EUTHANASIA OF RODENTS

PERFORMANCE STANDARD: When required for scientific data collection, clinical, or operational requirements, rodents will be humanely killed, as detailed in the approved protocol or according to this policy.

BACKGROUND: All species of animals used at Duke are to be euthanized in a humane manner which minimizes distress and maximizes smooth and event-free euthanasia. This policy covers only rodent species; there are separate polices for ‘non-rodent mammals,’ ‘amphibians & reptiles’, and ‘birds & aquatics’. The Animal Care Unit of the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture, the NIH Office of Laboratory Animal Welfare (OLAW), and the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) have issued guidance regarding their expectations on methods of euthanasia. Publication of the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition has further refined the expectations for euthanasia. A specific modification in the 2013 AVMA document involved CO₂ – a primary method for rodent euthanasia on campus.

The goal of the revised 2013 AVMA procedures for euthanasia using CO₂ is to minimize any associated pain and distress. The primary issue is that gaseous CO₂ is converted to carbonic acid when it contacts the moist mucous membranes of the nose, eye, trachea, and lungs, resulting in pain. At > 40% CO₂, humans report nasal pain and develop cardiovascular changes indicative of pain. Rats show similar changes in heart rate and activation of nasal pain receptors when exposed to CO₂ levels > 40%, and while we may not know that rodents sense pain exactly the same way as humans, based on these consistent observations, we must assume the same sensations are occurring in rodents.

Historically, institutions have used a ‘pre-filled chamber’ methodology, but current research indicates that animals placed in prefilled chambers likely experience a period of distress prior to becoming unconscious. The chart at the right shows the chronology of the ‘pre-filled chamber’ method, with the period of potential distress signified by the red rectangle.

The new methodology required by the AVMA Guidelines uses a chamber initially filled with atmospheric air, which is gradually filled with CO₂ over 5-6 minutes. The primary goal of the new methodology is to create unconsciousness prior to reaching CO₂ concentrations that might induce distress or pain (the period of potential distress is signified by the red rectangle).
In addition to these changes in methodology, all investigators should understand that unintended recovery of animals after euthanasia constitutes serious non-compliance with PHS Policy and serious deviation from the provisions of the Guide for the Care and Use of Laboratory Animals. Duke is obligated to report such non-compliance to the NIH.

ROLES:
1. Researchers and animal care staff will abide by this policy as described below.
2. Researchers and animal care staff are expected to follow the methods of euthanasia as outlined and approved in their protocols (or approved animal care SOPs), unless alternate euthanasia methods are IACUC-approved for their protocol or SOP prior to their performance.
3. This policy outlines the procedures and methods generally considered to be acceptable for use by the Duke research community. However, other methods may be approved by the Duke IACUC for specific research situations and/or protocols given appropriate scientific justification.
4. All individuals who may or are expected to perform euthanasia should be familiar with the details in their approved protocol and with the clarifications outlined in this policy. Core training is available by web module. More detailed training in the proper methods of euthanasia is available by contacting DLAR veterinary staff at 919.681.6792.
5. All individuals listed on a protocol where CO2 euthanasia is approved must complete core web module training as a means of ensuring that all protocol participants understand the expected behavior and anticipated outcomes when using CO2.

DEFINITIONS:
1. **Humane Euthanasia** consists of a primary method and a secondary physical method. Failure to complete both methods results in failed humane euthanasia.
2. **Secondary Methods** consist of a physical disruption of tissues or organs. Acceptable secondary methods include bilateral thoracotomy, exsanguination, or decapitation. Cervical dislocation is not an acceptable secondary method.
3. **Euthanasia Chambers** that are used for exposure to euthanasia-inducing gases may be either the animal’s home cage (preferred) or a box specifically designed for the purpose of euthanasia. The Euthanasia Chamber must be a leak-proof, rodent cage and provide adequate exposure to allow unobstructed viewing of the animal during the euthanasia activity.

PROTECTIVE POSTURES REQUIRED: Personnel Protective Equipment (PPE) routinely required for normal animal care or use is required for euthanasia activities.

POLICY:
1. The PI and all protocol participants are responsible for the welfare of the animals assigned to their protocol(s), including the manner in which the animals are euthanized.
2. Any method listed in this policy may be used for euthanasia in Duke University animal activities, providing the method has been IACUC-approved on the protocol or SOP that covers those activities. In an emergency, any method listed in this policy may be used to end animal suffering and prevent further animal distress. Such ‘off-protocol’ activities must be reported to the IACUC as soon as practical, but within 48 hours (2 business days) of performing the euthanasia.

3. The general euthanasia principles that govern all euthanasia activities are:
   a. Individuals must be qualified in the technique they are performing.
   b. Euthanasia must follow AVMA guidelines, unless a specific exception has been approved by the IACUC.
   c. Euthanasia must follow methods approved in the Duke protocol.
   d. Death must be assured by a secondary physical method.

   NOTE: These ‘musts’ are requirements, not suggestions or simply good ideas.

Documentation of personnel qualifications and training is required, generally using the Personnel Qualifications Form (PQF) of the protocol and/or other documentation or training records.

4. Placing live animals in with deceased animals is prohibited unless scientifically justified in an IACUC-approved protocol.

5. The use of a secondary method of euthanasia is always required. Commonly used secondary methods include:
   a. Bilateral Thoracotomy;
   b. Decapitation;
   c. Collection of tissues sufficient to assure the animal will not recover; or
   d. Exsanguination;

   NOTE: Cervical dislocation IS NOT an approved secondary method for euthanasia.

   NOTE: If no method is specified in the protocol, then bilateral thoracotomy is the default and expected procedure for a secondary method of euthanasia.

6. Disposal of any carcass or parts (all animals) that are not immediately used requires:
   a. Placing the carcass (or parts) in a bag or container; and
   b. Labeling the bag/container with the date of euthanasia, the PI’s name, and the person who performed the euthanasia.

   NOTE: If animals are to be disposed of by DLAR, bags compatible with the tissue digester must be used.
7. **Acceptable Methods of Primary Euthanasia:** The following methods are considered 'acceptable' for euthanasia under the Duke University animal care & use program.

   a. **Carbon Dioxide:** Carbon dioxide (CO\textsubscript{2}) is acceptable for euthanasia in rodents. CO\textsubscript{2} should be considered ONLY when rodents are 15 days of age or greater. **DO NOT USE** CO\textsubscript{2} for euthanasia of any rodent younger than 15 days of age!

      **Note:** Neonatal rodents are resistant to hypoxia and may take up to 50 minutes or more to die from CO\textsubscript{2} exposure. Neonatal rodents may fall asleep during CO\textsubscript{2} exposure, suggesting they are dead, when in reality they are only sleeping.

      **Note:** **DO NOT USE** CO\textsubscript{2} for any rodent younger than 15 days of age!

   i. **Carbon Dioxide Source:** The AVMA Guidelines for euthanasia using CO\textsubscript{2} requires precise delivery, which can only be achieved using containerized or piped sources of CO\textsubscript{2}.

      1. Compressed CO\textsubscript{2} gas from house/building supply and/or from CO\textsubscript{2} cylinders are the **only acceptable sources** of carbon dioxide.

      2. Carbon dioxide generated by other methods such as dry ice, fire extinguishers, or chemical means (e.g., antacids) is **not acceptable**.

   ii. **Animal holding systems for carbon dioxide**:

      1. **Preferred:** Euthanasia in the home cage is the most desirable method since it does not require the potentially stressful transfer of mice to another cage. Euthanasia in the home cage is accomplished by placing a euthanasia lid over the home cage. Contact IACUC@Duke.edu for information regarding appropriate euthanasia lids for your caging.

      2. **Acceptable:** Euthanasia using a CO\textsubscript{2} chamber is acceptable. Since the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition require that animals are placed in an atmosphere containing 100% room air and transitioned to 100% CO\textsubscript{2} over several minutes, there are certain requirements when using a designated CO\textsubscript{2} chamber:

         a. The chamber must be completely flushed with room air for 1-2 minutes between each euthanasia event to dislodge captured CO\textsubscript{2} from the container. Smaller chambers should be turned on their side for 1-2 minutes between each euthanasia event. Turning the chamber on its side will allow
an opportunity for the pooled CO₂ to drain from the chamber.

b. The chamber must also be sanitized after each use. Leaving fecal material, bedding, or other refuse in the euthanasia chamber is not good sanitation.

NOTE: Failing to complete either of these actions is a non-compliance with the ‘Euthanasia Policy,’ and may have an impact approval for continued use of carbon dioxide as a means of euthanasia.

iii. Methodology for Humane CO₂ Euthanasia:

1. **A transparent chamber must be used.** Place the animals in a transparent chamber. If you cannot clearly observe the animals in the chamber, then the chamber is not acceptable. The purpose for observing the animal(s) progressing to euthanasia is to assure the transition is smooth and uneventful. Restless or excited animals may be experiencing dysfunctional CO2 delivery and the process should be modified to accomplish humane CO2 euthanasia.

2. **The chamber must not be overcrowded.** Every animal in the chamber must be able to place all four of its feet on the floor of the chamber at the same time, with enough additional space so that when it does become unconscious, it will lie down on the floor of the cage and not on top of another animal. Since animals in their home cage are limited by the number of animals which can be in the same cage (Duke animal density policy), this requirement is easily accomplished by using the home cage for euthanasia.

3. **Species must not be mixed** when performing euthanasia.
   a. If you must euthanize several species at the same time, you must use separate chambers. Rodent species (e.g., rats and mice) may be predator or prey depending on the context. Predation stress is not humane and counter to the principles of humane euthanasia.
   b. Euthansia in the home cage is the preferred method. Animals mixed immediately prior to euthanasia may be distressed as they try to re-establish social order. Social distress is counter to the principles of humane euthanasia. The preferred approach is use of the home cage for euthanasia; this minimizes distress associated with the event. If cages of the same species are mixed, they should
be euthanized as soon as possible after mixing.

4. **The chamber must be filled gradually.** Place the animals in the home cage (or chamber) before initiating the flow of CO₂.

The overarching guideline for filling the chamber is provision of a CO₂ flow between 20% and 30% of the chamber volume. **DO NOT EXCEED A FLOW RATE OF 30%**.

   a. The formula for the flow rate calculation is:

   - Volume (in L) = \( \frac{\text{(height in cm) x (width in cm) x (length in cm)}}{1000} \)
   - Maximum acceptable flow rate = (Cage Volume in Liters) X 0.3

   b. Using the common Duke cage sizes, a 30% flow rate calculates to:

   i. Standard Duke mouse cage = 2 liters/min
   ii. Standard Duke rat cage = 7 liters/min
   iii. Most DLAR euthanasia chambers = 3.5 liters/min.

   **NOTE:** This works for chambers which are 20cm x 20cm x 30cm (8” X 8” X 12”). Other sized chambers will require a new calculation.

   c. The IACUC must document proper euthanasia. The following will be regarded as a non-compliance:

   i. Failure to have a flow meter in the CO₂ set-up, or
   ii. Failure to post the calculated maximum flow rate; or
   iii. Failure to stay below the calculated maximum flow rate.

d. While the goal is to provide exactly the calculated flow rate, if you cannot be on target, be below. Example: Calculated rate is 3.85L/min. the flow meter only shows 0.5 L increments; therefore provide 3.5 L/Min.

   **NOTE:** Exceeding the calculated flow rate is non-compliance with both the AVMA euthanasia guidelines and
with our institutional agreements with NIH.

5. **There must be sufficient time in a CO\textsubscript{2} atmosphere to ensure death.** The Duke position is that the time required in a CO\textsubscript{2} atmosphere for euthanasia is from the point where cessation of breathing has been observed, plus an additional 2 minutes!

   a. Using these guidelines:

      i. Breathing will generally stop between 4-5 minutes after beginning the procedure.

      ii. The heart will stop beating at between 5-6 minutes after beginning the procedure.

      iii. For safety, wait an additional 2 minutes after cessation of breathing (a total of 6-7 minutes) to assure the animal is dead.

   b. If fresh tissue is required for laboratory tests (e.g., fresh pancreas for RNA analysis); animals may be removed from the CO\textsubscript{2} chamber following sustained cessation of breathing (4-5 min), provided a physical secondary method is performed immediately (e.g., organ removal, major vessel being cut).

6. A secondary physical method of euthanasia that disrupts the physiological and anatomical integrity must be performed to insure that euthanasia is irreversible. It is critical that animals not recover unintentionally when euthanasia is the intended event. Recovery after intended euthanasia is a non-compliance which must be reported to oversight and funding agencies, if applicable. Acceptable secondary methods include:

   a. **Bilateral Thoracotomy:** This procedure opens both sides of the rodent’s chest, preventing the lungs from re-expanding. The procedure may be performed by entering the lungs from the posterior direction and through the diaphragm, cutting the diaphragm and the ribs in an anterior fashion; or crossing the chest at about the 5th rib and cutting across the chest to open both lung fields. Since a percentage of animals may have a complete mediastinum, the procedure chosen must invade both sides of the thorax, preventing any lung lobe from inflating!

   b. **Exsanguination:** This procedure results in the release of blood from the cardiovascular system. Exsanguination is
most commonly performed when an organ (e.g. liver, heart, kidney, etc.) is removed for bench-top procedures after euthanasia. Options include:

i. When removing an organ from the body, the artery and the vein connecting the organ to the cardiovascular system are cut, releasing the remaining blood from the circulation.

ii. Performing a deep cut across a main vessel in the body which allows complete blood loss to occur.

c. **Decapitation**: A procedure where the head is completely removed from the body. Initiate the procedure from the back of the neck, cutting the spinal column, then the softer tissues below to assure complete decapitation. When the head remains attached to the body—by soft tissue, bone, or skin—it is not decapitation

**NOTE:** Cervical dislocation consists of breaking of the animal's spinal cord, and is not approved as a secondary method of euthanasia.

7. Disposal of any carcass or parts (all animals) that are not immediately used requires:
   a. Placing the carcass (or parts) in a bag or container; and
   b. Labeling the bag/container with the date of euthanasia, the PI's name, and the person who performed the euthanasia.

**NOTE:** If animals are to be disposed of by DLAR, bags compatible with the tissue digester must be used.

b. **Barbiturates**: The advantages of using barbiturates for euthanasia in small animals far outweigh the disadvantages. Barbiturates include pure barbiturate (e.g. pentobarbital) and combination products (e.g. Euthansol®, etc.). Pentobarbital is a DEA Schedule II agent while mixtures such as Euthasol are DEA Schedule III. The pharmacologic properties and recommended use of combination products that combine sodium pentobarbital with lidocaine or phenytoin are interchangeable with those of pure barbituric acid derivatives. A secondary method of euthanasia is **required** to assure death when using barbiturates.
i. **Dosage for barbiturate euthanasia:**

1. When using pure barbiturate or a barbiturate combination mixture, the dose should be high enough to induce respiratory and cardiac arrest, not deep anesthesia.

2. Barbiturate derivatives: Use the dose recommended on the bottle (e.g. generally 10 ml/pound body weight or published dose) plus 10%. A secondary method is required.

ii. **Recommended routes of barbiturate administration:**

1. **Intraperitoneal injection** is the most common route used in rodents and is preferred in situations when an intravenous injection would be distressful or even dangerous.

2. **Intravenous injection** is also acceptable for animals with pre-existing venous access (e.g. at the end of a non-survival surgical procedure).

3. **Intracardiac injection** may be used if the animal is heavily sedated, unconscious, or anesthetized. DO NOT give an intracardiac injection in an alert or awake animal.

iii. **Non-pharmaceutical grade barbiturate:** According to the Public Health Service (NIH/OLAW) Policy, agents for sedation, analgesia, or anesthesia should be veterinary or human pharmaceutical-grade compounds, when available, unless the use of a non-pharmaceutical chemical or formulation is scientifically necessary, appropriately justified and approved by the IACUC. The use of a non-pharmaceutical-grade euthanasia agent must meet the same standards. If no equivalent veterinary or human drug is available for experimental use, then the highest-grade equivalent chemical reagent should be used and formulated aseptically with a non-toxic vehicle as appropriate for the route of administration. The NIH has determined that exorbitant cost increases for pentobarbital have placed it logistically into the unavailable category.

The Duke IACUC has granted institution-wide approval for the use of non-pharmaceutical grade pentobarbital (e.g. from a reagent or analytical-grade powder) when prepared by a knowledgeable individual with assurance of appropriate storage and handling. The stipulations and conditions for use of non-pharmaceutical grade pentobarbital for euthanasia include documentation in the controlled substance cabinet which includes the following information:
1. The name of the knowledgeable individual mixing the product;
2. The date the product was mixed;
3. The anticipated date of expiration of the product (generally 30 days post mixing);
4. The concentration of the mixed product;
5. The method of filtration of the mixed product; and
6. The beginning volume and a record of continued use.
c. **Halogenated gaseous agents** (e.g. isoflurane, sevoflurane, etc.) with or **without nitrous oxide** are acceptable for euthanasia of rodents. Agents may be provided by a closed pre-charged container (e.g. bell jar pre-charged with anesthetic) or by a continuous flow vaporizer. Animals must never come in direct contact with the agent. An optimal flow rate should displace at least 20% (range is 20%-30%) of the chamber volume per minute. Maximal dosage should be obtained by 5 minutes. Loss of consciousness should be induced rapidly by exposing animals to the maximum agent concentration possible. If using a vaporizer, gas flow may be turned off after cessation of breathing, but animals should remain in the chamber for at least 2 additional minutes. A secondary method is required.

d. **Cervical Dislocation under anesthesia** is acceptable when post-euthanasia tissue procurement is necessary and the use of CO₂, barbiturate, or another inhalational method would result in tissue degradation or interference with research. The effectiveness of dislocation must be verified by separation of cervical tissues. When the spinal cord is severed, a 2-4 mm space will be palpable between the occipital condyles and the first cervical vertebra (or between thoracic vertebrae). Cervical dislocation under anesthesia requires a physical secondary method.

e. **Decapitation with anesthesia** is acceptable when post-euthanasia tissue procurement is necessary and the use of CO₂, barbiturate, or another inhalational method would result in tissue degradation or interference with research. The equipment used to perform decapitation must be maintained in good working order and serviced on a regular basis to ensure sharpness of blades. The use of plastic cones to restrain animals is recommended to reduce distress from handling, minimize the chance of injury to personnel, and improve positioning of the animal in the guillotine.

f. **Exsanguination under deep anesthesia** is also acceptable for euthanasia of rodents. Because anxiety is associated with extreme hypovolemia, exsanguination is not acceptable for euthanasia of non-anesthetized animals.

1. **Acceptable Methods of Primary Euthanasia for fetal or neonatal rodents** (rodents up to 14 days of age):

   g. **Fetuses in utero**: Euthanasia of the dam will cause euthanasia of fetuses that remain in utero. No additional actions are necessary when fetuses remain in the uterus.

h. **Fetuses up to 14 days in gestation when removed from the uterus**: Neural development at this stage is minimal and pain perception is considered unlikely. Euthanasia of the mother or removal of the fetus from the uterus ensures rapid death of the fetus due to non-viability of fetuses at this stage of development.
i. **Fetuses 15 days in gestation to birth when removed from the uterus:** The literature on the development of pain pathways suggests the possibility of pain perception at this time. Fetuses at this age are less sensitive to inhalant anesthetics than are adults. Thus, decapitation with surgical scissors is the preferred method of euthanasia. When chemical fixation or rapid freezing (e.g. immersion in liquid nitrogen) 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 of the fetus, by injection of the fetus with a chemical anesthetic, or by deep anesthesia of the mother with a chemical agent that crosses the placenta, e.g., pentobarbital. The university veterinarian should be consulted for considerations of fetal sensitivity to specific anesthetic agents. When fetuses are not required for study, the method chosen for euthanasia of a pregnant mother must ensure rapid death of the fetus.

j. **Neonates from birth to 14 days of age:** The recommended method for euthanasia is decapitation (complete separation of the head from the body). For alternatives, contact an OAWA/DLAR veterinarian. IACUC approval is required for all methods of euthanasia.

k. **Animals 15 days of age and older:** Follow guidelines for adults described earlier in this policy.

1. **Alternate Methods of Euthanasia may be IACUC-approved for use under specific conditions.** Euthanasia methods are classified in The 2013 AVMA Guidelines classifies euthanasia methods as acceptable, acceptable with conditions, and unacceptable.

l. Acceptable methods are those that reliably and easily cause humane euthanasia.

m. Unacceptable techniques are those methods deemed inhumane under any conditions. These methods of euthanasia are prohibited by the Duke IACUC.

n. Methods acceptable with conditions are those techniques that may require certain conditions to be met to consistently produce humane death, may have greater potential for operator error or safety hazard, are not well documented in the scientific literature, or may require a secondary method to ensure death. Methods acceptable with conditions are equivalent to acceptable methods when all criteria for application of that method can be met. The Duke IACUC has determined that most of these methods of euthanasia continue to require scientific justification as well as assurance that the required special conditions are being engaged. The methods included in this category for which scientific justification and specified conditions are required include:
i. **Cervical Dislocation without anesthesia:** Manual cervical dislocation can be a humane technique for euthanasia of mice, and rats weighing < 200 g, when performed by individuals with a demonstrated high degree of technical proficiency. This technique should be used only when scientifically justified by the user and approved by the Institutional Animal Care and Use Committee. A secondary method of euthanasia is required to assure death.

ii. **Decapitation without anesthesia:** This technique may be used only when required by the experimental design and approved by the Institutional Animal Care and Use Committee. The equipment used to perform decapitation must be maintained in good working order and serviced on a regular basis to ensure sharpness of blades. The use of plastic cones to restrain animals appears to reduce distress from handling, minimizes the chance of injury to personnel, and improves positioning of the animal in the guillotine.

1. **Activities not specifically addressed in this policy:** Application of review and approval will remain consistent across species, research activities, and applications of procedures. The Duke IACUC may authorize alternate methods, when scientifically justified and on a case-by-case basis. If a specific issue is not addressed in this or other IACUC-approved policies, please contact the IACUC and/or OAWA for clarification.
References

- FAQ #1; OLAW Website: Acceptability of CO2 for rodent euthanasia (NOTICE: NOT- OD-02-062) Release Date: July 17, 2002
- American College of Laboratory Animal Medicine Report of the ACLAM Task Force on Rodent Euthanasia (ACLAM)
- American Veterinary Medical Association Guidelines for Euthanasia (2007) (AVMA)
- Guide for the Care and Use of Laboratory Animals (Guide) – the 8th Edition
- National Institutes of Health Guidelines for Euthanasia of Rodents Using Carbon Dioxide (NIH)
- 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)