Guidelines and Policy for Carbon Dioxide (CO₂)
Euthanasia of Rodents - Recommended Best Practices

Animals being euthanized should experience minimal pain, fear, or other significant stress prior to their death. Carbon dioxide (CO₂) is a frequent used euthanasia agent for small laboratory animals due to its rapid onset of action, safety and ready availability. Marquette University 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 use of CO₂
2. Promote best practices and ensure that pain and distress are minimized in euthanized laboratory animals

The PHS Policy that Marquette University has on file requires that euthanasia be conducted according to the American Veterinarian Medical Association (AVMA) Guidelines for Euthanasia (2013 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.

This guideline focuses on the following topics regarding the use of CO₂ euthanasia:

1. Action of CO₂ euthanasia
2. Species covered under CO₂ euthanasia
3. Administering CO₂
4. Confirmation of death

1. Action of CO₂ euthanasia

The following information is from the AVMA guidelines:

CO₂ inhalation causes respiratory acidosis and produces a reversible anesthetic state by rapidly decreasing intracellular pH. Inhaled concentration of 30% or higher cause deep anesthesia, and death should occur with prolonged exposure. CO₂ has the potential to cause distress via three different mechanisms:

1. Pain due to formation of carbonic acid or respiratory and ocular membranes.
2. Production of a feeling of “breathlessness”.
3. Direct stimulation of ion channels within the amygdala, believed to be associated with the fear response.

The discomfort associated with CO₂ is believed to occur starting at approximately at 15% inhaled concentration. Potential pain and distress caused by CO₂ inhalation can be mitigated in the animal loses consciousness before the chamber is at 15% concentration. Therefore, an appropriate gradual displacement of room air with carbon dioxide into the chamber will cause the animal to lose consciousness before the CO₂ is aversive.
A gradual fill rate of 10-30% chamber volume per minute displacement is expected for CO2 euthanasia at Marquette.

To calculate the flow rate of CO2 for a 20% displacement per minute:

a. Chamber Volume (in L) = \((\text{height in cm}) \times (\text{width in cm}) \times (\text{length in cm})\) \(\div 1000\)

b. Acceptable flow rate (in L/min) = (Chamber Volume in Liters) \(\times 0.20/\text{min}\)

**Standard rat cage at Marquette**

a. Volume (L) = \(20.32\, \text{cm} \times 48.26\, \text{cm} \times 26.67\, \text{cm} = 26.15\, \text{L}\) \(\div 1000\)

b. Flow rate \(\text{L/Min}\) = 26.15(L) \(\times 0.20/\text{min}\) = \(5\, \text{L/Min}\)

**Standard mouse cage at Marquette**

a. Volume (L) = \(19.05\, \text{cm} \times 12.71\, \text{cm} \times 29.21\, \text{cm} = 7.07\, \text{L}\) \(\div 1000\)

b. Flow rate \(\text{L/Min}\) = 7.07(L) \(\times 0.20/\text{min}\) = \(1.5\, \text{L/Min}\)

2. **Species**

Any rodent used for research may be euthanized by CO2 by following the guidance described below in administering CO2. Examples include mice of the genus Mus and rats of the genus Rattus.

3. **Administering CO2**

- All personnel administering CO2 to rodents must be properly trained. All Principal Investigators (PI) must assure that their research staff are properly 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 available from the Animal Resource Center (ARC).

- Compressed gas is the only acceptable source of CO2 for euthanizing rodents. Dry ice, fire extinguishers and other sources of CO2 are not acceptable and may not be used.

- The animals’ home cage or an empty rat/mouse cage are acceptable chambers to be used for euthanizing rodents and are best practice (AVMA). Gas must be delivered in a predictable and controllable fashion, at a low-flow rate of 10-30% volume displacement per minute.

- This guideline and any SOPs for CO2 euthanasia must be posted where CO2 euthanasia occurs.
• Euthanasia should occur in a procedure room or laboratory, away from other rodent housing.

• If using a chamber other than the animals’ home cage the chamber may not be pre-filled with CO\(_2\) prior to placement of animals into the chamber. CO\(_2\) is denser than room air, thus the chamber will need to be “purged” (dumped) between groups of cages.

• Because CO\(_2\) first anesthetizes animals and then, only after adequate exposure time, will result in death by CO\(_2\) narcosis, **rodents must be exposed to the gas until respiration has ceased, within the euthanasia chamber with CO\(_2\) continuing to flow.**

**Mouse and Rat Fetuses up to 14 days gestation**

Neural development during this stage is minimal and pain perception is considered unlikely. Euthanasia of the mother or removal of the fetus should ensure rapid death of the fetus due to loss of blood supply and non-viability of fetus at this stage of development.

**Mouse and Rat Fetuses over 15 days gestation**

The neural development during this time supports that pain may be perceived. Methods to euthanize include injection of anesthetics, decapitation with sharp surgical scissors or scalpels, or cervical dislocation.

**Euthanasia of Rodent Neonates**

**Mouse and Rat Neonates up to 10 days of age**

Acceptable methods include inhaled agents, injection of anesthetics, decapitation with sharp surgical scissors or scalpels, or cervical dislocation. Resistance to hypoxia results in a prolonged time to unconsciousness when CO\(_2\) is used. Prolonged exposure to inhalant anesthetics may be necessary. The use of CO\(_2\), inhalant anesthetic agents, or chemical anesthetics requires a physical method to ensure death (i.e. followed with decapitation with sharp surgical scissors or scalpels, or cervical dislocation).

4. **Confirming Death**

**AVMA** – Inhalation of CO\(_2\) produces a reversible anesthetic state, thus animals that are prematurely removed from the cage/chamber prior to death can recover to consciousness.

**Guide for the Care and Use of Laboratory Animals** – Death must be confirmed by personnel who have been specifically trained to recognize cessation of vital signs in rodents.

Therefore, all animals being euthanized with CO\(_2\) overdose must also receive a confirmatory method of euthanasia to ensure death
Marquette University IACUC confirmatory methods to be performed after CO₂ overdose include:

i. Exsanguination

ii. Decapitation

iii. Cervical dislocation (adult mice only)

iv. Bilateral thoracotomy

v. At least 50% additional time in the euthanasia cage/chamber filled with 30% CO₂ (adults only)

**Death of the animal must be ensured prior to disposal of the rodent carcass in the freezer.**

**Compliance Regulation**

NOTE: Failure to confirm death of a euthanized rodent is a significant non-compliance, reportable to the appropriate regulatory and accrediting agencies.

**References**

American Veterinary Medical Association Guidelines for Euthanasia (2013)

Guide for the Care and Use of Laboratory Animals

Penn Animal Welfare IACUC Guidelines for CO₂ Euthanasia