POLICY

Animal welfare regulations require that the Attending Veterinarian (AV) shall provide guidance to the Principal Investigator (PI) and staff regarding euthanasia, and that the Institutional Animal Care and Use Committee (IACUC) must approve the method of euthanasia to be utilized for research animals. The method of euthanasia and the criteria used to assess pain and distress in animals must be described in detail in the approved IACUC application. Additionally, in the event that an animal associated with an approved protocol shows evidence of illness or pain requiring emergency care including euthanasia, the AV/designee will exercise professional judgement in the interests and well-being of the animal.

Euthanasia is the act of inducing humane death in an animal by a method that induces rapid loss of consciousness and death with a minimum of pain, discomfort, or distress. The euthanasia method should utilize minimal restraint, be reliable and irreversible, and avoid risk and aversion for personnel. The method must be appropriate for the age and species of the animal and conform to the most recent AVMA Guidelines on Euthanasia. Any deviations from this policy and/or the AVMA Guidelines must be scientifically justified in writing by the PI and approved by the IACUC. Euthanasia of animals should occur in a procedure room or laboratory, not in an animal housing room.

To ensure a painless death that satisfies research requirements, animals must be euthanized by trained personnel using appropriate technique, equipment, and agents. It is the responsibility of the PI to ensure that personnel performing euthanasia have been properly trained and adhere to IACUC approved protocols and policies. UNO Animal Care and Use Program (ACUP) offers training to PIs and staff on an as-needed basis.

Following euthanasia, and **before disposal, death must be ensured** by performing a secondary physical method to ensure that euthanasia is irreversible. Animals found alive after apparent death following euthanasia constitutes serious regulatory non-compliance with the PHS Policy and a serious deviation from the provisions of the Guide for the Care and Use of Laboratory Animals. Failure to ensure death prior to carcass disposal will result in reporting to oversight and funding agencies, as applicable, and possible sanctions placed on the investigator.

Carcasses are never to be placed into the regular trash. Carcasses are to be securely placed in two leak proof bags. All bags with animal carcasses must be labeled with the following information: IACUC #, Method used to ensure death, Date, and Initials of person disposing of the carcass. The bags are to be placed into a properly labeled dedicated refrigerator, freezer, or cold room that is easily sanitized. Final carcass disposal must be in accordance with federal, state and local regulations.

Animals experimentally treated with hazardous materials must be disposed of according to regulations and guidelines for the agent(s) used.

REGULATION

The Public Health Service (PHS) Policy in accordance with IV.C. 1. g - Methods of euthanasia used will be consistent with the recommendations of the American Veterinary Medical Association (AVMA) Panel on Euthanasia, unless a deviation is justified for scientific reason in writing by the investigator.

United States Department of Agriculture (USDA) Animal Welfare Act (AWA) 2.31 (d)(5) – Animals that would otherwise experience severe or chronic pain or distress that cannot be relieved will be painlessly euthanized at the end of the procedure or, if appropriate, during the procedure. 2.33 (b)(4) – AV will provide guidance to principal investigators and other personnel involved in the care and use of animals regarding handling, immobilization, anesthesia, analgesia, tranquilization, and euthanasia.

Office of Laboratory Animal Welfare (OLAW) Guidance NOT-OD-02-062 PHS Policy on Humane Care And Use Of Laboratory Animals Clarification Regarding Use Of Carbon Dioxide For Euthanasia Of Small Laboratory Animals.

American Veterinary Medical Association (AVMA Guidelines for the Euthanasia of Animals), Current - Distress vocalizations, fearful behavior and release of certain odors or pheromones by a frightened animal may cause anxiety and apprehension in other animals. Therefore, for sensitive species, it is desirable that other animals not be present when individual animal euthanasia is performed.

American Veterinary Medical Association (AVMA Guidelines for the Euthanasia of Animals), Current – Neonatal animals appear to be resistant to hypoxia, and because all inhaled agents ultimately cause hypoxia, neonatal animals take longer to die than adults. Inhaled agents can be used alone in unweaned animals to induce loss of consciousness, but prolonged exposure time or a secondary method may be required to kill the unconscious animal.

The Guide for the Care and Use of Laboratory Animals – Unless a deviation is justified for scientific or medical reasons, methods should be consistent with the current Report of the AVMA Panel on Euthanasia. ADDITIONAL GUIDANCE

A Report of the ACLAM Task Force on Rodent Euthanasia. Artwohl J, Brown P, Corning B, and Stein S. 2006, JAALAS 45(1): 98-105.

PROCEDURE

1) **CO2 Inhalation**
    a. CO2 inhalation is the most common method of euthanasia used for mice, rats, guinea pigs, gerbils, and hamsters. Compressed gas is the only

acceptable source of CO_2 as this allows the inflow of gas to the induction chamber to be controlled.

b. Since the anesthetic effects of CO_2 are reversible, animals that are removed prematurely from the chamber prior to death can recover so please follow the procedures below carefully.
c. Procedures:
    i. Euthanasia chambers should be constructed of clear material and must be kept free of debris and excreta.
    ii. When possible, rodents should be transported and euthanized in their home cages.
    iii. Regrouping of animals from multiple cages into a single chamber is not recommended to minimize social aggression.
    iv. The chamber/cage must not be pre-charged with CO_2.
    v. Place the animals in the chamber (if using their home cage- place the euthanasia lid on the cage). Do not over-crowd the chamber/cage. All animals must be able to make normal postural adjustments.
    vi. Gradually introduce CO_2 at a flow rate of 30-70% of the chamber volume per minute. Sudden exposure of conscious animals to CO_2 concentrations of greater than 70% can be distressful and painful.
    vii. Once all animals are unconscious, the flow rate can be increased to minimize the time to death.
    viii. Following CO_2 euthanasia, a physical method to ensure death must be performed prior to carcass disposal.

2) Anesthetic Inhalation

a. The primary action is central nervous system (CNS) depression. The agents are nonflammable and nonexplosive under ordinary environmental conditions. Occupational exposure to inhalant anesthetics constitutes a human health hazard. (e.g. enflurane, isoflurane)
b. Since the anesthetic effects of inhalant anesthetics are reversible, animals that are removed prematurely from the chamber prior to death can recover so please follow the procedures below carefully.
c. Waste anesthetic gases must be appropriately scavenged to ensure a safe working environment in a laboratory setting.
d. A dedicated exhaust system, charcoal canisters, or a vented fume hood must be used to capture waste gases.
e. If using an open-drop system (application of isoflurane to an absorbent material then placed into the bottom of the chamber), the animal must be physically separated from the gas by a physical barrier.
f. Procedures:
    i. Chambers used for inhalational anesthetics must be of appropriate size as to prevent overcrowding of the chamber.

ii. All animals must be able to make normal postural adjustments.
iii. Loss of consciousness should be induced rapidly by exposing animals to the maximum agent concentration possible.
iv. Once the animal(s) is/are euthanized, a physical method to ensure death must be performed prior to carcass disposal.

3) Injectable Anesthetics

a. Injectable anesthetics can be effectively used to anesthetize animals prior to performing a physical method of euthanasia.
b. Since the anesthetic effects of injectable anesthetics are reversible, animals can recover so please follow the procedures below carefully.
c. Procedures:
    i. Administer the anesthetic overdose as described in the approved IACUC protocol and allow sufficient time for the animal to lose consciousness.
    ii. Once the animal(s) is/are euthanized, a physical method to ensure death must be performed prior to carcass disposal.

4) Cervical dislocation (Under Anesthesia)

a. Cervical dislocation is a humane technique when performed by individuals with a high degree of technical proficiency.
b. Cervical dislocation is limited to rodents weighing < 200 grams.
c. Cervical dislocation in unanesthetized rodents is permitted only if its use is scientifically justified.

5) Decapitation (Under Anesthesia)

a. Decapitation when performed properly is nearly instantaneous and is considered humane.
b. Guillotines that are designed for decapitation in adult rodents are commercially available. Equipment must be maintained in good working order and serviced on a regular basis to ensure sharpness of blades. The use of plastic cones to restrain animals is recommended as it reduces distress from handling, minimizes the possibility of personnel injury, and improves positioning of the animal in the guillotine.
c. Decapitation in unanesthetized rodents is permitted only if its use is scientifically justified.
d. Refer to the Policy for Guillotine Use and Maintenance for further information.

6) Other Acceptable Methods of Euthanasia In Rodents

a. Microwave Irradiation: microwave irradiation in a specially designed and approved apparatus.

b. IV administration of KCl under anesthesia.
c. IV or IP administration of a barbituric acid derivative.

7) Euthanasia of Rodent Fetuses

a. Mouse, Rat, and Hamster up to 14 days gestation; Guinea Pigs up to 35 days gestation
    i. 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 fetuses at this stage of development.
b. Mouse, Rat, and Hamster Fetuses over 15 days gestation; Guinea Pigs over 35 days gestation through birth
    i. The neural development during this time supports that pain may be perceived. Methods to euthanize include injection of anesthetics or decapitation with sharp surgical scissors or scalpels.
    ii. If chemical fixation of the whole fetus is required, fetuses must be anesthetized by hypothermia or by injection with an anesthetic prior to immersion in fixative.

8) Euthanasia of Rodent Neonates

a. Mouse, Rat, and Hamster Neonates up to 10 days of age
    i. Acceptable methods include CO2 inhalation, inhalant anesthetic agents, injection of chemical anesthetics or euthanizing agents, or anesthesia followed by decapitation with sharp surgical scissors or scalpels.
        1. Resistance to hypoxia results in a prolonged exposure time to unconsciousness when CO2 or inhaled anesthetics is used.
    ii. **The use of CO2, inhalant anesthetic agents, injectable chemical anesthetics or euthanizing agents requires a physical method to ensure death prior to carcass disposal**. Cervical dislocation is an AVMA approved method of euthanasia for neonatal rodents. However, due to the small size of altricial (hairless) neonates it can be difficult to perform aesthetically. Therefore, the IACUC and the Attending Veterinarian require decapitation instead of cervical dislocation as a more aesthetic method of euthanasia for neonatal rats, hamsters and mice up to 10 days of age. If there are specific experimental reasons that cervical dislocation must be used in neonates before eleven days of age, the reasons must be listed in the protocol and reviewed and approved by the IACUC.

iii. Additional methods include immersion in, or perfusion with chemical fixatives or immersion in liquid nitrogen. These methods should only be performed if preceded by anesthesia.
iv. Anesthesia in neonatal rodents may be induced by inhalant or injectable anesthetics. Prolonged exposure to inhalant anesthetics may be necessary. Alternatively, hypothermia may be used to induce anesthesia in pups 6 days of age or less.

b. Guinea Pig Neonates
   i. Follow guidelines for adult rodents.

9) Euthanasia of Non-Rodent Mammals

a. Intravenous injection of a barbituric acid derivative is the preferred method of euthanasia of non- rodent mammals. Intraperitoneal injection may be used in situations when an intravenous injection would be distressful or even dangerous. Intracardiac injection must only be used if the animal is unconscious, or anesthetized.
b. Once the animal(s) is/are euthanized, a physical method to ensure death must be performed prior to carcass disposal. Common methods approved by the IACUC include bilateral thoracotomy, exsanguinations, and removal of vital organs.

10) Euthanasia of Reptiles, Amphibians, Fish

a. Ectothermic vertebrates require special consideration because these animals may normally exhibit very low heart rates, are very tolerant to hypoxia, and can hold their breath for long periods of time. Absence of a heartbeat and/or breathing will not necessarily provide confirmation of death. Once the animal is/are euthanized a physical method to ensure death must be performed prior to carcass disposal. Common methods approved by the IACUC to ensure death include pithing and decapitation, removal of organs, and exsanguination.
   1. MS 222 alone is not effective for euthanasia of zebrafish eggs, embryos, or larvae (<14 days old), and other methods should be used for these life stages.
b. Zebrafish and other fish
   i. Rapid chilling: Zebrafish and other fish may be euthanized via rapid chilling (2-4ºC) until loss of orientation and cessation of opercular movement. Subsequent additional exposure of the fish to chilled water is dependent on the size and age. It has been suggested that rapid chilling in water associated with an ice slurry is a suitable killing method for small tropical and subtropical fish species 3.8 cm in length

(tip of the snout to the posterior end of the last vertebra) or smaller, having lower lethal temperatures above 4°C.

1. Fish should be placed in a cold-water ice slurry that does not exceed 2-4ºC. The entire surface of the fish must be exposed without direct contact with ice. A probe should be used to ensure temperature.
2. Zebrafish: adult zebrafish should be exposed to a minimum of 10 additional minutes following the loss of opercular movements; fry 4-7 days after fertilization (dpf) should be exposed for at least 20 additional minutes.
3. Rapid chilling is not an effective method of euthanasia for embryos <3 dpf.

ii. Immersion in diluted sodium or calcium hypochlorite solution (500mg/L) is acceptable for embryos up to 7 days of age.

iii. Use of MS 222:

1. The solution must be buffered with sodium bicarbonate to a pH between 7.0-7.5.
2. Stock solutions should be protected from light and refrigerated or frozen if possible. The solution must be replaced monthly and any time a brown color is observed.
3. Fish should be left in this solution for at least 30 minutes following cessation of opercular movement. Large fish may be removed from the water, a gill cover lifted, and a concentrated solution from a syringe flushed over the gills.

c. Amphibians and reptiles: The application of two or more euthanasia procedures is typically recommended for amphibians and reptiles as differences in metabolism, tolerance to hypoxia, and the ability for reptilian and amphibian hearts to beat after brain death can complicate confirmation of death.

i. Injectable agents

1. Direct injection into the brain through the parietal eye while under anesthesia has been described in some lizard species.
2. Sodium pentobarbitol (60-100mg/kg of body weight) can be administered IV, intracoelomically, in the subcutaneous lymph spaces, or in the lymph sacs, although doses vary by species. Doses as high as 1,100 mg/kg (500 mg/lb) of sodium pentobarbital with sodium phenytoin administered intracoelomically may be required for euthanasia of some species such as *X. laevis*. Time to effect may vary, with death occurring instantaneously or up to 30 minutes later

3. Dissociative agents such as ketamine hydrochloride or combinations such as tiletamine and zolazepam; inhaled agents; and IV administered anesthetics, such as propofol, or other ultra–short-acting barbiturates, may be used for poikilotherms to induce rapid general anesthesia and subsequent euthanasia, although application of an adjunctive method to ensure death is recommended.
4. Buffered MS 222 may be administered via water baths (amphibians), or injected directly into the lymph sacs (amphibians) or the coelomic cavity (small amphibians and reptiles). Prolonged immersion (as long as 1 hour) may be required for 5- to 10-g/L water baths.

ii. External or topical agents: Benzocaine hydrochloride, a compound similar to MS 222, may be used as a bath or in a recirculation system at concentrations ≥ 250 mg/L or applied topically to the ventrum as a 7.5% or 20% gel for euthanasia of amphibians. A dose of 182 mg/kg of benzocaine gel (20% concentration, 2.0-cm X 1.0- mm application) has been reported as effective for euthanasia of adult *X. laevis*.

iii. Inhalant agents: Many reptiles and amphibians are capable of breath holding and shunting of their blood, which permits conversion to anaerobic metabolism for survival during prolonged periods of anoxia (up to 27 hours for some species). Because of this, induction of anesthesia and time to loss of consciousness may be greatly prolonged when inhaled agents are used. Death may not occur even with prolonged exposure. Lizards and most snakes do not hold their breath to the same extent as some of the chelonians, and are therefore more likely to have a clinical response to inhaled agents

1. Carbon dioxide may be considered for euthanasia of amphibians and reptiles if alternate methods are not practical and where the limitations of this method are understood and addressed. Due to the potential lack of response to this method by many species and the requirement for a prolonged exposure time, other methods are preferable. Death by CO2 must be verified, and preferably, assured by application of a secondary lethal procedure.

iv. Rapid freezing—Reptiles and amphibians can be euthanized by rapid freezing when it results in immediate death. Based on rodent models, it is likely that this can be achieved by placing animals < 4 g (0.1 oz) in liquid N2. However, due to a dearth of empirical evidence supporting this method, operators should consider a secondary method to ensure that recovery does not occur. The technique should not be used for

species that have adapted freeze tolerance strategies, as this method may not result in instant death. Placement of animals ≥ 4 g in liquid N2 or other uses of hypothermia are not acceptable. An ice slurry is not an appropriate method for rapid freezing of reptiles and amphibians as the process of freezing can cause the formation of ice crystals in tissues that may cause pain.

1. Freezing is likely appropriate for newly oviposited eggs

11) Physical methods commonly approved by the IACUC to ensure death prior to carcass disposal:

a. Cervical dislocation (rats must be < 200g)
b. Decapitation (required for neonatal rats, hamsters and mice < 10 days old)
    i. Decapitation followed by pithing can be used a physical method of ensuring death for fish. Decapitation alone is not considered a humane approach to euthanasia.
    ii. The central nervous system of amphibians and reptiles is tolerant to hypoxic and hypotensive conditions. Decapitation must be followed by pithing or another method of destroying brain tissue. Decapitation should only be performed as part of a 3-step euthanasia protocol (injectable anesthetic, decapitation, pithing).
c. Bilateral Thoracotomy
d. Exsanguination
    i. Animals may be exsanguinated to obtain blood products, but only when they are anesthetized.
e. Removal of Vital Organs
    i. Animals may require vital organs to be collected, but only when they are anesthetized.
f. Pithing (Fish and Amphibians Only)
g. Maceration that results in instantaneous death is an appropriate method for the euthanasia of newly oviposited reptile or amphibian eggs. Later stages may be destroyed using methods that are acceptable for adult animals.

12) Carcass Disposal:

a. Animal users within the UNO facilities are to follow all applicable UNO carcass disposal standard operating procedures.
b. All carcasses are to be placed in body bags and labeled with the following information: IACUC #, method used to ensure death, date and initials of person disposing of the carcass.

LINKS TO RELATED FORMS, RECORD LOGS, AND SOPS

UNO Carcass Disposal Standard Operating Procedure – Contact the IACUC office at iacuc@unomaha.edu.