PURPOSE

To the greatest extent possible, animals being euthanized should not experience pain, fear, or other significant stress prior to their death. Carbon dioxide (CO2) is a frequently used euthanasia agent for small laboratory animals due to its rapid onset of action, safety and ready availability. The IACUC has adopted the following guidelines to assist the research community by clarifying the specific procedures relating to the use of CO2 and to promote best practices to ensure that pain and distress is minimized in euthanized laboratory rodents.

POLICY

Rodents must be euthanized by trained personnel using appropriate technique, equipment and agents. Death must be induced as painlessly and quickly as possible. Upon completion of the procedure, death must be confirmed by an appropriate method, such as ascertaining cardiac and respiratory arrest or noting an animal’s fixed and dilated pupils. A secondary physical form of euthanasia must be performed following CO2 euthanasia. Whenever practical, euthanasia should not be performed in the animal housing room. 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.

For the purposes of this policy, mice and rats are defined as animals greater than 10 days of age. Neonates are defined as mice and rats from 0-10 days of age. CO2 inhalation is the most common method of euthanasia for adult mice and rats. A few important aspects of this procedure are:

1. All personnel who will be performing CO2 euthanasia must be properly trained. That training will include face-to-face training by one of the LACU veterinary staff or their designee. That training will be affirmed by each trainee signing a statement they have been properly trained, understand the guidelines, and agree to follow the specified procedure.

2. When using an automated euthanasia chamber or device, users are expected to follow the posted standard operating procedure. An SOP that is specific for that automated euthanasia device will be posted immediately adjacent.
3. **Species must not be mixed.** The euthanasia chamber must allow ready visibility of the animals.

4. **Do not overcrowd the chamber or box used for euthanasia;** all animals in the chamber must be able to make normal postural adjustments.

   **Requirement:**
   a. When animals are euthanized in a standard mouse box (75 or 78 sq. in) using a Euthanex lid or in a Euthanex chamber, the maximum number of animals per standard mouse box allowed is 10; for a large mouse (157 sq. in.) box the maximum number of animals allowed is 20.

5. **Compressed CO2 gas in cylinders is the only recommended source of carbon dioxide** as it allows the inflow of gas to the induction chamber to be controlled. Dry ice as a source of CO2 or pre-filled chambers are not acceptable. “Either USP Grade A (medical) or Grade B (industrial) carbon dioxide may be considered acceptable as they each provide a minimum purity for carbon dioxide of 99.0%.”

6. **Without pre-charging the chamber, place the animal(s) in the chamber and introduce 100% carbon dioxide. A fill rate of 10 - 30% of the chamber volume per minute with carbon dioxide, added to the existing air in the chamber should be appropriate to achieve a balanced gas mixture to fulfill the objective of rapid unconsciousness with minimal distress to the animals.** (Example for a 10-liter volume chamber, use a flow rate of 3 liter(s) per minute.) Sudden exposure of conscious animals to carbon dioxide concentrations of 70% or greater has been shown to be distressful.

   **Requirement:**
   a. All euthanasia chambers provided in LACU are automated to provide a CO2 fill rate consistent with the AVMA recommendations.

7. **Expected time to unconsciousness is usually within 2 to 3 minutes.** Observe each rodent for lack of respiration and faded eye color. Maintain CO2 flow for a minimum of 1 minute after respiration ceases. If both signs are observed, then remove the rodents from the box; otherwise continue exposing them to CO2. If unconsciousness has not yet occurred within 2 to 3 minutes, the chamber fill rate should be checked. The system should also be examined for a defective flow meter, absence of CO2 supply, and/or leaks. Appropriate CO2 concentrations and exposure times will prevent unintended recovery.

   **Requirement:**
   a. All euthanasia chambers provided in LACU are automated to provide a CO2 fill rate consistent with the AVMA recommendations, maintain flow of CO2 for an appropriate period of time to ensure death, and provide a timer mechanism to signal when the cycle with appropriate wait time has been completed.
   b. When the CO2 euthanasia system cycle has been completed, the operator must verify death of all animals in the chamber and use a secondary method as appropriate and
approved in their protocol, properly dispose of the carcasses, take the soiled box to the properly location, and clean up behind themselves.

c. CO2 euthanasia stations outside of LACU facilities must have a detailed SOP for operation that is approved by the IACUC.

8. **Confirmation of death is required.** Since the anesthetic effect of CO2 is reversible, animals that are prematurely removed from the chamber prior to death can recover (OLAW). Furthermore, death should be confirmed by personnel who have been specifically trained to recognize cessation of vital signs in rodents (Guide). Therefore, all animals being euthanized with CO2 overdose must also receive a confirmatory method of euthanasia to ensure death. These confirmatory methods, to be performed after CO2 overdose, include exsanguination, decapitation, cervical dislocation or bilateral thoracotomy.

   **Requirement:** 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.

   “Unintended recovery of animals after apparent death from CO2... constitute[s] serious noncompliance with the PHS Policy and... the IACUC, through the Institutional Official, must promptly provide OLAW with a full explanation of the circumstances and actions taken.” (PHS)

9. **An accepted and common practice is to group animals for euthanasia.** The process of grouping animals immediately prior to euthanasia must provide each individual animal with the ability to make normal postural adjustments. For animals grouped into a standard mouse box for euthanasia recommendations are listed below. Best practice for animal welfare is that animals should be euthanized in their home box whenever possible. When euthanizing successive groups of animals using the same box/container, the euthanizing container should be cleaned between uses to remove the potential distress secondary to remaining pheromones, etc. Alternatively, a newunused container should be used with each group.

   **Requirement:**
   a. The maximum number of mice occupying a standard mouse box (75 sq. in.) for the purpose of CO2 euthanasia is 10.
   b. The maximum number of mice occupying a large mouse (157 sq. in.) box for the purpose of CO2 euthanasia is 20.
   c. For rats, the IACUC approved rat housing density policy must be followed.

10. **Neonatal animals (up to 10 days of age) are resistant to the effects of CO2 and require prolonged exposure to CO2.** Alternative procedures including decapitation and anesthetic overdose are recommended for rat and mouse neonates up to 10 days of age.

    **Requirements for neonates:**
    a. Isoflurane overdose using a bell jar. Procedure must be conducted in appropriate ducted biological safety cabinet, fume hood, or with other approved waste anesthetic
scavenging device and followed by a secondary form of euthanasia, such as decapitation.

b. Decapitation using sharp scissors.

References

Useful Reviews
- Klaunberg BA, O’Malley J, Clark T, Davis JA. Euthanasia of Mouse Fetuses and Neonates. Contemporary Top Lab Anim Sc 2004, 43:(5) 29-34.
- Wong D, Makowska IJ, Weary DM. Rat aversion to isoflurane versus carbon dioxide. Biology letters, 2013, 9 (1).