Standard Guidelines for Carbon Dioxide Euthanasia of Rodents — Recommended Best Practices

Animals being euthanized should experience minimal pain, fear, or other significant stress prior to their death. Carbon dioxide (CO$_2$) 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 CO$_2$
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 CO$_2$ euthanasia:

1. Action of CO$_2$ euthanasia
2. Species covered under CO$_2$ euthanasia
3. Administering CO$_2$
4. Confirmation of death

1. Action of CO$_2$ euthanasia
The following information is from the AVMA guidelines:

CO$_2$ 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. CO$_2$ 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 CO$_2$ is believed to occur starting at approximately at 15% inhaled concentration.

Potential pain and distress caused by CO$_2$ 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 CO$_2$ 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) = (height in cm) x (width in cm) x (length in cm) / 1000

b. Acceptable flow rate (in L/min) = (Chamber Volume in Liters) x 0.20/min

**Standard rat cage at Marquette**

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

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

**Standard mouse cage at Marquette**

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

b. Flow rate \( \left( \frac{\text{L}}{\text{Min}} \right) \) = \( 7.07 \text{ (L)} \times 0.20/\text{min} = 1.4 \text{ L/Min} \)

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 CO₂ prior to placement of animals into the chamber. CO₂ is denser than room air, thus the chamber will need to be “purged” (dumped) between groups of cages.

• Because CO₂ first anesthetizes animals and then, only after adequate exposure time, will result in death by CO₂ narcosis, rodents must be exposed to the gas until respiration has ceased, within the euthanasia chamber with CO₂ 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 CO₂ is used. Prolonged exposure to inhalant anesthetics may be necessary. The use of CO₂, 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 CO₂ 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 CO₂ 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.

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**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