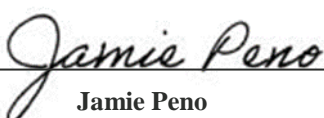
 <div style="display: inline-block; vertical-align: middle;"> <b>DIVISION OF RESEARCH &amp; INNOVATION</b>  <small>Research Integrity &amp; Compliance</small> </div>		<b>Institutional Animal Care and Use Committee</b>  <b>Standard Operating Procedures</b>	
<b>Title:</b> Humane Endpoints and Methods of Euthanasia			
<b>Effective Date:</b>	December 22, 2020	<b>Document Number:</b>	IACUC-SOP-02-11.00
<b>Approval/Date:</b>  <div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;">   <hr style="width: 100%;"/> <b>Jamie Peno</b>              Director, Research Integrity and Compliance           </div> <div style="text-align: center;"> <hr style="width: 100%;"/>             12/22/2020  <b>Date</b> </div> </div>			
<b>REVISION HISTORY</b>			
<b>Date</b>	<b>Section</b>	<b>Author</b>	

## PURPOSE

The purpose of this document is to provide guidelines involving humane endpoints and approved methods of euthanasia that reduce animal pain and distress, while still meeting research objectives when animals are used for biomedical research and teaching. Euthanasia techniques should be consistent with the AVMA Guidelines for euthanasia unless the deviation from these guidelines is justified and approved in the protocol for scientific or medical reasons.

## SCOPE

It is the responsibility of all investigators, staff, students and animal care staff using animals in research or teaching at UNT to follow these procedures.

Investigators are expected to:

1. Use alternative endpoints when possible.
2. Minimize animal numbers within statistical constraints.
3. Have experimental animals monitored at least twice daily, i.e., early morning and late afternoon, during the workweek. On weekends and holidays, animals will be monitored on a once-daily basis unless animals are expected to be in a morbid state.
4. Euthanize any animals found in a moribund state except when death is the endpoint as approved by the UNT IACUC.

## DEFINITIONS AND ABBREVIATIONS

UNT- University of North Texas, Denton

IACUC- Institutional Animal Care and Use Committee

## **PROCEDURES**

### **I. Humane Endpoints**

#### **A. Euthanasia as an alternative to death as an endpoint in Rodents:**

1. Legal, regulatory, and moral guidelines require that animal pain, distress, and suffering be minimized in any experiment. For these reasons, investigators are strongly encouraged to administer euthanasia, if experimental validity will not be compromised, in death-end-point experiments prior to actual death of the animals. These objectives assume that investigators can differentiate between animals that are morbid (i.e., affected with disease or illness), and those that are moribund (i.e., in the state of dying)
2. The IACUC believes that an investigator can judge and should perform euthanasia on moribund rodents based on objective signs or symptoms of dying depending on experience with the animal model, professional judgment, and the experimental protocol. The combination of signs or symptoms indicating euthanasia may vary with experimental endpoint.
3. The IACUC guidelines indicate that animals found moribund should receive euthanasia, but if experimental death itself is the required endpoint, the investigator may receive consideration for approval to conduct such studies by providing appropriate justification in the approved IACUC Protocol. Inconvenience or increased costs alone are not justifiable reasons, but the IACUC will otherwise, generally, accede to the scientific judgment of the investigator. Investigators are expected to make a good faith effort to justify their end points, or agree they can judge when to perform euthanasia on animals found moribund in a particular protocol. Moreover, all investigators are expected to continue to monitor experimental animals at least daily (including weekends and holidays), to euthanize any animals which they judge should receive euthanasia, to use alternative end points to death when possible, and to minimize animal numbers within statistical constraints in general, but especially in death-end-point protocols.
4. The following shall be included in the protocol if death is an endpoint:
  - a) Written justification when appropriate including:
    - i. Discussion of alternative endpoints
    - ii. Literature citation
    - iii. Copies of pertinent publications where appropriate
  - b) Justification of the number of animals to be included
  - c) Justification for the withholding of analgesics, if applicable
  - d) At least twice daily monitoring once animals exhibit abnormal signs (at least 6-8 hours apart)
  - e) Maintenance of written records of monitoring
5. Suggested Signs and Symptoms for Judging Morbidity (disease/illness) in Rodents:
  - a) Rapid breathing rate
  - b) Breathing rate very slow, shallow, and labored
  - c) Rapid weight loss
  - d) Hunched posture
  - e) Hypo- or hyperthermia
  - f) Ulcerative dermatitis or infected tumors
  - g) Anorexia (loss of appetite)

- h) Diarrhea or constipation
- 6. Suggested Signs and Symptoms for Judging the Moribund Condition (state of dying) in Rodents. Signs and symptoms of morbidity will be observed plus:
  - a) Impaired ambulation (unable to easily reach food or water)
  - b) Evidence of muscle atrophy or other signs of emaciation (body weight is not always appropriate, especially since tumors may artificially increase body weight)
  - c) Any obvious illness including such signs as lethargy (drowsiness, aversion to activity, lack of physical or mental alertness), prolonged anorexia, bleeding, difficulty breathing, central nervous.
  - d) Inability to remain upright
- 7. When an animal meets any of the following criteria, it should be considered for euthanasia:
  - a) Rapid weight loss (animals should be weighed a minimum of twice per week) of 15% or greater body weight
  - b) Debilitating diarrhea
  - c) Labored breathing
  - d) Lethargy
  - e) Persistent recumbence
  - f) Significantly abnormal neurological signs
  - g) Bleeding from any orifice
  - h) Self-induced trauma
  - i) Impaired mobility
  - j) Difficulty obtaining food and water
  - k) Inability to maintain normal body temperature
  - l) Clinical signs of pain that do not respond to analgesics
  - m) Tumor greater than 2 cm (measured in any direction)
  - n) Necrotic or ulcerated tumors
  - o) Animal refuses to eat or drink for a significant length of time

## **II. Methods of Euthanasia**

- A.** All laboratory animals must be euthanized in a timely manner to prevent/ alleviate animal suffering, either as described in the approved protocol according to experimental endpoints, or as soon as necessary if established criteria for humane endpoints are reached.
- B.** All IACUC approved protocols must include a description of the methods for euthanizing the animals, and how death will be confirmed, especially in animals receiving anesthetics or CO<sub>2</sub> prior to euthanasia.
- C.** Animals must be euthanized only by trained personnel using appropriate techniques, equipment, and agents. This is necessary to ensure a painless death that satisfies research requirements.
- D.** Death should be induced as painlessly and as quickly as possible.
- E.** In an effort to reduce stress in animals, euthanasia should not be performed in the animal housing room.
- F.** The euthanasia method must be appropriate to the species, approved in the animal study proposal and conform to the most recent AVMA Guidelines on Euthanasia.
- G.** The use of inhalant agents for euthanasia must observe the conditions and precautions spelled out in the pertinent sections of AVMA Guidelines. Administration of inhalant overdose may result in deep depression of all life signs prior to death. It is possible that animals could revive from this state, which can be mistaken for death during a cursory examination.
- H.** Common methods of euthanasia for laboratory animals:
  - 1. Injectable Agents

- a) Injectable barbiturate combinations: Injectable barbiturates, such as pentobarbital, are often used in combination with local anesthetics and anticonvulsants. An adequate dose of barbiturate is the most important component in these combinations.
  - i. Pentobarbital and formulations containing pentobarbital, are controlled substances, which are regulated by the Drug Enforcement Agency (DEA). A single lethal IP (poultry, birds, mice, rats, hamsters and guinea pigs) or IV (rabbits) administration of pentobarbital of  $\geq 120$  mg/kg is sufficient for euthanasia.
  - ii. Pentobarbital sodium generally comes in various pharmaceutical formulations, either as pentobarbital (e.g. Nembutal) or as a mixture labeled for euthanasia only (e.g. FatalPlus, Beuthanasia, etc.).
- b) Dissociative agent combinations: Lethal doses of dissociative agents such as ketamine can be used.
  - i. In rodents, ketamine should be used in combination with an  $\alpha$ -adrenergic receptor agonist such as xylazine
  - ii. Doses and volumes of drugs may vary, but at least 4-5 times the anesthetic dose should be used for euthanasia.
- c) To ensure death, administration of an injectable overdose must be followed by a secondary method of euthanasia such as:
  - i. Cervical dislocation in poultry, birds, mice, hamsters, rats (200 g). Animals with a greater muscle mass in the cervical region makes manual cervical dislocation physically more difficult and shall not be done.
  - ii. Decapitation
  - iii. Exsanguination
  - iv. Exsanguination as part of perfusion
  - v. Bilateral thoracotomy
  - vi. Removal of organs for procurement
2. MS 222 (aquatic species): Available as tricaine methane sulfonate (TMS), MS 222 can be used for the euthanasia of amphibians, fish, and other aquatic species. Tricaine is a benzoic acid derivative and generally should be buffered with sodium bicarbonate. A 10 g/L stock solution can be made, and sodium bicarbonate added to saturation, resulting in a pH between 7.0 and 7.5 for the solution. The stock solution should be stored in a dark brown bottle, and refrigerated or frozen if possible. The solution should be replaced monthly and any time a brown color is observed. For euthanasia, a concentration  $\geq 250$  mg/L is recommended:
  - a) Fish: Fish should be left in this solution for at least 10 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.
  - b) Amphibians: Amphibians should be left in this solution for at least 10 minutes following cessation of movement. MS 222 may also be injected into lymph spaces and pleuroperitoneal cavities.
  - c) Death must be assured by a second form of euthanasia by one of the following methods as secondary methods to ensure euthanasia of aquatics:
    - i. Pithing
    - ii. Decapitation
    - iii. Removal of multiple organs for tissue procurement
    - iv. Exsanguination
3. Inhalants
  - a) Carbon Dioxide: Carbon dioxide (CO<sub>2</sub>) inhalation is the most common method of

euthanasia used for poultry, birds, mice, rats, guinea pigs and hamsters. CO<sub>2</sub> exposure using a gradual fill method is less likely to cause pain due to nociceptor activation by carbonic acid prior to onset of unconsciousness. CO<sub>2</sub> must be used as follows:

- i. The euthanasia chamber should allow ready visibility of the animals. Do not overcrowd the chamber. All animals in the chamber must be able to assume normal postural adjustments.
- ii. Compressed CO<sub>2</sub> gas in cylinders is the only recommended source of carbon dioxide because gas inflow to the chamber can be precisely regulated.
- iii. An optimal flow rate for CO<sub>2</sub> euthanasia systems should displace 30% to 70% of the chamber or cage volume/min. Placing conscious animals in a pre-filled chamber is not acceptable as this is distressful to the animals.
- iv. Animals should be euthanized in their home cage. If their home cage cannot be used, chambers should be emptied and cleaned between uses.
- v. Animals should be left in the container until clinical death has been ensured (wait at least 1 minute after the last animal's last breath). Unintended recovery must be prevented by the use of appropriate CO<sub>2</sub> concentrations and exposure times or by other means as defined below.
- vi. The use of dry ice for CO<sub>2</sub> euthanasia is not permitted. The use of dry ice is a potential source of injury or distress if permitted to directly contact the animal.
- b) Inhalants (anesthetics other than CO<sub>2</sub>)
  - i. Inhaled agents may be useful in cases where physical restraint is difficult or impractical.
  - ii. When used as a sole euthanasia agent delivered via vaporizer of anesthetic chamber (open-drop technique), animals may need to be exposed for prolonged time periods to ensure death.
- c) To ensure death, administration of carbon dioxide or an inhalant anesthetic overdose in poultry, birds, mice, rats, guinea pigs and hamsters must be followed by a secondary method of euthanasia such as:
  - i. Cervical dislocation in poultry, birds, mice, rats (birds, mice, rats (<200 g), and rabbits (<1kg)
  - ii. Decapitation
  - iii. Exsanguination
  - iv. Exsanguination as part of perfusion
  - v. Bilateral thoracotomy
  - vi. Lethal IP (poultry, birds, mice, rats, hamsters and guinea pigs) or IV (rabbits) administration of pentobarbital ( $\geq 120$  mg/kg)
4. Physical Methods:
  - a) Animals subjected to physical methods of euthanasia should be anesthetized or tranquilized prior to euthanasia. Physical methods without prior anesthesia or tranquilization must be scientifically justified in the approved protocol.
  - b) Cervical Dislocation of Conscious Animals: This conditionally acceptable technique is used to euthanize poultry, other small birds, mice, and immature rats (<200 g). It requires neither special equipment nor transport of the animal and yields tissues uncontaminated by chemical agents. Data suggest that electrical activity in the brain persists for 13 seconds following cervical dislocation, and unlike decapitation, rapid exsanguination does not contribute to loss of consciousness. The following guidelines must be observed when performing cervical dislocation in conscious animals:
    - i. Individuals performing this technique must have a demonstrated high degree of

- technical proficiency as determined by the IACUC (Veterinarian or designee will observe the procedure).
    - ii. In heavier rats (>200 g) the greater muscle mass in the cervical region makes manual cervical dislocation physically more difficult and shall not be done.
    - iii. It is the PI's responsibility to determine that all personnel have been trained to perform this technique, and to monitor that personnel consistently apply it humanely and effectively.
  - c) Decapitation of Conscious Animals: This conditionally acceptable technique is used to euthanize rodents in research settings. It provides a means to recover tissues and body fluids that are chemically uncontaminated. It also provides a means of obtaining anatomically undamaged brain tissue for study. Loss of cortical function following decapitation is rapid and occurs within 5 to 30 seconds as measured by a significant reduction in amplitude recordings of visual evoked responses and EEG changes. Although it has been demonstrated that electrical activity in the brain persists for 13 to 14 seconds following decapitation, more recent studies and reports indicate that this activity does not infer the ability to perceive pain, and in fact conclude that loss of consciousness develops rapidly. The following guidelines must be observed when performing decapitation in conscious animals:
    - i. Individuals performing this technique must have a demonstrated high degree of technical proficiency as determined by the IACUC (Veterinarian or designee will observe the procedure).
    - ii. The PI is responsible for confirming that equipment used to perform decapitation is maintained in good working order, kept clean and serviced on a regular basis to retain blade sharpness.
    - iii. The use of plastic cones to restrain animals reduces distress from handling, minimizes the chance of injury to personnel, and improves positioning of the animal for decapitation.
    - iv. It is the PI's responsibility to determine that all personnel have been trained to perform this technique, and to monitor that personnel consistently apply it humanely and effectively. Personnel should be trained on anesthetized and/or dead animals to demonstrate proficiency.
- 5. Euthanasia of Rodent Fetuses and Neonates
  - a) The following guidelines are based on recommendations by the NIH and are for the use of rodent fetuses and neonates:
    - i. Fetuses up to 14 days in gestation: Neural development at this stage is minimal and pain perception is considered unlikely. Rodent fetuses are unconscious in utero and hypoxia does not evoke a response. It is unnecessary to remove fetuses for euthanasia after the dam is euthanized.
    - ii. Fetuses 15 days in gestation to birth: The literature on the development of pain pathways suggests the possibility of pain perception at this time. Whereas fetuses at this age are less sensitive to inhalant anesthetics, euthanasia may be induced by the skillful injection of chemical anesthetics. Decapitations with surgical scissors or cervical dislocation are acceptable physical methods of euthanasia. When chemical fixation or rapid freezing (immersion in liquid nitrogen) of the whole fetus is required, fetuses should be anesthetized prior to immersion in or perfusion with fixative solutions. Anesthesia may be induced by hypothermia of the fetus, by injection of the fetus with a chemical anesthetic, or by deep anesthesia of the mother with a chemical agent that crosses the placenta, e.g., pentobarbital. The university veterinarian should be

consulted for considerations of fetal sensitivity to specific anesthetic agents. When fetuses are not required for study, the method chosen for euthanasia of a pregnant mother must ensure rapid death of the fetus.

- iii. Neonates up to 7 days of age: Acceptable methods for the euthanasia of neonatal mice and rats include: injection of chemical anesthetics (e.g., pentobarbital), decapitation, or cervical dislocation. Additionally, inhalant anesthetics (e.g., isoflurane used with appropriate safety considerations), may be used. However, neonates have a high tolerance for hypoxia, so exposure must be prolonged (>20 minutes) and death confirmed by a secondary means. Pups should be anesthetized prior to freezing with liquid nitrogen. Similarly, anesthesia should precede immersion or perfusion with chemical fixatives. Anesthesia may be induced by inhalant or injectable anesthetics; the Veterinarian should be consulted for appropriate agents and dosages. Alternatively, when adequately justified, hypothermia for anesthesia may be used to induce anesthesia in pups younger than six days. When using hypothermia, there must be a barrier (petri dish, plastic wrap, etc.) between the pup and the ice.
- iv. Neonates older than 10 days: Follow guidelines for adults.

## **REFERENCES**

1. The Guide for the Care and Use of Laboratory Animals
2. 2020 AMVA Guidelines for Euthanasia of Animals
4. 9 CFR Animal Welfare Act

## **APPENDICES**

IACUC Standard Operating Procedures