a total CO₂ 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.
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REFERENCES
Animal Research Advisory Committee Guidelines for the Use of Non-pharmaceutical Grade Compounds in Laboratory Animals (2013) (ARAC).
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).