Guidelines for the Use of Carbon Dioxide (CO_2) for Rodent Euthanasia

The University of Texas at Austin
Institutional Animal Care and Use Committee (IACUC)

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.

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SUMMARY: Carbon dioxide (CO_2) euthanasia must be performed by trained individuals using appropriate equipment. The use of CO_2 as a euthanasia method and the names of the individuals performing this procedure must be listed in the approved IACUC protocol covering the study. One of two methods (pre-fill or slow-fill) can be chosen based on the circumstances surrounding euthanasia. A secondary physical means to assure death **must be utilized** prior to disposal of the carcass when CO_2 is used for euthanasia.

TRAINING: Principal Investigators (PI) must ensure that all individuals responsible for administering CO_2 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 Training and Compliance Manager (phone: 471-3909)

GUIDANCE:

I. Characteristics

Carbon dioxide (CO_2) 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 CO_2 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. CO_2 generated from other sources, such as dry ice or fire extinguishers is unacceptable because gas flow cannot be regulated precisely in those circumstances.

II. Humane Considerations

Exposing animals to a CO_2 concentration of 70% or more can induce unconsciousness very rapidly, and the use of a 100% CO_2 atmosphere (by prefilling the chamber) will result in the quickest time to death. For this reason, prefilling is considered to be the most foolproof method of using CO_2. However, high concentrations of CO_2 can

cause a marked bradycardia in rats and mice, presumably via nasal chemoreceptors. Because humans perceive CO_2 exposure of the nasal mucosa at similar concentrations to be a noxious stimulus, it has been proposed that high concentrations of CO_2 should be considered distressful or even painful to rodents. This has led to the suggestion that it is more humane to expose rodents to a gradually rising concentration that will cause narcosis before the respiratory tissues are exposed to high concentrations. The downside of this approach is that it will prolong the time to narcosis and death, and recent studies have also identified the fact that rodents show strong aversive behavior when maintained in a conscious state in these lower CO_2 concentrations. Unfortunately, the literature is somewhat contradictory at this time, and although controlled studies are underway at multiple institutions, the pros and cons of these two methods (prefilling vs. slow infill) are difficult to resolve into a single best method. Each method may be best suited for certain circumstances, as described below.

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 for extended periods, and
3. Not assuring that animals have been completely killed prior to disposal.

Since carbon dioxide is 50 percent 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.

III. Methods

Equipment

Use of a compressed gas cylinder is required to administer CO_2, and the gas delivery equipment must include an appropriate regulator that controls the delivery of gas to an exposure chamber. 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 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 gasketed 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 **dessicator 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 which has an adjustable screw-type clamp placed to regulate the escape of air and carbon dioxide from the top of the chamber. CAUTION: Use of a typical dessicator (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.

4. If the need for euthanasia is infrequent, it is possible to fill a **heavy-duty plastic bag** directly from a hose attached to a regulator. This is most useful for prolonged gassing needed for neonatal rodents. Animals are placed in a transparent bag, the majority of the air is carefully squeezed out, and the bag is carefully filled with 100% CO_2 and then tied shut. The animals should be observed frequently during euthanasia until the bag is re-opened when death is assured using one of the methods listed below.

IV. Techniques

Both the pre-fill and slow infill methods are acceptable if done properly. One or the other, as suggested below will best serve certain situations. HOWEVER: the most important criteria are that the method used does not result in prolonged signs of distress. Experience has shown that different ages, sexes, and strains of rodents can show varying reactions to CO_2 exposure. If you feel that one method is not resulting in a humane death, you are urged to try the other and/or to contact the ARC for veterinary assistance in determining the best method.

1. Pre-fill

Pros: Most rapid onset of both unconsciousness and death; Simple administration; Allows sequential use of a chamber without removing residual CO_2. Delivery of 100% CO_2 to the empty chamber or when topping it off after animals are sedated does not require careful control of the gas flow.

Cons: Placing animals in a high concentration of CO_2 may cause brief distress prior to loss of consciousness

When to Use: Sequential euthanasia of a large number of grouped (but uncrowded) animals in a chamber other than the home cage; Situations where the regulator cannot be adjusted to provide a controlled slow inflow.

Procedure: Pre-filling of a closed empty chamber can be done rapidly by using a high-volume flow rate. 100% carbon dioxide gas should be admitted into the chamber for at least 45-60 seconds. The gas is turned off and the animal is then placed inside the chamber and the lid secured. Transfer of the rodents into the chamber should be done gently but quickly in order to minimize loss of CO_2 while the lid is

open. If pre-filling was adequate, the animal should show signs of losing consciousness within 10-20 seconds. Once the animal is sedated, the high-flow gas can be turned on again for 15 -30 seconds to purge any remaining oxygen. The animal is then observed until all muscle activity and breathing has been absent for at least 30 seconds. A physical method is then used to verify death (see below).

2. Slow-fill

Pros: Animals are not exposed to high levels of CO_2 until after they lose consciousness; Can be done without removing animals from their home cage environment.

Cons: Slower onset of unconsciousness and death; Careful adjustment of gas inflow required; Prolonged exposure to low levels of CO_2 may be distressful.

When to Use: Euthanasia of a small number of animals as a single batch in a chamber; Euthanasia of entire cages using the home cage as the chamber; Euthanasia of sequential batches of animals if the chamber is thoroughly aerated between groups to remove residual CO_2. Requires adequate time to perform this slower method, and careful adjustment of the CO_2 delivery rate.

Procedure: The animal(s) are either placed in an empty chamber, or a CO_2 delivery lid is placed on their home cage. The flow of CO_2 from the gas cylinder is started at a rate that will displace ~20% of the cage or chamber volume per minute (a very slow rate when a mouse or rat home cage is used). This 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. Gas flow can be discontinued when signs of respiration have ceased. The animal is then observed until all muscle activity and breathing has been absent for at least 30 seconds. A physical method is then used to verify death (see below).

V. Removal from Chamber and Verification of Death

Observation for vital signs

Under proper conditions, an exposure time of 3-6 minutes (depending on the fill rate) is generally adequate to kill adult animals. Before animals are removed, all visible movement (including breathing) should have stopped. 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 heart beat. 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.

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 CO_2 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:

- Cervical dislocation (for mice or rats no larger than 200 grams)

- Decapitation
- Thoracotomy (making a stab incision into the chest with a scalpel or sharp scissors to open up the lung cavity)
- 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

VI. Other considerations

Two-Step euthanasia (CO_2 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 CO_2. 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

The time required for CO_2 euthanasia may be substantially prolonged (e.g., 10-20 min or more) in neonatal rodents due to their inherent resistance to hypoxia. For this reason, CO_2 should not be used as the sole method of euthanasia in neonates unless the animal can be exposed long enough to ensure death. As mentioned above, the plastic bag method may be the best option for prolonged exposure. Alternatively, use of a two-step method (CO_2 exposure for sedation followed by decapitation. Due to the anatomy of neonates, decapitation is more practical than cervical dislocation. Decapitation alone can be used as an alternate primary method of euthanasia for neonates, but this requires specific IACUC approval.