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IACUC GUIDELINE:	EUTHANASIA OF ANIMALS
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The methods utilized to euthanize any animal on an IACUC-approved protocol must comply with the AVMA Guidelines for the Euthanasia of Animals: 2020 Edition[1]. This new edition describes methods that are "acceptable with conditions", which are those techniques that may require certain conditions to be met to consistently produce humane death, may have greater potential for operator error or safety hazard, are not well documented in the scientific literature, or may require a secondary method to ensure death. Methods acceptable with conditions are equivalent to acceptable methods when all criteria for application of a method can be met. If these conditions cannot be met, then the alternative criteria or conditions should be described by the investigator in the protocol, along with appropriate scientific justification, which will be reviewed by the IACUC. All methods that comply with the AVMA Guidelines for the Euthanasia of Animals: 2020 Edition[1] are considered humane and painless and should be listed in the protocol under USDA pain/distress category C (i.e. not painful).

This guideline aims to summarize and highlight the methods of euthanasia that are deemed acceptable and acceptable with conditions in the AVMA Guidelines for the species used in our animal care and use program.

Training

- For any method of euthanasia, personnel performing the procedure must be appropriately trained to ensure that they can adequately perform the selected method of euthanasia, and are capable of verifying the death of the animal. This training is usually provided by the PI or their designee. If necessary, training can be arranged through the Attending Veterinarian.
- For euthanasia methods where skill is required (e.g., physical methods), the protocol PI, or their designee, must ensure that personnel who perform those methods are monitored for

competence. Additionally, a demonstration of competency in this method may be required by the IACUC as a condition of protocol approval.

- Animal Resource Center (ARC) technicians are properly trained and qualified but are not working on a specific protocol. They are trained and supervised under the direction of the Attending Veterinarian, or his designee (e.g. backup veterinarian), may euthanize animals by AVMA-approved methods to alleviate serious pain and distress, or in emergency situations.

Euthanasia of laboratory mice and rats housed in the ARC

- Injectable barbiturate-containing euthanasia solutions are acceptable for use without conditions.
- Altricial is defined[1] as: "immobile, blind, naked young animals (including but not limited to birds and some rodents) requiring parental care and feeding."
- Altricial neonatal rodents represent a special challenge because of their resistance to hypoxia, which results in prolonged time to unconsciousness when CO_2 or isoflurane inhalation is used. Therefore, if these agents are used for euthanasia, then the animals must be exposed to CO_2 or isoflurane for approximately 20 minutes and until they are nonresponsive to painful stimulus (tail pinch), and death must be ensured by the use of an adjunctive physical method (e.g. decapitation, cervical dislocation, or thoracotomy).
- Non-sedated, non-anesthetized, altricial neonatal mice or rats may be euthanized with sharp scissors or sharp blades or scalpels.[2]

Methods of Euthanasia

Carbon dioxide

- Considered an acceptable agent of euthanasia for laboratory rats, mice, and guinea pigs with the following conditions.
    - With the animal(s) preferably still in its home cage, or in the euthanasia chamber that contains room air, gradually fill the home cage or the chamber with 100% CO_2 from a compressed gas cylinder at an optimal flow rate that displaces 30 - 70% of the chamber volume per minute until the concentration of CO_2 in the home cage or the chamber reaches 100% (e.g., if the flow rate of 100% CO2 displaces 50% of the chamber/cage volume per minute, then wait 2 minutes). Leave the animal in the home cage or chamber for at least 3 minutes (mice) or 11 minutes (rats) after achieving 100% CO2 concentration.[5]
    - Placing a conscious animal in a chamber that has been pre-filled with100% CO_2 is unacceptable.
    - As gas displacement rate is critical to the humane application of CO_2, an appropriate pressure-reducing regulator and flow meter or equivalent equipment

with demonstrated capability for generating the recommended displacement rates for the size container being utilized is absolutely necessary.

    - Animals from different cages or groups must not be mixed in the euthanasia chamber as this may cause distress or harm (i.e. from fighting) to the animals. Additionally, the animals must not be overcrowded in the euthanasia chamber, and the chambers must be kept clean and free of debris and excreta.

- CO_2 is generally not considered a safe or effective sedative for fish as it is slow-acting, difficult to apply uniformly and often results in adverse reactions including morbidity and mortality in the treated fish[2]. Therefore, the use of CO_2 to cause anesthesia or euthanasia in fish, especially marine or brackish water fish, is not recommended and requires IACUC approval based on strong scientific justification.

Decapitation

- Considered acceptable with conditions if performed correctly and may be used in research settings when its use is required by experimental design and approved by the IACUC. Decapitation without anesthesia will be limited to those instances, due to scientific necessity, where other approved methods cannot be used, except in the case of altricial rodent neonates.
- Commercially available rodent guillotines designed to accomplish decapitation in a uniformly instantaneous manner should be used. This equipment must be well maintained, and serviced on a regular basis to ensure sharpness of blades. The maintenance schedule for guillotines is dependent on the frequency of use, but should be performed and documented at least annually. Please refer to the IACUC's Maintenance of Equipment Used for Decapitation Guideline for additional details.
- Decapitation by guillotine is preferable to cervical dislocation due to the inherent technical difficulty in the latter method and the problem of assuring that actual dislocation at the cervical vertebrae has occurred.
- For fish, reptiles, and amphibians, decapitation should be performed by cervical transection using a knife or other sharp instrument inserted caudal to the skull to sever the spinal cord and cervical vertebrate, followed by pithing.

Cervical dislocation

- Limited to use in small rodents (rats that weigh less than 200 grams, and mice) or small birds. Further, cervical dislocation without anesthesia will be limited to those instances, due to scientific necessity, where other approved methods cannot be used and then only if personnel have demonstrated technical competency in the technique.
- Considered acceptable if the following conditions are met.
    - For mice and rats, the thumb and index finger are placed on either side of the neck at the base of the skull or, alternatively, a rod is pressed at the base of the skull. With the other hand, the base of the tail or the hind limbs are quickly pulled, causing separation of the cervical vertebrae from the skull.

    - For small birds, the legs of the bird should be grasped (or wings if grasped at the base) and the neck stretched by pulling on the head while applying a ventrodorsal rotational force to the skull.
- Personnel should be trained on anesthetized and/or dead animals, and need to demonstrate technical competency in this technique.

Rapid Chilling

- Rapid chilling is an acceptable method for some zebrafish and some other small-bodied, similarly sized tropical and subtropical stenothermic fish species, if the following conditions are met:
    - Rapid chilling by immersion in 2° to 4°C water until loss of orientation and operculum movements, or for a minimum of 10 minutes for adult zebrafish, or for a minimum of 20 minutes for fry 4 to 7 days after fertilization.
- Rapid chilling is not appropriate for temperate, cool, or cold-water-tolerant finfish, or for zebrafish embryos <3 dpf (days post-fertilization).

Common laboratory amphibian species (e.g., Xenopus sp.)

- Amphibian species are best euthanized via a physical method (decapitation) while fully anesthetized (immersion in anesthetic [MS-222] bath solution).

Other physical or chemical agents

- Immersion in liquid nitrogen (rapid freezing) should be performed only if preceded by anesthesia, or when the following conditions apply:
    - Altricial neonatal rodents <5 days of age, which do not have sufficient nervous system development to perceive pain.
    - Reptiles or amphibians <4 g in body weight for which rapid freezing will result in immediate death.
- Animals must be fully anesthetized before immersion in, or perfusion with, chemical fixatives.

Inhalant anesthetic agents

- Inhaled anesthetic agents, such as isoflurane, are acceptable with the following conditions.
    - When used as the sole euthanasia agent delivered via a vaporizer or anesthetic chamber (open-drop technique), animals may need to be exposed for prolonged periods of time to ensure death, and death must be confirmed prior to carcass disposal. Death may be confirmed by physical examination, or ensured by the use of an adjunctive physical method (e.g. decapitation, cervical dislocation, or thoracotomy).

- Animals from different cages or groups should not be mixed in the anesthetic chamber as this may cause distress or harm (i.e. from fighting) to the animals. Additionally, the animals must not be overcrowded in the anesthetic chamber, and the chambers must be kept clean and free of debris and excreta

- Personnel exposure to anesthetic waste gas should be limited by using various scavenging techniques, but active scavenging techniques are preferred.

Barbiturate combination euthanasia agents

- Euthanasia solution (e.g. Euthasol®), a pentobarbital and phenytoin combination, act quickly and smoothly to render animals unconscious and is considered an acceptable agent for euthanasia. The barbiturate dose for euthanasia is typically three times the anesthetic dose.
- Pharmaceutical-grade animal drugs approved by the FDA for euthanasia are available (i.e. Euthasol®). These drugs are considered controlled substances (Class III), and investigators must therefore obtain a DEA registration in order to acquire and use these drugs. Sodium pentobarbital from Sigma and some pentobarbital-containing euthanasia solutions (i.e. Fatal-Plus, Sleepaway) similarly require a DEA registration, but since these substances are not pharmaceutical-grade animal drugs approved by the FDA for euthanasia they should not be used to euthanize animals without strong scientific justification and approval from the IACUC.
- The intravenous (I.V.) route of administration is the preferred and most efficacious route.
- The intraperitoneal (I.P.) route of administration is acceptable in rodents, rabbits, and reptiles.
- The intracardiac (I.C.) route of administration is only acceptable in previously anesthetized or otherwise unconscious animals of any species.

Tricaine methanesulfonate (MS-222)

- MS-222 is the only anesthetic agent for fish and amphibians that is currently approved by the U.S. Food and Drug Administration's (FDA) Center for Veterinary Medicine. The FDA-approved product is Tricaine-S®. MS-222 from Sigma or similar chemical/reagent vendor, is neither FDA-approved, nor a pharmaceutical grade compound.
- Powder MS-222 is a hazardous agent causing irritation to the eyes, skin and respiratory tract. A safety datasheet for this hazardous agent can be found by following this link. Therefore, and in the interest of safety, all laboratory use of MS-222 powder must be conducted in a fume hood and personnel working with MS-222 powder must wear a lab coat, safety glasses, and gloves.
- Euthanasia with MS-222 is accomplished by immersing the fish or amphibian in a buffered solution (pH 7.0 – 7.5) of MS-222 at concentrations of 250 – 500 mg/L, or 5 to 10 times the anesthetic dosage. Euthanasia of large finfish and *Xenopus laevis* requires a higher concentration of MS-222 (5 g/L in the case of *Xenopus laevis*) and prolonged exposure (at

least 1 hour in the case of *Xenopus laevis*), or the application of a secondary method (such as decapitation) on the deeply anesthetized animals.

- Fishes or amphibians that are anesthetized or euthanized with MS-222 should not be immediately returned to the wild, or be harvested for food. A minimum 21-day withholding period is required before the animal can be returned to the wild.
- Disposal of MS-222 powder must be performed through the Campus Hazardous Waste Program. MS-222 solutions less than 5000 mg/L may be poured into a drain leading to the sewer system. Contact Campus Hazardous Waste for solutions greater than 5000mg/L.

Clove Oil and Benzocaine

- Clove oil (85-95% eugenol active ingredient concentration) is an acceptable agent for euthanasia of fish. It is recommended that, whenever possible, products with standardized, known concentrations of eugenol be used so that accurate dosing can occur. The generally accepted dose for euthanasia is >400 mg eugenol/L, and death should be confirmed through the application of a physical method on the fully anesthetized fish.
- Benzocaine hydrochloride gel and solutions are acceptable agents for euthanasia for fish and amphibians; however, they must be carefully buffered to avoid tissue irritation.
- Clove oil and benzocaine are not available as FDA-approved products and are unacceptable for animals intended for consumption (by humans or other animals). As with the use of any non-pharmaceutical-grade drug, an IACUC exception for scientific reasons is required.

Verification of Death

- Verification of death of the animals prior to disposal is especially important where the method of euthanasia has the potential for allowing animals to recover. This can include the use of CO_2 or the use of overdoses of anesthetic agents where respiratory depression/suppression occurs before death. Injection of drugs specifically labeled as euthanasia agents can sometimes result in a deep anesthetic state that may mimic death but from which animals can recover. To prevent the inadvertent disposal of animals that are still alive, death must be confirmed prior to disposal.
- Methods for assuring death after euthanasia include:
    - Rodents euthanized by CO_2 for less than the duration of exposure to 100% CO_2 described above (e.g., 3 min for mice), or an overdose of an inhalant anesthetic agent (i.e. isoflurane) should remain exposed to room air prior to disposal for an adequate amount of time, but at least five minutes, and death should be confirmed by at least two of the following criteria: lack of pulse, lack of breathing, lack of a corneal reflex, lack of any response to firm toe/tail pinch, or an inability to hear respiratory sounds and heartbeat by use of a stethoscope.
    - Physical methods such as thoracotomy, cervical dislocation, decapitation, or removal of organs vital to life may be used as an adjunct method to ensure death of any animal species.

- Exsanguination by removal of more than 60% of expected blood volume for the species at one withdrawal.
- Zebrafish euthanized by immersion in an MS-222 or ice water (rapid chilling) bath without the application of a secondary physical method must not be removed from the immersion solution until at least 30 minutes following cessation of opercular (i.e., gill) movement.
- To ensure death of zebrafish embryos <15 dpf (days post fertilization) following immersion in MS-222, an adjunct method such as sodium hypochlorite treatment should be used.
- For fish and amphibian species, a secondary physical method should be applied to any animal euthanized by immersion in an anesthetic solution (MS-222, benzocaine, or clove oil) to ensure death.

Animal Carcass Disposal

- Disposal of animal carcasses must be in compliance with all pertinent federal, state, and local regulations, and in a manner that will reduce the risk of disease transmission, prevent pests or scavengers from gaining access to the animal remains, and ensure human and environmental safety.
- For animals housed in the Animal Resource Center (ARC): the ARC will arrange for the removal and disposal of all animal carcasses using a licensed commercial medical waste disposal firm. Carcass freezers are located in the ARC vivaria to contain the carcasses prior to disposal.
- For animals (i.e. fish, birds, reptiles and amphibians) housed in satellite animal holding facilities: the carcass disposal method should be described in the animal facilities' SOP, which is reviewed by the IACUC.

References

1. AVMA Guidelines for the Euthanasia of Animals: 2020 Edition. (https://www.avma.org/sites/default/files/2020-01/2020-Euthanasia-Final-1-17-20.pdf)
2. Report of the ACLAM Task Force on Rodent Euthanasia. August 2005. (http://www.aclam.org/Content/files/files/Public/Active/report_rodent_euth.pdf)
3. AFS Policy Statement Regarding the Need for an Immediate-Release Anesthetic/Sedative for Use in the Fisheries Disciplines
4. Guidelines for Use of Zebrafish in the NIH Intramural Research Program (https://oacu.oir.nih.gov/sites/default/files/uploads/arac-guidelines/zebrafish.pdf)
5. Hickman, DL. 2022. Minimal Exposure Times for Irreversible Euthanasia with Carbon Dioxide in Mice and Rats. J Am Assoc Lab Anim Sci 61:283-286. DOI: 10.30802/AALAS-JAALAS-21-000113