Guidelines for the Use of Carbon Dioxide (CO_2) for Rodent Euthanasia
The University of Texas at Austin Institutional Animal Care and Use Committee

These guidelines have been written to assist faculty, staff, and students in performing vertebrate animal procedures in a humane manner and complying with pertinent regulatory requirements. Under some circumstances deviations from these procedures may be indicated but such variances must be approved in advance by the IACUC.

This document provides information to be used when planning and using carbon dioxide (CO2) for rodent euthanasia at the University of Texas at Austin. It is organized into seven parts:

Section A – Summary and Training

SUMMARY: Carbon dioxide (CO2) euthanasia must be performed by trained individuals using appropriate equipment. The use of CO2 as a euthanasia method and the names of the individuals performing this procedure must be listed in the approved IACUC protocol covering the study. CO2 gas must be delivered using a compressed gas cylinder, pressure regulator, and flowmeter. A secondary physical means to assure death must be utilized prior to disposal of the carcass when CO2 is used for euthanasia.

TRAINING: Principal Investigators (PI) must ensure that all individuals responsible for administering CO2 euthanasia are appropriately qualified and monitored, and that they adhere to IACUC-approved protocols and institutional policies. Training can be provided from within the lab group if the existing staff has adequate expertise. Additional training in these techniques is available from the Animal Resources Center (ARC). Personnel who will be performing these techniques (or their PIs) can arrange training by contacting the ARC Compliance and Training Manager (phone: 512-471- 3909)

Section B – CO_2 Characteristics

Carbon dioxide (CO2) is currently considered to be a safe and humane method of euthanasia that has long been the preferred technique for use with rodents. The gas is inexpensive, nonflammable, and nonexplosive. Use of an appropriate chamber allows groups of rodents to be rapidly euthanized simultaneously. It causes no accumulation of exogenous chemical residues in tissues nor does it produce observable histological changes (with the notable exception of pulmonary tissues). It can be administered using fairly simple equipment that can be located centrally in a facility or fixed to a mobile platform for portable use. Exposure to high concentrations of CO2 has an initial rapid depressant and anesthetic effect, which is followed by death through asphyxiation while the animal is unconscious.

Carbon dioxide must be purchased and utilized in compressed gas cylinders. CO2 generated from other sources, such as dry ice or fire extinguishers is unacceptable because gas flow cannot be regulated precisely in those circumstances.

Section C – Humane Considerations

Exposing animals to a CO_2 concentration of 70% or more can induce unconsciousness very rapidly. However, high concentrations of CO_2 can cause a marked bradycardia in rats and mice, presumably via nasal chemoreceptors. Humans perceive CO_2 exposure via the nasal mucosa at similar concentrations to be a noxious stimulus, and because rodents show distinct aversion to high concentrations of gas it is assumed that rodents find it distressful. For this reason, the 2020 edition of the American Veterinary Medical Association Guidelines for the Euthanasia of Animals concludes that CO_2 euthanasia is humane only when rodents are exposed to a gradually rising concentration that will cause narcosis (loss of consciousness due to the depression of cerebral activity) before the sensitive respiratory tissues are exposed to high concentrations. An optimal flow rate for CO_2 euthanasia systems should displace 30%-70% of the chamber (or cage) volume per minute.

The most common errors that have an impact on humane euthanasia when using CO_2 for euthanasia are:

1. Overcrowding animals in the chamber,
2. Using equipment or methods that cause the animals to be exposed to suboptimal concentrations, and
3. Not assuring that animals have been completely killed prior to disposal.

Since carbon dioxide is 50% heavier than air, chambers should be designed so that as they fill with gas they can vent from the top. This allows the air to exit at the top and be completely replaced by carbon dioxide. Incomplete filling of a chamber may permit tall or climbing animals to avoid exposure to an optimal concentration of gas, which can lead to prolonged distress to the animals.

Animals placed together in chambers should be of the same species. Chambers must not be overcrowded. In this regard, it is important to also consider that mixing unfamiliar or incompatible animals in the same container may be distressful. Chambers should be kept clean to minimize odors that might distress animals subsequently euthanized in the same chamber.

Section D - Equipment

Carbon dioxide and CO_2 gas mixtures must be supplied in a precisely regulated and purified form without contaminants or adulterants (e.g., from a commercially supplied cylinder or tank). As gas displacement rate is critical to the humane application of CO_2, an appropriate pressure-reducing regulator and flow meter must be used. Alternatively, equivalent equipment with demonstrated capability for generating the recommended displacement rates for the size container being utilized is appropriate. For example, commercially available combination pressure/flow regulators deliver a fixed flow rate that must be matched to a corresponding chamber size. Each CO_2 location must have a placard, posting, or other readily available source of instructions that list the chamber type(s) in use and the appropriate flow meter setting(s) for each one. More information regarding flow rates for different cages/chamber, vendor sources for flow meters, options for retro-fitting existing cylinders, etc. can be obtained from the Animal Resources Center (ARC).

The top or walls of the chamber must be transparent so that animals are visible and observed during euthanasia. The top of the chamber should be closed in a way that covers the cage or chamber and allows the pressure of incoming gas to drive out and replace the air in the chamber, but does not allow significant quantities of room air to leak back in when the gas is turned off. A sturdy lid held on the top of the chamber by gravity can act as a low- pressure one-way valve and is a simple solution that generally satisfies these requirements.

Examples of chambers:

1. Various commercially available or customized tops allow a glass aquarium or acrylic box to serve as a

euthanasia chamber. These tops seal the enclosure and include inlet/outlet ports that can be connected to the supply of CO_2. The simplest functional system is one that has a single hole through which a length of tubing connected to the regulator passes. When an inlet is present but no outlet, the lid should not be clamped down and sealed because air must be allowed to escape from around the rim. Similar tops are available to euthanize animals inside standard plastic rodent cages. This allows the animal to be euthanized in its home cage, which is a preferred method.

2. A large plastic or glass desiccator jar with a tubulature in the top can be used if a two-holed rubber stopper is inserted. The hose from a CO_2 regulator is connected to a six-to-eight-inch piece of rigid plastic or stainless steel tubing that passes through one hole of the stopper and allows the carbon dioxide to be admitted at the bottom of the chamber. A three-inch piece of plastic tubing is passed through the other hole and is connected to a short length of hose with an adjustable screw-type clamp. When the gas is running, the clamp is open to allow the escape of air from the top of the chamber. When the gas is stopped, the clamp can be closed to contain the CO_2 within the chamber.

   CAUTION: Use of a typical desiccator (single tubing inlet with a greased ground glass seal) is dangerous because the heavy, sealed lid can allow pressure to build up and subsequently blow the lid off.

3. With a minimum investment, an acceptable chamber can be made out of an inexpensive plastic container with a lid that can be securely attached but is not completely airtight. A small hole is punched in the lid to serve as an inlet port, through which can be passed a length of plastic tubing attached to the regulator from a carbon dioxide cylinder. If neither the chamber nor the lid is transparent, a replacement lid can be crafted out of a durable transparent plastic. Plastic bags can be used as liners, which will facilitate disposal and keep the chamber clean.

Section E – Technique

The animal(s) are either placed in an empty chamber, or a CO_2 delivery lid is placed on their home cage. NOTE: if euthanasia is not being conducted in the home cage, chambers should be emptied and cleaned between uses. The flow of CO_2 from the gas cylinder is started at a rate that will displace 30-70% of the cage or chamber volume per minute. As mentioned above, the appropriate flow rate for a particular chamber (e.g., 8 liters/minute) should be posted. This calculated rate will allow a slow increase in the concentration of CO_2 to develop but will not cause noise or be perceived as a harsh “wind” to the animals. As gas levels rise to 40-50%, unconsciousness will occur as indicated by a loss of the righting reflex. At this point, the flow of the gas can be increased if desired to more rapidly fill the chamber and decrease the time to death. CO_2 flow should be maintained for at least one minute after respiratory arrest.

Section F – Removal from Chamber and Verification of Death

Observation for Vital Signs

After the gas is stopped, the animal is then observed until all muscle activity and signs of life have been absent for at least 30 seconds. Eyes are generally dilated, and mucous membranes will no longer be pink. After removal, check again to confirm respiratory arrest. If possible, verify by touch or by using a stethoscope that there is no heartbeat. If an animal is found to still be conscious, it must be returned to the chamber and the gas flow restarted. If the animal is unconscious but still alive, it can either be returned to the chamber or killed via a physical method such as cervical dislocation.

Physical Methods to Assure Death

Death must be verified after euthanasia and prior to disposal. Since the anesthetic effects of CO_2 are reversible, animals that are prematurely removed from the chamber prior to death can recover.

Unintended recovery after the procedure will be very rare if appropriate CO2 concentrations and exposure times are used, however it is considered to be inhumane, and this must be prevented. The use of a secondary physical method to assure death is required. Examples of acceptable physical methods include:

1. Cervical dislocation (for mice or rats no larger than 200 grams)
2. Decapitation
3. Thoracotomy (making a stab incision into the chest with a scalpel or sharp scissors to open up the lung cavity)
4. Experimental procedures that assure death such as fixative perfusion, dissection and removal of the brain or other major organs, or exsanguination.

NOTE: Failure to assure death of animals can lead to the spontaneous recovery of the animal in the disposal area, which is considered a federally reportable compliance incident that requires notification of funding agencies and can lead to loss of animal protocol approval. The relevant federal guidance can be found at this link: http://grants.nih.gov/grants/guide/notice-files/NOT-OD-02-062.html

Section G – Other Considerations

Two-Step Euthanasia (CO2 narcosis followed by a physical method)

Rather than waiting until death is complete from asphyxiation, cervical dislocation or decapitation can be used to kill rodents after they have been sedated with CO2. Once the animal has been exposed long enough to lose consciousness and be unresponsive to a toe pinch (analogous to a surgical plane of anesthesia) the physical method can be performed. Use of a two-step process should be detailed in the IACUC protocol if this is to be used.

Euthanasia of Neonates

CO2 should not be used as the sole method of euthanasia in neonatal rodents, because the time required for death to occur may be substantially prolonged due to their inherent resistance to hypoxia and hypothermia. The use of a two-step method (CO2 exposure for sedation followed by decapitation) is the recommended technique.

NOTE: Due to the anatomy of rat and mouse neonates, cervical dislocation is difficult to perform adequately, especially when euthanizing mice and rats in the "pinky" stage before hair grows in at 7-10 days. For routine purposes (such as assuring death after CO2 exposure) decapitation must be used rather than cervical dislocation. If there are specific experimental reasons that cervical dislocation must be used in neonates before eleven days of age for a particular project, this will require specific IACUC consideration and approval.

Approval Date	Change(s) Approved
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