Policy 4 – Rodent Euthanasia
Version 2.0
Approval Date: 1/22/07, 3/5/13

Purpose – The purpose of this policy is to provide investigators with acceptable and conditionally methods of euthanasia for various species and ages of rodents, along with methods that are not acceptable.

Background –

“…methods [of euthanasia] should be consistent with the AVMA Guidelines on Euthanasia (AVMA 2007 or later editions).” – the Guide

“Standardized methods of euthanasia that are predictable and controllable should be developed and approved by the Attending Veterinarian and IACUC.” – the Guide

Classification of method of euthanasia: The AVMA categorizes each method of euthanasia as acceptable (methods which consistently produce a humane death when used as the sole means of euthanasia), conditionally acceptable (methods which by the nature of the technique or because of greater potential for operator error or safety hazards might not consistently produce humane death or are methods not well documented in the scientific literature) or unacceptable (methods deemed inhumane under any conditions or that the panel found posed a substantial risk to the human applying the technique).

Policy –

Euthanasia quick reference chart:

<table>
<thead>
<tr>
<th>age</th>
<th>accepted</th>
<th>conditionally accepted</th>
<th>not accepted</th>
<th>comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>early embryos (E-1 to E15)</td>
<td>death of dam is sufficient, no other methods are needed</td>
<td>N/A</td>
<td>N/A</td>
<td>dam needs to be euthanized using accepted methods</td>
</tr>
<tr>
<td>late embryos to neonates (E16-P-9)</td>
<td>decapitation, cervical dislocation, chemical injection</td>
<td>N/A</td>
<td>CO₂ inhalation, hypothermia</td>
<td>animals are resistant to hypoxia at this age, CO₂ inhalation is not accepted</td>
</tr>
<tr>
<td>P-10 to adult</td>
<td>Gradual exposure to CO₂, anesthetic overdose (chemical injection, inhalation)</td>
<td>Cervical dislocation, decapitation, need IACUC approval</td>
<td>CO₂ from dry ice, hypothermia, smothering, blow to head</td>
<td>inhalation method must be followed by a physical euthanasia method</td>
</tr>
</tbody>
</table>

Overview:

Section 1: General Information Regarding Rodent Euthanasia
Section 2: Euthanasia of Adult Rodents (≥10d)
Section 3: Euthanasia of Rodent Feti and Neonates (E16-P9)
Section 4: Effects of Various Methods of Euthanasia on Rodent Physiology
SECTION 1: General Information

Euthanasia is the act of humanely killing animals by methods that induce rapid unconsciousness and death without pain or distress. Public Health Service policy requires that the Institutional Animal Care and Use Committees (IACUC) determines that methods of euthanasia utilized in research proposals are consistent with the recommendations of the 2013 American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (1).

The criteria used as the basis for the AVMA’s recommendations include:

1. Minimum pain, distress, anxiety or apprehension
2. Minimum delay until unconsciousness
3. Reliability and irreversibility
4. Safety of personnel; emotional effect on personnel
5. Species and age limitations

IACUC approval of any deviation(s) from the 2013 AVMA Guidelines must be project-specific and include critical review of assertions of scientific necessity. If conditionally acceptable techniques are planned, they must be scientifically justified and approved by the IACUC prior to implementing.

Euthanasia must be performed in a compassionate manner to avoid animal distress. Depending on the species involved, animals being euthanized may vocalize, release pheromones or behave in a manner, which may be distressing to other animals. For these reasons, animals may not be euthanized while crowded or in the presence of animals not being euthanized.

Additional required practices to ensure death in rodents - Death must be verified after euthanasia and prior to disposal. Confirmation of death is achieved by absence of respiration, heartbeat and toe/tail pinch reflexes. However, the assessment of heart beat and respiratory pattern can be very difficult in rodents due to their small size. Consequently, these criteria may be difficult to apply to these species and there is a risk of animals recovering. To ensure the irreversibility of inhalant euthanasia, it is required that animals undergo a secondary, physical method of euthanasia (i.e., exsanguination, thoracotomy, cervical dislocation, or decapitation).

Principal Investigators are responsible for ensuring all personnel performing animal euthanasia in an IACUC approved protocol have been properly trained to consistently apply the technique(s) in a humane and effective manner.
SECTION 2: Euthanasia for Rodents ≥10 Days Old (adults)

Inhalant Agents (Acceptable)

When possible, inhaled agents should be administered under conditions where animals are most comfortable (i.e., for rodents, in their home cage).

Carbon dioxide (2): Carbon dioxide has a rapid depressant, analgesic and anesthetic effect. Carbon dioxide is nonflammable, nonexplosive, and poses minimal hazard to personnel when used with properly designed equipment. Because CO₂ is heavier than air, incomplete filling of a chamber may permit animals to climb or raise their heads above the higher concentrations and avoid exposure.

High concentrations of CO₂ may be stressful to some species when awake. Accordingly, pre-filling the chamber is forbidden. Carbon dioxide systems should displace 10-30% of chamber volume per minute. Chambers must not be overcrowded; animals should be able to stand on the floor of the chamber with all four feet and have sufficient space to turn around and perform normal postural adjustments. In this regard, it is important to also consider that mixing unfamiliar or incompatible animals in the same container may also be stressful. Chambers should be emptied and cleaned/disinfected between uses.

Compressed CO₂ gas is the only permitted source of carbon dioxide because the inflow to the chamber can be regulated precisely. Carbon dioxide generated by other methods such as from dry ice, fire extinguishers, or chemical means (i.e., antacids) is forbidden.

Inhalant Anesthetics: Inhalant anesthetics are particularly valuable for euthanasia of smaller animals or for animals in which venipuncture may be difficult. Since the liquid state of most inhalant anesthetics is irritating, animals can only be exposed to vapors and must be prevented from contacting the anesthetic agent in its liquid form. In order of preference, halothane, enflurane, isoflurane, sevoflurane, and desflurane are generally acceptable for euthanasia of small animals (<7 kg). Halogenated anesthetic agents should only be used if they are appropriately scavenged to avoid personnel exposure, i.e., activated charcoal canister, fume hood or exhausted biosafety cabinet class II, type B.

For all inhaled forms of euthanasia, death must be verified after euthanasia and prior to disposal. Unintended recovery must be obviated by the use of appropriate CO₂ concentrations and exposure times or by other means. To ensure the irreversibility of the procedure after apparent death from CO₂, animals must further undergo a physical method of euthanasia (i.e., exsanguination, thoracotomy, cervical dislocation or decapitation; see physical methods listed below).

Inhalant Anesthetics (Conditionally Acceptable)

Ether is irritating to the mucous membranes and poses serious risks associated with its inflammability and explosiveness. Explosions have occurred when animals, euthanatized with ether, were placed in an ordinary (not explosion proof) refrigerator or freezer and when bagged animals were placed in an incinerator. Ether can only be used after IACUC approval in carefully controlled situations in compliance with all applicable safety policies and regulations.
Injectable Pharmaceutical Agents (Acceptable)

**Barbiturates**: A primary advantage of barbiturates is speed of action, which depends on the dose, concentration, route, and rate of injection. Barbiturates induce euthanasia smoothly, with minimal discomfort to the animal. Intravenous injection is the preferred route of administration, however intraperitoneal injections may be used in situations when intravenous injections would be stressful or impractical. Intracardiac injection can only be used if the animal is heavily sedated, unconscious, or anesthetized. Barbiturates may be administered intraperitoneally and induce rapid, smooth euthanasia with minimal animal discomfort. As with all controlled substances, barbiturate usage requires having appropriate licensure and registration, ensuring secure storage and maintaining accurate drug accountability.

**Potassium chloride in conjunction with general anesthesia**: Although unacceptable when used in unanesthetized animals, the use of potassium chloride administered intravenously or intracardially in animals under general anesthesia is an acceptable method of euthanasia. It is important for personnel performing this method of euthanasia to be trained and knowledgeable in anesthetic techniques, and competent in assessing anesthetic depth. Administration of potassium chloride requires animals to be in a surgical plane of anesthesia characterized by loss of consciousness, loss of reflex muscle response, and loss of response to noxious stimuli.

Physical Methods (Conditionally Acceptable)

Physical methods of euthanasia when properly used by skilled personnel with well-maintained equipment may result in less fear and anxiety and be more rapid, painless, humane, and practical than other forms of animal euthanasia. Physical methods are not generally allowed as a sole means of euthanasia (exceptions granted from the IACUC), but are required as adjuncts to other agents or methods. Personnel performing physical methods of euthanasia must be trained and monitored for each type of physical euthanasia technique performed. Since most physical methods involve trauma, there is inherent risk for animals and humans, therefore extreme care and caution should be used. Methods not performed correctly can result in animal and personnel injuries. Inexperienced persons must be trained by experienced personnel and should practice on carcasses until they are proficient in performing the method properly and humanely.

**Cervical Dislocation**: Cervical dislocation is a technique that is rapidly accomplished and can induce rapid loss of consciousness without chemically contaminating tissue, but requires technical proficiency. Cervical dislocation is a humane technique for euthanasia of rodents weighing \( \leq 200 \) g when performed correctly. In lieu of demonstrated technical competency, animals must be unconscious or anesthetized prior to cervical dislocation.

This technique can be used as a primary means of euthanasia only when scientifically justified by the PI, and approved by the IACUC. Those responsible for the use of this technique must ensure that personnel performing cervical dislocation techniques have been properly trained and consistently apply it humanely and effectively.

**Decapitation**: Decapitation is a technique that is rapidly accomplished and induces near instantaneous death without chemically contaminating tissues. Personnel performing this technique should recognize
the inherent danger of the guillotine or other sharp instruments and take adequate precautions to prevent personal injury. This method of euthanasia can only be used when its use is required by the experimental design and approved by the IACUC (except for animals younger than 10 days old). The equipment used to perform decapitation should 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 appears to reduce distress from handling, minimizes the chance of injury to personnel, and improves positioning of the animal in the guillotine. Those responsible for the use of this technique must ensure that personnel who perform decapitation techniques have been properly trained to do so.

**Euthanasia in Anesthetized, Laparotomized (Non-Survival Surgery) Animals:**

While animals are fully anesthetized, they may be euthanized using one of the following acceptable methods of physical euthanasia:
- exsanguination - great vessels severed, cardiac perfusion, or removal of vital organs
- incision of the chest cavity or diaphragm to produce a pneumothorax (collapsed lung) and cessation of respiration
- decapitation
- cervical dislocation

**Unacceptable Methods of Euthanasia**

The following methods of euthanasia are expressly forbidden under any circumstance: air embolism, blow to the head/stunning, burning, chloral hydrate, chloroform, cyanide, decompression, drowning, formalin, household products and solvents such as acetone, quaternary compounds (including CCl₄), laxatives, clove oil, dimethyl-ketone, quaternary ammonium products (i.e., Roccal D Plus), antacids (and other commercial and household products), hypothermia, neuromuscular blocking agents, rapid freezing, and strychnine.

Any exceptions to this policy must have IACUC and Veterinary approval

**References:**

1. 2013 AVMA Guidelines for the Euthanasia of Animals

SECTION 3: Euthanasia of Rodent Fetuses and Neonates (Embryonic day 15 - postpartum day 9)

The AVMA Guidelines on Euthanasia provides limited recommendations for the euthanasia of prenatal or neonatal animals. Regarding prenatal and neonatal euthanasia, the 2013 Guidelines state: “When ovarian hysterectomies are performed, euthanasia of feti should be accomplished as soon as possible after removal from the dam. Neonatal animals appear to be resistant to hypoxia” (1). However, the Report of the ACLAM Task Force on Rodent Euthanasia provides greater detail for these young animals (2). In all cases, the person performing the euthanasia must be fully trained in the appropriate procedures.

Feti: At approximately 60% of the gestation period, the neural tube has developed into a functional brain and the likelihood that a fetus may perceive pain should be considered. Reflexive behavior in response to painful stimuli has been observed in fetuses and correlates with adult behaviors. However, fetal behavioral arousal and awareness may be suppressed by low arterial oxygen limiting higher cortical processing.

Mouse, Rat and Hamster fetuses up to 14 days (E14) and Guinea Pig fetuses up to 34 days (E34) gestation: Neural development at 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 fetuses due to loss of blood supply and non-viability of fetuses at this stage of development. Therefore, it is unnecessary to remove fetuses for euthanasia after the dam is euthanized.

Mouse, Rat and Hamster fetuses 15 days gestation (E15) to birth and Guinea Pig fetuses 35 days gestation (E35) to birth: The neural development at this stage supports the likelihood that pain may be perceived. When feti are required for study, euthanasia of individual feti may be induced by the skillful injection of chemical anesthetics. Decapitation with surgical scissors or cervical dislocation are acceptable physical methods of euthanasia. Rapid freezing, without prior anesthesia, as a sole means of euthanasia is not considered to be humane. Animals should be anesthetized prior to freezing. When chemical fixation of the whole fetus is required, feti should be anesthetized prior to immersion in or perfusion with fixative solutions. Anesthesia may be induced by hypothermia of the fetus (3), or by injection of the fetus with a chemical anesthetic. The Veterinarian should be consulted for considerations of fetal sensitivity to specific anesthetic agents. Fetuses at this age are resistant to hypoxia and require extended exposure to inhalant anesthetics.

When fetuses are not required for study, the method chosen for euthanasia of a pregnant mother should ensure rapid cerebral anoxia to the fetus with minimal disturbance to the uterine milieu minimizing fetal arousal. Recommended methods for euthanasia of the mother are CO₂ exposure followed by cervical dislocation. Death of the mother must be verified after euthanasia and prior to disposal. The Veterinarian should be consulted for considerations of other euthanasia agents.

Neonates: Maturation of nociceptors and the development of excitatory and inhibitory receptor systems occur during the period just prior to birth and into the second week of postnatal life (P13-16). Resistance to hypoxia at this age results in a prolonged time to unconsciousness when CO₂ is used as a euthanasia agent and so this method is not permitted. Death must be verified after euthanasia and prior to disposal.
Mouse, Rat and Hamster up to 10 days (P10) of age: Acceptable methods for euthanasia include injection of chemical anesthetics (e.g., pentobarbital), decapitation, or cervical dislocation. The Veterinarian should be consulted for appropriate alternatives if needed.

Guinea Pig: Follow guidelines for adults (precocial young).

References -

1. 2013 AVMA Guidelines for Euthanasia of Animals
   http://www.avma.org/issues/animal_welfare/euthanasia.pdf


SECTION 4: Effects of Various Methods of Euthanasia on Physiology

Table 1: Biologic effects of decapitation (3, 5, 16, 49, 56, 60, 66)

<table>
<thead>
<tr>
<th>Effect</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increase in plasma sodium</td>
<td>Hemolysis</td>
</tr>
<tr>
<td>Increase in plasma potassium</td>
<td>Continued postmortem neurochemical alterations</td>
</tr>
<tr>
<td>Increase in GABA concentrations (brain)</td>
<td></td>
</tr>
<tr>
<td>Increase in Alanine (brain)</td>
<td></td>
</tr>
<tr>
<td>Increase in plasma ascorbic acid (30-40% &gt; resting state)</td>
<td></td>
</tr>
<tr>
<td>Increase in blood catecholamine levels</td>
<td></td>
</tr>
<tr>
<td>Increased plasma calcium, magnesium</td>
<td></td>
</tr>
<tr>
<td>No change in vasoactive intestinal peptides (brain)</td>
<td></td>
</tr>
<tr>
<td>No change in neuropeptide Y (brain)</td>
<td></td>
</tr>
<tr>
<td>Alteration in rat heart mitochondria function</td>
<td></td>
</tr>
<tr>
<td>Increase in serum corticosterone</td>
<td>Stress stimulus → mobilization from tissues to blood; generalized metabolic</td>
</tr>
<tr>
<td></td>
<td>response secondary to sympathoadrenal response; some handling related</td>
</tr>
<tr>
<td></td>
<td>stimulation.</td>
</tr>
<tr>
<td></td>
<td>Possible handling stress</td>
</tr>
</tbody>
</table>

Table 2: Effects of physical and pharmacological euthanasia methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Physiologic effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methoxyflurane and decapitation (10)</td>
<td>Increase in prostacyclin (vasodilator that inhibits platelet aggregation)</td>
</tr>
<tr>
<td></td>
<td>Vascular contractility suppressed</td>
</tr>
<tr>
<td></td>
<td>Decreased vascular contractility</td>
</tr>
<tr>
<td>Ether and decapitation, or decapitation alone (50)</td>
<td>No statistical difference in prolactin levels or LH/FSH secretory properties of</td>
</tr>
<tr>
<td></td>
<td>cultured anterior pituitary cells</td>
</tr>
<tr>
<td>Ether and decapitation (74)</td>
<td>No change in estrogen receptors/progesterone receptors in rat uteri</td>
</tr>
<tr>
<td>Ketamine and decapitation (50, 74)</td>
<td>No change in estrogen receptors/progesterone receptors in rat uteri</td>
</tr>
<tr>
<td>Pentobarbital and decapitation (4)</td>
<td>Increase in acetylcholine release in the brain</td>
</tr>
<tr>
<td>Halothane and decapitation (21)</td>
<td>Increase in plasma ascorbic acid</td>
</tr>
<tr>
<td></td>
<td>Increase in plasma catecholamines</td>
</tr>
</tbody>
</table>

Table 2: Effects on reproductive hormones: The following combinations may be unsuitable for studies of serum androgens

<table>
<thead>
<tr>
<th>Decapitation in combination with agents listed below (49, 71)</th>
<th>Immature</th>
<th>Male rats</th>
<th>Mechanism: direct effect on testes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LH</td>
<td>FSH</td>
<td>Prolactin</td>
</tr>
<tr>
<td>Xylazine</td>
<td>-</td>
<td>-</td>
<td>↓</td>
</tr>
<tr>
<td>Biotol</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Thiopental</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pentobarbital</td>
<td>-</td>
<td>-</td>
<td>↓</td>
</tr>
<tr>
<td>Ketamine</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Halothane</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Ether (tested on castrated rats)</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
</tr>
</tbody>
</table>

↓ = decreased  ↑ = increased  - = no change
<table>
<thead>
<tr>
<th>Method of Euthanasia</th>
<th>Induced by Pharmacologic and/or Physical Methods</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injectable Pentobarbital¹² (5, 53, 61)</td>
<td>Decreased muscular contractility in isolated muscle prps Decreased Q1 smooth muscle contractility when given orally or intravenously; not seen in intra-peritoneal route Intraperitoneal administration causes increased colonic contractility in response to acetylcholine Decreased spontaneous and drug induced vascular smooth muscle contractility Decreased catecholamine levels Increased partial pressure of CO₂ in arterial blood Increased serum activity renin Increased plasma aldosterone Splenic enlargement Increased plasma glucose and insulin Increased liver glycogen Decreased plasma triglycerides Increase in plasma insulin</td>
<td>Decreased calcium transport</td>
</tr>
<tr>
<td>Cervical dislocation/cervical fracture (32, 68, 72)</td>
<td>Decreased coronary flow; decreased contractile function in isolated perfused heart preparations Normal lymphocyte proliferation High levels of serotonin in lung Increase in granulocyte and macrophage colony forming cell counts in murine bone marrow cultures</td>
<td>Possible decreased sensitivity of B-adrenergic receptors secondary to cervical fracture Entrapment of platelets in pulmonary capillaries Apparent alteration of marrow stem cell pool</td>
</tr>
<tr>
<td>Cervical dislocation and methoxyflurane (32)</td>
<td>Increased mitogen induced lymphocyte proliferation Normal cytolytic T lymphocytes (CTL) response</td>
<td></td>
</tr>
<tr>
<td>Cervical dislocation and pentobarbital (32)</td>
<td>Increased mitogen induced lymphocyte proliferation Decreased CTL response</td>
<td></td>
</tr>
<tr>
<td>Cervical dislocation and halothane (32)</td>
<td>Normal mitogen induced lymphocyte proliferation Decreased CTL response</td>
<td></td>
</tr>
<tr>
<td>CO₂ and cervical dislocation (32)</td>
<td>Normal mitogen induced lymphocyte proliferation Decreased CTL response</td>
<td></td>
</tr>
<tr>
<td>CO₂ and decapitation (4, 23, 66)</td>
<td>Normal LH, FSH, prolactin, corticosterone Activity of cholinergic markers identical to decapitation only Altered GABA, receptor function</td>
<td>Due to rapid inactivation of metabolizing enzyme</td>
</tr>
<tr>
<td>Focused beam microwave irradiation (FBMI)³ (41, 45)</td>
<td>Best technique for measuring adenosine levels Decreased brain amino acids: alanine, GABA, γamino butyric acid (GABA), aspartate, glutamate, glutamine, tyrosine, phenylalanine, glycine, aspartate Increased levels of reduced glutathione, glutamate 5 fold decrease in d prostaglandin and Thromboxane B₂ (mouse brain) Twice the concentration of substance P, neurokinin A, and neuropeptide in brain tissue compared to decapitation</td>
<td>Possible enzyme inactivation by microwave irradiation causing increased recovery of peptides Possible disintegration of neuropeptide containing tissue compartments, or decreased binding of carrier proteins, releasing more peptides</td>
</tr>
<tr>
<td>CO₂ (8, 52, 69)</td>
<td>100% CO₂: decreased mean corpuscular hemoglobin (MCV) Increased total leukocytes and granulocytes (PLT) Decreased liver glycogen, pyruvate, ATP No change in platelet counts</td>
<td>CO₂ causes acidosis that affects RBC parameters</td>
</tr>
<tr>
<td>CO₂ or CO₂/O₂ (8, 27, 34, 46, 52, 69)</td>
<td>Increased hematocrit, mean corpuscular volume No change in serum norepinephrine, dopamine, serotonin, corticosterone Decreased serum creatine kinase, aspartate aminotransferase Significant decrease in liver glycogen stores Increased serum glucose Decreased activity of enzymes regulating branched chain amino acid degradation Decreased mean erythrocyte hemoglobin, mean corpuscular hemoglobin concentration</td>
<td>CO₂ causes acidosis that produces stimulation of enzymes of the glycolytic pathway</td>
</tr>
</tbody>
</table>
ADDITIONAL FACTORS THAT INFLUENCE THE OUTCOME OF EUTHANASIA: (6, 7, 18, 22, 38, 56)

- **Handling**: May cause sympathoadrenal discharge, which affects plasma glucose, progesterone plasma catecholamines. Habituating the animals to handling may mitigate this effect.
- **Environmental stimuli** (e.g., noise) can increase plasma corticosterone concentrations.
- **Sequence**: The order of euthanasia for rats housed in pairs produced significant differences in plasma tryptophan and unesterified fatty acids, plasma corticosterone, plasma protein lactate levels, substance P, cholecystokinin, somatostatin.
References