

## **INSTITUTIONAL ANIMAL CARE AND USE POLICY**

### **Rodent Euthanasia Methods**

Previous Approval Date: April 6, 2020

Approval Date: 7/17/2023

Next Review Date: 4/1/2025

### **Purpose**

The purpose of this policy is to ensure that euthanasia procedures for rodents comply with the [American Veterinary Medical Association \(AVMA\) Guidelines for Euthanasia of Animals: 2020 edition](#). To facilitate completion of the IACUC protocol, sample narratives for each method are provided for investigators to include in their IACUC protocols for review.

### **Background**

Animals being euthanized should not experience pain, fear, or other significant stress prior to their death. The term euthanasia is from the Greek word eu (good) and thanatos (death). Per the AVMA Guidelines, “a good death is tantamount to the humane termination of an animal’s life.” The Public Health Service [Policy on Humane Care and Use of Laboratory Animals](#) requires that euthanasia be conducted according to the [AVMA Guidelines \(OLAW\)](#). Based on the best literature and empirical evidence, these guidelines set criteria for euthanasia, specify appropriate euthanasia methods and agents, and detail conditions that must be met when using specific methods and agents. The criteria that have been used for determining that death is painless are rapid loss of consciousness followed by cardiac and respiratory arrest. Following these guidelines assures that all animals are humanely euthanized with a minimum of pain and distress.

This policy summarizes key aspects of rodent euthanasia as set forth in the [AVMA Guidelines](#) and practiced at SUNY Downstate Health Sciences University:

- General Concepts for All Methods
- Carbon Dioxide (CO<sub>2</sub>) Euthanasia (Above Ten Days of Age)
- Inhalant Anesthetics
- Injectable Agents
- Physical Methods without Anesthesia
- Perfusion under Anesthesia
- Euthanasia of Neonates
- Euthanasia of Fetuses
- Confirmation of Death
- Operation & Maintenance of Guillotines

For further detail, the reader is encouraged to consult the succinct, well-organized, and highly-informative [AVMA Guidelines](#) directly.

### **General Concepts for All Methods**

- The method of euthanasia must be appropriate for the species and the age of the animal.
- All methods of euthanasia must be detailed on the IACUC-approved protocol to which the animal is assigned.
- All animal euthanasia must be performed by appropriately trained personnel ([Guide](#) and [AVMA](#)), listed as performing euthanasia on the IACUC-approved protocol to which the animal is assigned.

- When animals are euthanized together, they must be of the same species.
- Animals must be continually observed and never be left unattended while succumbing to any euthanasia method. Methods involving the use of chambers (e.g., inhalant carbon dioxide or isoflurane) must use chambers constructed of a clear material and not overcrowded, providing sufficient floor space for each animal to ambulate and make normal postural adjustments.
- All methods used must result in the confirmed death of the animal; for several methods, this requires a secondary physical method of euthanasia to ensure death.
- Animal carcasses and tissues must be properly disposed of after euthanasia.

### **Carbon Dioxide (CO<sub>2</sub>) Euthanasia (Above Ten Days of Age)**

- Inhalation of high concentrations of CO<sub>2</sub> by rodents older than ten days results in a rapid decrease of intracellular pH (respiratory acidosis), which in turn leads to decreased brain function and death after prolonged exposure.
- Medical grade compressed gas is the only acceptable source of CO<sub>2</sub> for euthanizing rodents; **dry ice or other sources of CO<sub>2</sub> are not allowed.**
- The euthanasia chamber must not be pre-filled with CO<sub>2</sub> prior to placing rodents in the chamber. Sudden exposure of conscious animals to CO<sub>2</sub> concentrations of 70% or greater has been shown to be distressful and potentially painful. Gas must be delivered in a predictable and controllable fashion using a gradual-fill method, at a flow rate of 30-70% volume displacement per minute.
  - All Division of Comparative Medicine (DCM) systems are preset to deliver a fixed flow of CO<sub>2</sub> within this range.
  - PIs who have received IACUC approval to use euthanasia systems in their laboratory must use either a fixed flow system similar to [DCM's](#) or a customized system utilizing both a regulator *and* a flow meter. Calculate and post the [Proper steps for CO<sub>2</sub> euthanasia of rodents using a custom flow meter](#) sign in the laboratory.
- CO<sub>2</sub> is denser than room air and will remain at the bottom of the chamber, thus the chamber will need to be emptied (e.g. placed on its side) between groups or cages.

#### *Sample Protocol Narrative –*

Animals will be placed in a clear chamber for continual observation and have CO<sub>2</sub> delivered at a 30-70% fill rate until breathing stops. Death will be ensured by a physical method identified in the protocol, and carcasses will be placed in DCM freezers for proper disposal. If used for subsequent animals, the chamber will be cleaned after use and placed on its side to purge the chamber of CO<sub>2</sub>. Whenever possible, animals will be euthanized in their home cages and not comingled with animals from other cages to minimize distress.

### **Inhalant Anesthetics**

- Agents such as isoflurane, sevoflurane, and other halogenated gases may be used as a means to euthanize rodents when delivered as an overdose by either an anesthetic vaporizer or a bell jar set-up.
- If any procedure (e.g. blood collection or terminal surgery) is to be performed, a bell jar must not be used; instead, a more refined, controlled method to deliver the anesthetic must be used, i.e., a vaporizer.

#### 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.
- Appropriate waste gas scavenging system must be in place (e.g., properly placed and maintained charcoal canister filters).

*Sample Protocol Narrative –*

Animals will be placed in a clear chamber for continual observation. The oxygen flow rate will be 1.0-1.2 L/min and the vaporizer setting 3%-4%. Once the appropriate anesthetic depth is achieved, the vaporizer setting may be increased to 5% in order to induce death. Death will be ensured by a physical method identified in the protocol, the chamber cleaned, and carcasses will be placed in DCM freezers for proper disposal. When possible, animals will not be comingled with animals from other cages to minimize distress.

Use of Bell Jars with Inhalant Anesthetics

- Bell jar refers to any small, transparent, sealable container that is filled with a volatile anesthetic via a soaked absorbent 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.
- The bell jar must be used in a fume hood for proper waste anesthetic gas scavenging.

*Sample Protocol Narrative –*

Placement of the <<*insert specific anesthetic agent here*>>-soaked material (e.g. cotton or gauze material) into the bell jar will occur immediately prior to placement of separator and rodents into the bell jar to prevent pre-charging of the chamber and direct contact of the animal with the anesthetic. Animals will remain in the bell jar until breathing has ceased or until anesthetic depth has been achieved, as confirmed by lack of response to stimulus such as withdrawal to toe pinch. Death will be ensured by a physical method identified in the protocol, the chamber cleaned, and the carcasses placed in DCM freezers for proper disposal. Whenever possible, animals will not be comingled with animals from other cages to minimize distress.

## **Injectable Agents**

### Barbiturates

- Intraperitoneal injection of a barbiturate, such as pentobarbital, is an acceptable method of euthanasia for rodents. Commercial barbiturate euthanasia formulations are also appropriate.
- Barbiturates result in euthanasia by depressing the CNS producing sequential unconsciousness, deep anesthesia, apnea, and cardiac arrest.
- Use of non-pharmaceutical grade pentobarbital is rarely acceptable, in cases where its use is scientifically justified, reviewed & approved by the IACUC.
- The recommended dosage of sodium pentobarbital is 150 mg/kg for larger rodents and 250 mg/kg for mice (three times the anesthetic dose) and should be included in the drug chart of the protocol.
- Sodium pentobarbital is a Schedule II drug and Sodium pentobarbital-combinations (i.e. Euthasol) is a Schedule III drug which is regulated by the Federal and New York State (NYS) Drug Enforcement Agency (DEA). Federal and NYS regulations require maintenance of records including the date, purpose, and amount of agent used.

*Sample Protocol Narrative –*

Sodium pentobarbital, or a sodium pentobarbital containing agent, will be administered intraperitoneally. Animals will be monitored until breathing has ceased or until anesthetic depth has been achieved, confirmed by lack of response to stimulus such as withdrawal to toe pinch. Death will be ensured by a physical method identified in the protocol, and carcasses will be placed in DCM freezers for proper disposal.

### Dissociative Agent Combinations

- An overdose of ketamine and other dissociate agents, in combination with an  $\alpha$ -adrenergic receptor agonist (e.g., dexmedetomidine, xylazine) or a benzodiazepine (e.g., diazepam); can be administered as a means of euthanizing rodents under certain conditions.
- Doses and volumes of drugs may vary, but at least four times the anesthetic doses of ketamine combinations should be used and included in the drug chart of the protocol.

#### *Sample Protocol Narrative –*

<<**Insert specific drug(s) here**>> will be administered intraperitoneally. Animals will be monitored until breathing has ceased or until anesthetic depth has been achieved, as confirmed by lack of response to stimulus such as withdrawal to toe pinch. Death will be ensured by a physical method identified in the protocol, and the carcasses will be placed in DCM freezers for proper disposal.

### **Physical Methods without Anesthesia**

- These techniques may only be used when required by the experimental design and approved by the IACUC (except for fetuses or mouse, rat, and hamster neonates  $\leq$  ten days of age – see below).
- All personnel performing these methods are required to be trained by the Division of Comparative Medicine (DCM) and demonstrate proficiency with the technique prior to using it on experimental animals.

### Cervical Dislocation without Anesthesia

- Manual cervical dislocation can be a humane technique for euthanasia of mice, and rats weighing less than 200 grams.

#### *Sample Protocol Narrative –*

The animal will be restrained in a normal standing position on a firm, flat surface and grasped by the base of the tail with one hand. A sturdy implement (e.g., metal rod) or the thumb and first finger of the other hand are placed against the back of the neck at the base of the skull. To produce the dislocation, the hand or object restraining the head is quickly pushed forward and down while pulling with the hand holding the tail back and up at a 30 degree angle from the table. The carcasses will be placed in DCM freezers for proper disposal.

### Decapitation without Anesthesia

- Specialized rodent guillotines are available and must be kept clean and in good condition with sharp blades. See “Operation & Maintenance of Guillotines” below.
- The use of a species-appropriate restrainer (e.g., Deception) will reduce stress from handling, minimize the chance of injury to personnel, and improve the positioning of the animal in the guillotine.

#### *Sample Protocol Narrative –*

The animal will be restrained with a DecapiCone and its head securely placed through the guillotine opening. The guillotine level is then rapidly depressed. The carcasses will be placed in DCM freezers for proper disposal.

### **Perfusion under Anesthesia**

- The anesthetic and perfusion agents and associated doses (the volume of perfusion agent to be administered can be listed in the dose field) must be listed in the drug table of the IACUC protocol.

- Please note that since perfusion is a terminal surgery (Category D procedure), barbiturate combinations (i.e. Euthasol) cannot be used to induce anesthesia prior to the fixation procedure.
- Use of agents such as formaldehyde presents a risk to the health and safety of the user. As such, their use requires review and approval of an Institutional Biosafety Committee (IBC) Application Form by the IBC.

#### *Sample Protocol Narrative –*

Animals will be under a deep surgical plane of anesthesia, confirmed by loss of reflexes (e.g., toe pinch to confirm the absence of a withdrawal reflex), before any incisions are made and until the heart stops. The fur is clipped over the ventral chest and the area disinfected with 70% ethanol. The thoracic cavity will be opened to expose the heart. After inserting the perfusion needle through the left ventricle into the ascending aorta and nicking the right ventricle, sufficient physiological saline to flush out all blood is perfused, followed by a similar volume of fixative. After perfusion and sample collection is complete, the carcass must be properly disposed of by placement within a plastic bag and returned to the DCM freezer designated for collection of regulated medical waste for proper disposal.

### **Euthanasia of Neonates**

#### Mouse, Rat & Hamster Neonates Older than Ten Days

Follow any of the above methods.

#### Mouse, Rat and Hamster Neonates $\leq$ Ten Days Old

- Mice, rats, and hamsters of this age are developmentally resistant to hypoxia, resulting in the need for prolonged exposure (up to 50 minutes) for inhalant agents to be effective.
- Acceptable methods of euthanasia for these animals include the following which are detailed above in this document:
  - Carbon dioxide
    - *Sample Protocol Narrative –*  
Pups  $<10$  days of age will be placed in a clear chamber for continual observation and have CO<sub>2</sub> delivered at a 30-70% fill rate for at least 50 minutes. Death will be ensured by a physical method identified in the protocol, and carcasses will be placed in DCM freezers for proper disposal. If used for subsequent animals, the chamber will be cleaned after use and placed on its side to purge the chamber of CO<sub>2</sub>.
  - Injectable agents
    - *Sample Protocol Narrative –*  
See [samples](#) above for adult animals.
  - Cervical Dislocation without anesthesia
    - *Sample Protocol Narrative –*  
See [sample](#) above for adult animals.
  - Decapitation without anesthesia
    - See “Operation & Maintenance of Guillotines” below if using a guillotine.
    - For neonates  $\leq 7$  days, clean sharp scissors can be used. Neonates older than 7 days of age must be decapitated using a guillotine.
    - *Sample Protocol Narrative –*  
The animal will be restrained either manually or within a DecapiCone. Decapitation will be performed with either clean sharp scissors or by rapid depression of the guillotine level once its head securely placed through the guillotine opening. The carcasses will be placed in DCM freezers for proper disposal.

### Mouse, Rat and Hamster Neonates $\leq$ Ten Days Old

- The methods described above for these species at  $\leq$  ten days of age are appropriate.
- Hypothermia may also be used to induce anesthesia in pups  $\leq$  seven days of age followed by a physical method to ensure death.

#### *Sample Protocol Narrative –*

Animals  $\leq$  seven days of age will be placed in a clear open container (to prevent escape but allow air movement) within a refrigerator or on ice to induce hypothermia. Animals will never come into direct contact with the cooling agent. Once anesthetized, a secondary physical method listed in the protocol will be performed to ensure death, and the carcasses will be placed in DCM freezers for proper disposal.

### Guinea Pig Neonates

Guinea pigs have precocial pups. All guinea pigs, regardless of age must therefore be euthanized by one of the above methods.

### **Euthanasia of Fetuses**

- Rodent fetuses are unconscious in utero and do not respond to hypoxia.
- If the dam is euthanized and the abdomen is not penetrated, the fetuses will subsequently die.
- If the dam is euthanized and the abdomen is penetrated, the fetuses must be individually euthanized based upon the following criteria:

### Mouse, Rat and Hamster Fetuses at Greater than 15 Days of Gestation; Guinea Pig Fetuses at Greater than 35 Days of Gestation

Acceptable methods of euthanasia includes:

- skillful injection of chemical anesthetic overdose,
- decapitation with surgical scissors, or
- cervical dislocation

#### *Sample Protocol Narrative –*

Rodent fetuses  $\geq$  fifteen days of gestation will be manually restrained. Decapitation will be performed with clean sharp scissors. The carcasses will be placed in DCM freezers for proper disposal.

When rapid freezing (immersion in liquid nitrogen) or chemical fixation of the whole fetus is required, fetuses should be anesthetized prior to immersion in liquid nitrogen or perfusion with fixative solutions. Anesthesia may be induced by-

- Hypothermia of the fetus,
- Injection of the fetus with a chemical anesthetic, or
- Deep anesthesia of the dam with a chemical agent that crosses the placenta, e.g., pentobarbital.

#### *Sample Protocol Narrative –*

Fetuses will be anesthetized with hypothermia by submerging the fetus (with the amniotic sac intact) in cold (4-8°C/35-39°F) physiological saline until the fetus becomes completely immobile and then rapidly frozen by immersion in liquid nitrogen while anesthetized. If at any point the fetus is allowed to breathe, it must be decapitated with sharp scissors or a scalpel blade.

### Mouse, Rat and Hamster Fetuses $\leq$ 15 Days of Gestation; Guinea Pig Fetuses $\leq$ 35 Days of Gestation

- No further method of ensuring or confirming death of such fetuses is required.

### **Confirmation of Death**

Death of the animal must be confirmed via a secondary method prior to disposal of the rodent carcass. CO<sub>2</sub> has anesthetic-like properties and consequently the effects are reversible.

Failure of the animal to move or show a reflex response is not sufficient to confirm death. Inadequate exposure time to CO<sub>2</sub> or anesthetic agents may result in animals that appear dead but wake up from the deep anesthesia later on. Confirming death by verifying the loss of vital signs (heartbeat, respiratory movements) is unreliable due to the rodents' small size. A secondary physical method of euthanasia to ensure death is therefore required for all animals after use of CO<sub>2</sub>, inhalant anesthetics, or injectable euthanasia agents, prior to carcass disposal. The protocol should indicate which procedures will be followed to ensure death – multiple selections can be made to provide flexibility for personnel. One of the secondary methods below must be used to ensure that the rodent will not recover:

- Decapitation
- Cardiac perfusion
- Remove of vital organs (e.g. heart, lungs, brain)
- Opening of the chest cavity to induce bilateral pneumothorax
- Cutting the major blood vessels to induce exsanguination (e.g. aorta, vena cava)
- Cervical dislocation in adult rodents (not permitted for rats  $\geq$  200 g, per *AVMA Guidelines for the Euthanasia of Animals: 2020 Ed.*)

### **Operation & Maintenance of Guillotines**

There are many factors that could impact the frequency in which a guillotine requires maintenance (including sharpening of the blade), such as blade quality, the species and size of animals being decapitated, and the volume and frequency of use. Thus, the maintenance interval may vary widely and should be based on overall performance, including ease of use, e.g., force required, smoothness of operation. For laboratories that use the same species and size of animals, guillotine performance should be used to establish a temporal maintenance interval.

### Guidelines and Procedures

- For unanesthetized rodents, the use of plastic cones (e.g. DecapiCones) or another size-appropriate holding device is required to restrain animals to reduce distress from handling, minimize the chance of injury to personnel, and improve positioning of the animal in the guillotine.
- When in use, a properly maintained guillotine of appropriate size will decapitate the animal cleanly with minimal force. Before each use of a guillotine, it should be checked for rust, lack of visible nicks or other damage to the cutting edges and cleanliness. The operator should ensure that the action is smooth with no perceptible binding or resistance. Since there are varying sizes of guillotines available, the equipment chosen should be appropriate for the size of the animal.
- The IACUC also recommends testing the guillotine for sharpness on suitable materials before its use on live animals. A sharp blade will cut the test material cleanly with minimal force without dragging it between the blades or showing signs of sticking.
- Devices should be cleaned after each use, maintained in good working order, and serviced on a regular basis. Depending on species involved and volume of use, investigators may need to have devices sharpened more frequently.

- Devices may require lubrication with silicone or silicone-teflon aerosol spray after cleaning.
- If the equipment is found to be in less than good working condition, alternative properly maintained guillotines or approved euthanasia procedures must be used.

#### Guillotine Maintenance Recordkeeping

A maintenance record should be kept for all guillotine(s) and include information related maintenance, inspections and sharpening for each device. The records should be in close proximity to the device and made available for review as part of the IACUC's semi-annual site visits, during post-approval monitoring, inspections performed by internal and external stakeholders and self-assessment activities.

#### Non-Compliance

Failure to confirm death of a euthanized rodent is a significant noncompliance that may be reported to the appropriate regulatory and accrediting agencies, and will require corrective actions (e.g., re-training) and possible additional steps (e.g., prohibition of certain personnel from performing CO<sub>2</sub> euthanasia) depending on the specific circumstances of the noncompliance.

#### **REFERENCES**

- [American Veterinary Medical Association Guidelines for Euthanasia of Animals: 2020 Edition](#) (AVMA)
- [American College of Laboratory Animal Medicine: Report of the ACLAM Task Force on Rodent Euthanasia](#) (ACLAM)
- [Animal Research Advisory Committee Guidelines for the Euthanasia of Rodent Fetuses and Neonates](#) (2019) (ARAC)
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